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HUMAN OSTEOBLAST PROLIFERATION IN CULTURE FOLLOWING A NANOSECOND PULSED ELECTRIC FIELD (nsPEF)

by

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A Thesis Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirement for the Degree of

MASTER OF SCIENCE

BIOLOGY

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May 2010

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ABSTRACT

HUMAN OSTEOBLAST PROLIFERATION IN CULTURE FOLLOWING
A NANOSECOND PULSED ELETRIC FIELD (nsPEF)

Leonard Joseph Carinci, Jr.
Old Dominion University, 2010
Director: Dr. R. James Swanson

Osteoblasts are mononucleate bone forming cells responsible for the deposition of new bone. Application of mechanical stress on bone reveals its ability to produce and release electric potentials across the cell membrane called piezoelectricity. The electric potentials produced in response to mechanical stress may have a direct correlation on osseous cells and the signaling pathways that regulate proliferation. Nanosecond pulsed electric fields (nsPEFs) are high intensity, ultrashort pulses which have the ability to maintain the integrity of the cell membrane by avoiding traditional electroporation. We delivered 8 nsPEFs (0.5 Hz) of a 25 kV/cm or 35 kV/cm electric field strength for a 60 ns duration while maintaining the viability of the cell which allowed us to measure the subcellular effects of these pulses on enhancing osteoblast proliferation rates (mitotic activity) in culture. The use of flow cytometry along with a specialized cell tracing dye allowed computerized analysis of cell proliferation rates over a seven day post pulse period. The results indicate a significant difference between treatment and control groups in the percentage of divided cells (25 kV/cm: $p \leq 0.011$; 35 kV/cm: $p \leq 0.002$). As the electric field was increased the percentage of osteoblast division decreased. The experimental results will allow future researchers to fine tune pulse duration and intensity when studying the cell signaling mechanisms involved in osteoblast proliferation rates.

I dedicate this thesis to my family with great pride, for all their love, support and encouragement throughout all my academic years. Thank you all for believing in me and giving me the strength to accomplish my goals. You have all played a part in making me into the man I have become today and for that I will be forever grateful.

ACKNOWLEDGMENTS

I would like to express many thanks to my academic advisor and mentor Dr. R. James Swanson for all his support, assistance, and guidance that he provided to me while completing my graduate degree. I appreciate all the knowledge he has passed down to me and I will never forget this experience. In addition, I would also like to thank my committee members for their continued support during my time at Old Dominion University; Dr. Christopher Osgood and Dr. Juergen Kolb. I wish to extend my thanks to the entire staff of The Frank Reidy Center for Bioelectrics for all their input, assistance and patience put into my thesis research.

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INTRODUCTION

The Anatomy of Bone

The adult human body has 206 bones that have a functional role in maintaining the structural integrity of the skeletal system. The other main components of the human skeletal system include cartilage and ligaments. The skeletal system has many vital functions which act as the framework of the body. Some of the main functions of the skeletal system include, but are not limited to the following: support, protection, movement, blood formation, electrolyte balance, acid-base balance, and detoxification (10). Many of the bones in the body help maintain its flexibility allowing it to move in all positions. Bones also provide vital protection to many of the body's most valuable organs including the brain, spinal cord, and lungs. Skeletal muscle relies on bone as its structural support to provide the body with sufficient range of motion and movement. The immune system relies on bone marrow for its major production of red and white blood cells required by the body to function properly. Bone also acts as a storage unit for the body's calcium and phosphate reservoirs. Bone has the ability to release calcium and phosphate according to the body's needs. Another very important function of bone is the role it plays in maintaining body pH by absorbing or releasing alkaline salts. Bone tissue allows for detoxification of the circulating blood by removing unwanted elements such as metals, which can protect the body against their harmful effects (10).

As can be seen from the above list, bone is a crucial part of the human body and plays a role in many of the body's physiological functions. The five main types of bone found in the body include long, short, flat, irregular and sesamoid. Long bones are primarily used for movement of the body and include bones such as the femur, humerus, tibia, etc. Short bones are essentially the same width as they are long and only provide limited movement. Some short bones found in the body include the carpals and tarsals which work together to allow movement of the wrist and

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The journal model for this thesis is Infection and Immunity.

ankle. Flat bones are responsible for the protection of organs. Irregular bones have abnormal shapes and various functions in the body. Bone is composed of bone tissue, also known as osseous tissue, which is a connective tissue allowing bone matrix to become calcified or mineralized by hydroxyapitite. Other tissues that support bone and are found within its structure include blood, bone marrow, cartilage, adipose tissue, nervous tissue and fibrous connective tissue (10). Long bones contain a hard condensed cylinder of osseous tissue in its periphery which encapsulates the medullary cavity containing the bone marrow. Unlike the middle shaft (diaphysis) of long bones, the ends (epiphyses) are composed of less loosely packed osseous tissue called cancellous bone. Cancellous bone is usually found in the center and enclosed by compact bone. The two epiphyses provide the point at which two adjoining bones meet and the joint is supported by cartilage, ligaments, and tendons. Blood circulation thorough the bone is provided by blood vessels which travel through holes in the bones referred to as nutrient foramina. The outermost layer of bone is composed of periosteum which contains a tough outer collagen layer. The inner layer of the periosteum is where bones cells can be found either depositing or reabsorbing bone. Bone forming and destroying cells are also found in the interior of bone called the endosteum (10). Refer to Figure 1 for a detailed look at the anatomy of long bone.

The four main type of cells found in bone include osteogenic cells, osteoblasts, osteocytes and osteoclasts. Osteogenic cells originate from embryonic fibroblasts and are found in the endosteum. Osteogenic cells have the ability to undergo mitosis and differentiate into osteoblasts. Osteoblasts are mononucleate bone-forming cells responsible for deposition of new bone. These cells are cuboidal shaped and can be found in inner periosteum and endosteum. Osteoblasts have the ability to secrete osteoid which is a protein composed of Type I collagen, the main component of the matrix of osseous tissue (9). Other components of bone matrix include protein-carbohydrate complexes like glycoproteins (10). The matrix eventually becomes calcified becoming hydroxyapatite which is mainly composed of a calcium phosphate salt. Osteoblast function and characteristics will be discussed later in this chapter. After the osteoblasts secrete collagen to form the bone matrix they become entrapped in their matrix

secretion, they are now called osteocytes. Osteocytes are located in small cavities called lacunae. These lacunae are connected by small channels called canaliculi. The osteocyte can then reach through the canaliculi channels with their cytoplasmic processes to interact with other osteocytes located in the same vicinity. This interaction between osteocytes allow for passage of nutrients and waste removal. Osteocytes also have the ability to interact with osteoblasts located on the bone surface to signal bone deposition when needed. The final type of bone cells are called osteoclasts. Osteoclasts are large multinucleated cells located on the bone surface in pits called Howship's lacunae. Osteoclasts are formed from stem cells found in bone marrow that produce blood monocytes.

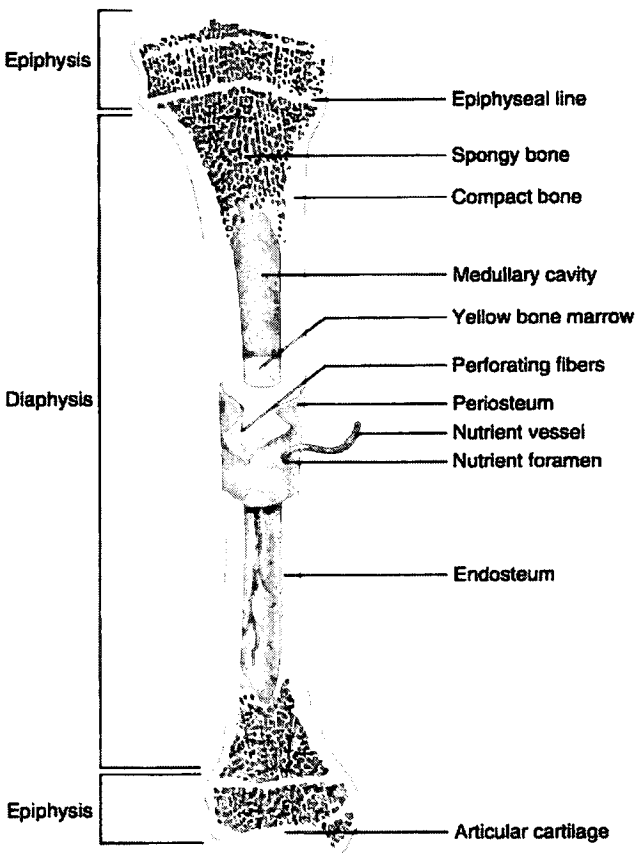

FIG. 1. Anatomy of Long Bone (10)

The formation of osteoclasts occurs when a few of these marrow cells become fused into each other explaining why they may contain up to four nuclei. The main function of osteoclasts is bone resorption. Osteoclasts use specific enzymes such as tartrate resistant acid phosphatase (TRAP) to work in the breakdown of bone mineralization (9). Refer to Figure 2 for an illustration of bone cell lineage and development (10).

The two types of bone, compact and spongy, differ in structure and function. Compact bone matrix is lined in a circular direction (concentric lamellae) around a central, Haversian canal. The concentric lamellae are connected to each other through canaliculi. The combination of the Haversian canal and its corresponding lamellae makes up an osteon, or the complete Haversian system. The collagen of the matrix penetrates down the matrix in a helical arrangement (10). The nutrient foramina are the channels responsible for allowing blood vessels and nerves into the bone. The nutrient foramina eventually lead into perforating or Volkmann canals. The canals have the ability to travel through the bone matrix and connect into the central canal. As blood carries nutrients up the central canal the osteocytes located within that particular central canal absorb nutrients from it and transfer them to other osteocytes within their vicinity through gap junctions. Osteocytes do not only transfer nutrients between each other, but they also transfer waste to the innermost osteocytes to allow for them to be removed through the bloodstream in the central canal. Figure 3 gives a detailed look at bone histology.

Spongy bone has some similar characteristics to compact bone, but significant differences exist. Spongy bone is still mineralized, but the spaces are filled with bone marrow. Since most osteocytes are close to bone marrow within the trabeculae of spongy bone, there is little need for central canals, thus a very limited number of osteons are found in spongy bone.

