ELECTROCEUTICAL TECHNOLOGY: ANTI-INFLAMMATORY EFFECTS OF 40-160
T/S INDUCTIVELY COUPLED ELECTRICAL STIMULATION (ICES) IN THE ACUTE
INFLAMMATION MODEL

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ABSTRACT

DEVIN KERRY HUBBARD: Electroceutical Technology: Anti-inflammatory Effects of 40-160 T/s Inductively Coupled Electrical Stimulation (ICES) in the Acute Inflammation Model
(Under Direction of Robert G. Dennis)

Electromagnetic therapies (*sic*: electroceuticals) have been studied and used for many years as a treatment for many ailments including chronic and acute pain, inflammation, muscle atrophy, non-union bone fractures, as well as peripheral and central neuropathies¹. Presently we seek to explore the realm of inductively coupled electrical stimulation (ICES) which is a subclass of pulsed electromagnetic field therapies (PEMFs) that uses rapidly changing electromagnetic fields to induce current flows in tissues. Such fields are hypothesized to act via various mechanisms. However, in the present we seek to clarify the often ambiguous and confusing literature regarding ICES mechanisms by conducting a scholarly review by which we then provide a dose reporting scheme for accurately describing the relevant parameters required to fully define ICES treatments. Based on our review and experience, we hypothesize that ICES requires very specific parameters to function appropriately. We seek to ascertain the efficacy of 40-160 Tesla/second (T/s) ICES stimulation as an anti-inflammatory therapy. A specific mechanism explored is the calcium/calmodulin (Ca/CaM) pathway implicated in the literature²⁻⁹. Our results bring into question the scientific methods of previously reported results by suggesting that nitric oxide levels in vitro fall below detection limits of commonly used methods for gauging Ca/CaM modulation by ICES. In an effort to elucidate the efficacy of ICES as an anti-inflammatory, we make use of the well-established carrageenan footpad edema (CFE) model in rats. The

CFE model provides a spontaneously-resolving, acute inflammation model with a very well understood progression and biochemical mechanism. Our results indicate that 40-160 T/s ICES provides statistically significant, repeatable CFE reduction ($P < 0.05$) as measured by plethysmometry with no observed detrimental side-effects. Further work should focus on elucidating mechanism, evaluating safety, and exploring other potential applications—such as treating chronic conditions. The present studies support ICES as having the potential to provide life-changing therapy to individuals suffering from acute inflammation and pain. Given carefully conducted research in the future, we feel that ICES may revolutionize modern medicine not only from a treatment standpoint, but from the standpoint of understanding basic human electrophysiology and biochemistry.

To my family and friends, you are my inspiration and reason to do well and good in this
world.

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LIST OF ABBREVIATIONS

ANOVA – Analysis of variance

ARFI – Acoustic radiation force impulse

ATP – Adenosine triphosphate

BMP-2 – Bone morphogenic protein 2

Ca²⁺ – Calcium (2+) ion

CaM – Calmodulin

CFE – Carrageenan footpad edema

Cl⁻ – Chloride

CLR – C-type lectin receptor

CNS – Central nervous system

DAMP – Damage-associated molecular pattern

DNA – Deoxyribonucleic acid

eNOS – Endothelial nitric oxide synthase

FBS – Fetal bovine serum

FGF-2 – Fibroblast growth factor 2

fMRI – Functional magnetic resonance imaging

HMSC – Human Mesenchymal derived stem cell

ICES – Inductively coupled electrical stimulation

IFN- λ – Interferon gamma

IL1- β – Interleukin-1 Beta

IL-6 – Interleukin-6

iNOS – Inducible NOS

IU – International units

K⁺ – Potassium

L-NMMA – N-monomethyl-L-arginine

Na⁺ – Sodium

NASA – National Aeronautics and Space Administration

NLR – NOD-like receptor

nNOS – Neuronal NOS

NO – Nitric oxide

NOS – Nitric oxide synthase

NPIR – Non-phagocytic inflammatory response

NSAID – Non-steroidal anti-inflammatory drug

PAMP – Pathogen-associated molecular pattern

PBS – Phosphate buffered saline

PEMF – Pulsed electromagnetic field

PET – Positron emission tomography

PIR – Phagocytic inflammatory response

PNS – Peripheral nervous system

PO – *per os* (by mouth)

PPS – pulses per second

PRF – Pulsed radio frequency

PRR – Pattern recognition receptor

rCIA – Rat collagen-induced arthritis

RLR – RIG-1-like receptor

RNA – Ribonucleic acid

rTMS – Repetitive transcranial magnetic stimulation

SAID – Steroid anti-inflammatory drug

TENS – Transcutaneous electrical nerve stimulation

TLR – Toll-like receptor

TVEMF – Time-varying electromagnetic fields

UNC TCF – University of North Carolina Tissue Culture Facility

α -MEM – Alpha minimum essential medium

α -SMA – Alpha smooth muscle actin

LIST OF SYMBOLS

μL – Microliter

μM – Micromolar

μs – Microsecond

CHAPTER 1 : A HISTORY OF ICES

Introduction

Over at least the past seventy years, many different forms of pulsed electromagnetic field (PEMF) therapy have been reported in the scientific literature. Among these, techniques that employ the use of inductively coupled electrical stimulation (ICES) therapy have been reported in peer-reviewed scientific literature as an effective means of reducing pain and inflammation in a wide variety of conditions while often promoting healing^{3,10-16}. Other forms of PEMF therapy have also been reported as effective, such as transcutaneous electrical nerve stimulation (TENS), however, the focus of this work will be on ICES rather than on other forms of PEMF treatment because of the many advantages of ICES.

One conceptual advantage of the use of ICES over other types of PEMF technology is that it does not require the assumption that magnetic fields themselves interact directly with living tissues by means of some form of magic, or a complex and poorly-understood quantum physical effect. Rather, the pulsed magnetic fields of ICES act through the well-understood physical mechanism of electro-magnetic induction, wherein the external pulse generator uses non-invasive electric coils to create time-varying magnetic pulses that penetrate living tissue essentially without any strong direct interactions because the living tissue is essentially “transparent” to the magnetic fields themselves. These pulses are inductively coupled across space to the structures within living tissues that have conductive paths, for example around the cell membrane in the paracellular space, or around organelles within cells. These conductive paths within the tissue act as the “secondary” coil of what can

essentially be viewed as an air-core electrical transformer, the primary coil being the external ICES. Based on the well-understood Law of Induction, one of the four classical Maxwell Equations, electrical currents are induced in and around the cells within the living tissue within the conductive paths in and around cells. This takes the form of ions in solution being forced to move, driven by the induced fields¹. Thus, ICES is essentially a means by which electromagnetic induction allows the *electrical* stimulation of deep living tissues without requiring the use of invasive electrodes. For this reason ICES has advantages over the much more common use of direct conductively coupled electrical stimulation, sometimes designated TENS. Even though the scientific literature strongly supports the effectiveness of treatments such as TENS, ICES has several other very important advantages over direct methods. For example, ICES does not have to overcome the resistance of skin tissue, and the induced fields generated in deep tissue by ICES-based systems are more widely and more uniformly distributed across the tissues being stimulated. TENS stimulation energy generally follows the path of least resistance, resulting in highly non-uniform energy distribution within the tissues being stimulated. One final advantage of ICES over more general forms of PEMF stimulation is that when properly applied, ICES pulses are transformed into induced electrical signals that themselves mimic known electrical signals within living tissues, such as those involved in excitation-contraction coupling of striated muscle (skeletal and cardiac muscle), therefore ICES can take advantage of native signal reception and amplification mechanisms within living cells/tissues, thus requiring only very low stimulation energy to achieve the desired cellular response. For these reasons, the remainder of this dissertation will focus on ICES rather than on other forms of PEMF.

The physics of ICES gives a clear and understandable mechanism by which electrical energy can be induced within deep tissues by the Law of Induction. It remains to be elucidated how the induced fields are transduced into useful biological signals. Observations and mathematical models suggest that one of the primary anti-inflammatory mechanisms of ICES is via the Calcium-Calmodulin ($\text{Ca}^{2+}/\text{CaM}$) dependent nitric-oxide synthase pathway^{2,5,7-9,17-21}. Specifically, it is hypothesized that electromagnetic pulses of appropriate parameters will preferentially induce calcium binding to CaM ⁷. Regardless of the mechanism, of utmost importance are the waveform parameters—with the most effective parameters reportedly falling within a range producing induced electrical fields on the order of 1 V/cm^{7,22}.

Unfortunately, the majority of the PEMF and ICES literature fails the basic scientific requirement of repeatability. By our accounting, more than 90% of all published reports fail to include adequate waveform parameters to fully define the dosimetry of the applied treatment. This shortcoming in the literature is very unfortunate as it tends to drive reputable clinicians and scientists away from the scientific study and clinical acceptance of PEMF in general and ICES specifically, even though there is strong evidence to suggest that ICES, when properly applied, is safe and can be very effective at reducing inflammation and pain while also accelerating healing of otherwise refractory injuries.

Herein we seek to review past and current technologies, effective waveform parameters, and propose a summary of the current theories regarding the mechanism of ICES. Our goal is to establish clearly those experiments which are properly executed and have well-described stimulation parameters, and show that ICES is an effective treatment for pain and inflammation given the appropriate stimulation waveforms.

The concept of using pulsed electromagnetic fields (PEMF) has been explored as a clinical therapeutic since the 1950's^{12-14,23-25}. Since then, PEMF has been used to treat critical bone-gap defects (including spinal non-fusion), wound healing, inflammation, as well as various psychiatric disorders^{1-3,6,7,9-16,20,22,25-64}. Macroscopically, there are no properties that would warrant the expectation that a static magnetic field would interact directly with tissues (as can be demonstrated by the fact that a static magnetic field can pass nearly unattenuated through tissue). However, there is reason to expect that a changing magnetic field would (and will) interact with any medium in which there are charge carriers—including tissues—to produce a flow of charge. Given that all biochemical interactions—from enzymes binding ligands to ions flowing through membranes—are driven by electrostatic interactions, it follows that a rapidly changing electromagnetic field should induce flow of current in tissues in the form of movement of ions that could have effects on the binding interactions of biochemical compounds. It is also very well established that tissues are constantly exposed to electrical fields, and that electrical fields play a key role in bio-signaling. Thus, because we hypothesize that the efficacy of PEMF is due to its inductive electrical signal within tissues, we prefer to use the term inductively coupled electrical stimulation (ICES) rather than PEMF. A second reason to use the term ICES is that the term PEMF has been used widely in a large body of literature that paints a less than replete picture of mechanism and effects. There is simply too much negative attention drawn to PEMF because most investigators do not understand that both PEMF and ICES should only work within a very specific range of stimulation parameters^{7,18,19}, namely those that induce signals that can be interpreted by existing signaling mechanisms within cells and tissues. Our research in muscle tissue engineering has established similar results, showing that only in a particular range of

stimulation parameters can one successfully provide signals to which tissues will respond in a favourable way. Herein, we seek to provide objective evidence that ICES, when applied appropriately, can provide significant and repeatable anti-inflammatory effects in an acute inflammation animal model.

Background

Electromagnetic therapies have been in use for many years. Electrical stimulation of tissues has been studied since Galvani's experiments using electricity to stimulate contraction of the muscles in dissected frog legs⁶⁵. The systematic study of the effects of electrical and magnetic fields on living and dead tissues began with Galvani in the late 18th century, whose research led to the discovery that one of the primary methods of information transfer within nerve and muscle tissues is via electrical pathways. In the middle of the 20th century, it was discovered that bone is piezoelectric in nature, and therefore was hypothesized to also transduce information electrically^{23,24}. Soon thereafter, many experiments demonstrated that directly-applied electrical currents can be employed to induce bone formation and remodeling^{12-14,25}. One problem with these early methods of direct electrical stimulation of bone tissue was that they required the implantation of electrodes into and around the bones to be stimulated. The deeply invasive nature of direct electrical stimulation of bone lead to the development of non-invasive methods, such as the use of induced electrical fields. These inductive methods employ magnetic fields from external magnets or solenoids that change over time to induce the desired electrical fields within the tissues, based on the well-understood Faraday's Law of Induction⁶⁶. Electrical fields induced in this non-invasive manner were subsequently shown to be effective in eliciting accelerated bone formation and healing¹². With the advent of inductive stimulation methods came the study of the effects of

non-depolarizing electromagnetic fields on tissues other than bone. Non-depolarizing electric fields are those which are too low to induce overt depolarization of the cell membrane as in the case of an action potential, but strong enough to presumably have other effects on molecular mechanisms within cells and in the extracellular space. Such mechanisms have been widely hypothesized to mediate the signals involved in functional adaptation of musculoskeletal tissues, and are the subject of ongoing mainstream scientific research. These signals are generally thought to be very small in magnitude compared to action potentials in excitable tissues, and many competing mechanisms have been hypothesized.

Nerve regeneration is also thought to be subject to similar signaling mechanisms that induce accelerated healing and repair as it was shown that non-depolarizing electromagnetic pulses could improve nerve lesion healing. Further studies showed that inflammatory factors could be reduced in tissue inflammation in humans post operatively^{10,11}. Pilla and colleagues developed a theory of interaction between pulsed radio frequency (PRF) waves and tissues which makes use of the frequency response of tissues and places lower bounds on waveform parameters based on the thermal noise threshold^{2,4,5,7,17–19,67,68}. More recently, ICES has been studied in terms of behavioural modulations—specifically the effects of ICES on bipolar-disorder, autism spectral disorder (ASD), Alzheimer’s, and Parkinson’s disease^{29–37,46}. Prior to discussing the effects of ICES on cells, tissues and systems, it is necessary to discuss the important parameters which govern how tissues will respond to electromagnetic radiation.

Types of biologically relevant signals

There are three key levels of signals that need to be specified in order to properly define the waveform parameters that are to be used when inductively stimulating:

1. Current flowing into the coils from the stimulation unit. This is the original driving signal that is produced by the electronic circuit within the ICES device to drive the coil that will then produce the magnetic field.
2. The time-varying magnetic flux in and around the coils resulting from the electrical current driving the wire coils.
3. The induced electric field in the tissue volume resulting from the time-varying magnetic flux generated by the coils.

Based on our detailed review of the literature, we have determined that in most cases investigators report only a partial description of the original driving signal emanating from the electronic circuit (#1 above), but do not measure, calculate, report, or estimate the resulting magnetic field vs. time (#2 above) or the electrical fields that are ultimately induced within the target tissues (#3 above). For the most part, the second level signal—magnetic flux—is the most relevant signal to specify because it is prone to deviate from theoretical values when calculated based upon the presumed driver circuit performance, it is readily measured using modern analog signal Hall effect sensors, and when measured accurately yields good estimates of the induced field within the tissues. It should be noted that it is the final signal—the electric field induced within the tissues—which is the hypothesized mediator of the responses seen in vivo and in vitro, but that it is difficult to directly measure these induced fields within tissue.

Current flowing into the coils (primary or first-level signal)

In time-varying magnetic field stimulators it is the primary signal from the electronic device that drives the coil(s) to produce the magnetic field. For the purposes of this

discussion we will not consider “static” magnetic devices such as permanent magnets or solenoids driven by steady DC current. In static cases the magnetic fields are largely steady and non-varying over time, so their ability to induce electrical fields is essentially zero because the first time derivative of the magnetic flux in steady magnetic fields is by definition equal to zero. That is not to say that such devices would have no biological effects, because they certainly may have effects through such mechanisms as the Hall Effect, in which charged particles (ions) ubiquitous in biological systems would be influenced as they move through the steady magnetic field. The induction of electrical fields within tissues requires magnetic fields that vary in time, and typically this is accomplished using a computer or a microcontroller-based platform to drive current waveforms through solenoid coils. To induce the desired electrical fields it is essential to control the slew-rate (rate of change or first time derivative of the magnetic flux) of the signal. Thus, it is of utmost importance that the primary driving electronics have adequate dynamic performance. However, since most investigators do not measure or report the second- or third-level signals (above) they generally cannot guarantee that the primary driver electronics have adequate dynamic performance to achieve the desired biological effect. The primary signal also allows one to determine the upper limit of the overall stimulus signal power. Basically the maximum power into the system can be calculated by knowing the maximum current flowing into the coils and the impedance of the coils (though empirically, the power transfer to the body is much lower because of inefficiency). Because the undesirable effects of non-ionizing radio frequency (RF) energy generally are regarded to arise from thermal effects within the tissue, it is conservative and correct to consider the total ICES system power when determining the upper limit of potential harmfulness of any ICES or RF stimulation system, and the power

consumption of the primary driving electronics provide a direct and convenient opportunity to measure and determine the upper boundary for power for the entire system.

