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Effect of Pulsating Electromagnetic Fields (PEMF) on Collagen Synthesis in Fetal Rat Calvarial Organ Culture

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ON \textbf{COLLAGEN SYNTHESIS IN FETAL RAT CALVARIAL ORGAN CULTURE}

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Master of Dental Science Thesis

THE EFFECT OF PULSATING ELECTROMAGNETIC FIELDS (PEMF) ON COLLAGEN SYNTHESIS IN FETAL RAT CALVARIAL ORGAN CULTURE

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1981
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I. Introduction

The use of electrical stimulation to accelerate osteogenesis in human subjects has had a widespread clinical impact in providing a non-invasive approach to dealing with non-healing fractures and congenital pseudarthroses.

As of July 1, 1981, worldwide results on the use of pulsating electromagnetic fields (PEMF) indicated an overall success rate of 75% in treating over 2100 patients (Third EBI In Vitro Conference, 1981).

Osteogenesis, in vivo, is a complex process involving a general sequence of events:

i) migration of mesenchymal cells to the appropriate site
ii) proliferation of these "progenitor" cells
iii) differentiation of these cells into specialized types, such as, osteoblasts
iv) expression of the differentiated phenotype
   a. matrix synthesis of collagen and proteoglycans
   b. calcification of the matrix

These events occur in a defined spatial orientation referred to as morphogenesis.

The fact that PEMF have been demonstrated to clinically enhance osteogenesis raised the question as to whether this stimulus has positive effects only at specific stages, or acts at all steps of the osteogenic process.

The objective of this research project was to test whether PEMF are capable of inducing increased collagen synthesis in an in vitro system using fetal rat calvaria.
II. Review of the Literature

A. Bioelectric Effects

The impetus for studying the stimulatory effects of electrical perturbations on osteogenesis was the demonstration that bone possesses piezoelectric properties, that is, bone deformation results in the generation of an electric current. Early classical studies demonstrated that not only could bone produce a separation of an electrical charge (direct piezoelectric effect), but that the application of an electric field produced strain or mechanical deformation in the bone (indirect or converse piezoelectric effect) (Fukada and Yasuda, 1957).

In fact, it has been demonstrated that regions of bone under compression, which tend to be concave, are usually negatively charged, and regions under tension, which are convex, are positively charged (Fukada and Yasuka, 1957), and that bone formation occurs on negatively charged regions (Bassett, 1965).

The importance of electrical energy in terms of biological effects has been suggested by limb regeneration experiments in amphibian.

It has also been shown that electrical stimuli can produce a partial regenerative response in mammals, specifically rats (Becker and Spadaro, 1972; Becker, 1972). This response was manifested by the appearance of an organized tissue structure containing cartilage, bone or muscle which
appropriately reflected the morphology of the surrounding area.

B. Relationship Between Electrical Stimulation and Osteogenic Response.

The demonstration of these bioelectric effects helped serve as a catalyst for further research into the relationship between electrical stimulation and osteogenic response. This work included in vivo animal and human studies as well as in vitro studies.

One of the earliest studies on electrical current effects on bone demonstrated that weak DC currents, in the range of 10-100 μA, were capable of causing osteogenesis in dogs (Bassett et al, 1964). In fact, when electrodes were implanted in the marrow cavities of femora, endosteal bone formation occurred around the cathodes.

An attempt by other investigators to reproduce these results proved inconclusive (O'Connor et al, 1969).

Further experimentation with dogs suggested that electrical stimulation in the order of 3-4 μA DC induced more rapid healing in cortical bone defects, and once again it was in the area of the negative electrode that the rate and amount of osteogenesis were greatly increased (Pawluk and Bassett, 1970).

Studies on rabbits were consistent in demonstrating that osteogenic electrical effects predominate around cathodes, and that DC currents of 2.5 - 3.5 μA caused a two-fold
increase in bone formation (Lavine et al, 1971). Another study found that an optimum current of 0.5 - 20 μA resulted in maximum osteogenesis, and that while bone formation at the cathode was both osteoblastic and metaplastic in nature, the lesser bone formation in the anodal region was virtually all metaplastic (Friedenberg et al, 1970).

Non-traumatized bone, specifically medullary cavities of rabbit tibiae, was also shown to have positive response to constant direct current; this osteogenic response was current-dependent, in that 20 μA levels produced the greatest response, while levels over 30 μA caused osteonecroses (Friedenberg et al, 1974).

More recent studies have demonstrated that low-level currents within dog mandibles (3-5 μA, 50 μA/cm in situ) result in increased osteogenesis in the region of the cathode, and that increases in cellularity and mitotic activity suggest that the electrodes stimulated cellular activity rather than cellular migration (Zengo et al, 1976).

While low frequency, pulsed electric fields (1.5 V, 0.2 - 2.5 μA) resulted in an increased osteogenic response, no preferential growth was noted around specific electrodes; it was suggested that firing rate and operating time, rather than current density, were the determining factor for the degree of osteogenic response (Levy and Rubin, 1972).

Constant direct currents averaging 20 μA applied via transfixion pins in dogs, resulted in periosteal proliferation which accelerated fracture healing (Connolly et al, 1977).
Comparisons of the effects of continuous versus interrupted direct currents on fracture healing in rabbits suggested that a 10 μA periodically interrupted DC current was as effective as a continuous current in stimulating osteogenesis around the cathode (Stefan et al, 1976).