Hormones That Control Bone Growth

The body contains many hormones, growth factors, and vitamins that play a vital role in the formation or resorption of bone. Many of these specific hormones act directly as a result of specific blood calcium concentration. A significant increase in bone formation can be seen during

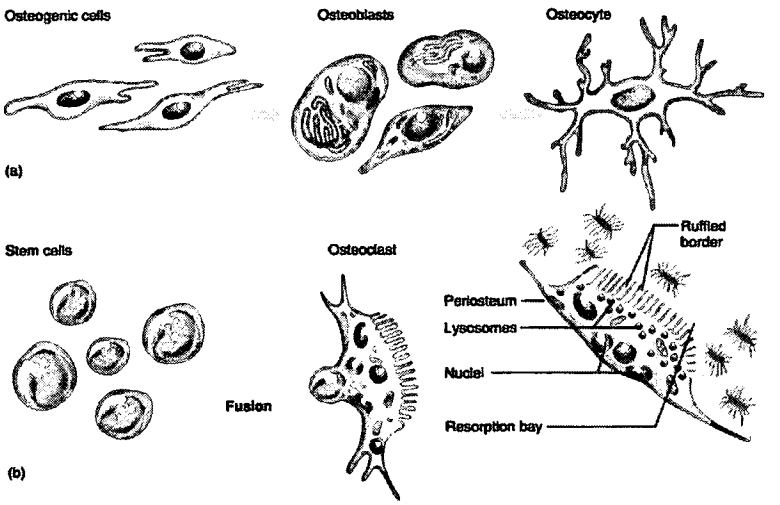

FIG. 2. Bone Cells and Their Development. (a) Osteogenic cells give rise to osteoblasts, which deposit matrix around themselves and transform into osteocytes. (b) Bone marrow stem cells fuse to form osteoclasts. (10)

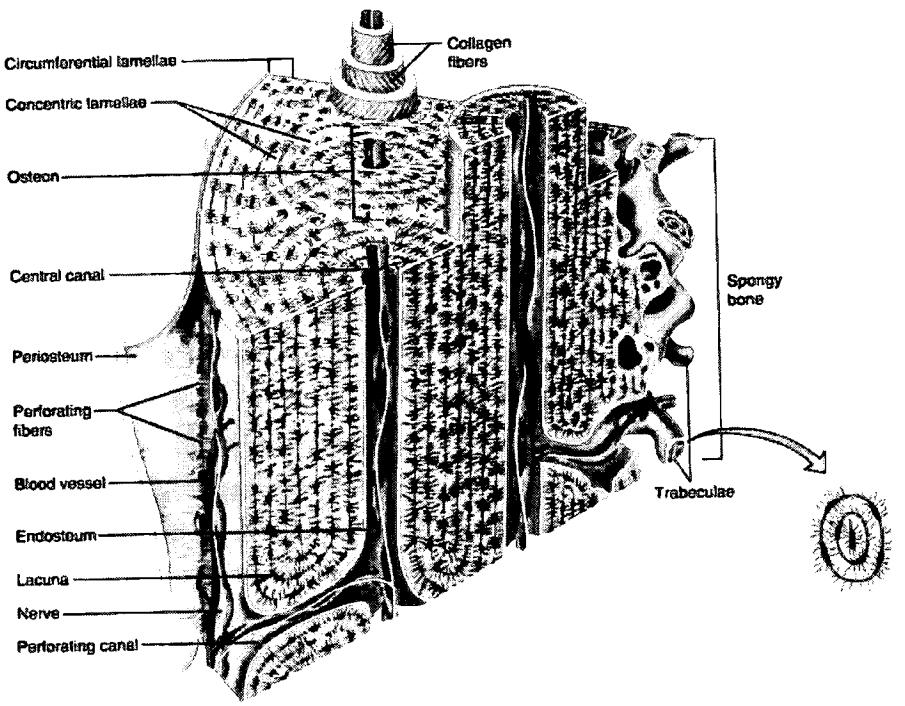

FIG. 3. The Histology of Bone. The three-dimensional structure of compact bone. (10)

adolescence when puberty causes the release of specific growth hormones as well as the secretion of estrogen and testosterone. These hormones have a significant role on the metabolism and ossification of bone. The mechanisms by which each of the hormones activates or inhibits bone growth are not well understood. Activation or suppression of specific bone cells is the main target for many of these hormones. Some of the major hormones that regulate the growth or breakdown of bone as a result of blood calcium levels include: calcitonin, calcitriol, cortisol, estrogen, growth hormone, insulin, parathyroid hormone, testosterone, and thyroid hormone (10). Calcitonin is a hormone secreted by calcitonin cells located in the thyroid gland which can be found in the neck. Calcitonin is responsible for inhibiting osteoclast activity and promoting osteoblast activity. When blood calcium levels are too high, the thyroid gland releases calcitonin into the body to begin to lower the calcium blood levels. Osteoclasts break down mineralized bone and cause higher levels of calcium into the blood. Calcitonin causes a dramatic decrease in osteoclast resorption and increase osteoblast deposition of bone tissue. Enhanced osteoblast activity will cause the release of osseous tissue (collagen) which will begin to use the calcium from the blood for bone mineralization. Figure 4 details how calcitonin regulates and corrects high blood calcium levels (hypercalcemia). Calcitonin also increases calcium excretion from the urine.

Parathyroid hormone (PTH) acts in the complete opposite manner of calcitonin. PTH is secreted by the parathyroid glands which are located behind the thyroid gland. When blood calcium levels drop below the normal levels required by homeostasis, the parathyroid glands will begin to release PTH into the body which will increase blood calcium back to a normal level. PTH has the ability to bind to specific receptors found on osteoblasts. PTH then causes the osteoblasts to release specific activation factors that cause an increase in osteoclast levels. Osteoblasts have the ability to regulate the amount of mature osteoclasts found in bone (the mechanism will be discussed later). As the osteoclast levels increase, more bone resorption causes the breakdown of mineralized bone, specifically the inorganic crystal hydroxyapitite, releasing calcium back into the blood. PTH also has the ability to cause more reabsorption of calcium in the kidneys which will be released back into the blood, thus not secreted in urine.

Parathyroid hormone can also halt the activity of osteoblasts which stops collagen formation, thus no new bone formation. PTH is also necessary, with vitamin D as a co-factor, for Ca^{++} absorption for the GI track. Figure 4 illustrates how parathyroid hormone regulates and corrects low blood calcium levels (hypocalcemia). As mentioned, Vitamin D (calcitriol) is a co-factor with PTH, increasing low blood calcium levels. Calcitriol causes the intestines to absorb more calcium. This vitamin also acts like PTH in that it causes osteoblasts to activate osteoclasts. The increase in osteoclasts causes more calcium to be released into the blood due to the breakdown of the mineralized bone.

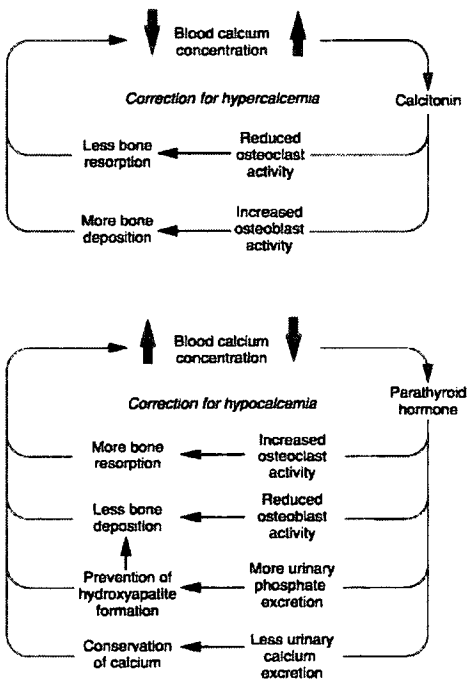

FIG. 4. Negative Feedback Loops in Calcium Homeostasis. (a) The correction of hypercalcemia; calcitonin. (b) The correction of hypocalcemia; parathyroid hormone (10).

Gonadal steroids such as estrogen and testosterone play a role in the development and growth of bone. These steroids are produced by the ovaries and testes respectively. Bone contains receptors for these steroids and they are believed to play a role in the contribution to bone strength.

Growth hormone (GH) is produced by the pituitary gland and stimulates bone formation. This hormone causes more calcium to be released through the renal system, but it also causes high absorption of calcium by the intestines. Other hormones such as thyroid hormone (TH) are necessary for bone to grow and mature. TH causes increased productivity of growth factors. Growth factors are found directly in bone and they are responsible for communicating with surrounding bone cells to activate/deactivate bone metabolism. Glucocorticoids (cortisol) are produced by the adrenal gland. Cortisol can halt bone formation and allows more bone to be broken down. Cortisol also has the ability to allow more calcium to be released in the urine by causing less renal absorption. Additional vitamins such as A and C play a role in enhancing stronger linking of collagen fibers in the deposition of new bone.

There are many growth factors that have the ability to regulate the function of osteoblasts. Some of the major factors that regulate osteoblasts directly include bone morphogenetic protein (BMGs), transforming-growth factor-β1 (TGFβ1) and TGFβ2, insulin-like growth factor (IGF1) and IGF2, fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and WNT (9). Some factors do not affect osteoblasts directly, but rather alter the bone environment in which they are located; an example of this is vascular endothelial growth factor (VEGF). Signaling pathways between cells allow them to communicate with each other through the use of these growth factors which regulate proliferation and differentiation. RUNX2 and osterix are vital transcription factors that are necessary for bone formation and growth (9). Growth factors such as BMP2 and FGF can activate RUNX2 and osterix which allow osteoblasts to differentiate and proliferate. Figure 5 illustrates signal transduction pathways used to regulate osteoblast function. Osteoblasts have receptors for all of the growth factors listed above. Bone morphogenetic proteins bind to its complementary receptor located on the osteoblast. The binding of the BMP receptor forms the appropriate complexes required to phosphorylate proteins named SMAD1 and SMAD5. The addition of a phosphorus group to these proteins causes the upregulation of RUNX2 and osterix, which will lead to bone formation (9). BMP2 also has the ability to cause the activation of mitogen-activated protein kinase (MAPK) which causes an increase in RUNX2 transcription (9). TGFβ is very similar to BMP in that it adds a phosphorus group to SMAD2 and SMAD3. TGFβ

also has a direct effect on the activation of MAPK. Fibroblast growth factors bind to receptors named FGFR1 to FGFR4. Additional growth factors such as insulin-like growth factor (IGF1) binds to a receptor named IGF1R. Endothelin-1 (ET1) bind to a G-protein named ETa. IGF1 and ET1 play a role on the direct activation of MAPK which activates RUNX2. After FGF binds to its receptor found on the osteoblast it activates p42, 44 MAPK and protein kinase C (PKC). PKC activation leads to RUNX2 transcription. 44 MAPK and p42 cause RUNX2 to become activated which allows genes that regulate osteoblast function to become transcribed. Some of the specific genes that regulate osteoblast function include alkaline phosphatase and osteocalcin (9). WNT is a protein growth factor that uses receptors LRP5/6 and Frizzled to activate β– catenin in the cytoplasm of the cell. β– catenin is then transferred to the nucleus of the osteoblast to ultimately play a role in the regulation of additional unknown genes that cause bone formation (9).