Magnetic flux produced by coils (secondary or second-level signal)

Because there are electrical (Ohmic and reactive) energy losses in driving the primary signal through the coils, it is most accurate to directly measure the dynamic magnetic flux produced by the current flowing into the coils. From these measures one can disregard the need to correct for dynamic limitations of the primary driver circuit, and the induced electric field within the tissues can be more accurately estimated. Faraday's law of induction shows that the induced circular electric field in a conducting surface is proportional to the inverse of the rate of change of the magnetic flux (defined as the magnetic field strength times the area through which it is passing). The key parameters involved with the induced electric field are the rate of change of the magnetic field (i.e. $d\mathbf{B}/dt$, which is the first time derivative of the magnetic flux $\mathbf{B}$) and the radius around which one examines the field of interest. Specifically, the larger the rate of change of the magnetic field, the larger the possible induced electric field. Maxwell's relationship explains why the driving electronics must have good dynamic performance: to provide adequate magnetic flux slew rate to induce the desired electric field in the tissue. For a given magnetic flux change, the larger the radius of interest (up to the inner radius of the stimulating coil), the larger the induced field, and the smaller the radius, the smaller the induced field. The induced electric field for a Helmholtz coil decays linearly to zero within the boundaries of the coils and falls off as the inverse of the distance from the outer edge of the coil outside of the boundaries of the coils (Figure 1.1). The internal surface of the graph in Figure 1.1 is a cone, representing the induced electrical field strength between the coils where the induced electric field decreases toward zero linearly as the radius of

curvature of the induced field drops to zero in the x-y plane. The inner conical surface is perhaps most relevant because it is the volume of tissue between or within the coils that generally is intended to undergo treatment with ICES.

Figure 1.1: Induced electric field caused by Helmholtz coils. (Left) Representative plot of induced (tertiary or third level) electric field strengths within a conducting surface as caused by a Helmholtz configured set of ICES coils. Any path within the circumference of the coils with radius less than the coils will have an induced electric field dictated only by its radius, not its axial position within the coils. Outside the circumference of the coils, the radius of interest must be concentric with the axis of stimulation in order for the plot above to apply. Note that the peak magnetic field is induced around a pathway of radius equal to the stimulating coils. (Right) Representative 2-dimensional slice of the surface on the left showing a cross section of the conical interior and $1/r$ behaviour of the induced (tertiary or third level) electric field in a conducting surface. The diameter of the representative coils is 50 mm and the plot is constructed for a magnetic flux slew rate of 80 T/s.

Induced electric field within tissues (Tertiary or third-level signal)

Finally, it is necessary to discuss the induced electric field—specifically with regard to the tissue volumes of interest. The induced field can be calculated simply using equation 1 below:

$$E(t) = -\frac{dB}{dt} \frac{r}{2} \quad (1)$$

For example, if one considers a stimulation volume on the order of 10 μm (average cell diameter), then with a magnetic flux slew rate of 1,400,000 Gauss/second (=140 Tesla/second), a magnetic pulse will induce a peak electric field of approximately 3.5×10^{-4} V/m around the perimeter of a typical cell. If one considers thermal noise averaging, and cellular response, then the predicted threshold induced field for a measureable response is on the order of $10^{-3} - 10^{-5}$ V/m⁶⁹. Thus, a 140 T/s stimulus should cause a measurable physiological response. Furthermore, assuming the low-end of the stimulation threshold to be approximately 10^{-5} V/m, the smallest signal that one might expect to use and still observe a physiological response is approximately 4 T/s. However, if one considers a conduction pathway on the order of the radius 35 mm (ex: the outer edge of a 6-well plate well), then the peak electric field produced by the same, 140 T/s magnetic pulse is on the order of 1.23 V/m—well above the detection threshold for tissues. We would like to point out that in fact, the model being used to explain the induction of electric fields within a tissue volume is identical to the model of eddy currents⁶⁶. In the case of eddy currents within a tissue, one can consider the conducting pathways to be represented by the fluid in the pericellular space, just outside the cell membrane and between cells and thus, circular pathways around cells are those of interest. Since there are many cells in a tissue mass, there are various conducting

pathways, some circular, but most are not. Considering that the field strength in a plane varies with respect to the radius of interest, one can determine that if cells meet in locations where the cross sectional radii are not identical, then the currents where the cells meet will not cancel, and there will be a net flow of current around the larger radius of interest. However, if two cells meet at a location such that their cross-sectional areas are approximately the same (and they are both relatively circular cross sections) then circular currents flow around each cell, and should approximately cancel where the cells meet—producing a conducting path around both cells (Figure 1.2). Because of these geometric effects, it is possible that amplification effects might be seen for signals that fall below theoretically calculated stimulation thresholds. Such circumstances may dominate the geometry in tissues with relatively high cellular density such as muscle and skin in which the cells occupy well over 50% of the volume in any representative sample of tissue. In the case where cells are separated by relatively larger distances, the induced electric fields in the pericellular fluid spaces surrounding each individual cell may not interact as shown, each cell being subjected to an induced electric field. If all cells in the target tissue have approximately the same geometry, then each individual cell in the target tissue would be stimulated very nearly uniformly throughout the tissue within the coils. This geometry could dominate in tissues with relatively lower cellular density with widely distributed (not clumped) cellular arrangement, such as bone, tendon, all types of cartilage, ligament and crucially, the interfaces where these tissues meet²⁸.

Figure 1.2: A cartoon of the current flow induced around cells. Top.) Cells of equivalent radius have offsetting electric fields between them, resulting in a net current flow around the perimeter of both cells, but not between them. Bottom.) Cells of different radii of intersection will have a net current flow around their perimeter and in the direction between them determined by the larger cell.

The above arguments generally hold true for the simplest of cell geometries: 10 micron diameter spherical cells. Though this assumption of simple geometry may be adequate for many estimations of tissue properties, such as estimating the number of cells in a given volume of tissue, the spatial details of cell membrane geometry and receptor distribution may well dominate when considering the mechanisms that relate to electrochemical transduction and mechano-transduction in cells and tissues. The assumption of a simple and smooth cellular geometry thus runs the risk of falling into the scientific error

known as the "assumption of a spherical cow", which takes the form of a joke based upon commonly encountered scientific over-simplifications that make calculations easier at the cost of ignoring the most important details of the system being studied. Many cells in the musculoskeletal system are known to have a complex surface structure containing thin filaments that stretch out into the space between cells. For example, osteocytes (cells in bone tissue) are known to have cytoplasmic processes which extend into canaliculi (tiny canals) in the hard bone matrix^{27,28}. These thin extensions of the bone cell membrane are known to be involved in the collection of nutrients and elimination of waste, but it is hypothesized that osteocytes may detect mechanical loads through the detection of signaling that arises from the mechanically-induced flow of fluids and ions through the lacuno-canicular network surrounding each osteocyte²⁷. It is one of our working hypotheses that ICES systems work at this pericellular level to emulate the mechanical signals in musculoskeletal tissue systems that would normally induce a functional adaptive response, such as bone growth and remodeling to increase bone density as a result of exercise. We further hypothesize that the emulation of these signals by ICES stimulators has the added benefit of employing the natural signal amplification systems within the musculoskeletal system without actually applying the mechanical loads to the tissues being stimulated, thus allowing musculoskeletal tissues to adaptively respond to the emulated signals without also being subjected to the structural micro damage that would otherwise occur from the mechanical loads.

ICES as a biological signal

Biologically relevant signals often have the property of being very low-level; either very low amplitude, low energy, infrequent, or otherwise subtle. As a result, these signals are often difficult to detect experimentally. Through millions of years of evolution, the molecular

and cellular responsiveness to low-level signals has evolved in many cases to become highly specific and responsive only to a very precisely defined signal, so as to prevent amplification of spurious background noise that might elicit inappropriate cellular or molecular response. Within the receptive bandwidth of such low-level biological signals the signal itself must have a high signal-to-noise ratio (SNR), with minimal energy being expended upon parameters of the signal that do not contribute to the intended message. This evolved SNR allows all other signals that fall outside of the receptive bandwidth to be essentially ignored. The evolutionary process tends to make good use of such highly selective and efficient processes once they have passed the test of natural selection, so it is reasonable to hypothesize that a signal that might elicit a functional adaptive response in one tissue, for example bone, might also be employed by other tissues for similar purposes. This would be especially true for tissues within the same functional groups such as musculoskeletal tissues, cardiovascular tissues, nerve tissues, etc. On the basis of this reasoning we hypothesize that specific signals that induce tissue growth and regeneration in one tissue in musculoskeletal system might elicit the same general response in many or all other tissues of the musculoskeletal system. So a specific signal that is known to elicit acceleration of bone repair might also elicit accelerated repair in cartilage, ligament, tendon, and muscle as well. Our review of the literature reveals that this general assumption may be implicit, but is generally not explicitly articulated in the description of any of the ICES technologies that have been reported. In most cases we believe the ICES signals that are employed, often referred to as ICES "waveforms", have been arbitrarily selected and often not developed and refined based upon the aforementioned line of reasoning. Therefore many ICES technologies do not take advantage of the inherent natural mechanisms of biological signal amplification,

preferring instead to use a brute-force approach to coerce the target tissue toward the desired response rather than employing high fidelity signals that work with innate biological filters and amplifiers. The literature suggests that this latter brute-force approach, though crude, is in fact effective to a limited degree. However, such a crude approach has no basis from which to develop increasingly sophisticated, efficient, and effective ICES signals. Thus, most commercially-available ICES technologies simply are not improved over time. Once treatments can be demonstrated to be statistically significant in their intended biological effects, the evolution of the ICES waveform protocols toward increasingly better signals generally does not occur. This process of accepting the first guess that “works” is exacerbated by modern regulatory practices, such as those enforced by the FDA relating to medical devices. Once a medical device has been proven “safe and effective”, it is exceedingly difficult and costly to make any changes to the stimulation parameters. The cost of making even a slight change to PEMF or ICES parameters on an approved medical device can rise as high as \$180M and can take from 3 to 5 years to complete because the entire pre-market approval (PMA) process must be repeated for each change to any waveform parameters, according to current FDA policy related to PEMF devices, which have been in force since May 28, 1976. [[I think I have an actual court case that you can include as a reference for this, I will search for it.]]. Imagine the chilling effect of similar regulatory restrictions on any other technology. Imagine where computer technology would be if innovation were frozen in place by a federal regulatory agency such that computers proven to be “effective” in 1976 could essentially not be incrementally improved upon without. In this regard, in addition to protecting the public against ineffective and or dangerous medical device technologies, FDA device regulations have also had the unintended consequence of

putting the “NO” firmly back into inNOvation. This helps to explain why health and medicine have generally not enjoyed the same pace of technological progress that other non-medical technologies have enjoyed for the past 4 decades, including computing, communication, entertainment, transportation, safety equipment, and basically every other aspect of our lives.

The unintended consequence of this over-regulated, inefficient, and crude approach to the development of ICES waveforms has been that most ICES systems remain very inefficient, bulky, costly, and they subject the target tissue to unnecessary levels of electromagnetic energy. More rational approaches to ICES waveform design are certainly possible.

ICES waveform shapes

Many different methods exist for inducing an electric field within tissues and all of these have been employed at various times by different ICES systems. Waveforms can be divided into four distinct waveform categories: pure sinusoidal, triangular/sawtooth/trapezoidal/square, asymmetric pulses, and pulsed radio frequency (PRF)/Modulated signals. Stead (DC) magnetic fields will not be considered, though they are frequently employed, for the reasons stated previously. One must also keep in mind that there are three levels of signals, as discussed above. For the following discussion the signal waveforms refer to signals in level #2: the magnetic field generated by the coils.

Sinusoidal waves

Sinusoidal stimulation is by far the most common form of ICES stimulation, based upon a pure sinusoidal magnetic waveform. In the literature it is well established that tissues

typically respond to radio frequencies (RF) from 0 Hz to 10 kHz—outside of this range, tissues and cells are essentially transparent (with the exception of PRF signals). The smallest wavelength of such signals in an electrolyte environment is on the order of 3000 meters—thus cells are unlikely to be acting as antennae at such frequencies. Furthermore, a frequency of approximately 30 THz would be required to induce resonance in a cell of size on the order of 10 μm in a saline solution. Interestingly, because tissues have been found to be responsive in such a low frequency range, one must consider the mechanisms by which cells or molecules might transduce these signals. Much of the biological response is dependent upon the bulk electrical properties of tissues (direct and reactive impedances), which dictate how electrical energy is absorbed through a medium. In the case of a magnetic field, because the vast majority of mammalian tissues are not known to interact with magnetic fields, one must consider magnetically induced electric field pathways as the primary method in which magnetic fields can interact with tissues. Because the induced electric field is proportional to the rate of change of the magnetic field, the amplitude and frequency of the magnetic field dictates the strength of the cellular response. Thus, higher frequency and amplitude signals should be more effective in eliciting a response. It should be noted that there is significant theoretical evidence that suggests that there is a lower bound for frequencies as well due to the thermal noise threshold^{19,69,70}. Interestingly, there have been a number of studies that find effects well below the theoretical frequency and amplitude limits predicted mathematically, suggesting either a placebo effect or an alternative transduction mechanism^{29–40,69,71}.

Triangular/Trapezoidal/Square waves

Triangular, trapezoidal and square waves fall into a similar category because they represent Fourier sums. While the multi-frequency aspect of such signals may be a reason

that they are effective, it may equally be the case that their efficacy is due to the high slew-rates that can be produced. For practical purposes, pure square waves are impossible to create electronically: there is always a finite rise-time and fall-time for the primary electrical signals, they cannot change instantaneously. Thus, this category of three waveforms can be collapsed into triangle and trapezoidal, which includes square waves which are actually trapezoids because their rising and falling slopes are not perfectly vertical. Both triangular and trapezoidal waveforms provide bipolar induced fields, which depend upon the slope of the sides of each trapezoidal waveform—the main difference being that there is a delay between positive and negative peaks in a trapezoidal pulse given by the length of the signal plateau.

Asymmetric pulses

Asymmetric pulses are typically triangular or trapezoidal in nature, but have a differing rising and falling edge. Such waveforms can be useful for inducing non-equal bipolar induced electric fields. Examples of asymmetric pulses include saw-tooth waves such as those shown in Figure 1.3.

PRF/Modulated signals

Pulsed radio frequency (PRF) signals provide a high-frequency method for encoding low-frequency signals, similar to the way in which an FM radio works. Because tissues will integrate low-frequency signals (i.e. they act as a high-pass filter), they can demodulate pulsed PRF signals. The advantage of such a stimulation paradigm is that tissue penetration can be increased. Since radio frequency signals can penetrate tissues easily, PRFs can provide an effective means of stimulating deep tissues without using very strong external

fields. The efficacy of PRF stimulation has been explained by Pilla et al. on the grounds of a proposed biochemical model^{2,3,5,7,10,18,19,68}. Under appropriate stimulation parameters, PRFs can modulate first order kinetics of ion binding to enzymes. Pilla's work is focused on modulating calcium binding to calmodulin in vivo—providing a method by which downstream targets such as endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) can be affected⁷.

Figure 1.3: Representative ICES waveforms. A.) Sinusoidal waveforms have smoothly varying edges, and can also be pulsed at high frequencies to produce PRF signals. B.) Trapezoidal and square waveforms represent waveforms with large rising and falling edge slopes and non-changing peaks and troughs. C.) Asymmetric pulses, such as the saw-tooth waveform shown, represent waveforms that have large rising and/or falling edge slopes, but provide non-symmetric induced electric fields within tissues of interest. A description of the numbered portions above can be found in Table 1-1.

Table 1-1: Summary of important descriptive ICES waveform parameters. All numbered items are labeled in Figure 1.3 on their respective waveform type letter. Unlabeled components are those which cannot easily be drawn on a figure, however are absolutely necessary. Trapezoidal waveforms are assumed to be constructed of straight lights—if lines are curved, a function may be required to define the edge slopes. It should be noted that this table is not comprehensive, as more complicated waveforms may require additional information to fully define one full cycle of stimulation.

Summary of Important Secondary Wave Structures Necessary for Fully Defining ICES Waveforms			
Sinusoidal (A)	PRF (A)	Trapezoidal (B)	Asymmetric Pulse (C)
Period (1) Amplitude (2) Peak Slope	Bulk Pulse characteristics Carrier Period (3) Amplitude (2) Encoded frequency Peak Slope Pulse rate (pps)	Amplitude (2) Positive Rising Edge Slope Positive Rising Edge time (4) Time at Max (5) Positive Falling Edge Slope Positive Falling Edge time (6) Time at Zero (7) Negative Falling Edge Slope Negative Falling Edge time (8) Time at Minimum (9) Negative Rising Edge Slope Negative Rising Edge time (10) Peak Slope Pulse rate (pps)	Period (1) Amplitude (2) Rising Edge Slope Rising Edge time (4) Falling Edge Slope Falling edge time (6) Peak Slope Pulse rate (pps)

Waveform parameters

All waveform categories and shapes are defined by a set of waveform parameters. These include amplitude, frequency, slew rate, and other parameters. Some waveforms are well described by only two parameters, such as continuous pure sine waves which can be defined by the two parameters *amplitude* and *frequency*. Other waveforms are more complex and may require six or more parameters for a complete description. An example of this is asymmetric trapezoidal waves that are generated in short bursts of pulses followed by periods of no stimulation. In this case the waveform would be fully defined by at least twelve parameters: start time, initial slope, peak amplitude, duration (time) held at peak amplitude, final slope, terminal amplitude (can be zero or have opposite sign for bipolar pulses), duration of zero or opposite-sign plateau, return slope (if non-zero), time between pulses, number of pulses in each burst, dwell time between bursts, and at least one additional parameter to define the periodicity of the bursts of asymmetric trapezoidal pulses.