Oscillating electric fields (20 V/30 Hz) applied to rat femora which were immobilized for 4 weeks, prevented the development of disuse osteoporosis, and caused electrically stimulated bones to increase in size relative to their contralateral controls (Martin and Gutman, 1978).

It has been reported that inductively-coupled electromagnetic fields of low frequency and strength enhanced bone repair in canine osteotomies (Bassett et al, 1974). This study also demonstrated some aspect of signal specificity, in that pulses of 150 μs duration repeated at 65 Hz and producing 20 mV/cm peak voltages were significantly more effective in augmenting bone repair than pulses of 1 ms duration repeated at 1 Hz and producing peak voltages of 2 mV/cm.

The success of direct currents on osteogenesis in animal systems helped promote the use of electrical stimulation to accelerate osteogenesis in human subjects.

Healing time of crural fractures in humans decreased by 30% in groups stimulated by slow pulsing asymmetrical direct currents (Jorgensen, 1972).

Clinical studies on the effect of direct current stimulation of non-union fractures and congenital pseudarthroses
showed that currents of 10-20 μA resulted in a success rate of 60% for the complete healing of non-unions (Brighton et al., 1975). The positive effects of direct currents in accelerating healing in non-union fractures were confirmed in studies by Connolly et al. (1977).

In a review of the experiments aimed at producing an osteogenic response by implanting electrodes, it was reported that currents below 5000 μA and above 3 μA were capable of stimulating osteogenesis; those above 50 μA caused osteonecrosis (Marino and Becker, 1977). It was suggested that currents above 2-3 μA were non-specific stimuli acting through injury, while those in the low range acted by some other mechanism.

The problems involved with the use of direct currents are primarily the need for a surgically invasive approach to implant the electrodes and a requirement for multiple electrodes in the treatment of large bones. As a result, infection can be a potential problem. Based upon the success of pulsing electromagnetic fields in augmenting bone repair in animal investigations, this treatment modality was adopted for use on humans. These pulsing electromagnetic fields of low energy and frequency have been reported to promote osteogenesis in surgically resistant pseudarthroses and non-union fractures with an overall success rate of 70% (Bassett et al., 1977).

The obvious advantage of this technique is that
the electromagnetic coils are placed externally obviating the need for surgical implantation of electrodes.

It should be noted that one cannot compare the currents produced by measuring with an electrical monitoring device as part of the circuit unless the resistance of the conducting medium is known. Therefore, the most obvious criticism of the aforementioned articles is that the current levels cited may not be directly comparable.

The effects of electrical stimulation have also been examined in vitro. In vitro continuous electric fields (electrostatic) and pulsed fields (electrodynamic) were reported to increase both DNA and collagen synthesis in 3-T-6 fibroblasts, and electrodynamic fields seemed to be more effective in elevating collagen synthesis levels (Bassett and Herrmann, 1968).

The growth pattern of rat calvaria in organ culture was modified by unidirectional, pulsating electric fields, with more dense growth occurring on the negatively charged bone surfaces (Norton and Moore, 1972).

Pulsed electric fields were shown to have a stimulatory effect on the growth rate of embryonic chick tibiae in vitro, whereas invariant fields were incapable of causing such an effect (Watson et al, 1975).

Other investigators have demonstrated that an in vitro epiphyseal plate model (costochondral junction of rats) showed consistent accelerated growth in response to an electrical field of 1500 V/cm (Brighton et al, 1976). Parameters with significant increases were percent elongation of the explant,
incorporation of $^{35}$S, $^{45}$Ca and tritiated thymidine.

Electrical perturbations were reported to produce cyclic AMP changes in embryonic chick tibia epiphyseal cells; an electric field of 900 V/1.5 cm increased cyclic AMP content only when the longitudinal axis of the tibia was oriented parallel to the electric field (Norton et al, 1977).

Oscillating electric fields (1166 V/cm, 5 Hz) were shown to enhance DNA synthesis in bone cells from rat calvaria and chondrocytes from chick epiphyses (Rodan et al, 1978). This response was tissue specific in their experimental system, since chick skin fibroblasts and rat lymphocytes were not stimulated by the electric field.

It has also been recently reported that electromagnetically induced pulsating currents stimulated DNA synthesis in rat calvaria cells in culture (Norton et al, 1980). The effect seemed to be stimulus specific in that signals with different pulse characteristics varied in their degree of stimulation of DNA synthesis. In addition, the effect seemed to be cell state specific since responsiveness to the electrical stimuli decreased with time in confluent culture.

This concept of stimulus specificity has been derived from impedance measurements in toad bladder, where membrane responses to pulsating electrical currents depend on pulse duration, pulse frequency, and the ratio between the positive and negative polarity of the pulse (Pilla and Margules, 1977). By varying these parameters one can alter the membrane conductivity or change the charge density on the membrane surface.
It has also been reported that chick-limb rudiment cells (chondroblasts, chondrocytes, and osteoblasts) release significant amounts of calcium in response to current pulses or electromagnetic fields (Bassett et al, unpublished data).

To summarize, it has been demonstrated that electrical perturbations are capable of eliciting an osteogenic response in both human and animal experiments. In vitro systems have also confirmed that a stimulatory potential of electrical signals exists.

The most notable characteristic of the entire field in bioelectric research is the empiricism present. Much of the selection procedure for stimulatory electrical signals was through trial and error. Stimuli of various pulse shapes, durations and amplitudes and repetition rates were tested and discarded before signals were developed which could consistently provide therapeutic results. It has now been determined that the most effective treatment regimen with electrical stimulation involves careful determination of the placement of the electrodes or electromagnetic coils, intermittent exposure of 12-16 hours per day and immobility of weight-bearing bones.