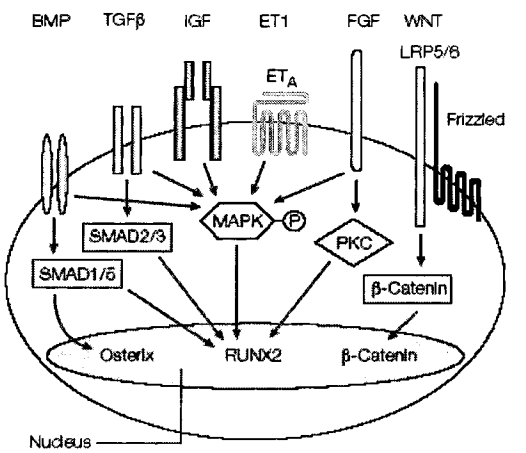

FIG. 5. Signal Transduction Pathways That Regulate Osteoblast Function. (9)

Piezoelectricity in Bone

The application of mechanical stress to certain objects reveals their ability to produce and release electric potentials across themselves. Objects such as wood and hair have a piezoelectric effect. As an outside stress is applied to the object, the piezoelectricity can be

measured as a voltage in the material. E. Fudaka and I. Yasuda are credited with the discovery of the piezoelectric effect in bone. The electric voltage released from bone is produced as a result of the crystalline structure the collagen forms through mineralization in the matrix (14). The collagen fibers found in bone matrix form a spiral arrangement which travels throughout the compact bone. The fibers found in each lamella move in the opposite direction in relation to the lamella located right next to it. The alternating and opposite direction of the fibers in each lamella provide the bone matrix with more strength. The pattern of the collagen fibers in bone causes it to have symmetry. Piezoelectricity in bone only appears when the application of shear stress or force causes the collagen fibers in the bone to begin to slip past each other (6). Shear stress is defined as the application of pressure parallel to the face of the object being examined. In 1957, E. Fudaka and I. Yasuda preformed an experiment to prove and measure the piezoelectric effect in bone. The researchers were interested in proving that the amount of piezoelectricity released from bone was directly related to the angle at which the stress/pressure was applied in relation to the axis on the particular bone being studied. To conduct the study, plates of bone were cut from the outer layer of the femurs from a man and an ox. Pieces of bone were dried before application of pressure. The amount of moisture in a specimen when tested can alter its response to mechanical stress (5). Drying increases bone ultimate tensile strength, modulus of elasticity, and superficial hardness while decreasing tensile strain (% of elongation) (5). The apparatus by which the pressure was applied and piezoelectricity was measured is fully explained in the research paper written by Fudaka and Yasuda (6). The application of pressure on the bone was used to measure the direct piezoelectric effect. The application of electric field on the bone was used to measure the converse piezoelectric effect. Direct piezoelectricity describes the ability to produce electricity due to an amount of stress on an object. Converse piezoelectricity describes the presence of strain when an electric field is applied. Refer to Figure 6 to see the direct piezoelectric effect of bone. The more pressure that is applied the more polarization that can be observed. Polarization describes the ability of the bone to produce an electric field of its own in opposition to the stress applied to it; this is why bone is described as dielectric. Figure 6 shows the direction in which the pressure was applied to the bone plate through the use of circles and

triangles. A linear relationship exists between the amount of pressure applied and the amount of polarization seen in the bone. Refer to Figure 7 to see the converse piezoelectric effect of bone. The application of an electric field is shown to produce a strain or stress on the bone. As the electric field increases so does the strain measured on the bone. The angle and direction in which the pressure is applied affects the magnitude of the piezoelectric constant.

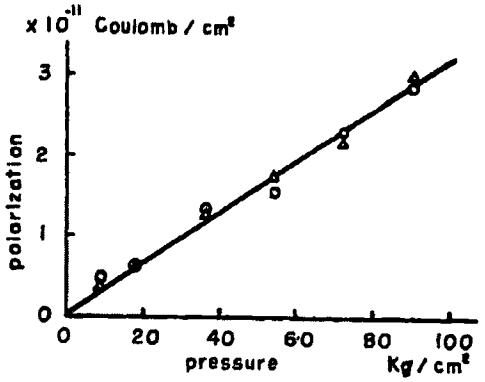

FIG. 6. Direct Piezoelectric Bone Effect. (6)

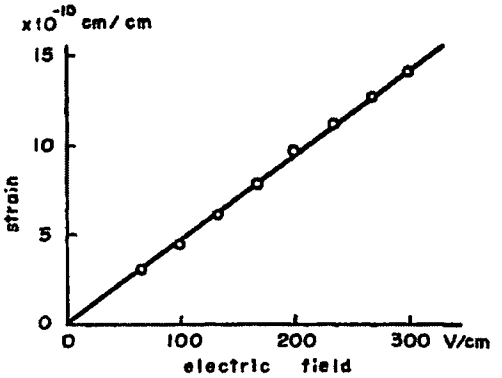

FIG. 7. Converse Piezoelectric Bone Effect. (6)

Refer to Figure 8 to see the relationship in the amount of piezoelectricity measured as the angle is altered from which the pressure is applied in relation to the axis of the femur bone of a man. Refer to Figure 9 to see the same results on the femur bone of an ox. The angle at which the pressure is applied has a significant impact on the amount of piezoelectricity produced. Some pieces of the ox bone were first boiled and then dried at 120 degree Celsius to check if the piezoelectric effect was due to biological origin. The boiled pieces of bone are seen on Figure 8 by the black dots. The figure shows that the black dots do not deviate significantly in piezoelectricity from bone that has not been boiled, this evidence states that this phenomenon is not strictly due to the biological characteristics of bone. Additional research has shown that areas of compression on bone cause the development of negative potentials when compared to other areas of that same bone (1).

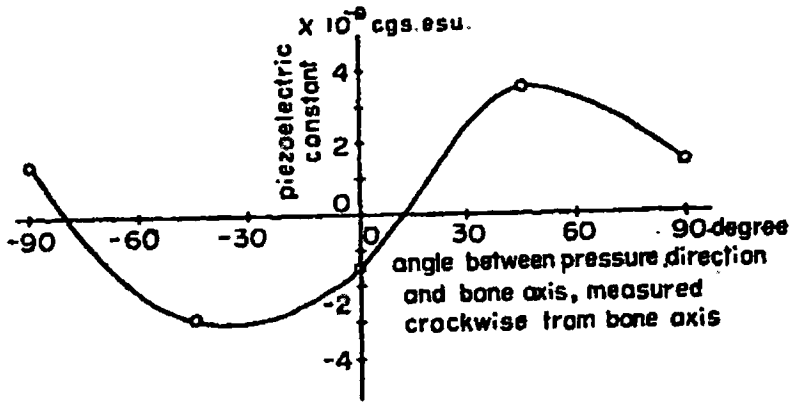

FIG. 8. Dependence of the piezoelectric constant of a femur of a man upon the angle between the pressure direction and the bone axis. (6)

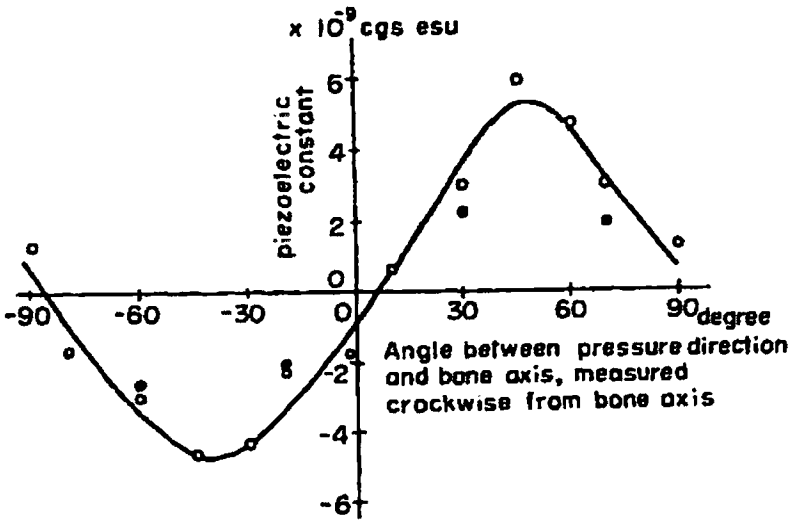

FIG. 9. Dependence of the piezoelectric constant of a femur of an ox upon the angle between the pressure direction and the bone axis. (6)

The electric potentials produced in response to mechanical stress may have a direct effect on the osseous cells and the signaling pathways that regulate them directly (1). Electrodes were placed on the anterior and posterior portion along the midshaft of a feline fibulae. The electrodes were used to measure the electric potentials generated by the bone. Stress was applied to cause

compression on the bone so that it bowed in a concave posterior direction. The posterior electrode generated negative electric potential when compared to the anterior electrode and remained until the stress was released (1). When the bone was bent in the opposite direction, the anterior portion of the bone began to produce a negative potential in comparison to the posterior electrode (1). The compression area is typically electronegative when compared to the tension area of the bone (4). Negative potentials generate bone deposition, while positive potentials cause bone resorpbtion (4). As a bone is compressed and bent, the compression causes osteoblast activation to try to remodel the bone back to its normal shape (4). As the osteoblasts work to deposit bone, the tension side of the bone is being reabsorbed, this is called bone remodeling (4).

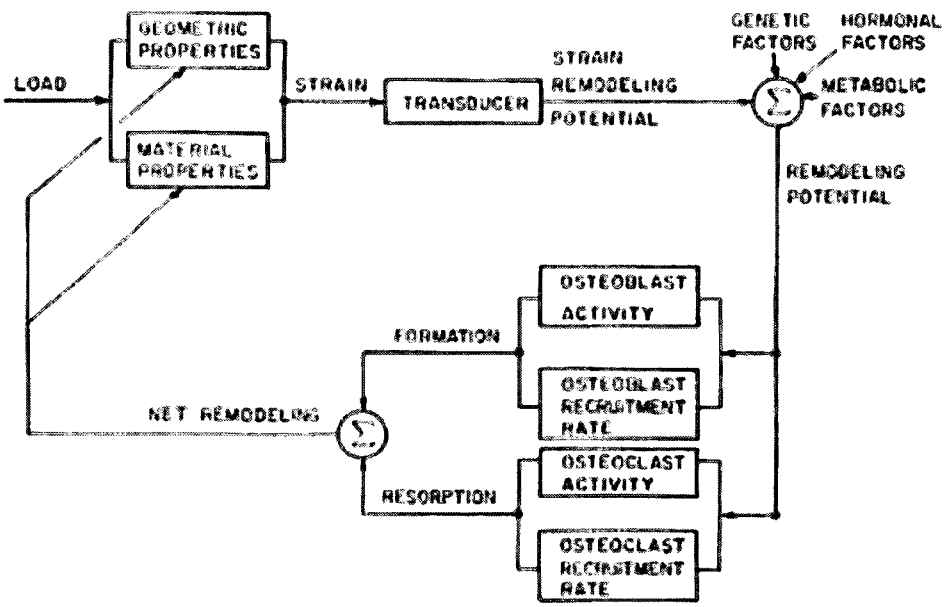

FIG. 10. Functional Dependence of Bone Remodeling on the Strain Field Based on Cell Activity. (7)

This is another example of the ability of bone to piezoelectrically react to different stresses on itself. Refer to Figure 10 for an illustration of bone remodeling feedback system. The continued

balance between the deposition of new bone and the breaking down/resorption of old bone directly determines the new amount of bone remodeling that will occur in a particular bone (7). The geometric properties of a load such as the angle at which it is applied to the bone alters the amount of strain seen by the bone. The osteoblast has the ability to act as a transducer cell that converts mechanical stress/strain into electrical potential. This piezoelectric potential produced has a direct effect on the genetic, hormonal, and metabolic factors that were previously discussed. The combination of all these factors regulates and recruits osteoblasts/clasts to either lay or break bone down. The net modeling can continue as long as the load is being applied to the bone.

Process of Osteogenesis

The process of forming new bone is called osteogenesis. As previously explained, the process of osteogenesis depends solely on the balance between osteoblast bone deposition and osteoclast bone resorption. Osteogenesis occurs during development in bone by one of two ways: 1) intramembranous ossification or 2) endochondral ossification. The flat bones of the skull are produced by intramembranous ossification while most all other bones of the body including the limbs are a product of endochondral ossification.

Intramembranous ossification occurs when mesenchyme is compressed into a sheet-like structure. The cells found in this sheet of tissue transform into osteogenic cells. At this point, portions of mesenchyme sheet start becoming spicules of trabeculae which make up spongy bone. Osteogenic cells differentiate into osteoblasts in the trabeculae network. As the spongy bone continues to grow in size, the osteoblasts will deposit osteoid tissue that will begin to mineralize with the deposition of calcium phosphate. The osteoblasts will eventually become osteocytes as they become engulfed in the collagen they secrete. The secretion of collagen in the network of trabeculae forms some permanent spongy bone. Osteoclasts can also be found in the trabeculae of the spongy bone during development forming a canal for bone marrow. The spongy bone found on the outer surface of the canal will further mineralize and form compact bone. The anatomy of a typical flat bone will have spongy bone sandwiched in between outer layers of compact bone (10).