Amplitudes

The mechanism for the biological effects of ICES as they relate to magnetic flux peak amplitude, and thus the relative importance of this parameter, remains slightly ambiguous at this point because there is a large range of experimentally effective amplitudes that fall well below thermal noise limits. However, generally speaking, larger amplitudes are more effective in direct tissue stimulation until high amplitudes that begin to cause collateral tissue damage are reached. This damage is most likely because more energy is dissipated into the tissues in each unit of time. Energy per unit time yields the physical units of power, and electromagnetic power is associated with tissue damage when the power level begins to reach

a level with significant thermal effects within the tissue. This effect is put to positive use in modern surgery when radio ablation is utilized to destroy tumors or other unwanted tissues. Assuming that the RF power is below a damaging level, we have noted in a wide variety of literature that induced electric fields on the order of 0.01 – 10 V/m appear to be most effective in treating chronic pain and inflammation. Generating such field strengths can be done using several magnetic waveforms. It should also be noted that much lower amplitude magnetic fields, on the order of picotesla, (10^{-12} T) have been reported to be clinically effective for treatment of multiple sclerosis and Parkinson's patients²⁹⁻⁴⁰. So, one can conclude that waveform amplitude certainly plays a role in both the efficacy and the potential risk involved in the use of ICES stimulators, but the precise role and the underlying biological and electromagnetic mechanisms remain to be elucidated.

Frequency

The waveform frequency parameter is also considered of vital importance when considering periodic signals. The precise role of frequency is somewhat obfuscated by the imprecise use of this very well-defined engineering term. As noted previously, tissues typically only respond to frequencies below 10 kHz, with the exception being that FM signals can be demodulated by tissues, provided the low frequency encoding falls below 10 kHz. Because the frequency of a sinusoidal magnetic flux signal dictates the time derivative dependence, and therefore the induced electric field magnitude, it follows that higher frequency signals are capable of inducing electric fields with greater peak amplitudes in the target tissue. However, as we shall see later, there are theoretical limits that help narrow down the range of frequencies that would be theoretically effective. For example, a 1 Hz wave would require a peak amplitude of approximately 100 tesla in order to induce an

electric field on the order of 1.75 V/m around the perimeter of a 35 mm disk. This peak field strength is approximately 100 times higher than the average field produced in a clinical MRI unit, which is a very large amplitude indeed. One tesla = 10,000 gauss, so a 100 T field = 1 MG, which is about 1.5 million times the average magnetic field strength of the Earth. At higher frequencies the calculus, a simple derivative of the sinusoidal waveform, indicates that significantly lower magnetic flux amplitudes could theoretically become biologically effective. For example, by increasing the frequency from 1 Hz to 1 kHz, the required peak magnetic field becomes approximately 0.1 T, which is more reasonable and technically is much easier and less expensive to achieve—but it remains very large.

Slew rate

As an alternative strategy to employing magnetic fields of very high amplitude it is both possible and sometimes advantageous to use high rates of change (steep slopes) coupled to otherwise low frequency pulses. It is in this use of the term *frequency* that confusion sometimes arises. For pure sine waves the meaning of the term *frequency* is defined as "the first time derivative of phase angle", whereas the meaning of the term *frequency* in reference to non-sinusoidal pulses is "how frequently the individual pulses are generated". Improper or imprecise use of the term frequency can lead to considerable confusion when defining the precise parameters for non-sinusoidal magnetic pulse waveforms. Trapezoidal and triangular magnetic pulses can be generated individually with long periods of inactivity between pulses, but it is possible by this approach to generate very large induced electric fields by driving the trapezoidal waveforms with very steep rising and falling edges, that is, incorporating large slew rates to each edge of each trapezoidal or triangular pulse. Such signals are easily capable of producing 1.5 V/m induced signals while keeping peak magnetic field strength

well below 0.1 T provided the pulse can be delivered in a short enough time (approximately 100 μ s). Frequency modulated signals provide an alternative method for producing high slew-rate signals by encoding low frequency signals in high frequency (1-27.12 MHz) sinusoidal carrier waves.

The thermal noise threshold

An interesting and important discussion must be had regarding the thermodynamic effects of electric fields. Specifically, as one decreases the magnitude of the induced electric field, there comes a point where thermal fluctuations due to random motion within the sample can easily produce field strengths large enough to mask the applied signal. This masking is referred to as the thermal noise threshold and is on the order of 9×10^{-2} V/m when signal averaging is not taken into account. Thus one would expect that any induced field below the thermal noise threshold should not induce a physiologic response. However, cells are able to integrate applied signals, which allows the theoretical noise threshold to fall even further to levels as low as $10^{-3} - 10^{-5}$ V/m⁶⁹. Observations in literature support the claim that ICES efficacy can be observed orders of magnitude below the thermal noise threshold—a feat that is attributed to the ability of tissues to integrate and amplify very small signals.

Review of past literature

Bone studies

The majority of the evolution of ICES therapy in the 20th and 21st century has been driven by the development of bone-growth stimulators. When Fukada and Yasuda discovered that bone is piezoelectric and subsequent studies implicated that bone remodeling could be driven by this property, it was only a matter of time before people began exploring the

possibility that applied electromagnetic fields could drive other biological processes. Thus, much of the pioneering work done by Bassett, et al. laid the foundation for subsequent work in other tissues.

Cell studies

Effects of ICES on cells have been studied extensively in those cells of bone or cartilage-derived lineage. In vitro studies on both primary and immortalized cells have been conducted, and there is evidence to suggest that each responds differently to ICES⁷². Cell studies done on osteoblast-like cells have mainly focused on the nitric oxide synthase (NOS) pathway of cells such as MC3T3-E1 cells^{8,9}. Proliferation in several different cell types has been extensively studied and found to be increased in the presence of low-magnitude ICES on the order of 0.002 V/m^{20,45,73–76}. In addition to modulating proliferation, ICES has been implicated in the up regulation of DNA synthesis, IGF-2 (osteosarcoma)^{77,78}, FGF-2 (endothelial cells)⁷⁴ and BMP-2 mediated osteoblastic differentiation in human mesenchymal stem cells (HMSCs)⁷⁹. In addition to the studies on bone, there have been questions as to the efficacy of ICES in nerve regeneration. In particular, a study conducted at NASA by Goodwin, McCarthy and Dennis showed that human neuronal cells could be modulated by time-varying electromagnetic fields (TVEMF). They found differences in cell morphology as well as proliferation rates in cells that were cultured in the presence of TVEMFs^{71,80}. While cell culture studies are important to understanding biochemical and cell-level responses to ICES, they cannot provide the tissue and organism level responses that can be gleaned from in vivo, animal and human studies.

Soft tissue studies

To understand the effects of ICES therapy on a system level, we feel it is easiest to break the existing literature into the broader categories of nerve healing, and anti-inflammatory studies. Because ICES is so well established as an effective treatment in bone-healing, we choose not to review that literature—however the reader should be aware that there is a vast literature concerning bone remodeling (A good reference with which to start is the 1974 Bassett reference).

Nerve healing

To understand the effects of ICES on nerve regeneration, we have broken the in vivo studies into three broad categories: peripheral, spinal cord and cortical studies. We have chosen to separate the cord from central and peripheral studies because it is the junction point for both central and peripheral nerves, and thus has the potential to affect both simultaneously.

The focus of the majority of peripheral nerve studies has been to examine the ability of ICES to temper pain and stimulate regrowth. As previously mentioned, the studies performed at NASA by Goodwin et. al. indicated that neuronal proliferation could be significantly affected by low frequency pulses much lower in magnitude than the earth's magnetic field. Studies performed by Raji et al. have shown that rat peroneal nerve regeneration can be enhanced by the use of ICES^{43,44}.

The majority of the published, controlled laboratory studies on peripheral nerves examine the effects of ICES on sciatic nerve lesions. Significant evidence from animal studies suggests that ICES is potentially effective in accelerating sciatic nerve healing.

Square wave pulses (~600 T/s magnetic flux rate), as studied by Siskin et al., seem effective in increasing sciatic nerve regeneration regardless of the orientation of the Helmholtz stimulation coils⁴⁷. However, Baptista et. al. showed that there was no significant effect from treating sciatic crush lesions in Swiss mice using a stimulation protocol that induced a 20 kT/s magnetic flux rate—a relatively large stimulus⁸¹.

Finally, it is important to discuss the potential cortical effects of ICES. Cortical effects should be considered from two different views: direct stimulation (ex: rTMS, low magnitude ICES, etc.) which stimulate the brain directly, and indirect stimulation that causes cortical remapping or modulation through plasticity by stimulating peripherally. Direct stimulation methods such as those used in the studies published by Sandyk et al. have indicated that very small induced fields may be effective in alleviating some of the difficulties associated with multiple sclerosis and Parkinson's disease²⁹⁻⁴⁰. However, it should be noted that the field strengths in question fall far below the thermal noise threshold and that the majority of these studies are case studies, not controlled laboratory studies. Unfortunately the literature regarding the central effects of peripherally applied ICES on central nerve function is rather sparse. Because peripheral neurons play a very large role via the feedback mechanism in cortical plasticity, it follows that if ICES affects these neural feedback loops, then fMRI and PET studies would reveal potentially significant effects of peripherally applied ICES on cortical plasticity.

Inflammatory pathways

In order to properly understand the potential mechanisms by which ICES may interact with the body, it is vital to explore the processes of inflammation. Inflammation typically

follows a well-defined series of steps that are mediated by specific molecules and cells in the body. Acute inflammation can be distinguished from chronic inflammation by molecular and cellular differences (however, the transition from acute to chronic is still poorly defined).

The inflammatory process is initiated by a noxious stimulus—such as a burn, cut, blunt trauma, chemical exposure, auto-immune response, or infection. In all situations, the cascade begins when a pattern recognition receptor (PRR) on the surface of an immune cell detects the presence of a pathogen-associated molecular pattern (PAMP) or a damage-associated molecular pattern (DAMP). Molecular patterns are molecules that are released in response to a noxious stimulus. Upon detection of a pattern, the PRRs trigger the release of various inflammatory factors which lead to increased blood flow, vascular permeability, and hyperalgesia—the inflammatory factors released depend upon the type of stimulus as well as the duration of the stimulus (i.e. acute vs. chronic). These inflammatory factors cause the classic signs of inflammation: Rubor (redness), calor (heat), tumor (edema/swelling), dolor (pain), and function laesa (loss of function).

Pattern recognition receptors are a family of receptors that are specifically tuned to respond to pathogens and damage-associated molecular patterns (PAMPs and DAMPs). A recent review highlights that there are four accepted PRR families: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIG)-1-like receptors (RLRs), and NOD-like receptors (NLRs)⁸². These four families can be classified as belonging to either the transmembrane protein family or cytoplasmic protein family. Specifically, TLRs and CLRs are both families of transmembrane proteins, and RLRs and NLRs are cytoplasmic proteins. Most TLRs respond to bacteria, viruses, and protozoa; CLRs

typically respond to fungal infections; NLRs respond to bacteria, and RLRs respond to RNA and DNA viruses⁸².

Pathogen and damage associated molecular patterns are the specific ligands that trigger a response in PRRs of the immune system. Lipopolysaccharides such as those found on bacteria, are examples of PAMPs that can trigger an immune response. Damage associated molecular patterns are groups of molecules that are released in response to physical damage to cells and tissues. It should be noted that PAMPs and DAMPs have become a more recent focus of study, and at this point, are relatively poorly understood.

Figure 1.4: Chemical structure of λ-carrageenan. A representative chemical structure drawing of a single molecule in a polymer chain of the algal-derived sulfonated polysaccharide λ-carrageenan. A bolus of a 1% λ-carrageenan solution is given via injection to induce inflammation in the carrageenan-induced rodent footpad edema model of acute inflammation.

A very well-established model for the study of the anti-inflammatory efficacy of new drugs is the carrageenan-induced footpad edema (CFE) model^{83,84}. This model makes use of an injection of λ -carrageenan (an algal-derived sulfonated poly-saccharide, Figure 1.4) into the footpad of a rodent (typically rat or mouse). The CFE model is an acute model of inflammation, and therefore typically resolves spontaneously within the course of 8-12 hours while displaying mostly acute inflammatory-associated cytokines and proteins. It has been shown that the CFE model displays two inflammatory responses: a non-phagocytic inflammatory response (NPIR) and a phagocytic inflammatory response (PIR). The NPIR occurs first, and is initiated by the trauma associated with a subcutaneous injection, and is dose-independent for carrageenan. The second, more prolonged, PIR occurs approximately 60 minutes post-injection and involves recruitment of neutrophils and macrophages⁸⁴. Furthermore, it has been shown that pleural injection of carrageenan does not result in an NPIR, but rather a PIR only—thus implicating the damage associated with an injection into solid tissue as the cause for the NPIR⁸³. To control for NPIR it is common to inject the contralateral (non-treatment) hind footpad with an equivalent volume of sterile PBS—this triggers NPIR but does not trigger a PIR, allowing one to subtract the swelling caused by NPIR from the CFE induced footpad.

An important aspect of the CFE model is that it is strongly suppressed by the introduction of steroid anti-inflammatory agents such as dexamethasone. Dexamethasone inhibits production of arachadonic acid—a phospholipid derivative which is necessary for the production of leukotrienes, prostaglandins and thromboxanes. Figure 1.5 shows a representation of the arachadonic acid inflammatory pathway. A bifurcation in the use of arachadonic acid occurs which has downstream implications for inflammation. The

lipoxygenase pathway causes downstream production of leukotrienes which are heavily involved in neutrophil chemotaxis. The cyclooxygenase pathway produces prostaglandins and thromboxane as downstream products. Prostaglandins have many systemic effects, but are specifically associated with smooth muscle contraction modulation, platelet aggregation modifications, hyperalgesia and pyrogenesis. Prostaglandins have also been implicated in the modulation of neuronal sensitivity to pain. The end effects of prostaglandins are on average to increase blood flow (via vasodilation), reduce platelet aggregation, increase pain, and increase temperature. Thromboxane on the other hand has a tendency to increase platelet aggregation (and thus cause clotting), while also causing vasoconstriction which reduces blood flow.

Figure 1.5: Diagram of the arachadonic acid inflammatory pathways. A bifurcation in the pathway explains the differences between dexamethasone anti-inflammatory treatments and commonly available over the counter non-steroidal anti-inflammatories (NSAIDs). Dexamethasone-like drugs inhibit arachadonic acid formation, while NSAIDs inhibit the cyclooxygenase pathways.

A final note should be added regarding two other important inflammatory pathways that are involved. First is the platelet activating factor pathway—a pathway which leads to a change in vascular permeability as well as contributing to leukocyte trapping. A second pathway is the nitric oxide pathway—which is responsible for changes in vasodilation. Specifically nitric oxide causes smooth muscle relaxation which leads to an increase in vasodilation. Because nitric oxide synthase pathways are activated in inflammatory

responses, swelling is (typically) increased as a result of edema caused by increased blood flow. The nitric oxide synthase pathway is a specific pathway of interest in the study of ICES as it has been implicated by several researchers as the primary mechanism by which ICES operates physiologically^{6,7,10,18,19,67}.

Anti-inflammatory effects of ICES

There are two notable studies that shed significant mechanistic light on the anti-inflammatory and pain reducing effects of ICES: those of Per Hedén et al. and Christine Rohde et al^{10,11}. Both studies examined the post-operative effects of ICES on breast augmentation and breast reduction patients respectively. In the former, a pilot study of patients undergoing breast augmentation, ICES (2-ms bursts of 27.12 MHz PRF, 3.2 V/m peak applied for 30 minutes every 4, 8 or 12 hours on different post-operative days) was shown to significantly reduce pain scores¹¹. The second study, performed by Rohde et al. using similar ICES parameters showed significant pain reduction, and interestingly a drastic reduction in IL1- β levels in wound exudate as compared to sham groups¹⁰. Reduction in inflammatory factors suggests at least one possible biochemical mechanism—perhaps the Ca^{2+} /CaM dependent NOS pathway suggested by Pilla et al⁷. It is interesting to note that although these reports and others have demonstrated very significant and repeatable reduction in post-operative pain when ICES is correctly applied without evidence of adverse side effects, while the use of narcotics to manage pain has severe and well documented health and social effects, there does not appear to be any increase in the clinical acceptance of ICES stimulation for the management pain. Essentially—despite growing support in the peer-reviewed literature, the availability of many commercial ICES products, and the lack of

evidence indicating adverse effects—the use of ICES for any form of pain management remains outside even the fringe of standard medical practice.