While a variety of electrical signals have been able to potentiate a positive response, it now appears that oscillating or pulsed electromagnetically-induced electrical fields and direct current fields possess similar ability to produce an osteogenic response.
C. Collagen Structure and Types

Collagen is the most prevalent human protein as it constitutes approximately one-half the total body protein in fully developed adult organisms (Gay and Miller, 1978). Its primary function is as a structural component in a variety of connective tissues such as skin, tendon and bones.

The collagen molecule is composed of three coiled polypeptide chains called α chains which are further coiled around a central axis to form a rope-like triple helix. This coiled-coil structure is stabilized by interchain hydrogen bonds. Each chain has non-helical NH₂-terminal and COOH-terminal regions at opposite ends. It should be noted that collagen is synthesized and assembled intracellularly as a procollagen molecule which contains additional amino acid sequences at both ends. The procollagen molecule undergoes several post-translational modifications both within the cell and extracellularly.

Intracellular modifications include:

a) removal of certain sequences
b) hydroxylation of proline and lysine residues
c) glycosylation of hydroxylysine residues and other propeptides
d) chain association and disulfide bonding
e) folding into triple helix

Extracellular processing following transport and secretion includes:

a) conversion of procollagen to collagen
b) spontaneous ordered aggregation  
c) cross-linking via oxidation of lysine and hydroxylysine residues and synthesis of covalent cross-links  
(Prockop et al, 1979).

There are at least five structurally and genetically distinct types of collagen each consisting of three \( \alpha \) chains. Besides having different molecular forms, these collagen types vary in their tissue distribution. Table I illustrates this point (from Bronstein and Sage, 1980 and Prockop et al, 1979).

It should be noted that two or more collagen types can be found simultaneously in different tissues. For example, Types I and III have been demonstrated concurrently in lung, heart, uterus, liver, nerve, placenta, tooth, periodontal ligament, skin and kidney; Type I collagen is found associated with Type II collagen in articular and epiphyseal cartilage in the perichondrium.

D. Collagen Biosynthesis and Regulation

Because of the genetic diversity of the collagen types, the first control over collagen biosynthesis involves the process of transcribing the selected genome which codes for a particular procollagen polypeptide chain.

Although there appears to be tissue selectivity with regard to the collagen type synthesized, such as Type II in cartilage and type IV in basement membrane, it is likely that most tissues contain multiple collagen types. This raises the question as to whether collagen producing cells are capable of
simultaneously synthesizing more than one type of collagen, or
whether separate populations of cells are responsible for this
phenotypic diversity. While it has been demonstrated by Gay
et al (1976) that human skin fibroblasts are capable of simul­
taneously synthesizing types I and III collagen, this does not
imply that this is a universal property of all collagen pro­
ducing cells. Other cells may require a cell division in order
to permit their progeny to select for a different collagen
type.

1) Factors Affecting Modulation of Collagen Phenotype

It has been convincingly demonstrated in vitro that
collagen phenotype can be modulated in response to various
culture techniques and environmental factors.

The relation between in vitro senescence and phenotypic
alteration has been described in chondrocytes (Mayne et al,
1976b) and in osteoblast-like cells (Scott et al, 1980).

The relative proportions of collagen types can change
during chondrocyte subculture as the percent synthesis of
Type II collagen declines (Cheung et al,1976; Benya et al, 1978).

The transfer of primary monolayer chondrocytes to
secondary spinner cultures can cause a reversion to normal
type II synthesis (Norby et al, 1977).

Primary chick chondrocytes grown in the presence of
embryo extract (Mayne et al, 1976b) or 5'-bromo-2'-deoxyuri-
dine (Mayne et al, 1975) change their collagen phenotype.

The modulating effect of fetal calf serum (Narayanan and
Page, 1977) and of metabolites such as calcium and pyrophos-
phate (Deshmukh and Kline, 1976; Deshmukh et al, 1976) have been described.

Mediators of inflammation such as lysosomal enzymes have been shown to have the ability to alter collagen phenotype (Narayanan and Page, 1976; Deshmukh and Nimni, 1973).

It has been demonstrated that during embryonic chick limb development and morphogenesis there are stage-dependent transitions in collagen type (Linsenmayer et al, 1973a, 1973b; Van der Mark et al, 1976).

This modulation of phenotype also occurs in the developmental sequence of matrix-induced endochondral bone formation, following allogeneic subcutaneous implants of demineralized rat bone matrix (Reddi et al, 1977; Foidart and Reddi, 1980).

2) Stability of Collagen Phenotype

Contrasted with this concept of instability of collagen phenotype in culture, was the demonstration of a stable collagen phenotype in organ culture (Benya and Nimni, 1979) or in cultures where high cell density or tissue matrix was maintained (Muller et al, 1977). These two studies imply that cell-matrix interaction and low proliferation with concomitant cell-cell interaction confer stability of collagen phenotype in the in vitro situation.

In addition it has been demonstrated that cultured lung fibroblasts can maintain a stable collagen phenotype and this qualitative and quantitative stability of collagen production
existed during both the growth phase and prolonged subculture 
(Hame and Crystal, 1977).

It has also been shown that under conditions of a 
constant extracellular milieu, collagen production in human 
lung fibroblasts is stable and independent of cell growth and 
population doublings (Breul et al, 1980).