In contrast to intramembranous ossification, endochondral ossification does not begin with the direct ossification of the mesenchyme. The mesenchyme instead becomes hyaline cartilage which forms a model which holds the preliminary shape of the bone in which will be formed. The cells found in the cartilage called chondrocytes multiply and enlarge in the middle of the model formed by the hyaline cartilage. The center of the model where the cartilage cells gather is called the primary ossification center. A thick layer of connective tissue around the cartilage model eventually gives rise to osteoblasts. The osteoblasts will provide a temporary boney structure on the perimeter of the hyaline cartilage model to provide support for the outline. The surrounding layer of connective tissue around the cartilage is now called the periosteum. The connective tissue of the periosteum eventually breaks thorough the cartilage and invades the primary ossification center to form the primary marrow space. The osteogenic cells found in periosteum now have access to the primary marrow space and will differentiate into osteoblasts. The osteoblasts will now deposit osteoid tissue which will become mineralized to form compact bone. Osteoclasts will invade the marrow space as well; destroying the newly formed bone to only leave compact bone on the outer portion of the new bone. Between the cartilage found on the head of bones and the shaft where bone is deposited there is a transitional zone called the metaphysis. The five zones of the metaphysis are as follows: 1) zone of reserve cartilage, 2) zone of cell proliferation, 3) zone of cell hypertrophy, 4) zone of calcification, and 5) zone of deposition (10). As chondrocytes proliferate and hypertrophy in zones two and three respectively it causes the zone of reserve cartilage (closest to the head of bone) to push the bone further out causing the increase in bone length. Mineralization by calcium and phosphate of the cartilage in zone four will eventually lead to the death of the chondrocytes which will be removed by osteoclasts. Osteoblasts will begin to deposit bone in the zone of deposition. Secondary ossification centers appear at birth and increase the length of bone in the same manner.

Osteoblasts regulate the transition between pre-osteoblasts to fully matured osteoclasts that can break down bone. Pre-osteoclasts are found in circulating blood and bone marrow. Pre-osteoblasts contain RANK receptors on the surface of the cells. Osteoblasts have the ability to secrete the RANK ligand that binds to the receptors on the pre-osteoblasts. Once the ligand is

bound into the receptor the pre-osteoclast becomes a mature osteoclast that has the ability to break down mineralized bone. Osteoprotergerin is a cytokine found in bone marrow that has the ability to regulate pre-osteoclast maturity. Osteoprotergerin has the ability to also bind to the RANK ligand found on osteoblasts. If osteoprotergerin binds to the RANK ligand before the RANK receptors on pre-osteoclasts then it will not mature into a fully functional osteoclast. By blocking the interaction between pre-osteoclast and osteoblasts, osteroprotegerin can essentially regulate the amount of bone resorption. After the resorption of bone by mature osteoclasts through the secretion of enzymes such as acid phosphatase, the osteoclast will undergo apoptosis (programmed cell death).

The osteoblasts caught in their own mineralized matrix of collagen are called osteocytes. These osteocytes will secrete factors that bind to receptors found on the osteoblasts to inhibit more bone growth when not needed. Sclerostin is an example of a factor that binds to receptors LRP5/6 used in the Wnt pathway for increased bone formation. The binding of sclerostin to the LRP5/6 receptors inhibits the pathway from telling the osteoblast to produce more bone. When a bone cracks the osteocytes in the vicinity of that stress will begin to die. The osteocyte can no longer inhibit bone deposition through the secretion of sclerostin. The cells lining the broken bone will eventually form a connection with the nearest blood vessel to allow the entry of pre-osteoclasts into the broken area of the bone. Growth factors now cause the stromal cells to give rise to pre-osteoblasts and additional pre-osteoclasts. The pre-osteoblasts will proliferate with the use of many growth hormones and bone proteins. The pre-osteoblasts with secrete RANK ligand to attach to the RANK receptors found on the pre-osteoclasts formed from stromal cells and from the circulating blood. The binding of the RANK ligand to the RANK receptor forms mature osteoclasts which chew away the portion of the bone where the initial stress was. After the osteoclasts is finished breaking down the bone it will undergo apoptosis. The pre-osteoblasts will begin to mature into fully functional osteoblasts. Osteoprotergerin will now bind to the RANK ligand on the osteoblasts to prevent osteoclasts from being able to remove any more bone. The continued proliferation of the osteoblasts will now allow the cells to line the perimeter of the portion of bone that is missing due to osteoclast activity. The osteoblasts will begin to deposit

bone matrix which will be mineralized to form compact bone. The osteoblasts that are trapped in the matrix while rebuilding will become the new osteocytes in that area of the reformed bone. The new osteocytes will network with surrounding osteocytes to take the place of the cells that were previously there. The mechanism will repeat itself if need be in the same exact manner.

Pulsed Electromagnetic Fields: Introduction to Previous Literature

Pulsed Electromagnetic Fields (PEMF's) have been proven to be an effective method for bone repair and pain relief in bone related medical conditions in many clinical research trials. The use of PEMF's has taken electrical stimulation of bone to greater heights. Pulsed Electromagnetic Field therapy has proved to be a very effective, yet simple method for delivering electrical stimulation to the bone. One of PEMF's biggest upsides is that it is non-invasive, and can be extremely user friendly. The introduction of PEMF devices to the medical community has yielded significant positive results with users around the world. Application of the treatment can be administered by the patient in most cases which explains its worldwide popularity and convenience. PEMF devices are an affordable solution to provide quicker recovery and more bearable lifestyles to people suffering with bone and joint related complications. The future of this type of treatment is extremely promising as current research continually states. Current research is still investigating the specific mechanisms in which bone relief and formation due to PEMF's are carried out. Numerous research studies provide various explanations on how PEMF's actually cause bone healing/relief. Electrically induced osteogenesis is continually being researched to help provide insights into how to make the clinical uses of PEMF more effective and understood. Some research has stated PEMF and electrical stimulation causes the secretion of more growth factors which in turn cause bone growth. Others simply state that the stress applied by the electric fields as well as the environment activates more osteogenic activity in certain targeted areas. Though PEMF treatment has been proven successful, many questions still linger about application times, levels, and methods on how PEMF can yield the quickest, most successful results.

Pulsed Electromagnetic Fields Effect on Osteoblast Proliferation

Osteoblasts are bone cells responsible for the formation and deposition of bone. As previously explained, PEMF treatment can induce osteogenesis under the right parameters (intensity/time), but too much exposure has been shown to have negative effects on the proliferation of osteoblasts in bone. Osteoblastics cells were placed in polylacticcoglycolic (PLGA) scaffolds and exposed to different densities of magnetic field over an 18 day period (16). The wave that the cells were exposed to were narrow 300μs quasi-rectangular pulses with a repetition rate of 7.5Hz and the flux densities of the magnetic field were 0.13mT, 0.24mT, and 0.32mT (16). The cells were either exposed two or eight hours per day. In each group there was also a control which was never exposed to a PEMF. Figure 11 shows the osteoblast cells that were exposed to 0.13mT. The cells treated for 2 hours per day continually proliferated, whereas the cells that were exposed eight hours daily were initially proliferating, but eventually fell below the control group at day 18.

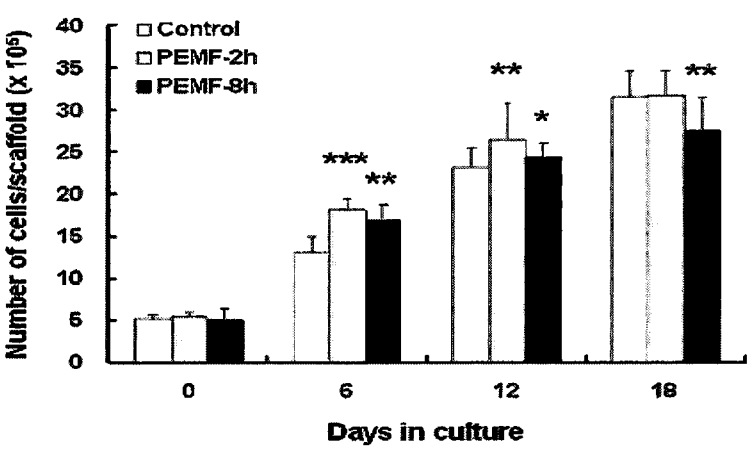

FIG. 11. Number of osteoblasts with/without PEMF stimulation (0.13mT) at 0, 6, 12, and 18 days. (16)

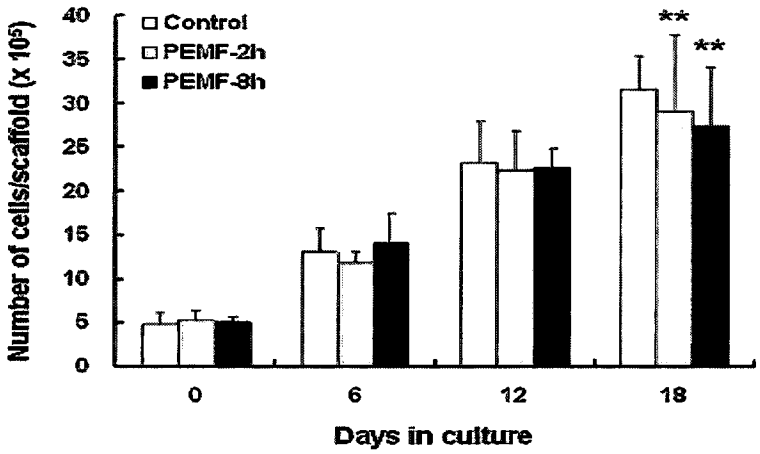

FIG. 12. Number of osteoblasts with/without PEMF stimulation (0.24mT) at 0, 6, 12, and 18 days. (16)

This is an example of how overexposure can potentially lead to a decreased effect in bone cell proliferation, thus slower bone growth/healing time. Figure 12 shows the osteoblasts that were exposed at 0.24mT. Both the two and eight hour treatments proved to be effective, but were still smaller than the proliferation levels seen in the control group that received no treatment. These results indicate that time exposed to the PEMF may not be the treatment complication, but rather the wave parameters in the electric field may have an effect on how efficiently cells proliferate (16). Figure 13 shows the osteoblast cells that were exposed to 0.32mT. This figure shows that the groups exposed to PEMF for two and eight hours per day still do not yield as effective osteoblast proliferation levels as the control group. The overall conclusion of this research is that certain parameters in wave frequency and intensity can have an effect on inducing osteoblast proliferation (16). These findings can be extremely useful because it has the potential to allow medical professionals to better develop more effective PEMF treatments. Longer exposure to PEMF treatment did not yield higher osteoblast proliferation levels.