Possible mechanisms of inflammation reduction

While there are many possible mechanisms by which ICES could influence cells, tissues, organs and whole systems—there only a few basic mechanisms that are adequately explored in the scientific literature.

For low-amplitude magnetic fields, a Larmor precession model is discussed which states that the Larmor precession behaviour of certain atoms or molecules (such as water) can be modulated in the presence of a magnetic field. In the case of water, modifying the Larmor precession can impact the ability of thermal fluctuations to drive chemical reactions—shifting the amount of energy required by a ligand to displace water from a binding site on a target molecule^{5,17,85}.

Next is an implicit theory which is not always discussed explicitly: Eddy current interactions with signaling proteins. The fundamental idea of this first theory is based on Faraday's law of induction which states that electromagnetic eddy currents can be induced in a conducting surface (such as a slice of tissue) by a time-varying magnetic field. In the presence of a changing magnetic field, the electrolyte surrounding cells can act as a conducting medium and eddy currents can flow in these spaces. If there are free ions in solution, presumably they could be placed into organized motion and their frequency of interaction with their receptors of interest might be preferentially increased or decreased, causing a cell response. Another possibility is that proteins are affected directly—since every biochemical reaction is driven fundamentally by the electromagnetic force, it follows that

protein binding pockets could be modulated by induced EMFs or eddy current flow. A more specific proposed mechanism is that put forth by Pilla and his collaborators, which states that ICES of the appropriate waveform and pulse duration (specifically pulsed radio frequencies) is able to modulate the Michaelis-Menten binding kinetics of the Calcium-Calmodulin dependent nitric oxide synthases. The theory that ICES functions via modifying Ca/CaM binding has been supported in the literature for cell-free preparations, as well as in vitro and in vivo studies^{2,5,7,10–13,18,19,68,86}. Modulating such a fundamental pathway could result in modulated levels of NO production and therefore have very drastic downstream effects in the body.

Another consideration to be made is that electrical stimuli are known to have well-understood effects on muscle, bone and nerve tissues. Muscles, bones and nerves all require electrical stimuli on various scales of time and signal intensity from millisecond time base curing events of excitation and action potential generation, to much lower levels signals that are hypothesized to be essential to the maintenance of homeostasis and functional adaptation—thus it is not a far stretch to suppose that ICES acts to promote homeostasis or facilitate functional adaptive responses in cells/tissues that have been injured. Because the ICES protocols used in the following experiments are based on stimulation patterns found in healthy, adult-phenotype muscles and nerves, it may be that part or all of the effects seen for ICES are a result of an electrically-mediated restoring force towards physiological homeostasis. Tissues have evolved such that they respond to specific stimulation patterns and selectively ignore all other stimuli which are not relevant. Evidence of the previous claim can be found in the fact that tissues are essentially transparent to static magnetic fields, and the majority of electromagnetic phenomenon that are either in the ambient background or those

that are man-made. Thus, it is very reasonable to assume that appropriately chosen stimulation patterns should be interpreted by electrically responsive tissues as homeostatic in nature and could thus promote a reduction in inflammation (i.e. promote a lack of change from homeostasis).

A different viewpoint is that cells can be modeled as living finite state machines. Cells could find themselves locked into perpetual pathologic states even after the insult/injury has long passed. Thresholds for transition from state-to-state may change with age of the organism in question. This is one explanation for why exercise response changes with age: the threshold for promoting a transition from homeostasis to tissue repair or functional adaptation to exercise may change with age along with the threshold for pathologic chronic response to injury, such that under certain circumstances, such as advanced age, it becomes easier to induce a state of chronic pathological response while it becomes more difficult to elicit the desired adaptive response from the cells/tissues. It is possible that ICES with appropriate stimulation parameters reinforces the transition to the positive adaptive state while reducing the response to a change to the undesirable chronic pathologic state. And crucially, if ICES facilitates adaptive signaling that emulates the so-called “exercise response” without the need for physical stimuli such as exercise, the beneficial effects may be enhanced simply because the exercise response can be elicited without the risk of actual physical injury. Such a mechanism might allow bedridden patients to heal without overexerting their capabilities. Furthermore, ICES might also be used to help prevent bone and muscle atrophy in astronauts and individuals under conditions wherein they do not receive the necessary physical stimulation to maintain bone density and muscle mass.

Central and peripheral nervous system components should be considered as well. Pain is perceived through signals transmitted from the peripheral nervous system to the central nervous system. To some extent, pain transduction is poorly understood, but generally speaking, pain is detected by free nerve endings which sense changes in heat, pressure and chemicals. When inflammation occurs, local pressure gradients are changed, resulting in physical forces on nerves (pressure) which can cause irritation and pain. Furthermore, tissue damage releases cytosolic components that are known to irritate nerves and cause pain. Long-term activation of inflammatory pathways is known to lead to cortical remapping and chronic pain^{87,88}.

These theories are far from complete or comprehensive; however, they serve as a good beginning to the development of an understanding of the basic mechanisms to elucidate the effects of ICES on the body.

Possible mechanisms of pain reduction

In order to understand the possible mechanisms by which ICES might modify pain in the body, it is important to understand the central and peripheral organization of pain. Peripherally, pain is typically described in terms of the nerve types that carry the pain data. There are two classic peripheral pain pathways in the body: “Slow” pain, conducted by unmyelinated C-fibers; and “fast” pain, conducted by myelinated A δ fibers.

Slow pain—typically described as visceral, deep, achy, nauseous pain—is conducted peripherally through C-fibers that carry information at a rate of 0.5-3m/s. The unmyelinated nerve endings synapse in the spinal cord within laminae layers II and III (collectively known as the substantia gelatinosa) of the dorsal horns. From the substantia gelatinosa, the signal is

carried to layer V, where it then travels up the paleospinothalamic pathway to the brain. In the brain, slow-fiber pain neurons terminate in the thalamus, reticular nuclei, tectal area and periaqueductal gray regions prior to transmission for higher-level processing in the somatosensory cortex (3a)⁸⁷.

Fast pain—typically described as shallow, sharp and well localized—is conducted peripherally through A δ fibers that carry information at a rate of 2-30 m/s. From the periphery, primary A δ fibers travel to the spinal cord where most synapse with secondary nerves in Lamina I of the dorsal horn. From Lamina I, the secondary nerves cross to the contralateral side in the anterior commissure prior to traveling superiorly through the neospinothalamic pathway to the thalamus. From the thalamus, the pain signal travels to SI area 1/3b for processing.

The central nervous system also has the ability to modulate the pain response peripherally through analgesic pathways. These pathways tend to originate from the periaqueductal grey matter as well as the periventricular nuclei. Stimulation of these nerves provides enkephalin-based signaling to the Raphe Magnus, where they synapse with secondary serotonergic neurons that travel through the dorsal lateral funiculus, making connections with enkephalin neurons that project to the dorsal horn and provide inhibitory connections to laminae I, II, and V. Additionally, there are projections from the medulla and pons that provide additional inhibition by projecting to laminae I and V⁸⁹. These feedback mechanisms are known to project from the central nervous system because stimulation of the periaqueductal grey matter has a strong analgesic effect while also blocking spinal-cord reflex responses to painful stimuli.

Additionally, it should be noted that neurons in laminae I and V likely receive afferent input from non-nociceptive A β fibers. It has been shown that A β fibers projecting to lamina V have inhibitory effects on nociception by stimulating inhibitory neurons that project to lamina II. This inhibition tends to inhibit nociception. However, it should be noted that nociceptive inputs from A δ and C fibers also have effects on the inhibitory interneurons in lamina II⁸⁹—which is to say that A δ and C fibers feed back on A β fibers and vice versa. Simply put, nociception can be inhibited by non-nociceptive (typically mechanical) stimulation, and mechanical perception can be inhibited by nociception.

Finally it is worth noting that there are various central pathways via which opiates and other chemical analgesics can affect pain, however since this discussion is mostly concerned with how ICES might interact with pain perception, we choose to simply mention that these pathways can be stimulated electrically and chemically, as well as inhibited by drugs such as Naloxone. Stress response in humans is a known factor that can cause activation of the opiate pain reduction pathways.

The general organization of pain typically regards the thalamus as the primary region through which the pain signal is relayed prior to higher-order processing in the somatosensory cortex. As mentioned previously, A δ and C fibers are currently understood to project to areas in S1 of the post central gyrus (Brodmann areas 1, 2 and 3). Specifically, A δ fibers are hypothesized to project to areas 1 and 3b, while C fibers are hypothesized to project to area 3a. The general interaction between central processing of peripheral inputs from A δ , A β and C fibers is shown in Figure 1.6.

Figure 1.6: Simplified illustration of central interactions between A δ , A β and C fibers.
 (Top left/blue) Activation of A δ fibers increases activation in area 3b/1 of SI, while at the same time inhibiting input from peripheral A β fibers and inhibiting central area 3a.
 (Bottom left/yellow) Activation of non-nociceptive A β fibers inhibits peripheral A δ and C fiber signals, while upregulating area 3b/1 and inhibiting area 3a of SI. (Bottom right/red) Activation of peripheral C fibers inhibits peripheral A β (non-nociceptive) input, while centrally activating area 3a and eventually inhibiting 3b/1 of SI.

Briefly, in the presence of afferent nociceptive input from A δ fibers, A β input is inhibited in the spinal cord in lamina II by inhibitory interneurons. A δ input is processed in area 3b/1, thus activation of this area is caused upon sharp nociception—simultaneously, 3a is inhibited⁸⁷. If A β fibers are activated peripherally, they cause a corresponding inhibition of both A δ and C fiber input from the periphery by activating inhibitory interneurons in lamina II that project from lamina V. A β fibers project to area 3b/1 in SI, and tend to have a net inhibitory effect on area 3a⁸⁷. Finally, C fibers inhibit A β afferents while stimulating area 3a and inhibiting 3b/1 in SI. The implications for the aforementioned pathways are that:

1. Mechanical stimulus carried by A β fibers can modulate the perception of pain both centrally and peripherally.
2. Nociceptive stimuli carried by C fibers can modulate the perception of mechanical stimulus centrally and peripherally. C fiber stimulus only modulates perception of A δ fiber input centrally by inhibiting area 3b/1 in SI.
3. Nociceptive stimuli carried by A δ fibers can modulate the perception of mechanical stimuli centrally and peripherally; however A δ activation only modulates nociception from C fibers centrally by activating area 3b/1 and inhibiting 3a.

In order to appropriately address the question of how external electromagnetic stimuli might modulate pain response, it is essential to ask the question: How is pain transduced? It is very well established that both C and A δ fibers are free nerve endings (i.e. they do not specifically “connect” to anything like a Pacinian corpuscle, etc.). Furthermore, it is still debated as to the exact mechanism by which nociceptors can distinguish between the different types of pain, degrees of pain and qualities pain. It is well understood that C fibers are typically responsible for transmission of heat information, but they are also responsible for other types of slow pain such as aches, nausea and deep, poorly localized pain. Similarly, A δ fibers have a similar problem: they seem to be able to describe several types of pain, yet they are all essentially identical. Recently, it has been proposed that one of the ways in which pain is transduced is by modifying the conditions in the cells surrounding the free nerve endings in question⁹⁰. Specifically, it is hypothesized that levels of nucleotides (specifically ATP) can significantly affect the ability of nerves to respond to stimuli⁹⁰. Specific receptors (such as TRPV1, as well as the P2X and P2Y families) are implicated directly in the transduction of signals by nerve endings. The P2 families of receptors in particular are

sensitive to extracellular ATP levels—allowing them to be capable of modulating nerve response. In addition to having nucleotide receptors that modulate nociceptive quality, it should be noted that all nociceptive stimuli are likely to elicit responses in surrounding non-nociceptive somatosensory afferents. Thus, when integrated in the CNS, perception of the quality of pain is likely the result of both nociceptive and non-nociceptive somatosensory input.

From a cellular standpoint, there are a plethora of factors involved in determining the ability of a nerve to send signals. In our case we are specifically interested in those factors that can be modulated peripherally. Perhaps the most obvious factor influencing neural activity is resting membrane potential. The resting membrane potential is developed by the presence of ion channels that provide an equilibrium state between [mostly] sodium (Na^+), potassium (K^+), and chloride (Cl^-) ions. The permeability of these and other channels (such as Ca^{2+}) is modulated at the time of activation to allow for an inversion (depolarization) of the membrane potential. Various mechanisms are involved in re-developing the membrane potential (repolarization) so that the nerve is reset after depolarization. Modulation of any of these three states can give rise to a different type of signal. The activity of neurons under normal conditions can be modulated in several ways: 1) Driving the membrane potential exogenously (e.g. direct electrical stimulation), 2) by input from other nerves (stimulatory or inhibitory), 3) by availability of electrolytes and signaling molecules, 4) by the ability of ions and neurotransmitters to interact with their respective target (e.g. the ability of Na^+ to flow through a channel, or the ability for NMDA to bind its receptor, etc.), and 5) by modifying the surrounding tissues which release modulating factors into the pericellular space. Each of

these modulating factors is a potential target of ICES, however only two of these four are likely, as discussed below.

One of the most obvious methods by which one might expect to be able to modulate the membrane potentials would be to directly drive the membrane potential electrically. This is the putative mechanism of primary signal transduction in the case of transcutaneous electrical nerve stimulation (TENS) and other direct electrical stimulation methods. The problem with direct electrical stimulation is that uneven stimulation occurs at a microscopic level within tissue. Local overstimulation occurs along electrically conducting pathways, and local under-stimulation occurs along pathways that do not carry current—thus there is both a risk of damage to cells from over-stimulation and simultaneous under-stimulation of cells outside of the conductive pathway. Inductive methods such as ICES result in a more uniform method of delivering electrical stimulus, as magnetic field lines are left relatively unaltered by transmission through tissues. Pulsed magnetic fields are able to develop circular eddy currents in a conducting plane proportional to the rate of change of the magnetic flux (or simply the field strength in the case where the stimulated area is not changing). In the case of transcranial magnetic stimulation (TMS), induced field strengths on the order of 0.03-0.15 V/m have been shown to have significant interactions with the CNS⁹¹. Across the width of a cell body, one might expect then that potentials on the order of 0.3 μ V could potentially affect neural function. ICES typically uses smaller magnetic field strengths than rTMS, but because it is capable of providing rapidly changing, low-magnitude magnetic fields, it is capable of inducing potentials on the order of 1-10 V/m around a cell diameter. Thus, while the magnitude of these ICES fields would not likely be large enough to elicit a depolarization in most cells, it is probable that they could produce a potential of 0.1 mV across a cell

body—leading to a modulation in the sensitivity of the neuron to firing. However, since a change of 0.1 mV is only about 0.1% change in the potential across the cell, it is unlikely that such a minute change would act directly on driving the membrane potential. Also note, the induced eddy currents would induce electrical potentials and ion flux transverse or parallel to the non-conducting membranes rather than across them, as membrane potential is defined. It is more likely that a small change in membrane potential would lead to a modulation in the permeability of membrane proteins responsible for developing the standing membrane potential. It is worth noting that one might expect a difference in the effects of ICES on cells based on whether or not they are myelinated. Since myelin is an insulator, it can act like a capacitor, thus an electric field developed across the myelin sheath could in fact cause longer-lasting effects than those on a non-myelinated sheath. Additionally, myelin may shield the nerve from some of the electrical signal, reducing the efficacy of ICES on heavily myelinated neurons. Finally, un-myelinated nerves are exposed directly to electric fields produced by ICES, as a result, they may be more strongly stimulated—however more likely in a more transient manner than myelinated cells. One reason to explain the lack of transience in a myelinated cell might be that because myelin is a dielectric, it can store energy in the form of an induced electric field (in precisely the same way as a capacitor dielectric stores an electric field). When an external stimulus is removed, there is a time-constant associated with the breakdown/relaxation of the internal dielectric field^{92–94}. Thus, prior to the complete relaxation of the dielectric myelin there can be transient induced fields that may very well cause extended effects of ICES on myelinated nerves. It should be noted, that these are simply conjectures. Experiments will be discussed later that might offer some insight for differentiating potential mechanisms.

A slightly less obvious mechanism by which ICES may interact with cells (including neurons) is by modifying the ability of target ions to bind their appropriate receptor. There is a large amount of literature to suggest that calcium is one ion that can be easily modulated by ICES^{1,2,4,7,10,11,17–19,67,68,86,95}. Specifically, calcium in the context of calmodulin activation has been studied—however the specific model developed by Markov and Pilla that predicts the interactions of calcium with its target calmodulin can be developed for nearly any ion and receptor in the body. Above a certain threshold stimulation intensity, the relevant parameters for modifying binding are frequency and pulse width⁷. Modifying the ability of ions to interact with their targets has obvious implications for both resting membrane potential as well as sensitivity to depolarization.

Modifying the availability of ions and neurotransmitters is unlikely to be a mechanism by which ICES interacts with neurons—with the exception of perhaps modifying the availability of neurotransmitters produced by other cells (such as nucleotides, etc. produced in non-nerve cells).