3) Intracellular Control of Collagen Production

While modulation of collagen phenotype is a well-described 
phenomenon, in certain in vitro and in vivo systems, it raises 
questions concerning the mechanism involved in cellular regu­ 
lation of collagen production from the standpoint of qualita­
tive and quantitative control with regard to collagen pheno­
type and amount, respectively.

a. Intracellular degradation

It is known that the production of functional collagen is 
not an accurate reflection of the degree of collagen synthesis. 
It has been demonstrated that up to 20-40% of newly synthesized 
collagen is degraded intracellularly prior to secretion, and 
that this degradative process is independent of collagenase­
mediated or phagocytic mechanisms (Bienkowski et al, 1978b and 
1978a). The exact role of this intracellular degradation has 
not been clearly elucidated although it has been suggested 
that it functions to control the quantity and fidelity of the 
collagen produced by each cell (Breul et al, 1980).
b. Transcriptional modulation and viral transformation

It has been demonstrated that concentration of procollagen mRNA correlates with rates of procollagen synthesis in chick embryonic tendon fibroblasts in situ, in culture, and in Rous sarcoma virus (RSV) - transformed fibroblasts, indicating that procollagen synthesis rates are determined at the transcriptional level and not by translational efficiency (Rowe et al, 1978). This association between procollagen synthesis and procollagen mRNA levels has been confirmed in independent studies (Howard et al, 1978; Sandmeyer and Bornstein, 1979).

Transcriptional rather than translational control has also been reported in a study on the regulation of procollagen synthesis during the development of chick embryo calvaria (Moen et al, 1979). The increases in procollagen synthesis were associated with a loss or inactivation of mRNA's for non-collagenase proteins.

c. Procollagen peptide feedback inhibition

It has been recently reported that procollagen peptides from the NH₂-terminal ends of type I or type III fetal calf skin procollagen are specific inhibitors of collagen synthesis via a feedback mechanism, whereas collagen degradation, collagen hydroxylation and synthesis of non-collagen proteins were unaffected (Wiestner et al, 1979). While these procollagen peptides also effectively inhibited
collagen synthesis in human fibroblast cultures, they did not affect synthesis of type II collagen. This suggests that there may be structural specificity between different types of pro-collagen peptides.

4) Environmental Control of Collagen Production

From the previous descriptions it is readily apparent that external influences can affect collagen phenotype. In addition, many environmental factors are capable of modulating the level of collagen synthesis.

a. Serum, cell density, acetate, ascorbate

It has been demonstrated that culture medium conditions can affect the maintenance of the differentiated state in primary cultures of avian tendon cells (Schwarz et al, 1976). Serum in excess of 0.5% decreased collagen synthesis; high cell densities overcame inhibitory serum effect. Higher lactate ion concentration (30 mM) raised collagen levels to their in vivo levels. High cell density and the presence of ascorbate permitted normal levels of collagen production, whereas low cell density or absence of ascorbate resulted in collagen being synthesized at only one third the normal level (Schwarz and Bissell, 1977).

b. Hormones

In vitro hormonal stimulation and inhibition of bone collagen synthesis has been well documented in a fetal rat calvaria organ culture system.

i) Insulin

Insulin has direct effects by increasing the labelling
of collagenase-digestible protein (CDP) from 60-115%, with the peak response occurring at a hormone concentration of $10^{-7}$M (Canalis et al, 1977). Insulin also increased the labeling of non-collagen protein (NCP) but its preferential effect on CDP caused a significant increase in percent collagen synthesis (PCS) in the insulin treated calvaria. The onset of this insulin effect occurred after 12-24 hours in culture and was lost within 3 hours of removal of the hormone. The effect was not due to decreased collagen breakdown since there was no difference in the amounts of CDP released to the medium.

ii) Parathyroid hormone (PTH)

PTH has been shown to decrease the incorporation of $[^3$H] proline into CDP but has little effect on NCP. Hormonal levels from $10^{-9}$ M - $10^{-6}$ M were effective inhibitors of collagen synthesis (Dietrich et al, 1976). After 24 hours of culture in $10^{-8}$ M PTH there was a decrease in both collagen synthesis and procollagen mRNA levels, but little effect on the release of collagen into the medium, suggesting that PTH regulation of collagen synthesis occurs primarily by affecting procollagen mRNA levels (Kream et al, 1980).

Inhibition of bone collagen synthesis was equally effective using native bovine PTH-(1-84), the active amino terminal portion bPTH-(1-34) or a synthetic analog of bPTH, and the effects were dose related (Raisz et al, 1979).

iii) Glucocorticoids

The effects of cortisol and related glucocorticoids on fetal rat bone collagen synthesis in vitro appear to have two different
effects. Cortisol (0.03 - 3 μM) stimulated percent collagen synthesis in short term cultures (48 hours). The long term effects were inhibition of collagen and non-collagen synthesis probably due to inhibition of precursor cell proliferation (Dietrich et al, 1979).

iv) Vitamin D metabolites

The effects of 1α, 25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) on bone collagen synthesis were to cause a dose-related inhibition of percent collagen synthesis by selectively inhibiting incorporation of label into CDP with no effect on the NCP fraction (Raisz et al, 1978).