Pulsed Electromagnetic Fields Accelerate Apoptosis in Osteoclasts

The effect that Pulsed Electromagnetic Fields has on osteogenesis is not only regulated by osteoblasts, but osteoclasts as well play a role in the process. Osteoclasts are bone cells responsible for bone reabsorption. Increased numbers of osteoclast cells can be seen in people suffering from osteoarthritis because their bone becomes fragile and reabsorbed. Targeting the mechanism by which these cells work and what effects PEMF can have on them has been a new area of interest for researchers. The formation of new bone is partially due to osteoblast proliferation, but osteoclast inhibition is a factor as well. In a recent experiment conducted by K. Chang et al (2006) osteoclast cells were exposed to a 7.5Hz PEMF with a pulse duration of 300μs for either a 1, 8, or 16 hour time period. The pulse duration was 300μsec. Osteoclasts were exposed to the treatment at specific time points after isolation (0 hours, 24 hours, 48 hours, and 72 hours). No significant enhancement of any apoptotic activity was seen in the osteoclast treated for 1 hour at any of the time points as seen on Figure 14 when compared to the control (3). Osteoclasts exposed to the PEMF for 8 hours did show increased apoptosis of osteoclasts 48 hours after isolation as seen in Figure 15 (3). The other duration times after isolation showed no difference from the control (24h and 72h). Osteoclasts in the 16 hour exposure groups also showed an increase in apoptotic activity of the osteoclasts as well. The other duration times (24h and 72h) did not show any difference in apoptotic rate in the osteoblast when compared to the control as seen in Figure 16. This evidence helped researchers conclude that PEMF's can cause apoptosis of mature (48 hour) osteoclast cells (3). There is a time frame when apoptosis of osteoclasts can occur and it is believed to be around the 48 hour mark. Twenty four hours after isolation may have proved to be too short a time to induce apoptosis on these cells, whereas 72 hours may have been too long. The figures show almost 100% apoptotic rate in osteoclasts at 72 hour in all three treatment groups. Most osteoclasts may already undergo cell triggered apoptosis at the 72 hour mark; this is why the treatment groups and the control yield very similar results. In conclusion, the mechanism between PEMF and apoptosis in osteoclasts is still rather unclear, but PEMF treatment did prove to accelerate apoptosis rates with certain durations of treatment at 48 hours after isolation.

This research may one day lead to remarkable applications that can be used to help treat and reduce bone related disorders such as osteoporosis or assist in bone formation with osteoblasts.

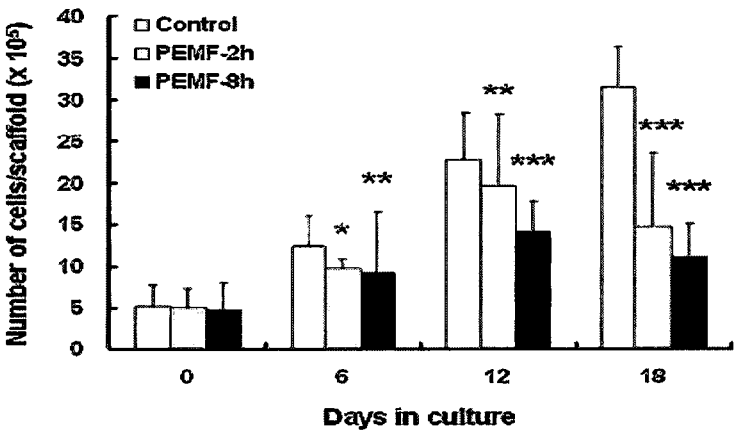

FIG. 13. Number of osteoblasts with/without PEMF stimulation (0.32mT) at 0, 6, 12, and 18 days. (16)

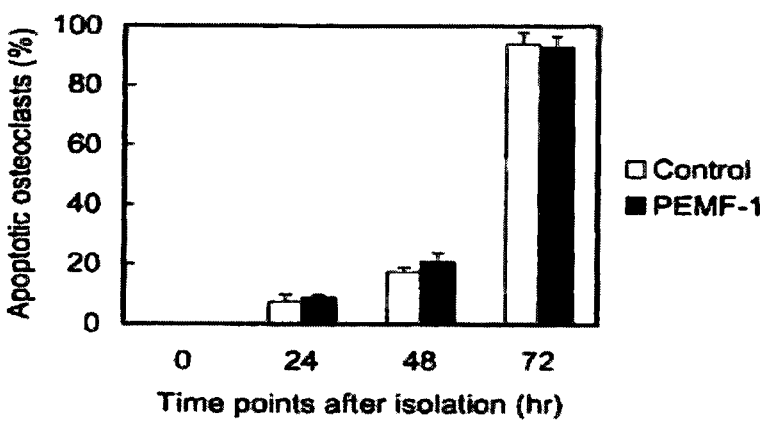

FIG. 14. Induction of apoptosis in mature rat osteoclasts by pulsed electromagnetic fields (PEMF) stimulation for 1 hr. Results are for apoptotic osteoclasts remaining attached to culture dishes with condensed and/or fragmented nuclei, observed after staining with PI, as a percentage of all osteoclasts on culture dishes. (3)

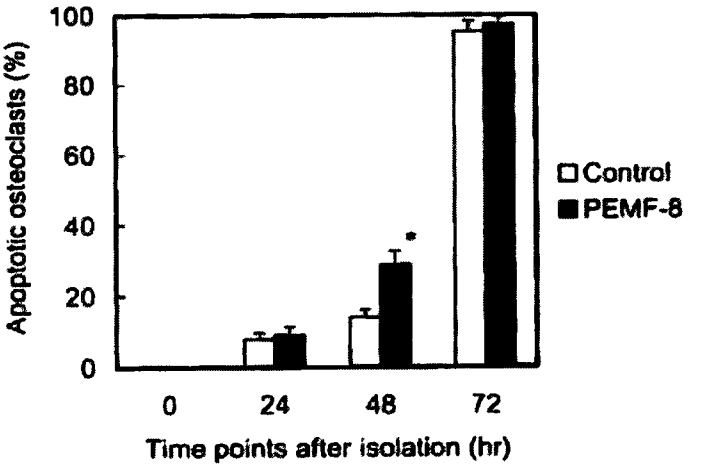

FIG. 15. Induction of apoptosis in mature rat osteoclasts by pulsed electromagnetic fields (PEMF) stimulation for 1 hr. Results are for apoptotic osteoclasts remaining attached to culture dishes with condensed and/or fragmented nuclei, observed after staining with PI, as a percentage of all osteoclasts on culture dishes. *Significantly different from controls ($p < 0.001$). (3)

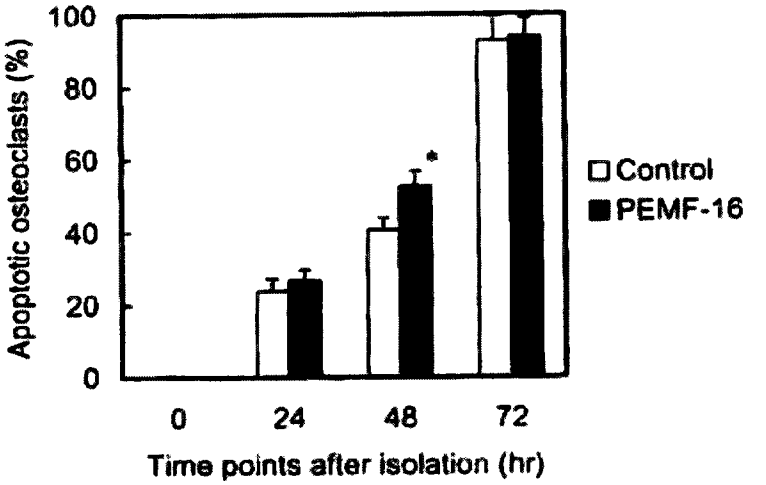

FIG. 16. Induction of apoptosis in mature rat osteoclasts by pulsed electromagnetic fields (PEMF) stimulation for 1 hr. Results are for apoptotic osteoclasts remaining attached to culture dishes with condensed and/or fragmented nuclei, observed after staining with PI, as a percentage of all osteoclasts on culture dishes. *Significantly different from controls ($p < 0.05$). (3)

Conclusion of the Review of Literature

In conclusion, the use of Pulsed Electromagnetic Fields in bone is a remarkable field of study that has proven results. The mechanism by which PEMF causes osteogenesis is still being researched today. Many additional areas of osteogenesis activation are being researched in conjunction with PEMF treatment to see which yields the most effective results. Wave size, frequency and pulse power are crucial variables that can affect the overall outcome of experiments. Growth factors, ion channels, and piezoelectricity are a few of the additional factors that play a role in osteogenesis/overall bone growth. Pulsed Electromagnetic Fields are beginning to attract more interest in an effort to solve some of the lingering problems that are occurring in each experiment. Many questions remain to be answered in this area of study, but PEMF has been proven to have therapeutic effects on bone. It is exciting to think about this field of study and the assistance it might bring to people around the world everyday in years to come.

Biological Implications of Nanosecond Pulsed Electric Fields on Cells

As can be seen from the evidence and supporting research listed above, the use of electric fields has been a promising area of research seeking for answers on the effects that the manipulation of electrical currents have on cells. Nanosecond pulsed electric fields (nsPEF's) has taken the use of electric fields on cells to new dimensions. The cytoplasm of a cell acts as a conductor in response to electric fields, whereas the plasma membrane along with intracellular organelle membranes acts as a dielectric, or a non-conducting substance (13). The non-conductive plasma membrane acts as the insulator for the cell. The nanosecond pulse electric field generators have the ability to slowly charge a capacitor and discharge the electrical energy stored extremely fast in the nanosecond to microsecond voltage range with amplitudes measured in several kilovolts. The cell's plasma membrane will act as a capacitor for the electric field where the energy can be stored. If the duration of the pulses applied to the plasma membrane is shorter than that of the charging time of the membrane, then we can expect to see potential differences generated across the subcellular membranes in the cell, these pulses are referred to as ultrashort (11). By generating ultrashort pulses the harmful effects on the plasma membrane and cell viability are increased because of the limited thermal effects produced by the nanosecond pulse.

The plasma membrane contains many voltage-gated channels that only respond to voltages that surpass the threshold required to open that channel. If the voltage applied to the plasma membrane exceeds 1 V then the membrane will undergo electroporation. Electroporation refers to the formation of pores in the plasma membrane that increases the permeability of the membrane allowing large molecule transport into the cell (2). The nanosecond pulses provide such high energy and fast discharge that the effects of electroporation can be reversed and the pores have the ability to reseal. The successful use of nsPEF's on the subcellular membranes of a cell has lead to exciting primary and secondary effects that may lead to groundbreaking evidence in an effective treatment to cancer.

The plasma membrane serves as a boundary between extracellular fluid and intracellular components. If the membrane integrity is jeopardized through the use of nsPEF's then substances can be introduced into the cell that may have otherwise been kept out. Propodium iodine (PI) is a dye that if introduced inside of a cell will bind to nucleic acids located inside of the nucleus and will emit fluorescence (13). 60 nanosecond, 26 kV/cm pulses were applied to HL-60, a leukemic cell line, to observe the uptake of PI into the cell. Twenty minutes after the electrical stimulation of the cell, PI was observed being taken up by the HL-60 cells (13). The uptake of PI into the cell after electrical stimulation provides evidence for the temporary loss of plasma membrane integrity. In addition, Beebe et al (2003) provided evidence that the plasma membrane has the ability to reseal after nanosecond pulsed electric field stimulation. Ethidium homodimer is a dye that acts exactly like PI. Jurkat cells have the ability to uptake ethidium homodimer directly after nsPEF treatment, but become nonpermeable 5 minutes later (13). This research provides evidence that the short duration of the nsPEF's does not terminally destroy the cells ability to regulate transport through the plasma membrane.

The first evidence of the effect of nsPEF's on subcellular electroporation was shown in human eosinophils. The eosinophils were loaded with calcein to define the intracellular boundaries with an intact cell membrane. Calcein exists in two forms: 1) free calcein which is highly fluorescent and membrane impermeable and 2) calcein-AM which is non- fluorescent and membrane permeable. As the calcein-AM enters the cell, esterases cleave off the acetocymethyl

ester to allow fluorescence and the formation of free calcein (11). The free calcein cannot permeate any of the intracellular membrane-bound components because it is membrane impermeable. Eosinophils exposed to 60 nanoseconds, 3.6 and 5.3 MV/m during multiple nsPEF's shrank in size, as well developed a "sparkler" morphology (11). Eosinophil granules became very fluorescent (called "sparkler cells") and there was retention of the staining in the cells cytoplasm. These conclusions provide evidence of selective disruption (poration) of the eosinophil granule membrane during nsPEF application (11). Since the cytoplasmic staining was not effected it was concluded that the plasma membrane was kept intact (11).