Modifying inputs from other nerves would likely be an indirect feedback mechanism caused by a direct interaction of ICES with a peripheral nerve. For example, if ICES modulates the activity of a peripheral neuron that projects to an area of the CNS that provides a feedback response to peripheral nociceptive neurons (such as the central feedback mechanisms described above), then pain could be amplified or inhibited by the interactions of ICES with peripheral nerves.

Neural firing is contingent upon signals received from surrounding cells. If ICES interacts with cells as described by Pilla et al.⁷, then it follows that metabolic activity of cells

surrounding neurons should be modulated by ICES. Furthermore, if there are pericellular current flows within innervated tissues, one would expect a corresponding change in tissue metabolism¹. Thus, if one modifies the conditions of the surrounding tissue using ICES, a neuron might be expected to behave differently given the same stimulus. This also implies that if nociception is typically proportional to tissue damage, that if inflammation is reduced by ICES, then the intensity of nociception should decrease^{10,11}.

Possible cutaneous wound healing mechanisms

ICES has been implicated in wound healing in the literature, and may hold the potential to modify scar formation pathways. Scar formation is typically governed by a well understood mechanism involving fibroblasts laying down a well-organized collagen matrix. There are three well understood types of scarring—hypertrophic, keloid and atrophic. The typical scar is formed in the following steps:

1. A wound is created (cut, scratch, surgery, lesion, etc.)
2. Inflammatory mechanisms are activated (inflammatory factors released, vasodilation, increased blood flow, macrophage response, immune response, etc.)
3. Repair mechanisms are triggered to form a scar (fibroblasts and other repair cells start laying down new matrix)

Upon tissue damage, several events trigger fibroblast migration and differentiation. Chemical factors are released into the tissue surrounding the wound—cytokines from damaged cells trigger inflammatory and healing factors to be recruited to the site of damage. Additionally, because the existing extracellular matrix and connective tissue surrounding a

wound is disrupted, the mechanical stimuli to which the cells are accustomed in homeostasis change. Evidence has been collected to demonstrate that fibroblasts normally experience very little average extracellular mechanical stress due to the amount of crosslinking in the extracellular matrix in which they exist. In the presence of a wound (i.e. a source of high extracellular stress), fibroblasts are then hypothesized to undergo differentiation into myofibroblasts—a subset of fibroblasts that express α -smooth muscle actin (α -SMA)^{96–102}. This mechano-differentiation hypothesis is supported by the fact that stress fibers readily form in vitro when fibroblasts are plated on hard-substrates (such as polystyrene), but do not form when grown in soft hydrogels such as collagen⁹⁷. Once fibroblasts have moved into a wound, they begin expressing stress fibers in the form of cytoplasmic actins—this phase is called the “protomyofibroblast” stage¹⁰². These stress fibers connect to both integrins of cell-matrix junctions as well as N-cadheren-type adherens junctions¹⁰². In vivo, these stress fibers are eventually replaced with α -SMA when the pseudomyofibroblasts are exposed to TGF- β 1. As well as from the circulating fluids in a wound, TGF- β 1 is released by the existing and newly formed ECM when the pseudomyofibroblasts begin supplying a mechanical stress to the ECM¹⁰³. A wound can then be pulled closed by the stronger pull of α -SMA before scar formation begins as myofibroblasts begin laying down thick extracellular matrix.

Pulsed electromagnetic fields have also been conjectured to accelerate wound healing. There are several mechanisms by which one might expect PEMFs to accelerate wound healing. The first mechanism is that appropriate waveform parameters may induce microcurrents to flow in and around pericellular spaces¹. Such stimulation would obviously have repercussions in terms of modulating ion flow, activating extracellular proteins—this mechanism would most likely affect metabolic rate, as well as anti-inflammatory factor

expression. A second mechanism is that put forth by Arthur Pilla et. al. and suggests that ICES can modulate the rate at which calcium binds to calmodulin, thus increasing the rate at which eNOS and nNOS can produce nitric oxide intracellularly. Such a mechanism is proposed to directly reduce levels of interleukins as well as other inflammatory factors^{6-8,10,11,18,19,68}. A reduction in said inflammatory factors could rapidly increase the rate at which a wound can heal. It should be noted that both of these mechanisms could lead to pain reduction by reducing inflammation. A third possible mechanism (or at least contributing factor) is that ICES stimulation may electrically modulate epithelial secretion of neuroactive agents. Specifically it is hypothesized that nucleotide signaling is critical to nerve sensitization, and that an increase in ATP is likely to increase the ability of peripheral nerves to transduce pain signals to the central nervous system⁹⁰. In conjunction with the previous observation, it is important to consider the nervous system globally—any electrical stimulus is likely to have effects on resting membrane potential—resulting in hyperpolarization or depolarization. There are obvious impacts of modulating nervous system peripheral signals—the least of which is that modulation of signal transduction can affect the way the pain is perceived centrally. A reduction in pain reduces circulating stress molecules which can further lead to a reduction in inflammation—accelerating wound healing. Globally, however, the generally accepted mechanism by which ICES interacts with cells is based upon ion-interactions with different membrane bound and non-membrane bound enzymes.

Summary and the future of ICES

The ICES literature is rather sparse when one considers the vast continuum of electromagnetic frequencies and amplitudes. The problem of organizing and classifying effective ICES waveforms in tissues is similar to the problem faced by Mendeleev and other

chemists who faced the growing problem of classifying elements into the periodic table. More recently a similar problem was faced by subatomic particle physicists such as Glashow, Weinberg and Salam in trying to develop what we now call the Standard Model—a method for classifying and understanding the subatomic particles and their interactions. The problem faced by these influential scientists is not completely held in simply organizing information—it was in taking a large amount of completely unorganized information and convincing a scientific community of an effective means of organizing the information. The importance of such organization is twofold. First, organization helps to circumvent vicious arguments between those seeking answers to the same problems by giving objective grounds on which to make rational arguments. And secondly, possibly more importantly, it allows outside viewers—those not directly involved in the scientific community—the opportunity to understand clearly the methods and goals of the study. If organization at this level can be achieved, then the ICES community as a whole can make research progress at an incredible pace. As a research community it is important to apply several simple principles in experiments and articles that will help to alleviate the questions that are often generated by those not directly involved in the research. First, it is important to strive to be scientifically rigorous—any published experiment **MUST** include adequate information to completely replicate the experiment. Waveform parameters must be fully and carefully defined such that an induced electric field can be calculated. In some cases the parameters should be measured and determined experimentally, using well-calibrated instruments suitable to the task. Secondly, it is important that authors choose effective and specific titles for articles. Titles such as “PEMF is Not an Effective Means of Treating Rotator Cuff Injury” do not help the already confused literature (unless every single frequency, amplitude, waveform structure

and treatment regimen was tested). Consider the possible title for a study in which the drug aspirin (acetylsalicylic acid) was found to be ineffective in reducing post-operative pain. The resulting manuscript titled "Drugs are not effective in the treatment of post-operative pain" is non-specific to the point of being both misleading and incorrect. Just as there are many types of drugs, there are vast numbers of different ICES stimulation protocols. The lead advisor for this dissertation (Dennis) estimates the number of potential ICES stimulation protocols to be on the order of 10 trillion (unpublished estimate). Therefore, titles should include at minimum a descriptor of the magnetic field waveform such as "75 Hz, 250 mH Sinusoidal PEMF is Not an Effective Means of Treating Rotator Cuff Injury." This title allows those in the field to quickly isolate articles based on their treatment parameters, and it gives those outside the field an understanding that different ICES protocols are used for different reasons. Just as ultrasound has different clinically effective waveforms for different applications (imaging, targeted ablation, ARFI, etc.), both clinical practitioners and the educated public must understand that the same is likely true of ICES. Secondly, it is important that, as the ICES literature progresses and waveforms are grouped based on efficacy, we use consistent terms to define ICES stimulation protocol parameters (Table 1-1). Until a well-defined set of terms is established, understanding and forward progress in the use of ICES will be limited. Specifically, terms such as those found in Table 1-1 should be used to define a protocol in conjunction with the relevant magnetic slew rate. The *most* appropriate method for dosing ICES is by providing the magnetic slew rate and the pulse characteristics such that the power and/or length of time during which tissue is exposed to ICES may be calculated. An example dose might be 5 pulses per second (pps), 100 μ s pulse-length, at 80 T/s. Such a dosing allows one to calculate that an equivalent dose of 500 μ s of

80 T/s stimulation is provided. It is, however, *not* correct to infer that such a dose is equivalent to applying a static 0.04 T field for any length of time. If the scientific community is strict with definitions, clear in its methods, and scientifically approaches the many questions posed by the interactions of ICES with tissues, then it can take the field from being in a questionable and disorganized state toward a respected and organized body of knowledge that has earned the respect of scholars and physicians.

CHAPTER 2 : IN VITRO STUDIES

Overview

Herein we sought to examine the effects of inductively coupled electromagnetic stimulation (ICES) of specific parameters on the osteoblast-like MC3T3-E1 cell line. Previous research has indicated that ICES stimulation with the correct waveform characteristics can elicit a response from the calcium/calmodulin (Ca/CaM) mediated nitric oxide synthase (NOS) pathway⁷⁻⁹. ICES stimulation (80 T/s) was provided to MC3T3-E1 cells plated in 6-well plates using a custom apparatus. L-N-monomethyl arginine was used as a NOS inhibitor and negative control to test whether NOS is a pathway stimulated by ICES. No significant changes in NOS levels were measured between any of the treatment groups at any point during the experiment. All NOS levels were below detection limits, which calls into question previously reported data indicating that NOS activity can be modulated in MC3T3-E1 cells using ICES. Further research should be conducted to examine which pathways are modulated by ICES. It is possible that different types of ICES modulate different inflammatory pathways.

Introduction

Electromagnetic field therapy has been implicated as a healing agent for many years. While ancients document the use of presumed ferromagnets as an analgesic and healing agent, the efficacy of such treatments has been questioned for many years. More recently, other forms of electromagnetic therapy have been introduced as potential therapeutics for

various ailments—however the question as to the mechanism eluded scientists until the 1950's when pioneering work was done by Fukada and Yasuda in the subject of orthopaedics^{23,24}. Fukada and Yasuda hypothesized and discovered that bones are piezoelectric in nature—furthermore, it was shown that bone degradation and augmentation occurred on the electropositive (anodic) and electronegative (cathodic) sides of an implanted electrode respectively⁸⁵. The discovery that the piezoelectric properties of bone are directly linked to its augmentation and degradation led to the notion that bone healing could be accelerated using externally applied electric fields. Pioneering work by Bassett et. al. showed that healing of non-union fractures could be elicited and accelerated using externally applied electric fields¹²⁻¹⁴. In the mid 1970's, while the application of electric fields to bone regeneration were being studied, the notion that other tissues could be stimulated using externally applied electric fields came to light. Both capacitively and inductively coupled methods were explored and found to elicit cellular responses. It was also noted that non-piezoelectric tissues were responding to electrical stimulation. While the notion that externally applied electric fields can elicit responses is rather easy to conceptualize considering the large proportion of aqueous ions in the body, the exact transduction mechanism was unknown. In 1974, Arthur Pilla proposed a mathematical description of the interaction between tissues and electric fields based on the modulation of ion-dependent/triggered signal transduction pathways². In particular, Arthur Pilla expanded on his work by studying the Calcium-Calmodulin (Ca/CaM) interaction in cells. Since calcium in the body exists as an ion (Ca^{2+}), one might expect that it might be influenced to migrate in the presence of an electric field. Thus, Arthur Pilla built a mathematical model that examined the possibility of using capacitively and inductively coupled methods for stimulating

Ca/CaM binding in cells. Based on Michaelis-Menten style binding kinetics, Pilla proposed that ICES could be used to stimulate the Ca/CaM mediated nitric oxide (NO) anti-inflammatory pathway under the appropriate waveform conditions. Specifically, Pilla found that only particular frequencies, pulse lengths and amplitudes actually elicit a cellular response^{7,12,13,18,19,21}. Our conjecture is that the most important stimulation parameter in ICES is the rate of change of the magnetic flux. Our hypothesis is that higher rates of magnetic flux have the capacity to induce larger electromotive forces and therefore provide physiologically relevant signals to tissues. Herein we seek to replicate previously performed research in the field, while exploring the possibility that our ICES protocol acts via an established mechanism.

Materials and Methods

Cell culture

Osteoblast-like MC3T3-E1 cells (Passage 31) were obtained from the University of North Carolina at Chapel Hill Tissue Culture Facility (UNC TCF) in the Lineberger Cancer Research Center and stored over liquid nitrogen until use. Cells were grown in phenol-red-free α -MEM (Invitrogen/GIBCO, Carlsbad, CA, SKU 41061-029) obtained from the UNC TCF, supplemented with 10% FBS (Cellgro, Manassas, VA), 100 IU/mL penicillin, 100 μ g/mL streptomycin (Cellgro, Manassas, VA), and 50 μ g/mL ascorbic acid (Fisher Scientific, Pittsburg, PA). Some groups also received a 300 μ M dose of N-monomethyl-L-arginine (L-NMMA) as an inhibitor of NOS (Acros Organics, Geel, Belgium). Cells were grown in 15-cm petri dishes until approximately 80% of the surface was covered, then the

cells were split into 10, 10-cm petri dishes prior to being distributed into 6-well plates at a concentration of 78 cells/mm^2 ($5 \times 10^5 \text{ cells/mL}$).

Cells from the same passage (passage 32) were used in both the controls and the treatment groups to ensure no differences were as a result of passaging the cells. Furthermore, each 6-well plate contained both treatment and sham groups. Active coils (treatment group) were arranged in the configuration shown in Figure 2.1. Note that the inactive coil centers are approximately 40 mm from the treatment groups, and thus are not affected by the ICES fields. However, as a control, one set of 6-well plates had all active coils and another had completely inactive coils. Temperature was monitored using an infrared camera (FLIR Systems Inc., Sweden) so as to ensure that no inductive thermocouple/thermometer heating occurred.

ICES stimulation protocol

Cells plated in 6-well plates were placed into custom-designed tray that allowed 6 pairs (12 total) of coils (28 AWG, 80 turns, 35 mm average diameter) to be positioned concentrically above and below each of the 6 wells of the plate (Figure 2.1). Coils were energized to deliver 80 T/s stimulation through a custom built circuit as outlined in Figure 2.2. All electronics were housed outside of incubators, and ribbon cables were used to connect the stimulation coils to the pulse generating electronics.

Figure 2.1: Diagram showing the in vitro 6-well ICES stimulator (Left) 3-D model of the ICES 6-well plate stimulator. (Right) Photograph of the assembled ICES stimulator with individual driving circuits able to provide various amplitude doses. Stimulation parameters are shown in Figure 2.2.

Figure 2.2: Representative drawing of the ICES stimulation protocols used throughout the experiments presented herein. The first stimulation protocol is alternating positive and negative pulses every 200 ms. The second and third protocols consist of five rapid pulses in succession followed by a 1-second period of rest. Amplitude dictates the final dosing (40, 80, 120 or 160 T/s).

Cell count and viability assay

In order to assess the general growth and health of the cells used, cell counts and viability assays were conducted on the final day of culture (Day 15). Trypan blue was used to assess cell viability. Briefly, cells were trypsinized (0.05% trypsin-EDTA, Gibco 25300) and resuspended in 10 mL of growth medium, centrifuged in 50-mL conical tubes for 5 minutes at 100xg. Cells were then resuspended in 1 mL of PBS, and mixed with an equal part of 0.4% trypan blue dye. Cells were then counted after 3 minutes of incubation using a hemacytometer.

Nitric oxide assay

Since the proposed pathway of interest is the Ca/CaM mediated NO pathway, we found it pertinent to assess, indirectly, the amount of NO produced by the cultured cells. In order to further probe the claim that NO production is via the NOS pathway, we divided the treatment groups in two. One treatment group received a 300- μ M dose of L-N-monomethyl-L-arginine (L-NMMA), a selective NOS inhibitor, while the other did not. Since NO is an unstable free radical, the most accessible method available to measure the NO concentration in the cultured cells was by measuring the indirect byproduct of NO metabolism, nitrite. Nitrite levels were assessed using the Griess reaction (Griess paper reference) each time that the cells were fed (Day 0, 2, 4, 7, and 9). The Griess reagents were provided by Promega kit G2930 (Promega, Madison, WI). Briefly, conditioned media was harvested and 50 μ L from each sample was aliquotted into a 96-well plate in triplicate. To the samples was added 50 μ L of sulfonamide solution prior to incubation for 10 minutes in the absence of light at room temperature. Using a multichannel pipet, 50 μ L of NED solution was added to each well and incubated at room temperature for 10 minutes in the absence of light. Finally, absorbance measurements at 550-nm were taken and nitrite concentration determined from a standard curve.

Results

No changes in nitric oxide levels were observed in any of the treatment or control groups throughout the course of the experiment. All values observed were below the detection limits of the assay used throughout the entire course of the experiment. Figure 2.3 shows the results of the NOS assay over the time-course of the experiment. Cells were

checked for viability and no significant reduction in cell viability was noted in any of the groups.