While 1α,25(OH)₂D₃ is clearly the most potent in terms of its inhibitory capacity, other vitamin D metabolites also inhibited collagen synthesis (Raisz et al, 1980).

v) Prostaglandins

It has been demonstrated that prostaglandin E₂ (PGE₂) selectively inhibited incorporation of label into CDP and not into NCP (Raisz and Koolemans-Beynen, 1974). This effect did not occur immediately and was not due to increased release of collagen from bone to medium, suggesting that an inhibition of collagen synthesis had occurred.

c. Cyclic AMP, beta-adrenergic system

The effect of cyclic AMP on the intracellular degradation of newly synthesized collagen has been studied (Baum et al, 1980). An increase in intracellular levels of cyclic AMP in human lung fibroblasts, caused
by prostaglandin E1 or cholera toxin, resulted in decrease in collagen production due to an increase in intracellular degradation.

The regulation of collagen synthesis by the beta-adrenergic system has also been studied because the latter affects cyclic AMP levels by elevating adenyl cyclase activity (Berg et al, 1981). It was found that human lung fibroblasts incubated with the beta-agonists isoproterenol or epinephrine synthesized 30% less collagen. The mechanism for this action appears to be the beta-receptor since only the 1-isomer of isoproterenol was effective, and since propranolol, a beta-blocker prevented the cyclic AMP increase and concomitant decrease in collagen production.

d. Diphenylhydantoin (Dilantin)

The pharmacological effects of Dilantin therapy on collagen synthesis have been reported by Hassell et al (1976). Fibroblasts derived from dilantin-induced gingival hyperplastic tissue had higher percentages of collagen synthesis than fibroblasts from normal or non-induced gingival tissue (from epileptics on Dilantin who exhibited tissue overgrowth). This effect was not specific to collagen, but occurred as a result of an increase in overall protein synthesis in the dilantin induced hyperplastic tissue.

e. Growth Factors

It has recently been reported that autologous growth factors derived from organ and cell culture of fetal rat calvaria can
stimulate incorporation of $[^3H]$ proline into collagen and $[^3H]$ thymidine into DNA in organ culture of the same system (Canalis et al, 1980). This suggests intercellular and cell-matrix interaction in regulating collagen synthesis.
III. Objectives

Electrical perturbations are employed clinically as a treatment modality to enhance the repair of non-healing fractures. It was decided to select one aspect of the healing process, specifically collagen synthesis, and determine if, in organ culture, PEMF is capable of modulating the production of collagen.

The organ culture system employed in this study, fetal rat calvaria, has already been characterized with regard to its levels of collagen synthesis and its response to physiologic factors such as hormones, which act to potentiate or suppress collagen production.

The specific objectives of this study were to:

1) Isolate and maintain an organ culture system.

2) Examine the effect of PEMF upon collagen synthesis in this system.

3) Determine whether PEMF can modulate the response of the system to a known stimulator (insulin) and inhibitor (PTH).

4) Attempt to characterize the importance of various parameters of PEMF stimulus specifically, continuous or intermittent exposure and duration of exposure.
IV. Materials and Methods

1) Culture Techniques:

The frontal and parietal bones were removed from 21-day old fetal rats (Sprague-Dawley, Charles River Breeding Laboratories) and split along the sagittal suture. Half calvaria were cultured at 37°C under a humidified atmosphere of 5% CO₂/95% air in 25 ml Erlenmeyer flasks containing 2 ml of modified BGJ medium (Bingham and Raisz, 1974), supplemented with 5% fetal calf serum that had been heat inactivated at 56°C for 30 min, and with 100 μg/ml ascorbic acid. The concentration of proline in the medium was 1 mM. The flasks were sealed with rubber stoppers.

In the appropriate experiments either 10⁻⁸M synthetic bovine PTH 1-34 (Beckman) or 10⁻⁸M porcine insulin (Sigma) was added to the medium.

Calvaria were then exposed to pulsating electromagnetic fields (PEMF) for various periods of time (continuous, single 12 hr pulses or intermittent pulses of 12 hr on, 12 hr off, 12 hr on, 12 hr off). The PEMF is produced by pulsating currents through two opposite parallel Helmholtz coils 8-10 cm apart and the calvaria in flasks were placed between the coils. The magnetic field induces a current of approximately 2 μA/cm². The pulses were of a quasi-rectangular shape of 200-300 μsec duration with a narrow component of opposite direction of 15-30 μs (Fig. 1). The fields were delivered in the form of 5 msec - pulse trains at a frequency of 15 or 25 Hz. The PEMF coils used in this study correspond in waveform, pulse burst and frequency to the coils
most widely used in the treatment of non-healing fractures.

The calvaria were transferred to fresh medium at 24 hours. At 48 hours, 5 μCi/ml of [5'-3H] proline (7.8 Ci/mmol, New England Nuclear, Boston, Mass.) was added to each flask for 2 hrs. The cultures were terminated by removing the calvaria and extracting them with 3 washes of 5% TCA, 3 washes of acetone and 2 washes of ether. The calvaria were then dried and weighed on a Perkin-Elmer electronic balance. The bones were then homogenized in 1.0 ml of 0.5 M acetic acid with glass/glass homogenizers.

2) Analytical Methods:

An aliquot of the homogenate was incubated in re-purified bacterial collagenase (kindly provided by Dr. Barbara Kream) and the labeled proline incorporated into collagenase digestible protein (CDP) and non-collagen protein (NCP) was measured according to the method of Peterkofsky and Diegelmann (1971). The enzyme used had been repurified in a G-200 Sephadex column and did not contain any proteolytic activity on non-collagen substrates.