To better understand the effect that nsPEF's have on the nuclear membrane, a fluorescent dye called acridine orange (AO) was used. This dye has the ability to permeate through the plasma and intracellular membranes. The dye interacts with DNA by intercalation and RNA by electrostatic interaction (13). When AO interacts with DNA it fluoresces green and when it interacts with RNA it fluoresces red. HL-60 cells with AO exposed to 10 nanosecond, 65 kV/cm electric fields showed a decrease in the fluorescence in the nucleus (13). These results conclude that nsPEF's have the ability to cause poration in the nuclear membrane as well possibly produce conformational changes in DNA. Stacey et al (2003) pulsed Jurkat and HL-60 cells with 60 nanosecond, 60 kV/cm electric fields and observed DNA damage (15). The use of a comet assay allowed the researchers to measure DNA fragmentation caused by the nanosecond pulsed electric fields. Beebe et al (2002) used HL-60 and Jurkat cells to induce apoptosis, programmed cell death, through the use of nanosecond pulsed electric fields (12). The cells were exposed to 10, 60, and 300 nanosecond pulses with an electric field of tens of kV/cm up to 300 kV/cm. The number of pulses varied between 1 to 5. Through the use flow cytometry, cell function and viability was measured. Jurkat cells began to undergo apoptosis at 10 nanoseconds with an electric field of 300 kV/cm (12). An increased level of Annexin-V-FISC fluorescence indicates externalization of phosphatidylserine, which acts as an indicator for apoptosis in cells (12). Cells that were exposed to pulse durations of 60 nanoseconds began to show apoptotic signs at 40 kV/cm (12). The ability to induce apoptosis in cancer cells has been a remarkable finding in this research.

Beebe et al (2003) proved that the combination of classical plasma membrane long electroporation pulses combined with nanosecond pulses enhanced gene expression in HL-60 cells through the use of a green fluorescent protein (GFP) (13). GFP is used to directly measure gene expression. The HL-60 cells were exposed first to the long electroporation pulses and then followed thirty minutes later with a nanosecond pulse, which showed nine times the amount of fluorescence when compared to the control groups (13). Although the exact mechanism by which the enhanced gene expression occurred is unknown, the evidence is rather promising.

Intracellular calcium is stored in the compartments of the endoplasmic reticulum and the mitochondria. Calcium released from the mitochondria is associated with the start of apoptosis in a cell (12). Nanosecond pulsed electric fields have been proven to cause the release of intracellular calcium. Beebe et al (2003) loaded HL-60 cells with Fura-2 which is a calcium indicator. A 60 nanosecond pulse with 10 kV/cm amplitude caused an increase in the release of the intracellular stored calcium (12). The release of intracellular calcium in cells due to nsPEF may have a direct effect on osteoblasts. Hormones in the body that control osteoblast proliferation are controlled by feedback systems that rely on blood calcium levels for control.

NsPEF's provide an effective application to cells that can have a multitude of effects not only on the plasma membrane, but on the subcellular components as well. Pulse duration, intensity, amplitude and quantity all can play a direct role on how specific cell lines react to the exposure to these ultra short pulses. After reviewing all pertinent material on the subject of electrical stimulation of bone cells, I hypothesize that I will observe enhanced proliferation rates of normal human osteoblasts in culture after nanosecond pulsed electrical stimulation. Although all *in vivo* physiological parameters are difficult to regulate in culture, the supporting evidence gives me reason to believe that nanosecond pulsed electric fields may have an effect on normal human osteoblasts rate of mitotic division *in vitro*.

MATERIALS AND METHODS

Normal Human Osteoblasts (NHOst)

The bone cells used in this experiment are part of Clonetics® Normal Human Osteoblast Cell System (Lonza, Walkersville, MD). Normal human osteoblasts are an adherent cell line which when cultured attach to flask bottoms. The cells were purchased cryoperserved as opposed to in a proliferating state. Lonza ships all cryopreserved osteoblast cells lines in the second or third passage. Quality control tests on the cell line were preformed before shipping to ensure negative results for HIV-1, mycoplasma, Hepatitis B and C, bacteria, yeast and fungi.

Preparation of Supplements

To successfully maintain and culture the normal human osteoblasts I purchased the Clonetics® ReagentPack® (Lonza, Walkersville, MD) which contains 100mL of each HEPES Buffered Saline Solution, Trypsin/EDTA and Trypsin Neutralizing Solution. Ideal storage for all of the above materials is -20°C. In addition to the supplements needed for culture, 500 OBM Basal Medium was needed to feed the cells. The media was shipped at pH 7.7 with an osmolality of 275 mOsm/kg H_20. The preparation of the media required the Clonetics® OGM SingleQuot® Kit (Lonza, Walkersville, MD) which included 50mL of Fetal Bovine Serum (FBS), 0.5mL of an aqueous solution of Gentamicin Sulfate and Amphotericin-B, as well as 0.5mL of ascorbic acid. The FBS, GA-1000 and the ascorbic acid were all mixed with the OBM basal medium before use with the cells.

Culturing Osteoblasts

All osteoblasts were cultured in Nunc® T-175 nunclon treated flasks with blue filter caps for ventilation. The recommended seeding density for normal human osteoblasts is 5,000 cells/cm^2. Exhausted media was replaced in every culturing flask every 48 hours with 8mL of warmed media under a Nuaire® biological safety hood. Doubling times varied with the normal human osteoblasts but were typically in the range of 24-48 hours. When T-175 flasks were seeded lightly with cells, it took an average of one week to reach a confluency level of 80%. All cells were harvested at 80% confluency. Figure 17 is a micrograph of normal human osteoblasts at

95% confluency. If cells were to reach a confluency level > 80% they were discarded because they would begin to undergo physiological alterations and growth inhibition. All cells were incubated in a Nuaire® IR Autoflow CO_2 water jacketed incubator at 37°C and 5% CO_2. Cells were harvested at time of maximum confluency for either reseeding or use in an experiment. No osteoblasts were passaged more than 8 times to ensure the best quality results. At time of harvest, exhausted media was discarded and 5mL of warm HEPES buffered saline solution was inserted into the flask. The HEPES was slowly distributed throughout the flask to ensure all areas of the flask bottom were covered for a two minute period. The HEPES solution was then removed and replaced with 6mL of warm Trypsin/EDTA for cell detachment. The trypsin was distributed evenly and then the flask was placed into the incubator for no longer than a two minute time period. After incubation, the flask bottom was tapped vigorously to remove remaining attached cells. After 90% detachment, 8-10mL of tryspin neutralizing solution (TNS) was put on top of the trypsin that was still remaining in the flask. The TNS is responsible for neutralizing the effects of the tryspin completely. At this point, new flasks can be reseeded or cells can be transferred to 50mL centrifuge tubes to pellet. If desired, cells were frozen back with 80% media, 10% FBS and 10% dimethyl sulfoxide (DSMO) in liquid NO_2 at the concentration of 1,000,000 cells/mL for later use.

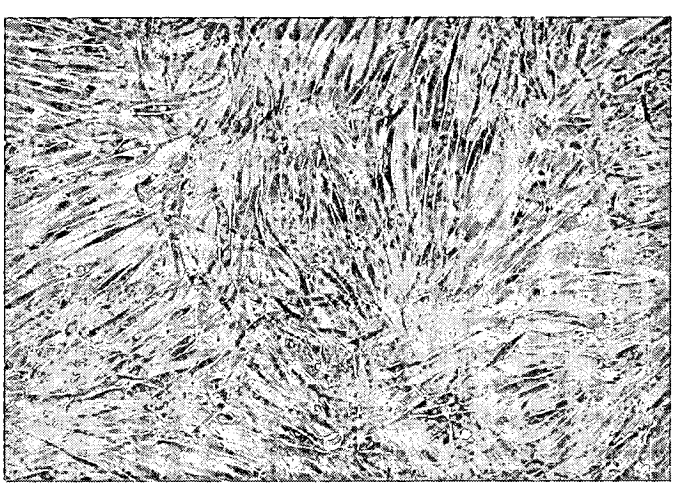

FIG. 17. Normal human osteoblasts at a 95% confluency level. (Lonza, Walkersville, MD)

Osteoblast Preparation

To achieve cell counts, the cells were placed in centrifuge tubes then spun at 1000rpm for five minutes in an Eppendorf® 5810R centrifuge to pellet. Once pelleted, the supernatant was removed and the pellet was resuspended in 1mL of fresh media then pipeted for the cells to disperse properly. Ten μL of the cells were mixed with 10 μL of trypan blue for hemocytometer loading. The number of cells per milliliter was determined with a hemocytometer. After the proper calculations, the cell stock solution was diluted with media to set the final concentration of cells to 500,000/mL.

CSFE Cell Proliferation Dye

CellTrace® CFSE Proliferation Kit manufactured by Molecular Probes® was used as a cell tracing reagent in this experiment. The kit contains vials of CFSE, carbonxyfluorescein diacetate succinimidyl ester (component A) which was diluted 1:18 into high quality DSMO (component B) to produce a 5mM stock solution of functional dye. The molecular weight of CFSE is 557.47. CFSE has the ability to passively enter cells without any color or fluorescence. Upon entry into the target cell, the acetate group of CFSE becomes cleaved off by intracellular esterases allowing the carbonxyfluorescein succinimidyl to become fluorescent. The dye is well retained by the target cells throughout the stages of division in meiosis. Every time a cell from the initial target population undergoes a division the fluorescent label is inherited by the successive generations for proliferation tracing. Each daughter generation will have half the fluorescence of the previous parent cell (F1 = 1/2, F2 = 1/4, F3 = 1/8). The amount of trace fluorescence in each of the cells allows FlowJo® to calculate how many cells in original population underwent division, and of those which did divide, the average number of divisions they underwent.

Before application of the proliferation dye to the osteoblasts, the cells were re-suspended in warm phosphate buffered solution (PBS) with 0.1% bovine serum albumin (BSA). Following re-suspension, 2 μL of the 5mM CFSE dye was added to every milliliter of cells for a final concentration of 10 μM. The osteoblasts were then incubated for 10 minutes following the dye application. After incubation, the staining of the osteoblasts was halted by the addition of 5mL of

cold media to the cells. The osteoblasts were then placed in a 4°C refrigerator for 5 minutes. The osteoblasts were then pelleted through centrifugation at 1000rpm for five minutes and washed with fresh media a total of three times before experimental use.

Nanosecond Pulsed Electric Field Generator

A 60 nanosecond transmission line pulse generator was used in this experiment to deliver the electrical energy to the osteoblasts. The components of the pulse generator consisted of: Tektronix® TDS3052B Two Channel Color Digital Phosphor Oscilloscope and a Glassman® High Voltage Series EH power source. Figures 18 and 19 give details of the pulse generator used in this experiment. The impedance or resistance of multiple cables when compared to one long cable is much lower thus allowing for more efficient transfer of the electricity.