Figure 2.3: Plot of nitrite vs. time for conditioned media from in vitro study. A plot of the NO concentration (in µM) for various time points throughout the experiment for each treatment group. All points are below the detection limit of the reaction (2.5 µM) used in this experiment.

Discussion

The results observed in this experiment bring in to question several previously observed results in literature. Specifically, in cell cultures of the size studied herein and of those studied in the literature one might question the appropriateness of using the Griess reaction to monitor nitrite levels. Specifically, previously reported values in literature are at or below the reaction detection limit for the Griess reaction. However, assuming the Griess reaction is sensitive enough to detect such small levels of NO as would be produced in the

cell culture environments used herein and in the literature, several alternative explanations exist for the discrepancies observed.

One possible explanation for the results observed is that the ICES stimulation protocols used in this study are significantly different from those previously studied in the literature. Perhaps the ICES protocol employed herein is not as effective at stimulation at the given dose as previously studied protocols. The result may be that the ICES stimulation used in this experiment does not result in significant increase in NOS activity or NO production. Alternatively, one might expect that there is a possibility that our ICES protocol does not stimulate the same Ca/CaM dependent NOS pathway—leading to a complete lack of difference in NO production between groups.

Ultimately, the goal of ICES is to help alleviate pain and inflammation. There are several pathways yet to be discussed that are directly involved in pain and inflammation—for example, the arachadonic acid pathway, a prominent and important pathway involved in many different inflammatory effects. Since in vitro studies often fail in their ability to translate directly to the clinic, it is important that future studies seek either translation of the in vitro results or focus on animal and human studies. In order to establish the efficacy of ICES as an anti-inflammatory, it is perhaps most pertinent to consider animal studies. A well-established model of inflammation, such as the carrageenan foot-pad edema model, should be chosen and the efficacy of ICES on such a model tested. Only then is there reasonable evidence to support further exploration of possible mechanisms.

CHAPTER 3 : ICES AS A MEANS OF ACUTE INFLAMMATION REDUCTION

Overview

Inductively coupled electrical stimulation (ICES) has been implicated as an effective means of treating various maladies, including inflammation. In this study we used a carrageenan footpad edema (CFE) model in the rat to examine the anti-inflammatory effects of 40 T/s ICES. Inflammation was measured using plethysmometry and the circulating inflammatory factors interferon-gamma (INF- γ), interleukin-1-beta (IL-1 β), and interleukin-6 (IL-6) were monitored. We found significantly reduced (31.8%, $P < 0.01$) disease burden measurements in groups treated with ICES compared to controls as measured using plethysmometry. However, circulating inflammatory factors did not show statistically different levels in any of the groups as compared to the negative control.

Introduction

Various sources in the literature have strongly supported the ability of ICES to reduce acute inflammation^{10,11}. Specific inflammatory factors, such as IL-1 β , have been implicated in the anti-inflammatory effects of ICES^{4,6,7,10,18,19}. To our knowledge, very few studies have been conducted which make use of CFE to study ICES efficacy¹⁰⁴, thus we implement the use of plethysmometry to track paw inflammation volume. The CFE model is an acute inflammatory model which makes use of an injection of the algal-derived sulfonated polysaccharide λ -carrageenan to induce a well understood inflammatory response in the footpad of a rat^{83,84}. Typically the inflammation volume can be tracked using paw volume

measurements (plethysmometry). Inflammation in the CFE model typically resolves spontaneously within 8-12 hours in untreated animals. Additionally, significant reduction in inflammation can be achieved using dexamethasone as a positive control. Thus, CFE provides an easily modulated model to testing the efficacy of anti-inflammatory therapeutics. The goal of this study is to establish the baseline efficacy of ICES as an anti-inflammatory therapeutic in order to outline dosing experiments as well as future experiments that could lead to the determination of specific mechanisms by which ICES works.

Materials and methods

Animals

This study made use of 6-8 week old adult male Sprague-Dawley rats that were given access to food and water ad libitum. ICES treatment was provided external to the cages in order to leave the animals undisturbed during the experiment. At the terminus of the experiment animals were anesthetized according to IACUC-approved protocols. All measurements and treatments were given in accordance with IACUC-approved protocols.

ICES instrumentation

In order to leave animals undisturbed during their treatment, a customized stimulation mat was developed which housed paired coils of alternating orientation so as to reduce destructive interference. The mat was placed underneath the cages of the animals with driving electronics to the side of the mat. The mat consisted of custom-wound, square-shaped coils laid in a pattern of alternating polarity (Figure 3.1). The stimulation protocol provided peak stimulation of 40 T/s magnetic flux change through the volume occupied by the animal.

Figure 3.1: Image of the mat-style ICES stimulation unit (Left) Representative drawing of the alternating coil pattern which provided uniform stimulation through closed magnetic loops. Arrows indicate direction of current flow through coils, x's represent a magnetic field pointing into the page, dots represent magnetic field lines coming out of the paper. (Right) Image of the actual setup upon which the cages were housed. Note that the drawing shows only a partial array of the 3x6 coils in the plane, whereas the actual test instrument had an array of 4x8 coils, as indicated in the photograph to the right. Instantaneous magnetic flux alternated between adjacent and diagonal coils in much the same way as dark and light squares alternate on a checkerboard. The electromagnetic pulses were applied only for 100 μ s per pulse, and pulse polarity was alternated between pulses so the direction of the magnetic flux toggled from on direction to the other with each polarity reversal.

Treatment groups

In this study, 30 animals were divided into 3 groups of 10. The negative control group received *per os* (PO) PBS, the positive control group received a 3 mg/kg (PO) dose of dexamethasone, and the remaining group was exposed to 40 T/s ICES with the following stimulation parameters:

Waveform shape: trapezoidal

Rising/falling edge slew rate: 40 T/s

Pulse width: 100 μ s

Pulse rate: 5 pps

Duty cycle: 0.0001s/0.2s = 0.05%

Stimulation length: Continuous while animals were in their cage.

Groups are displayed below in Table 3-1, which summarizes groups and dosing.

Animals were exposed to their respective treatments one hour prior to CFE induction—ICES stimulation continued throughout the duration of the experiment.

Table 3-1: Dosing chart for ICES CFE study. Group 1 received PO PBS as a negative control. Group 2 received 3 mg/kg PO dexamethasone as a positive control. Group 3 received 40 T/s ICES does via the mat-style unit. All animals received carrageenan in their right hind paw and a saline injection for the left hind paw.

Group #	N	Treatment	Dose	Route
1	10	PBS	N/A	PO
2	10	Dexamethasone	3 mg/kg	PO
3	10	ICES	40 T/s	External
Totals:	30			

Carrageenan challenge/CFE induction

One hour post-treatment, carrageenan footpad edema was induced in the right hind-paw of adult rats by injecting 0.1 mL of 1% carrageenan in saline. The contralateral limb was used as a control by injecting 0.1 mL of sterile saline into the footpad.

Plethysmometry measurements

A plethysmometer (PVP1001, Kent Scientific Corporation, Connecticut, USA) was used to measure change in paw volume as an indicator of inflammation. Inflammation from CFE is reported as a difference between right (carrageenan) and left (PBS control) paw volumes for each animal. Plethysmometry measurements were taken prior to carrageenan administration (0 hours), and then 1, 2, 4 and 8 hours post-carrageenan administration according to methods established by the Charles River Laboratories (Wilmington, MA).

Circulating inflammatory factor measurements

Concentrations of interferon-gamma (INF- γ), interleukin-1-beta (IL-1), and interleukin-6 (IL-6) were monitored to determine how select inflammatory factors were affected by ICES treatment. To measure the inflammatory factor levels, 0.2-mL blood samples were drawn sublingually under no anesthesia at 1 and 8 hours post-carrageenan administration in animals 1-5, and at 4 hours in animals 6-10 in each treatment group.

Results

Plethysmometry

Edema (i.e. CFE) was calculated as the difference between right and left hind paws for each animal at each time point. A plot of the 8-hour CFE time-course is shown in Figure

3.2. Groups treated with dexamethasone (3 mg/kg) had a disease burden reduction of 63.2% ($P < 0.001$) with respect to saline controls. Significance is based on two-way repeated measures ANOVA with a post-hoc Bonferroni test. Significance was observed in all time-points after 1 hour for dexamethasone as compared to PBS. Significance was observed in all time-points after 2 hours for 40 T/s ICES as compared to PBS.

Figure 3.2: 8-hour CFE time-course summary of inflammation. PBS, Dexamethasone (3 mg/kg) and 40 T/s ICES treatment are shown as diamonds, squares and triangles respectively. Significance: *= $P < 0.05$, *= $P < 0.001$.**

From the 8-hour time course, one can calculate the total disease burden, which is the area under the CFE time-course curve. This calculation is performed as a discrete integral

using the average value between time points (middle-sum discrete Riemann integral). It should be noted that each data point in a CFE curve is corrected by subtracting the baseline initial CFE volume for the given treatment group from all subsequent time-points. A plot of the total 8-hour disease burden is shown in Figure 3.3. Significance in disease burden was observed in both dexamethasone (1.25 ± 0.15 mL-h, $P < 0.001$) and 40 T/s ICES groups (2.32 ± 0.28 mL-h, $P < 0.01$). Significance was also observed between the dexamethasone and ICES group ($P < 0.005$).

By comparing disease burdens to the PBS negative control, disease suppression can be calculated as a percent difference between the given treatment and the PBS control. The explicit calculation is given as:

$$DS = \frac{DB_{PBS} - DB_x}{DB_{PBS}} = 1 - \frac{DB_x}{DB_{PBS}} \quad (2)$$

Disease suppression is shown for the dexamethasone positive control and the 40 T/s ICES treatment in Figure 3.4. Dexamethasone showed 64% disease suppression while ICES showed 32% disease suppression.

Figure 3.3: 8-hour total disease burden for ICES CRF study. Disease burden represents the base-line corrected area under the CFE curve for each group. Lower disease burden values correspond to a more positive outcome. Groups (left to right): PBS negative control, dexamethasone positive control (3 mg/kg), and ICES (40 T/s). Significance: **=P<0.01, *=P<0.001 as compared to PBS negative control. +=P<0.005 as compared to dexamethasone positive control.**

Figure 3.4: 8-hour disease suppression for ICES CFE study. Disease suppression, calculated from disease burden, represents the percent difference between a given treatment and the PBS negative control. Groups: (left) Dexamethasone (3 mg/kg); (right) 40 T/s ICES. Larger values are considered a more positive outcome.

Cytokine concentrations

No significant differences were observed in cytokine levels across groups. Values presented as 4.9 with no error bars are considered to be below detectable limits for the given assay and represent values that were less than 50% of the lowest concentration on the standard curve (9.8 pg/mL). Figure 3.5, Figure 3.6, and Figure 3.7 represent the 8-hour IFN- γ , IL-1 β and IL-6 summaries respectively.

Figure 3.5: 8-hour summary of IFN- γ concentration in CFE-induced rats. No significance found using two-way ANOVA with Bonferroni test as compared to PBS negative control.

Figure 3.6: 8-hour summary of IL-1 β concentration in CFE-induced rats. No significance found using two-way ANOVA with Bonferroni test as compared to PBS negative control.

Figure 3.7: 8-hour summary of IL-6 concentration in CFE-induced rats. No significance found using two-way ANOVA with Bonferroni test as compared to PBS negative control.

Discussion

The plethysmometry data supports our hypothesis that ICES is an effective treatment for reducing acute inflammation. Significant differences in CFE were observed between both dexamethasone and ICES treatment as compared to PBS negative controls. This observation strongly supports the hypothesis that ICES is an effective anti-inflammatory therapeutic. Furthermore, a significant difference was observed between ICES and dexamethasone which implies that the two may have different mechanisms of action, or that there is a dose-response associated with ICES. It is apparent that future work should focus on determining

the dose-response of ICES as this will most certainly assist in determining what type of mechanism is involved in the anti-inflammatory effects observed.

Circulating cytokines were not significantly different between any of the groups. One possible explanation for this observed behaviour is that the specific cytokines examined (IFN- γ , IL-1 β and IL-6) are associated more strongly with chronic inflammation than with the acute inflammation response. Perhaps a more telling indicator of the anti-inflammatory effects of ICES would be to examine histological differences between ICES, PBS and dexamethasone. Since dexamethasone specifically blocks production of both lipoxygenase (LOX) as well as cyclooxygenase (COX) at the cellular level, it inhibits the recruitment of many more inflammatory cells than an anti-inflammatory agent that inhibited only one of the two¹⁰⁵. Significant differences between ICES and PBS might be expected in neutrophil infiltration if, for example, ICES inhibited the LOX pathway—however, if ICES inhibited COX and not LOX, or neither, then neutrophil infiltration would be expected to be significantly different from the positive dexamethasone control but not from the negative control. Furthermore, additional factors, such as platelet activating factor and NOS levels could be monitored in future work to determine if alternative (non-COX/LOX) pathways could be affected. Literature strongly suggests that involvement of NOS modulation as the primary mechanism by which ICES functions^{7,10,11,19}. However, our previous in vitro work has called these NOS pathways into question. Another consideration to be made is the fact that initial inflammatory response is typically triggered by binding of pathogen associated molecular patterns (PAMPs) to pattern recognition receptors (PRRs) on the surface of immune cells. It is possible that the triggering of PRRs is inhibited or modulated by the presence of ICES stimulation—either molecular binding or down-regulation of PRR expression. Because

protein expression could be modulated by ICES to cause a change in molecular patterns, it's important that future work also include the potential to examine gene and protein expression.

ICES is herein verified as an effective means for reducing acute inflammation. Future work should focus on determining mechanism, dosing and safety. Such work can be done as dose-response studies that monitor histology and protein expression. Safety should also be studied in an effort to determine any side-effects that might be caused by ICES. Dose-dependence may shed light on the potential mechanisms as certain pathways are more effective at reducing swelling than others. If ICES can be further studied and understood, it may provide an alternative therapy for those requiring anti-inflammatory therapy while also furthering the understanding that we have of basic human physiology and electrophysiology.

CHAPTER 4 : ICES DOSE-RESPONSE IN CFE REDUCTION

Overview

Previous work has shown that a significant reduction in inflammation can be achieved by using low-dose inductively coupled electrical stimulation (ICES) in the acute carrageenan footpad edema (CFE) model. Presently we seek to investigate the possibility that ICES may have a dose-dependent response. We treated CFE-induced rats with 40, 80, 120 and 160 T/s ICES treatment provided by a custom-made stimulation unit meant to mimic sub-threshold in-vivo electrical patterns. Significant reduction in CFE at various sample times was observed in ICES treatment groups. Significant reduction in 8-hour disease burden was observed in all ICES treatment groups and significant 4-hour disease burden reduction was observed in all but the 80 T/s ICES treatment groups. While significant differences were not observed between treatment groups in terms of inflammation reduction, the data trends towards a dose-dependent response for ICES. Our data and observations herein confirm that ICES holds the potential as an extremely effective anti-inflammatory treatment that ameliorates the need for ingested, liver-dependent anti-inflammatory drugs.

Introduction

Our previous research, and the research of others, has strongly suggested that ICES is an emerging therapy that provides significant inflammation reduction^{1,3,10,11,22,49,56,64,106,107}. Specifically it has been shown that ICES, using the appropriate stimulation parameters, can provide significant reduction of inflammation in the acute rat CFE model. Such reduction in

inflammation has been achieved with levels as low as 40 T/s peak field strength. ICES is a poorly understood and researched therapy—a vast majority of the literature fails at least one of the fundamental requirements of the scientific paradigm. The two most frequently observed downfalls of ICES research in our observation are improper or non-existent controls and failure to provide repeatable results¹. For the very reason that the majority of ICES research has been poorly handled by the scientific community on the whole, there is a large amount of doubt and healthy skepticism that has emerged over time in the general populations' understanding of electromagnetic therapies. Thus, we have elected to perform research in as controlled a manner as possible, to the highest achievable scientific standards, so as to eliminate any bias, errors, or confounding variables that could negatively affect the quality of our measurements. In order to achieve such high quality research, the very well-established rat CFE model was chosen as the acute model of inflammation to study. Furthermore, expertise in working with the CFE model was sought out and the experiments run locally at a contract research organization with significant CFE experience.

The CFE model is a very well established method for studying acute inflammation. The model operates by injecting lambda carrageenan into the hind foot pad of adult Sprague-Dawley rats. The injection causes a biphasic response: an early, trauma-induced, non-phagocytic immune response (NPIR) and a later, phagocytic immune response (PIR). The NPIR is hypothesized to result from the trauma associated with the injection, while the PIR is associated with the immune response caused by the carrageenan itself^{83,84}. The PIR is marked by infiltration of neutrophils into the tissue, swelling, edema, redness and pain. Additionally, the CFE model responds reliably and repeatably to steroidal and non-steroidal anti-inflammatory drugs (SAIDs and NSAIDs) which block the arachadonic acid pathways

associated with COX-1 and COX-2. Thus, a positive control can be implemented as a comparison for inflammation reduction. The CFE model is an acute model that spontaneously resolves in most animals over the course of 8-12 hours—with peak CFE inflammation occurring around 4 hours in most cases.