Duplicate test tubes were set up for each bone sample. These were a CP tube (C if for collagenase digestible protein or the supernatant; P is for noncollagen protein or the precipitate) and a B tube (supernatant of this tube serves as an individual blank for each bone). The precipitate was discarded. In addition, there were blank CP and B tubes with 100 μl 0.5 M acetic acid (instead of the homogenate sample) and reference CP and B tubes containing an aliquot of collagen, to determine that the collagenase was working during the experiment. To each tube 100 μl of 0.5 M NaOH was added.
Collagenase and blank Tris mixtures were prepared based on the following recipe.

### Collagenase and Tris Mixture Recipe

<table>
<thead>
<tr>
<th>Samples</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase or CaCl₂ - Tris Buffer</td>
<td>µl</td>
<td>100</td>
<td>150</td>
<td>200</td>
<td>250</td>
<td>300</td>
<td>350</td>
</tr>
<tr>
<td>62.5 mM N-ethylmaleimide</td>
<td>ml</td>
<td>.40</td>
<td>.60</td>
<td>.80</td>
<td>1.0</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>0.025 M CaCl₂</td>
<td>µl</td>
<td>200</td>
<td>300</td>
<td>400</td>
<td>500</td>
<td>600</td>
<td>700</td>
</tr>
<tr>
<td>1 M HEPES</td>
<td>ml</td>
<td>4</td>
<td>5</td>
<td>6.7</td>
<td>8.4</td>
<td>10.0</td>
<td>11.7</td>
</tr>
</tbody>
</table>

To each CP tube was added 100 µl of vortexed homogenate sample and 200 µl of the collagenase mixture. To each B tube was added 100 µl of vortexed homogenate and 200 µl of the Tris-blank mixture.

All the tubes were capped, vortexed well, and were then incubated for 90 minutes in a 37°C Dubnoff incubator at 40-60 oscillations per minute. The tubes were then placed on ice and added immediately to each sample was 500 µl of TCA-TANNIC ACID mixture (made fresh during the incubation) and 100 µl of 1 mg/ml bovine serum albumin in water. Both these solutions were cold. The TCA-tannate mixture was a 10% solution of TCA with 0.5% tannic acid.

The tubes were vortexed well and were centrifuged at 2800 RPM for 15 minutes in the cold at 4°C. The supernatants were removed with a pasteur pipette to counting vitals containing 10 ml of ACS (these are the C and B tubes). The precipitates of the CP tubes were resuspended in 100 µl of demineralized water and 0.75 ml of NCS. The dissolved precipitates were transferred to counting vials containing 8 ml
Spectrafluor - toluene and POPOP and each tube was rinsed 2x with 1 ml of the spectrafluor-toluene and this was added to the counting vials to bring them up to 10 ml of scintillant.

All the tubes were capped and vortexed well and were counted for 10 minutes in an Isocap 300 counter made by Nuclear-Chicago. The cpms of each vial were converted to dpm using a correction factor to account for the counting efficiency of the machine. The data was analyzed as follows:

Collagenase Digestible Protein (CDP) = Cdpm - Bdpm
Non-Collagen Proteins (NCP) - Pdpm.

Specific Activity of Collagen was expressed as CDP/μg dry weight of bone sample.

Specific Activity of Non-Collagen Protein was expressed as NCP/μg dry weight of bone sample.

Percent Collagen Synthesis (PCS) was calculated after multiplying the dpm in NCP by 5.4 to correct for the relative abundance of proline in collagen and non-collagen protein.

\[ PCS = \frac{CDP}{CDP + 5.4 \times NCP} \times 100 \]
V. Results

1) Effect of Continuous PEMF (48 hr)

Initial experiments were carried out in which the calvaria explants were subjected to continuous exposure to PEMF for 48 hrs and the incorporation of $[^{3}H]$ proline into collagenase digestible (CDP) and non-collagen protein (NCP) was analyzed. These are summarized in Table 2.

Because of the variability in the labelling of various samples, percent collagen synthesis as a fraction of total protein synthesis is a more reliable measure of treatment effects. It therefore appeared that 48 hours of continuous exposure had no effect.

The clinical method of choice for PEMF application is one of intermittent exposure, 12 hr on and 12 hr off. This regimen has evolved empirically over the years as experience was gained with this modality of treatment. In fact, continuous exposure has proven to be less effective in comparison to this intermittent sequence of treatment.

Since the exact mechanism of electrically-induced stimulation is unknown, we elected to evaluate shorter and variable periods of PEMF exposure in order to more closely reflect the clinical situation.

2) Effect of 24 h PEMF exposure, 24 h rest period

The tissue explants were then tested for their response to an initial 24 h pulse with PEMF followed by a 24 h rest period outside the field presence. This data is shown in Table 3.
This treatment regimen consistently resulted in elevated levels for collagenase-digestible protein and percent collagen synthesis with little effect on non-collagen protein. While these increments in collagen synthesis were small and not statistically significant in single experiments, as a cohort they indicated PEMF-induced enhancement.

3) Effect of Intermittent PEMF Exposure (12 h on, 12 h off, 12 h on, 12 h off)

The next protocol tested was a 24 h exposure applied intermittently in 12 h installments. These results are shown in Table 4. In this case the field showed no effect in stimulating collagen synthesis.

4) Effect of 12 h Pulse on PEMF, 36 h Rest Period

To determine if the duration of PEMF treatment was a critical variable, further experiments were carried out with 12 h exposure to PEMF followed by a 36 h silent period outside the field. This data is presented in Table 5.