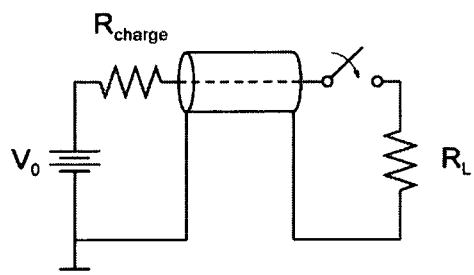

FIG. 18. Transmission Line Pulse Generator Circuit. (Frank Reidy Research Center for Bioelectrics, Norfolk, VA)

FIG. 19. Nanosecond Pulsed Electric Field Generator. (Frank Reidy Research Center for Bioelectrics, Norfolk, VA)

Prior to pulsing the osteoblasts, 140 μL of the cells were inserted into Biosmith® 0.1 cm cuvette and loaded into the pulse generator system. Pulse intensity for this experiment included the following: 25 kV/cm and 35 kV/cm. Eight pulses under each pulse intensity were delivered for each replicate. A total of 5 replicates were run in each experiment. Control groups consisted of 140 μL of cells that were inserted into the cuvette and removed without the application of an electric field. Upon completion of all pulsing experiments each replicate was labeled and placed into its own well of a six well plate along with 3 mL of warmed media for incubation. The cells were then incubated for a time period of 7 days post pulse. The media in each well was replaced every 48 hours with 3mL of fresh warmed media. On the seventh day, the cells were removed from each individual well using the culturing techniques explained above for analysis.

Flow Cytometry of Osteobloasts

The osteoblasts were run through a BD FACSAria® Flow Cytometer for further analysis. The cell sorter consists of a 488 nanometer blue laser. After data was collected from each replicate, the files were transferred into FlowJo® to determine the osteoblast proliferation rates.

RESULTS

Analysis of the flow cytometry data in FlowJo® revealed that the application of nanosecond pulsed electric fields between the ranges of 25kV/cm and 35kV/cm keep the Normal Human Osteoblast (NHOst) population viable at a 60ns pulse duration with an application of eight pulses. Although the cells are kept viable, the analysis also revealed that the use of nanosecond pulsed electric fields do not increase the proliferation rates of NHOst at these parameters. Due to these findings, I will accept the null hypothesis, which states, there is not a significant difference seen in cell proliferation rates consistent with an increase in osteoblast mitotic activity in pulsed cells.

FlowJo® Analysis

Through the use of the proliferation function in FlowJo®, I was able to track the NHOst generations as a result of the CSFE cell tracking dye®. The use of the proliferation option generated numerous cell population tracking functions based on the data files transferred from the flow cytometer for each variable. The functions in which this experiment's results were based on were division index, proliferation index and percent divided.

The division index is defined as the average number of cell divisions undergone by a cell which was present in the original population. The proliferation index is defined as the average number of cell divisions that cells which have divided from the original population underwent. This function allowed us to measure not only the responding cells in the original population, but also took into account the cells in population that did not undergo any divisions. The percent divided is defined as the percentage of cells from the original population that actually underwent a division. For example, if one third of the starting population underwent a division, then the percent divided would be 33.3%. The percent divided = (proliferation index) (percent divided). Table 1 gives the average results of the FlowJo proliferation analysis for each of the functions described above for each treatment group.

Division Index

The results for division index indicate the highest number of divisions amongst cells in the original population was apparent in the control group. The average cell in the control group

underwent 2.45 divisions. Individual replicate data for the control were as follows: Treatment #1: 2.29, Treatment #2: 2.62, Treatment #3: 2.45, Treatment #4: 2.32 and Treatment #5: 2.58. In contrast, the responding cells in the 25 kV/cm treatment group underwent an average of 2.02 divisions. Individual replicate data for the 25 kV/cm group were as follows: Treatment #1: 1.88, Treatment #2: 1.93, Treatment #3: 1.85, Treatment #4: 2.21 and Treatment #5: 2.23. According to my data, there was a slight increase in the average number of cell divisions among the responding cells between the 25 kV/cm and the 35 kV/cm. The responding cells in the 35 kV/cm treatment group underwent an average of 2.09 divisions. Individual replicate data for the 35 kV/cm group were as follows: Treatment #1: 2.23, Treatment #2: 2.03, Treatment #3: 1.7, Treatment #4: 2.31, Treatment #5: 2.12. Refer to Figure 20 to see the progression of cell divisions in the original population for each treatment group and replicate. The SPSS statistical package was used to determine the statistical relationship between each treatment group and variable. Before proceeding with the required tests to determine if the treatment groups were significantly different from each other, all assumptions were checked on the data set. The assumptions that were checked on the data results were Test of Normality using the Shapiro-Wilk Test and the Test of Homogeneity of Variance using Levene's Test. The data set was also checked for the presence of outliners. All requirements for assumption checking were met on this data. The Tukey HSD (Honestly Significantly Different) Test was used to determine if there was a significant relationship between the variable groups for division index. The test showed that there is a significant difference between the control group and the treatment groups for the division index results. The control group is significantly different from the 25 kV/cm treatment group ($p \leq 0.11$) and the 35 kV/cm treatment group ($p \leq 0.025$). The test also revealed that the results of the 25 kV/cm group are not significantly different then that of the 35 kV/cm group ($p \leq 0.885$). Table 2 gives the Tukey Test SPSS output for division index.

TABLE 1. Mean of treatment group data after FlowJo® analysis

	Percent Divided	Division Index	Proliferation Index
Control	86.79	2.45	2.82
25 kV/cm	73.44	2.02	2.77
35 kV/cm	69.93	2.09	2.84

Proliferation Index

In contrast to the division index, the proliferation index provides the average number of divisions that cells which initially divided from the original population have undergone. The proliferation index more accurately reflects the impact the nanosecond pulsed electric field had on the responding system (cells that responded), rather than the entire system as a whole. The results for proliferation index indicate that the highest number of average cell divisions in the original population (responding cells) was apparent in the 35 kV/cm treatment group. The average number of divisions a cell in the original population underwent in the 35 kV/cm group was 2.84 divisions. Individual replicate data for the 35 kV/cm group were as follows: Treatment #1: 2.69, Treatment #2: 3.16, Treatment #3: 2.66, Treatment #4: 2.9 and Treatment #5: 2.8. In contrast, the average number of divisions seen in the control treatment group was slightly less when compared to the 35 kV/cm group. The average proliferation index for the control group was 2.82. These results may be an indication that although there were less cells from the original population which divided, those that did respond underwent more divisions in the 35 kV/cm treatment group. The results may also show that the application of the 35 kV/cm nanosecond pulsed electric field may hinder more cells in the original population from dividing when compared to the control. Individual replicate data for the control group were as follows: Treatment #1: 2.64, Treatment #2: 3, Treatment #3: 2.92, Treatment #4: 2.69 and Treatment #5: 2.84. The average number of divisions in the original populations that received a 25 kV/cm application was the smallest when compared to the control and 35 kV/cm group. The 25 kV/cm group had an

average proliferation index of 2.77. This data shows that of all the cells in the stating population that divided, the 25 kV/cm treatment group saw the least, on average, number of osteoblast divisions. Individual replicate data for the 25 kV/cm group were as follows: Treatment #1: 2.77, Treatment #2: 2.72, Treatment #3: 2.86, Treatment #4: 2.68 and Treatment #5: 2.82. Figure 21 shows the progression of proliferation index in each treatment group for each replicate.

The SPSS results revealed that there is not a significant difference between the control group and the treatment groups for the proliferation index results. The control group is not significantly different from the 25 kV/cm treatment group ($p \leq 0.872$) and the 35 kV/cm treatment group ($p \leq 0.966$). The test also revealed that the results of the 25 kV/cm group are not significantly different than that of the 35 kV/cm group ($p \leq 0.739$). Table 3 displays the Tukey Test SPSS output for proliferation index.

Percent Divided

As described above, the percent divided is the percentage of cells from the original population which divided, assuming that no cell dies during the culturing steps. The results for percent divided indicate that the highest percentage of cell division in the original population was apparent in the control group. The mean percentage of cells in the original population that underwent a division in the control group was 86.79%. Individual replicate data for the control group were as follows: Treatment #1: 87.31%, Treatment #2: 83.81%, Treatment #3: 86.39%, Treatment #4: 91% and Treatment #5: 85.42%. In contrast, the average percent division seen in the 25 kV/cm group (73.44%) was less than that of the control group. These results may be an indication that electric fields at these parameters may hinder overall cell division in the original populations. Individual replicate data for the 25 kV/cm group were as follows: Treatment #1: 70.74%, Treatment #2: 64.90%, Treatment #3: 82.30%, Treatment #4: 79.16% and Treatment #5: 70.12%. The lowest average of percent division was seen in the 35 kV/cm treatment group. The 35 kV/cm group had an average percent division of 69.93%. Individual replicate data for the 35 kV/cm group were as follows: Treatment #1: 64.21%, Treatment #2: 63.85%, Treatment #3:

79.63%, Treatment #4: 75.49% and Treatment #5: 66.47%. Figure 22 shows the progression of percent division in each treatment group for each replicate.

The SPSS results revealed that a significant difference exists between the control group and the treatment groups for percent division. The control group is significantly different from the 25 kV/cm treatment group ($p \leq 0.011$) and the 35 kV/cm treatment group ($p \leq 0.002$). The test also revealed that the results of the 25 kV/cm group are not significantly different than that of the 35 kV/cm group ($p \leq 0.639$). Please refer to Table 4 for the Tukey Test SPSS output for percent division.

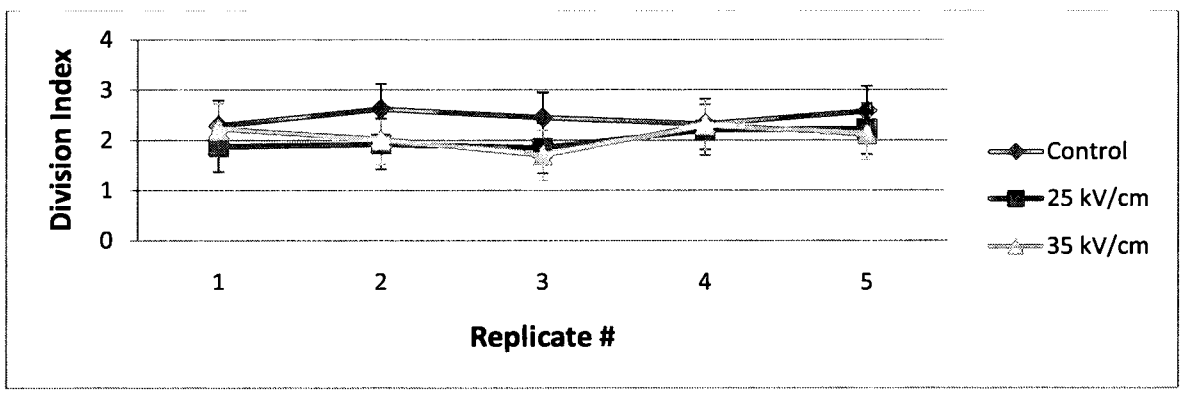

FIG. 20. Division index of treatment groups following a nanosecond pulsed electric field.