To test the efficacy of ICES in reducing inflammation in the CFE model, it is important to consider the potentially effective stimulation protocols. ICES protocols are even more poorly understood from a mechanistic standpoint than from an efficacy standpoint. Thus, in determining potentially effective treatment regimes, we explored the gambit of physiologically relevant signals that might be produced. Tissues in the body are constantly exposed to a variety of internally and externally developed electromagnetic fields. Some of these fields are strictly electric (DC E-fields), some are strictly magnetic (DC B-fields) and some are electromagnetic (A/C B-fields and E-fields). If one removes the veritable ocean of man-made electromagnetic stimulation provided by information and power transmission, one is left with only a small number of externally generated stimuli to which the body is exposed: Earth's magnetic field, electric fields generated by natural phenomenon (lightning, etc.), light from the sun and high-energy cosmic rays. Evolution naturally selects for physiological development that favours those who can survive in such conditions. Thus, it follows that the body should either ignore the signals completely, or use them to adapt. Given that there is little evidence to suggest that, with the exception of light from the sun, the body uses any of the aforementioned signals to survive or adapt, one must turn to the electromagnetic stimuli developed internally as a potential source to which tissues might respond. It is well established that in the absence of signals perceived as “normal,” tissues will begin to shift from homeostasis. In extreme conditions, such as loss of efferent nerve connection to a

muscle, tissues can begin to atrophy or even necrose in the absence of normally perceived signals. Most internally developed fields are set up by nerves, muscles and bones in the body^{108,109}. Such internally evolved fields can be considered to be a part of the homeostatic conditions in which tissues exist under normal, healthy conditions. Striving to maintain homeostatic electrical conditions would thus seem to be a potential method to reduce inflammation as it could help “trick” tissues into experiencing normal conditions and reduce the quantity of pro-inflammatory factors released or recruited. Therefore, our stimulation regimen is based upon field intensities and durations that are known to be native in the in vivo scenario while generally being absent from natural external ambient electro-magnetic fields.

There is very little rational basis upon which to suggest that a static magnetic field should have any effect on tissues. Specifically, the only physical arguments that might be made for a static magnetic interaction would, by necessity, be based on para and ferromagnetic particles in the body, the Hall Effect, or modification of Larmor precession. Paramagnetic and ferromagnetic materials do exist in the body. In fact, some complex animals are hypothesized to have specific structures to explain their ability to orient and navigate along the earth’s magnetic field lines by means of magnetoreception. Some microorganisms such as magnetotactic bacteria are known to harbour particles specifically to allow organization and orientation along magnetic field lines. However, there is very little literature to suggest that musculoskeletal tissues or other structural tissues of the body interact strongly enough with magnetic fields to produce measurable effects. Furthermore, there is no evidence to assume that tissues require static magnetic stimulation in order to survive—this is partially because no observations have been made that suggest that there are

any negative effects of removing a human from the earth's magnetic field. Furthermore, the body does not generate its own magnetic fields on a large enough scale that they would be significant as compared to thermal noise or the earth's magnetic field. There is a large amount of evidence that suggests that tissues require and generate their own electric fields in order to function and survive. From a physics standpoint, there are two simple ways of developing an electrical field: by direct (capacitive) electrical stimulation and by induced (inductive) electrical stimulation. Capacitive fields are prone to dangerous arcing, manipulation by metallic and conducting objects and pose the issue of providing current flow along a path of least resistance: this can cause over stimulation of a small area of tissue and under-stimulation of the large volume of surrounding tissues. Inductive methods, however, make use of rapidly changing magnetic fields—a type of magnetic stimulation by which any charge conducting materials could potentially be affected. Specifically changing magnetic fields have the ability to induce an electromotive force that can drive a current and produce an electric field. Thus, it is possible to introduce electric fields into the body by using inductive methods: this is precisely the theory of operation for ICES.

Materials and methods

Animals

This study made use of 6-8 week old adult male Sprague-Dawley rats that were given access to food and water ad libitum. Animals were housed in modified cages that were approved for use by an IACUC. Briefly, cages contained an insert that reduced livable floor area to 4"x 16"—this design allowed for enough room for an animal to move properly, while also providing a controlled space in which to achieve appropriate ICES dosing (Figure 4.2).

Animals were housed with one animal per cage in order to ensure comfort and room to turn around. At the termination of the study, animals were anesthetized to collect sample tissues according to IACUC-approved protocol.

Figure 4.1: Image of the mat style ICES treatment unit. (Top) A cartoon describing the direction of current flow in coils. Dots represent magnetic field lines pointing “out” of the page; x’s represent magnetic field lines pointing into the page. (Bottom): a photograph of the ICES mat setup showing driving electronics on the left and the mat surface to the right.

Figure 4.2: Depiction of cage-insert style ICES stimulation unit. (Top left) A 3-D representation of the cage inserts for the ICES units. The oblong disks represent coils or dummy coils used in the experiment. The animal is housed between the two plates upon which coils are mounted. (Top right) An actual image of the ICES insert used to provide high-dose treatment. (Bottom left and right) Side and top views of the actual ICES cage insert used to deliver treatment to CFE animals.

ICES treatment units

ICES was provided by two different types of setups. One was an external mat that was designed to provide even, low-dose stimulation (40 T/s) over the surface of the base of the cage (Figure 4.2). In this first setup, cages rested on top of the stimulation mat and an insert was placed into the cage to provide volume control that was the same as the volume control in the higher dose units. The higher dose units contained an insert that housed all electronics and stimulation coils (Figure 4.2). The high-dose units were capable of delivering stimulation levels of 40, 80, 120 and 160 T/s. Due to space and time restrictions, the high-

dose units were used only for the 80, 120 and 160 T/s treatment groups. The treatment units delivered three types of pulse stimulation protocols. These three protocols are shown in Figure 4.3.

Figure 4.3: Graphical representation of the electrical stimulus travelling into the ICES coils used in this study. Figure is not to scale. Pulse widths are 100 μ s wide.

Treatment groups

Animals were divided into 10 treatment groups, summarized in Table 4-1 below. One hour prior to CFE induction, animals were exposed to their respective treatment. Negative control was provided by a *per os* (PO) dose of phosphate buffered saline (PBS). Positive control was provided by a 3 mg/kg dose (PO) of dexamethasone. ICES treatment was provided at four various intensities: low (40 T/s), Medium (80 T/s), High (120 T/s), and Extra High (160 T/s). Groups are displayed in Table 4-1 below which summarizes groups and dosing. Animals were exposed to their respective treatments one hour prior to CFE

induction—ICES stimulation continued throughout the duration of the experiment. It should be noted, that for groups 7-10, the experiment was terminated at the 4 hour time point to provide a comparison to the 8 hour time point for the ICES low and ICES extra high animals.

Table 4-1: Dosing chart for CFE dose-response study.

Group #	n	Treatment	Dose	Route
1	10	PBS	N/A	PO
2	10	Dexamethasone	3 mg/kg	PO
3	10	ICES Low	40 T/s	External
4	10	ICES Medium	80 T/s	External
5	10	ICES High	120 T/s	External
6	10	ICES Extra High	160 T/s	External
7	6	PBS	N/A	PO
8	6	Dexamethasone	3 mg/kg	PO
9	6	ICES Low	40 T/s	External
10	6	ICES Extra High	160 T/s	External
Totals:	84			

CFE induction

One hour post-treatment, carrageenan footpad edema was induced in the right hind-paw of adult rats by injecting 0.1 mL of 1% carrageenan in saline. The contralateral limb was used as a control and 0.1 mL of saline was injected into the footpad.

Plethysmometry measurement

A plethysmometer (PVP1001, Kent Scientific Corporation, Connecticut) was used to measure change in paw volume as an indicator of inflammation. Inflammation from CFE is reported as a difference between right and left paw volumes for each animal. Plethysmometry measurements were taken prior to carrageenan administration (0 hours), and then 1, 2, 4 and 8 hours post-carrageenan administration according to methods established by the Charles River Laboratories (Wilmington, MA).

Tissue collection

At the end of the final time point (8 hours for groups 1-6 and 4 hours for groups 7-10), animals were anesthetized and tissues were collected. Both hind paws were collected for animals 1-6 in each of the groups. Paws from animals 1-3 in all groups were stripped of skin and fixed in 10% NBF for subsequent histology while paws from animals 4-6 in all groups were snap frozen in liquid nitrogen and stored for future RNA analysis. After a minimum of 48 hours of fixation, NBF fixed paws were transferred to 20x paw volume of Immunocal (Decal Chemical Corporation, Tallman, NY) for decalcification.

Histology

Upon completion of decalcification, paws were mounted in paraffin and sectioned in the sagittal plane. Three sections from the center of the foot, each 100 μ m apart, were mounted on slides and stained with H&E for standard light microscopy analysis. Sections were visually inspected at high magnification and evaluated for cellular infiltration.

Results

Results of the CFE study are summarized in the tables and charts below. Previous work has established that the peak time for inflammation differences between treatment groups are the 4 and 8 hour time points. Thus, groups 7-10 were terminated at 4 hours to provide limbs for RNA and histology comparison, while groups 1-6 were terminated at 8 hours in order to provide full time-course CFE profiles.

Plethysmometry

Edema (i.e. CFE) was calculated as the difference between right and left paws for each animal at each time point. A plot of the 4-hour and 8-hour CFE time-courses are shown in Figure 4.4 and Figure 4.5 respectively. Significance is based on standard ANOVA.

Figure 4.4: 4-hour CFE time-course summary for PBS, Dexamethasone, 40 T/s (ICES Low), and 160 T/s (ICES X High). Significance based on two-tailed t-test as compared to PBS measurement at the same time point. Significance: *=P<0.05, **=P< 0.005, *=P<0.001.**

Figure 4.5: 8-hour CFE time-course summary for PBS, Dexamethasone, 40 T/s, 80 T/s, 120 T/s and 160 T/s ICES doses. Significance based on two-tailed t-test as compared to PBS measurement at the same time point.

From the 4 and 8-hour time courses, one can calculate the total disease burden as the integral under the CFE time-course curve. This calculation is performed as a discrete integral using the average value between time points (Middle-sum discrete Riemann integral). It should be noted that each data point in a CFE curve is corrected by subtracting the baseline initial CFE volume for the given treatment from all time points in the respective treatment group. Plots of the total 8-hour and 4-hour disease burden are shown in Figure 4.6 and Figure 4.7 respectively. By comparing disease burdens to the PBS negative control, disease suppression can be calculated. Disease suppression (Figure 4.8) is calculated as the percent difference between the given treatment and the PBS control. The explicit calculation is given as:

$$DS = \frac{DB_{PBS} - DB_x}{DB_{PBS}} = 1 - \frac{DB_x}{DB_{PBS}} \quad (3)$$

Figure 4.6: 8-hour total disease burden for groups 1-6. Disease burden represents the base-line corrected area under the CFE curve for each group. Lower disease burden values correspond to a more positive outcome. Groups (left to right): PBS negative control. Dexamethasone positive control (3 mg/kg). Low dose ICES (40 T/s). Medium ICES (80 T/s). High dose ICES (120 T/s). Extra High dose ICES (160 T/s). Significance: *= $P < 0.05$, **= $P < 0.005$, *= $P < 0.0005$ as compared to PBS negative control. += $P < 0.05$, ++= $P < 0.005$ as compared to Dexamethasone positive control.**

Figure 4.7: 4-hour Disease Burden Summary. Disease burden represents the base-line corrected area under the CFE curve for each group. Lower disease burden values correspond to a more positive outcome. Significance: *=P<0.05, ** P<0.005, ***P<0.0005 as compared to PBS negative control. +=P<0.05 as compared to Dexamethasone positive control.

Figure 4.8: Disease suppression, calculated from disease burden, as compared to PBS negative control for each group in the 8-hour CFE time course. Groups (left to right): Dexamethasone (3 mg/kg) positive control, Low dose ICES (40 T/s), Medium dose ICES (80 T/s), High dose ICES (120 T/s), Extra high dose ICES (160 T/s). Larger values are considered a more positive outcome.

Histology

Below are representative histological samples from 4-hour time points of the CFE study (Figure 4.9). Right hind paws represent carrageenan injected paws, left paws are self-controls that were injected with saline.

Figure 4.9: Sagittal slice of a dexamethasone positive control right hind paw stained with H&E. A) View of the entire sagittal section of the paw with the heel facing to the right of the image. B) Close-up of the foot pad section showing skeletal muscle and connective tissues. C) Magnification of close-up in (B) showing dark stained nuclei, red stained muscles and connective tissue showing slight infiltration is seen in this section.

Figure 4.10: Sagittal slice of a PBS negative control right hind paw stained with H&E.

A) View of the entire sagittal section of the paw with the heel facing to the right of the image. B) Close-up of the foot pad section showing skeletal muscle and connective tissues. C) Magnification of Close-up in (B) showing dark stained nuclei, red stained muscles and connective tissue having distinct and heavy neutrophil infiltration in the footpad.

Figure 4.11: Sagittal slice of a 40 T/s ICES treatment right hind paw stained with H&E

A) View of the entire sagittal section of the paw with the heel facing to the right of the image. B) Close-up of the foot pad section showing skeletal muscle and connective tissues. C) Magnification of Close-up in (B) showing dark stained nuclei, red stained muscles and connective tissue having distinct and heavy neutrophilic infiltration.

Figure 4.12: Sagittal slice of a 160 T/s ICES treatment right hind paw stained with H&E. A) View of the entire sagittal section of the paw with the heel facing to the right of the image. B) Close-up of the foot pad section showing skeletal muscle and connective tissues. C) Magnification of close-up in (B) showing dark stained nuclei, red stained muscles and connective tissue as well as very heavy neutrophilic infiltration.

Figure 4.13: Sagittal slice of a dexamethasone positive control left hind paw stained with H&E. A) View of the entire sagittal section of the paw with the heel facing to the right of the image. B) Close-up of the foot pad section showing skeletal muscle and connective tissues. C) Magnification of close-up in (B) showing dark stained nuclei, red stained muscles and connective tissue showing little to no neutrophil infiltration.

Discussion

The plethysmometry data supports our hypothesis that the biological response to ICES treatment is dose-dependent. Specifically, it appears as if increasing ICES dose

provides increasing inflammation reduction. It is difficult to conjecture without further research whether or not the dosing plateaus at a particular value. However, it is clear that there are significant reductions in inflammation using sub-threshold stimulation provided by ICES. From the CFE data plots, it is clear that the highest ICES doses provide consistent statistically significant reductions in inflammation as early as 2-hours into treatment which persist throughout the remainder of the experiment. Furthermore, it is also observed that even the lower ICES doses (40 and 80 T/s) are able to produce statistically significant reduction in inflammation as early as 2-hours into the experiment—however because of the large variance and smaller relative difference in mean CFE from the PBS control, it is hard to consistently observe statistically significant differences in CFE reduction. An interesting consideration to make with regards to the efficacy of ICES is the time point at which efficacy may first be observed. It is well understood that there are two phases of inflammation associated with CFE: the first, immediate inflammatory response is associated with the trauma of injecting carrageenan. The second, delayed response is associated specifically with the immune response to the carrageenan, and is marked by increased neutrophil infiltration. Because the contralateral limb was used as a self-control for each animal and was injected with saline, the trauma associated with injection is theoretically subtracted from the CFE. Thus, our observations suggest that we are specifically able to inhibit the inflammation associated with the acute immune response—this gives insight as to the potential mechanisms. Further investigation is required to determine exactly which pathway is being inhibited or stimulated. ICES stimulation is designed as a means of mimicking the natural electrical signals that tissues normally see, thus it is a distinct possibility that normally unregulated pathways are in fact inhibited. A corollary to such a general pathway is that ICES treatment should only be

effective in situations where a tissue is under irregular physiological conditions (i.e. pathological states). Such a general pathway could also imply that the effects of ICES would be naturally asymptotic towards a value higher than dexamethasone. The reason for such an asymptotic behaviour is that the effect would not begin until the pathology had started to manifest. This situation is analogous to proportional feedback control in an electronic system—the result is that the controller reaches an equilibrium point that is different from the target value. However, this model is simply a conjecture and is based solely on supposition. Perhaps a more telling metric for overall inflammation reduction is the effect of ICES on the total disease burden.

The 4-hour disease burden indicated that all treatment groups, except 80 T/s ICES, provided statistically significant reduction in disease burden. Over the course of 8 hours, disease burden is significantly different for all ICES treatment groups as compared to the PBS negative control. Both the 4-hour and 8-hour outcomes provide strong evidence to suggest a dose response. There is no statistical significance between the ICES treatment groups, however they do trend toward a dose-response with higher doses providing a more effective treatment. The lack of difference between the 120 and 160 T/s stimulation does suggest the possibility that there is a dose plateau where additional stimulus would not provide a significantly improved outcome—however further research would be required to support such a claim.