Single 12 h pulses of PEMF elevated CDP and PCS levels. One of these experiments was statistically significant. These experiments more strongly suggested that PEMF has the ability to potentiate collagen synthesis.

5) Effect of Alteration of Flux Density

In previous experiments, the electromagnetic coils were maintained in a vertical position and the culture flasks were placed in between the coils with the long axis of each flask parallel to
the coils. Based upon the physical properties of electromagnetic coils, as current flows through the coil winding, an electromagnetic field is produced at right angles to the face (diameter) of the coil, and the number of field lines per square centimeter, termed the flux density, determines the voltage induced in an object placed in the field. The result was that with the experimental configuration employed so far, a smaller flux density was experienced by the tissue explants.

In order to increase flux density, the coils were maintained in a horizontal position with the flasks perpendicular to the coils. This allowed more surface area of medium and calvaria to be stimulated and thus increase the current density. It was elected to employ the protocol of intermittent PEMF application (12 h on, 12 h off, 12 h on, 12 h off) to most closely approximate the clinical situation. These results are shown in Table 6.

Under these conditions PEMF caused a significant increase in PCS.

6) Effect of Increased Flux Density, Intermittent PEMF and Hormonal Action

To obtain insight into the mode of action of PEMF, we tested the relationship between PEMF stimulation and collagen synthesis modulation by hormonal treatments of known effect. These included 10^{-8} M insulin, and 10^{-8} M PTH, a proven stimulator and inhibitor of collagen synthesis respectively (Canalis et al, 1977; Dietrich et al, 1976). This data is also presented in Table 6.
As is evident, insulin was a significant stimulator of PCS. In comparison the PEMF-induced effect was of the same order of magnitude as insulin alone. The elevation in PCS with the combination of insulin and PEMF exceeded that of either treatment alone, although the increase was not additive. The increase in collagen synthesis with combined insulin and PEMF may, however, be real. CDP levels in the combined treatment group were significantly higher than in the control group, while the elevation of NCP levels tended to mask any statistical significance in PCS in this combined treatment group.

The PEMF-induced enhancement was blocked by 10^{-8} M PTH, which completely abolished the stimulatory potential of PEMF.
VI. Discussion

These findings demonstrate several points. Firstly, PEMF is capable of eliciting a direct anabolic response in a bone forming culture system by enhancing collagen synthesis.

The use of high flux density combined with an intermittent PEMF exposure regimen resulted in a significant increase in percent collagen synthesis.

Secondly, the effect mediated by these electrical signals was quantitatively similar to the response of this system to hormonal stimulation by insulin (10^-8 M). The combined use of PEMF and insulin appeared to increase the response compared to each modality alone, although this stimulation was less than additive.

Thirdly, the stimulatory effect of PEMF was completely abolished in the presence of PTH (10^-8 M). This would indicate that PEMF acts upon the same population of cells which are responsive to PTH. While no probing of the mechanistic process was undertaken this finding would suggest that PEMF acts on one of the events in the sequence of events involved in the transduction of the hormonal effect of PTH. It has been previously demonstrated that populations of cells enriched in osteoblasts show a predominant response to PTH stimulation of cAMP (Luben et al, 1976). This would tend to provide supportive evidence that the target cell of PEMF may be the osteoblast.

Further evidence was obtained regarding several other points. While no systematic experimentation was carried out to test the importance of magnetic flux density, the data suggested that it plays a role in modulating the response. Maximization of flux
density resulted in highly significant stimulation of collagen synthesis and may indicate that a flux threshold must be reached in order to elicit a response.

It also appears that the silent period following stimulation may be an important factor in determining the potential success of the electrical perturbation. This point was illustrated by the failure of continuous PEMF stimulation to enhance collagen synthesis when compared to shorter and intermittent exposure periods followed by silent periods outside the field presence. The fact the PEMF effects are observed subsequent to these pause periods suggests that in vivo PEMF may affect an early event in the osteogenic process, such as proliferation or differentiation.

This correlates well with the in vivo situation. Empirically, the most successful clinical treatment modality involves intermittent stimulation via PEMF, following precise spatial placement of the coils in relation to the nonhealing fibrous defect. It should be noted that this principle of threshold flux levels and intermittent application providing for a maximum response is not unique to this system. An evaluation of the biological response to orthodontic tooth movement has indicated that minimum force levels are required for tooth movement to be initiated; in addition intermittent force application which provides the tissue with rest periods results in the most physiologic tooth movement with the minimum of undesired sequelae (Reitan in Graber and Swain, ed. 1976).

The biphasic nature of effects with continuous exposure to PEMF in that it may or may not be stimulatory has precedence in pharmacology, such as in cyclic AMP control of proliferation (and differentiation)
(Chlapowski et al, 1975; Deshpande and Siddiqui, 1976; Pastan et al, 1975).

An understanding of the electrical perturbations produced by PEMF in the responding cells is relevant to the discussion of PEMF-induced effects on collagen synthesis.

PEMF can generate a current in any conductive material exposed to it. This would include the electrolytic extracellular environment as well as any intracellular charged molecules.

Measurements of the current generated by the electromagnetic fields used in this study have shown that in a saline environment, they produce a range of current from 2-8 $\mu$A/cm$^2$ depending upon the radius of the circle of the culture dish (Sechaud and Pilla, 1981). It has also been calculated that the magnitude of the induced electric field was approximately 1 mV/cm.