TABLE 2. Tukey Test for division index

	(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Control (no pulse)	25 kV/cm	.4320*	0.12233	0.011	0.1056	0.7584
		35 kV/cm	.3740*	0.12233	0.025	0.0476	0.7004
	25 kV/cm	Control (no pulse)	-.4320*	0.12233	0.011	-0.7584	-0.1056
		35 kV/cm	-0.058	0.12233	0.885	-0.3844	0.2684
	35 kV/cm	Control (no pulse)	-.3740*	0.12233	0.025	-0.7004	-0.0476
		25 kV/cm	0.058	0.12233	0.885	-0.2684	0.3844

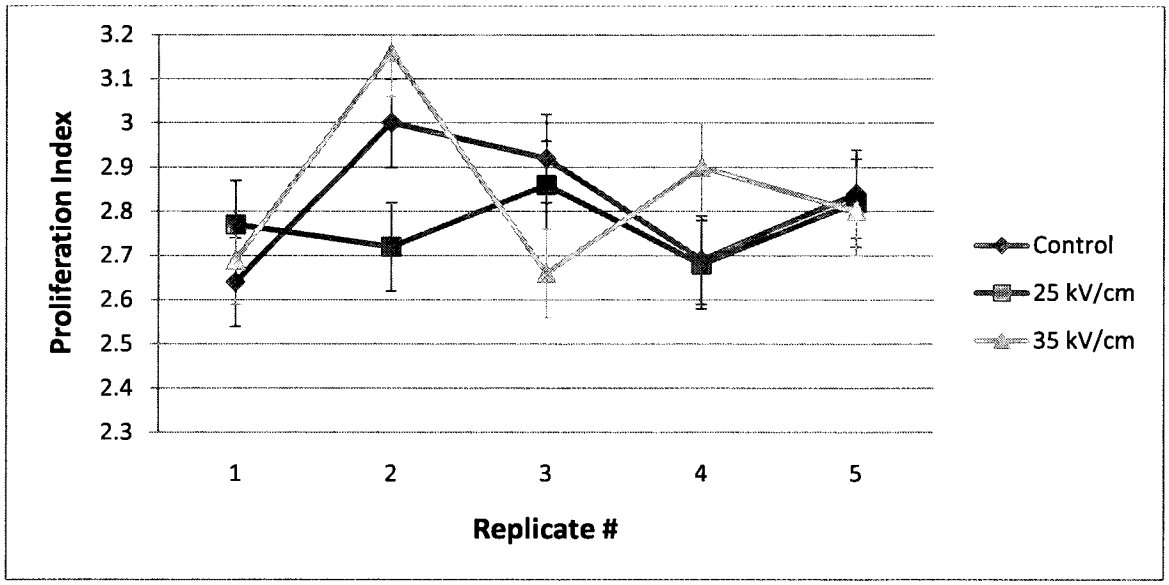

FIG. 21. Proliferation index of treatment groups following a nanosecond pulsed electric field.

TABLE 3. Tukey test for proliferation index

	(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Control (no pulse)	25 kV/cm	0.048	0.09587	0.872	-0.2078	0.3038
		35 kV/cm	-0.024	0.09587	0.966	-0.2798	0.2318
	25 kV/cm	Control (no pulse)	-0.048	0.09587	0.872	-0.3038	0.2078
		35 kV/cm	-0.072	0.09587	0.739	-0.3278	0.1838
	35 kV/cm	Control (no pulse)	0.024	0.09587	0.966	-0.2318	0.2798
		25 kV/cm	0.072	0.09587	0.739	-0.1838	0.3278

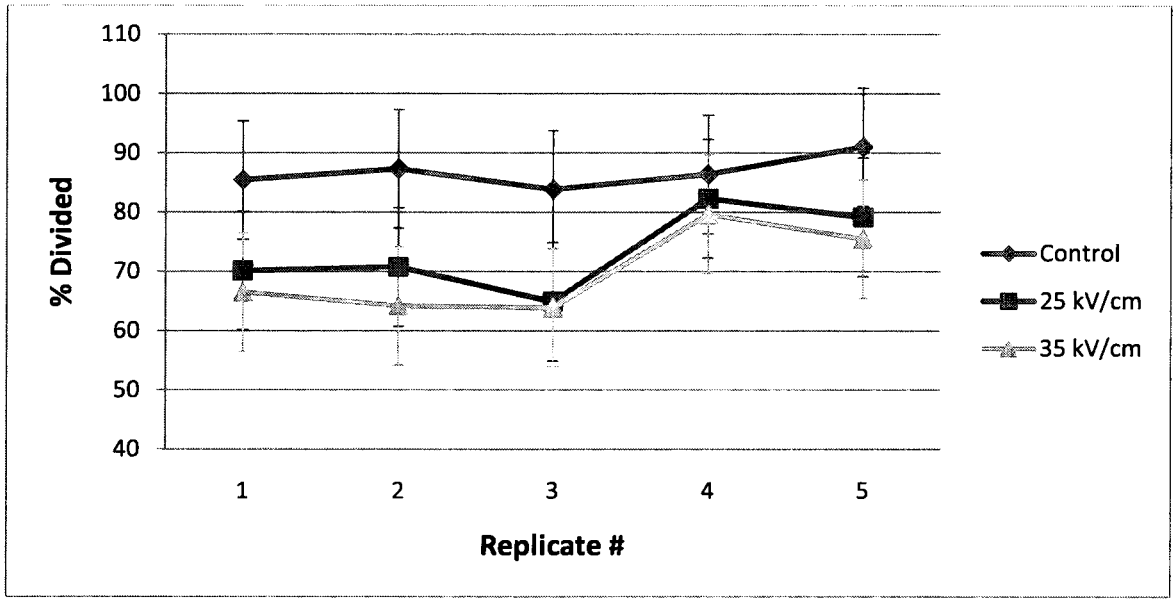

FIG. 22. Percent division of treatment groups following a nanosecond pulsed electric field.

TABLE 4. Tukey test for percent divided

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control (no pulse)	25 kV/cm	13.3420*	3.82114	0.011	3.1477	23.5363
	35 kV/cm	16.8560*	3.82114	0.002	6.6617	27.0503
25 kV/cm	Control (no pulse)	-13.3420*	3.82114	0.011	-23.5363	-3.1477
	35 kV/cm	3.514	3.82114	0.639	-6.6803	13.7083
35 kV/cm	Control (no pulse)	-16.8560*	3.82114	0.002	-27.0503	-6.6617
	25 kV/cm	-3.514	3.82114	0.639	-13.7083	6.6803

DISCUSSION

Previous research using pulsed electromagnetic fields to alter mitotic activity of bone cells has illustrated the potential for increasing the rate of cell proliferation or induction of apoptosis. The use of innovative technology involving nanosecond pulsed electric fields allow researchers to alter intracellular processes to manipulate cell functions while maintaining cell viability. The ability to expedite bone growth through the use of electric fields would facilitate more effective and advantageous clinical treatment applications for bone injuries and degenerative disease. The purpose of this research was to distinguish how effective the use of nanosecond pulsed electric fields was in inducing cell division outside of the body. By conducting my research in vitro, a focus of my experiment was to determine how reliant the process of bone deposition was on internal factors including, but not limited to: hormones, growth factors, vitamins and the cell signaling pathways in which they influence. A main objective of this research was also to determine the effect nanosecond pulsed electric fields have on osteoblasts outside of the crystalline structure of bone and if this was vital to inducing a response to external electrical stimuli.

The results indicated that the application of nanosecond pulsed electric fields at the chosen parameters did not accelerate cell division of osteoblasts in culture; in fact it may have done the opposite. The result that makes this determination most apparent is the percentage of cells divided. The results indicate that as the electric field was increased, the percentage of the original population of osteoblasts that actually divided drastically decreased. This is an indication that the use of nanosecond pulsed electric fields at this pulse intensity and duration may have potentially been interrupting or hindering mitotic activity. A proposed theory is that the application of these electric fields may have potentially damaged or directly altered the cell's ability to induce mitosis, ultimately halting the cell division. In addition, the cells in the original population of the the control group underwent the most, average number of divisions, which was 2.45. I conclude that the absence of the contributory factors found inside the human body resulted in the osteoblasts not exhibiting the expected enhanced proliferation rates; most notably the absence of calcitonin, growth factors and bone morphogenetic proteins. Each of these factors plays a vital

role in regulating and assisting the system that controls bone deposition and resorption. The process of bone reconstruction is primarily based on a feedback system controlled by the amount of calcium present in the blood. The absence of specific growth factors found inside the body may have disrupted the cell signaling pathways between osteoblasts which allow them to communicate in order to regulate proliferation and differentiation. As described earlier in this paper, osteoblasts have multiple cell receptors for growth factors which when present initiate bone growth. A potential for future investigation would include the application of nanosecond pulsed electric fields in the presence of the necessary growth factors or in vivo experimentation. Without the presence of the human growth factors required by osteoblasts to regulate cell division, the cell signaling pathways are not consistent to that of what they are in vivo. This system has multiple contributory factors which regulate whether differentiation occurs. It is now supported that without these factors present it is difficult to gauge the accuracy of the impact the nanosecond pulsed electric fields had on osteoblasts in culture.

Another proposed theory as to why osteoblast proliferation was not increased as a result of the nanosecond pulsed electric fields may include the absence of the crystalline structure found in bone which is responsible for piezoelectricity. As previously described, the electric voltage released from bone is produced as a result of the crystalline structure, hydroxyapitite, formed by the collagen fibers. This structure is a result of mineralization of the collagen in the bone matrix. Osteoblasts in culture lack the crystalline structure in which they typically are found in the human body. This research provides evidence that the overall structure of bone may play a vital role in the amount of osteoblast cell division. The nanosecond pulsed electric fields may have presented an external stress on the osteoblasts, but without the presence of the crystalline structure, no piezoelectricity would have been generated. This stress or strain on bone typically will activate genetic, hormonal and metabolic factors which are necessary for bone remodeling. The remodeling potential will determine how much resorption and deposition takes place in the bone, referred to as the net remodeling.

Future experimentation should take notice to how much of a role the contributory factors discussed play a role in the overall system of bone reconstruction. An effective approach may be

to possibly apply nanosecond pulsed electric fields in the presence of these factors to better understand which cell signaling pathways are vital for osteoblast division. Altering the parameters (pulse intensity and duration) may also provide insight on how tolerant the osteoblasts are to electric fields; this may help future researchers determine the ideal settings to induce cell activity. Bone reconstruction is complex, which as this research has proved is not one dimensional. My conclusion is that to more effectively measure the use of nanosecond pulsed electric fields on normal human osteoblasts, the use of in vivo experiments is necessary. To imitate the system as it is found in the human body has proved to be extremely challenging in an in vitro setting. The results of this experiment were conclusive in that the use of nanosecond pulsed electric fields at the chosen parameters were ineffective in increasing proliferation rates of osteoblasts in culture.

CONCLUSION

The use of a 60 nanosecond duration transmission line pulsed generator at a pulse intensity of 25 kV/cm and 35 kV/cm of eight pulses over four seconds has proved to be ineffective at increasing the rate of proliferation of normal human osteoblasts in culture. As the pulse intensity is increased, the percentage of the starting population that undergoes division decreased.

Normal human osteoblasts rely on a wide array of contributory elements unique to the physiological systems of the human body to communicate through cell signaling pathways including hormonal, genetic and metabolic factors that assist in the activation of bone reconstruction. In vitro experimentation of the osteoblasts lacks the presence of these factors which may prove to be challenging to replicate in a laboratory setting for future researchers.

Nanosecond pulsed electric fields may have potentially had an irreversible un-desirable effect on the nuclear membrane of the osteoblasts at these parameters. The electric field could have caused conformational changes in the DNA which halted osteoblast division, thus providing evidence to why the percentage of cells divided decreases as intensity increases. Future researchers may alter pulse parameters to investigate the effects the electric fields have on the subcellular membranes of the osteoblasts. Further examination of normal human osteoblasts, specifically their rate of proliferation, can potentially lead to increased effective clinical applications for quicker bone healing.

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