The disease burdens for the 120 and 160 T/s treatment groups are not significantly different than the dexamethasone positive control but they do indicate a trend towards being different. If sufficient data were available and perhaps more information available regarding the effects of even higher intensity ICES treatment (above 160 T/s), a solid conclusion could

be drawn regarding existence of a dose response. Furthermore, if the response of ICES does plateau before becoming equivalent to the inflammation reduction provided by dexamethasone, the implication would be that the ICES may work via a different pathway than dexamethasone, that the same pathways are less efficiently activated, or that ICES operates via some combination of mechanisms including those involved with dexamethasone. A more appropriate method for beginning to determine a mechanism is to observe histology.

The histological sections obtained from the experiment were stained with H&E, thus marking cells and nuclei. Dexamethasone is known to stop the production of arachadonic acid which leads to downstream reduction in both the cyclooxygenase pathway (associated with production of prostaglandins and thromboxanes), as well as the lipooxygenase pathway (associated leukotriene production and increased neutrophilic infiltration). From the images, it can be seen that the dexamethasone treated groups had visibly reduced infiltration as compared to the PBS treatment group. However, it appears as if the ICES treatment groups do not have significantly reduced infiltration as compared to controls.

ICES has repeatedly shown significant inflammation reduction in the acute inflammation model. Our results herein suggest the possibility of a dose dependent mechanism by which ICES may work while confirming that ICES provides significant inflammation reduction. The histological sections collected do not strongly implicate that ICES is capable of reducing neutrophil infiltration. Since one of the most strongly involved in promoting neutrophil chemotaxis is the LOX pathway, it seems unlikely that ICES strongly affects LOX. Furthermore, if LOX is not inhibited, then the arachadonic acid pathway must also not be affected. Specifically, phospholipase must remain largely

unaffected if the neutrophil levels are not significantly affected. It is possible that ICES is capable of modulating levels of nitric oxide, as has been suggested in the literature. Other possible mechanisms include reduction of platelet activating factor and cyclooxygenase pathway inhibition. Future research should focus on isolating which pathway(s) may be inhibited or up-regulated by the effects of ICES by monitoring local and systemic factors involved in inflammation. Regardless, ICES promises the potential to serve as an effective means of treating inflammation without the use of pro-drugs that can tax the liver or steroids which can cause undesirable long-term effects.

CHAPTER 5 : SUMMARY, DISCUSSION AND FUTURE WORK

The results of the experiments presented herein clearly support the claim that ICES holds the potential to ameliorate many inflammatory ailments. Presently it has been shown that ICES in doses of 40, 80, 120 and 160 T/s can be effective in significantly reducing acute inflammation. The models used in these experiments are very well established as reliable methods for gauging anti-inflammatory efficacy. The same models used herein are extensively used within the pharma-industry for early screening of potential new anti-inflammatory drugs. The question of the underlying mechanism, however, still remains somewhat elusive. As compared to dexamethasone, ICES does not seem to have nearly as strong an anti-inflammatory effect. Dexamethasone is known to act by inhibiting the arachadonic acid pathway, which subsequently inhibits lipoxygenase and cyclooxygenase pathways, both of which have very strong effects on inflammation. ICES, however, acts via an unknown mechanism. Previous authors have reported strong evidence to suggest that ICES delivered via PRF acts by modulating calcium binding interactions with calmodulin^{7-9,18,19}. The studies conducted herein were unable to replicate previous in vitro studies that implicated the Ca/CaM mechanism; however, it is worth noting that the ICES protocols used in the present studies made use of a different stimulation protocol.

The question then must be asked as to why the results of the present ICES in vitro studies did not concur with previous literature with regards to mechanism. First, and perhaps most obvious, is the fact that previously reported results made use of the Griess reaction, which is inadequate for detecting the low levels of nitrite reported in the literature, bringing

those reported results into question. However, in an effort to exactly replicate previously published results reported in literature, the studies herein used the same reaction and noted no significant differences in nitrite levels across any of the study groups. A second possibility for the disparity is that the stimulation parameters are different enough that perhaps the ICES protocols studied herein act via a different pathway. While a disparity in ICES protocols is possible, it does not fit with the working theory in the literature which states that tissues integrate signals and require rapidly changing magnetic fields to induce electric fields. Thus, the PRF used by previous authors should cause similar effects to the ICES protocols used in the studies herein presented. Finally, it is possible that the present work or previous work was done incorrectly—thus it is important that the present in vitro studies eventually be repeated with a more appropriate measure of NOS activity in order to verify previously reported mechanistic theory.

In conjunction with the in vitro studies presented, in vivo experiments were performed. These experiments were able to repeatedly demonstrate significant and consistent reduction in swelling in rats run through a CFE challenge. While significance was not established from a dose-response standpoint, there is strong evidence to suggest that there is trending toward a dose-dependent response for the ICES stimulation protocols used herein. An increase in sample size and the use of higher dosages than those used in the present studies could help establish whether or not there is a dose-dependent response seen with ICES stimulation. Based on the data collected it can also be suggested that ICES efficacy may begin to asymptotically approach a plateau at doses above 120 T/s. Additionally, the disease burden reduction at the potential plateau doses is less than that observed from dexamethasone positive controls, suggesting that ICES either acts via a different mechanism,

that it is less effective at stimulating or inhibiting the same pathways as dexamethasone, or that it has other as-yet undetected effects that manifest only partially as reduced inflammation while operating simultaneously through unrelated mechanisms to reduce pain and accelerate healing. In order to completely differentiate ICES mechanism from dexamethasone, drugs could be administered that might block the efficacy of ICES but not dexamethasone. For example, L-NMMA could be injected locally with carrageenan. If ICES acts via a NOS-dependent pathway, it might be expected that the efficacy of L-NMMA treated groups would decline as compared to dexamethasone or non-L-NMMA groups given ICES.

Regardless, ICES holds the potential to drastically and safely improve the lives of many who suffer from chronic inflammatory diseases. Future work should focus on three main aspects of ICES: Applications, elucidation of the underlying mechanism and safety.

From a safety standpoint, ICES delivers an extremely low-power signal to tissues such that there have been no noticeable thermal effects. The other two areas of potential safety concern involve the effect of ICES on sepsis and the generation or exacerbation of tumor growth. For the aforementioned reasons, studies on septic exacerbation, tumorigenicity and tumor growth should be carried out. If ICES promotes homeostasis, and if it acts via a mechanism that suppresses the immune response, then it is important to understand the potential effects on tumor growth. Furthermore, if tissue growth is accelerated by ICES, then it is important that clinicians be aware that carcinomas would present a contraindication to ICES application. Needless to say, ICES should be tested for safety prior to deployment in the clinical setting.

Specific applications will become apparent as mechanism and efficacy is elucidated. For example, one specific experiment that should be conducted to determine maximum efficacy would be to study the different possible ICES pulse parameters available that still provide the same dosing amplitudes as used in the experiments presented herein. For example, one might consider differentiating which of the three protocols, if any, used in the experiments above is more effective than the others. Alternatively, increasing the pulse frequency while maintaining amplitude dosing would provide valuable information regarding the power dosing of ICES.

For wound healing studies, an animal experiment could be conducted to study the formation of myofibroblasts in vivo in the presence of ICES. One would be interested in studying the rate at which myofibroblasts are formed, as well as the rate at which α -SMA is produced by the myofibroblasts. Markers for both myofibroblast and α -SMA formation are well documented in literature^{97–99,102,103}, and could be tracked throughout an experiment using stained biopsies. Such an experiment would likely use mice or rats. A surgically induced incision wound of known length and depth would be made in a location of the animal that could be easily monitored, and inaccessible to the animal. Four treatment groups could be created: Control (receiving no treatment), dexamethasone, ICES and ICES+dexamethasone groups. ICES therapy would be provided by instrumenting cages with appropriate coils to provide ICES stimulus approximately equal to those used herein. Wound healing rate would be monitored in several ways. At the biochemical and cellular levels, wound exudate could be harvested and H₂O₂ production could be monitored using Ampex Red as described by Zhou¹¹⁰. Biopsies taken from wound sites could be examined for rate and extent of formation of myofibroblasts. Additionally, histology could also be used to

determine the amount of α -SMA produced by said myofibroblasts. An increase in H_2O_2 would almost certainly imply an increase in intracellular Ca^{2+} levels—an important observation to correlate with wound healing rate. NO levels could also be monitored in wound exudate using available fluorometric kits from Cayman Chemical. Other inflammatory factors could be monitored in similar ways such as described by Rohde et al¹⁰. Wound healing rate could be assessed physically by measuring the size of the wound daily (or hourly if necessary) as well as monitoring inflammation (tumor), redness (rubor) and IR temperature (Calor), three of the five classic, clinical hallmarks of inflammation. If the aforementioned mechanisms are true of ICES, then one would expect that ICES could enhance the wound healing rate, and possibly reduce scar formation. In order to examine specific pathways, inhibitors of specific enzymes (such as eNOS) could be added to selectively inhibit specific steps in the proposed mechanism. L-N-monomethyl arginine (L-NMMA) is a common inhibitor of eNOS and nNOS, and could be added to test whether NO production in the cells (NOT mitochondria) affects wound healing in the presence of PEMF or dexamethasone.

Perhaps the most revealing wound healing experiments to perform would be to modify those performed by Hinz et al^{97,98}. Collagen or silicone gels of varying stiffness could be made in order to produce the necessary environment for hosting primary lung and subcutaneous fibroblasts. Previous experiments have shown that there is a marked difference in wrinkle formation between high α -SMA (lung fibroblasts) and low α -SMA (subcutaneous fibroblasts) staining cells on high-stiffness gels⁹⁸. It has been shown that α -SMA contraction strength is limited by calcium levels, however, it has also been shown that if the Ca^{2+} concentration is changed transiently, that the force developed also changes transiently⁹⁹.

Additionally, it has been shown that myofibroblast formation can be induced in vitro using TGF- β 1—thus if an appropriate model could be developed, then myofibroblast contraction force could be measured directly in vitro. All of the same assays could be carried out for the collagen/silicone matrices as were done for the in vivo studies. Specifically, H₂O₂ production, NO production, and α -SMA staining could all be assessed. Furthermore, micrographs of collagen and silicone matrices could be examined for deformation and quantified optically or using ESEM. Finally, a measure of cellular contraction could be useful to determine the forces imparted on the fibroblasts themselves. Specific inhibitors of enzymes could be added as challenges to determine exact pathways. Treatment groups for therapy could be divided into groups as in the in vivo studies: negative controls (pathway inhibitory drugs), positive controls (pathway stimulatory drugs), and ICES. According to Tomasek et al, the approximate force developed across a collagen matrix of an appropriate length (unfortunately, the relevant size info is not given, making the stress/strain impossible to determine) by protomyofibroblasts and myofibroblasts rises from 0 to approximately 200 dynes (2 mN). This force can readily be measured using an appropriate strain gauge⁹⁹. Various challenges, such as calcium, nitric oxide, as well as inhibitors could be added in order to examine the effects of various pathways on the force developed. If ICES does operate via calcium-dependent mechanisms, then one might expect that in the presence of low-calcium levels, contraction strength of the myofibroblasts would increase measurably in the presence of ICES as compared to negative controls.

From a neurological standpoint the first, and perhaps most obvious, experiment to test peripheral interaction of ICES with neurons would be direct electrode measurements. Under conditions of ICES, direct measurement of resting membrane potential, depolarization

potential, and the characteristics of each including repolarization could be measured. A change in the rate of repolarization, depolarization or resting membrane potential could then be directly detected for both myelinated and unmyelinated cells. Patch clamp experiments could also be used to elucidate the behaviour of ion channels in the presence of ICES. Direct electrical measurements could also be taken centrally—however since one might be interested in whole groups of neurons, imaging might be a more appropriate modality for studying central mechanisms.

Several imaging experiments come to mind when considering peripheral ICES and the effects on the CNS. First, fMRI, PET and optical imaging could all be used to explore stimulation of peripheral nerves in animals and humans. By stimulating far enough away from the imaging unit (for example, on the foot), one could examine the effects of ICES on the brain as a whole. If nociceptive nerve activation was being studied, then an experiment on squirrel monkeys could be done using ICES in conjunction with a nociceptive stimulus on the hand. Because previous studies have shown very specific interactions between areas 3b/1 and 3a with respect to nociceptive afferent input⁸⁷, one could easily compare results. Furthermore, a contralateral limb (or perhaps even the same limb) could be used as a control to examine the differences between ICES and non-ICES stimulated nociceptive and mechanical stimuli. If, for example, ICES mostly affected un-myelinated nerves, then that might explain the apparent reduction of pain in patients suffering chronic pain (often associated with C fiber or central mechanisms). Activation or inhibition in area 3a might be indicative of such a mechanism if it was seen under ICES stimulation conditions while delivering a noxious stimulus to an animal. Furthermore, if behaviour in 3b/1 was unchanged, it might suggest that myelinated nerves are relatively unaffected by ICES.

In the case of ICES, since it is applied peripherally, it is essentially implied that central mechanisms will only play a role inasmuch as they provide processing and feedback of the peripherally stimulated/modulated neurons. Thus, conditions that are hypothesized to be centrally mediated, such as fibromyalgia, epilepsy, stroke-induced pain, multiple sclerosis pain, and spinal cord injury pain, are unlikely targets for ICES therapy. However, clinical anecdotal data indicate that ICES is very effective at reducing or eliminating idiopathic chronic pain. These data raise the possibility that central mechanisms for interpreting pain signals could be modulated or even reset by peripheral and focal use of ICES therapy. It is possible that fibromyalgia could be affected by modulating the peripheral signals that are processed in areas that affect the locations in the brain that are responsible for fibromyalgia pain—however, the exact mechanism(s) of fibromyalgia is not understood. There is evidence to suggest that neurogenic urinary incontinence could be a target for therapy (unpublished data from Dennis). Application of ICES to the spinal cord region aggravated by inflammation has helped relieve urinary incontinence in dogs suffering from arthritis—typically these animals have other co-morbidities such as difficulty walking. Interestingly, the comorbidity is typically also cured with application of ICES. The exact mechanism by which ICES is effective in these situations is still not understood—perhaps being both neurological as well as anti-inflammatory in nature. Central lesions or ablations would not likely be candidates for ICES treatment as in the situation where 3b/1 is destroyed but 3a spared unless the mechanism of action for ICES results in a net attenuation of C fiber activity.

Peripherally, however, ICES has been shown to be effective in pain management and wound healing for both acute and chronic injuries^{1,7}. Potentially treatable conditions include: wound healing after surgery, chronic pain resulting from inflammatory conditions such as

arthritis, and neuropathies resulting from inflammation. An interesting case to consider would be phantom limb syndrome. Since phantom limb is hypothesized to be a centrally mediated condition, it is unlikely that ICES stimulation of a stump would have any significant pain-reducing effects, however, in cases where peripheral nerves are firing inappropriately and leading to pain, ICES could potentially be a way to reduce phantom limb pain.

In terms of future inflammatory studies, several important experiments should be performed. As ICES is a candidate for treating and relieving vast numbers of individuals suffering from chronic pain, it is of utmost importance to conduct a chronic inflammatory study. It is very well understood that different markers and pathways become involved if acute inflammation is left unchecked and leads to chronic inflammation. A well-established model of chronic inflammation is the rat collagen-induced arthritis model (r-CIA). Such a model uses an injection of foreign collagen to induce chronic arthritis in rats. The histological, and systemic markers are well studied in the r-CIA model, allowing it to be a potentially invaluable model for examining the ability of ICES to relieve chronic inflammatory situations. Obviously positive results would strongly indicate the use of ICES for individuals suffering from arthritis, as well as many other chronic inflammatory conditions.

A second inflammatory study to be considered is to examine the efficacy of ICES in conjunction with other anti-inflammatory agents. Currently ICES is employed in addition to drugs as a mechanism by which to assist with spinal fusion surgeries in patients at high risk for non-fusion. ICES may be a useful treatment in conjunction with commonly available NSAIDs such as naproxen, aspirin, and ibuprofen. Patients suffering from ailments such as

carpal tunnel syndrome, or those suffering sports-related injuries might find relief and healing from using ICES in conjunction with NSAIDs. Acute studies could be performed using the CFE model to test for the efficacy of NSAIDs with the addition of ICES as a treatment supplement. Particularly if ICES can augment NSAID efficacy, the implications would be twofold. First, in cases where high NSAID dose is required, ICES could be used in conjunction to provide additional alleviation. Furthermore, in patients suffering disorders preventing the safe metabolism of pro-drugs (such as those with liver enzyme dysfunction), it is possible that ICES could be used to lower the doses of NSAID necessary to maintain relief.

Herein it has been repeatedly and reliably shown that ICES of the appropriate stimulation parameters has the ability to significantly reduce acute inflammation. Further research holds the promise of expanding the realm of applications for ICES from the acute to chronic conditions. ICES holds the potential to reduce inflammation, healing time, scar formation and pain in many situations. If applied correctly, researched correctly and documented properly, ICES may revolutionize the way in which modern medicine is conducted.

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