The effect of direct current on development and tissue repair has been extensively reviewed by Jaffe and Nucitelli (1977). They indicated that the current affecting a biological system, such as the cell membrane can be inferred from the current passing through the extracellular medium.

Uninterrupted DC currents don't penetrate the cell membrane, but may act by translocating charged molecules on the cell surface (Poo and Robinson, 1977).

PEMF produces pulsating currents in the medium, and based upon the impedance properties of cell membranes, such currents penetrate the cell membrane (Pilla and Margules, 1977). Oscillating DC currents were shown to produce similar biological effects (alkaline phosphatase inhibition) to PEMF (Facklam, personal communication).
Since fracture repair has been achieved with both DC and oscillating AC currents, it raises the question as to whether they both act as similar modalities on the target cell or tissue. The projected hypothesis is that while continuous DC currents don't penetrate the cell membranes, they may be capable of causing electrochemical changes in the cell membrane due to ionic redistribution. This type of response would be similar to the changes in membrane permeability subsequent to hormonal binding to surface receptors, and may involve a second messenger such as cyclic AMP or cytoskeletal rearrangement for stimulus transduction (Rodan in Banner et al, 1981). Pulsating DC currents and PEMF induced oscillating electrical fields are capable of penetrating cell membranes. Therefore these stimuli could act either at the level of the cell membrane or could directly affect intracellular organelles.

A very brief and general discussion of how PEMF might modulate proliferation and/or phenotypic expression in skeletal tissue may help put the results obtained in this study into the broader context of the in vivo situation.

In terms of initiating normal fracture repair, PEMF could exercise control at several levels.

a) by promoting proliferation of osteogenically competent cells.

b) by increasing the probability for expressing differentiated properties in a precursor cell population.

c) by controlling the expression of the differentiated function with respect to matrix formation and calcification through activation of osteogenically committed cells.

Several of these concepts have been addressed by Owen (1978),
with regard to skeletal tissue possessing osteogenic precursor cells whose only option is bone formation, and inducible osteogenic precursor cells which require the presence of an inducing agent to trigger the differentiated state. It is possible for PEMF to be involved at several of these levels. For example, PEMF has also been shown to significantly increase lysozyme levels in chick cartilage cells and this step precedes matrix calcification (Norton, unpublished data).

PEMF may thus be a general stimulus whose specificity is determined by the responsive cell and its threshold to this stimulus.

It should be noted that no attempts were made in this study to address the question of the mechanism involved in PEMF-mediated effects; rather the objective was to determine whether PEMF is capable of inducing an anabolic effect on an organ culture model. With the demonstration that PEMF can indeed modulate collagen synthesis, future experimentation can be designed to further characterize the effect upon collagen synthesis and to investigate the mechanistic steps involved.
VII. Summary

This study on the effects of PEMF in organ culture has demonstrated the following:

1) PEMF has the ability to induce an anabolic effect in a bone forming system, specifically it can enhance the production of collagen.

2) The nature of this potentiation mimics hormonal action since its effects are equal and not additive to those of insulin (10^{-8} M) and are blocked by PTH (10^{-8} M).

3) The data suggest that intermittent exposure to electrical perturbation followed by a silent period is superior to other regimens of treatment and that effectiveness may be related to the flux density of the PEMF.
VIII. Tables and Figures

**TABLE I. GENETICALLY AND STRUCTURALLY DISTINCT COLLAGEN**

<table>
<thead>
<tr>
<th>Type</th>
<th>Tissue Distribution</th>
<th>Molecular Form</th>
<th>Chemical Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Bone, tendon, intervertebral disc, liver, kidney, cornea, visceral endoderm, tooth, gingiva</td>
<td>([\alpha_1(I)2\alpha_2])</td>
<td>Hybrid composed of 2 chains low in hydroxylysine and glycosylated hydroxylysine</td>
</tr>
<tr>
<td>Itrimer</td>
<td>Embryonic tendon, dentin, skin tumor</td>
<td>([\alpha_1(I)]_3)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Cartilage, bone growth plate, lung: tracheal and bronchial cartilage, intervertebral disc</td>
<td>([\alpha_1(II)]_3)</td>
<td>Relatively high in hydroxylysine and glycosylated hydroxylysine</td>
</tr>
<tr>
<td>III</td>
<td>Skin, arteries, uterus, tooth, gingiva</td>
<td>([\alpha_1(III)]_3)</td>
<td>High in hydroxyproline and low in hydroxylysine contains inter-chain disulfate bonds</td>
</tr>
<tr>
<td>IV</td>
<td>Basement membranes: alveolar, capillary, glomerular, placenta tumor, lens capsule, liver, skin</td>
<td>([\alpha_1(IV)]_3)</td>
<td>High in hydroxylysine and glycosylated hydroxylysine, may contain large globular regions</td>
</tr>
<tr>
<td>V</td>
<td>Basement membranes, alveolar, capillary cartilage bone, nerve dura, liver, placenta, tendon, gingiva, muscle</td>
<td>([\alpha_B \text{ and } \alpha_{AaC} \text{ or } \alpha_1, \alpha_2, \alpha_3])</td>
<td>Similar to Type IV</td>
</tr>
</tbody>
</table>
TABLE II. EFFECT OF 48 H PEMF ON THE INCORPORATION OF $[^{3}H]$ PROLINE INTO COLLAGENASE DIGESTIBLE (CDP) AND NON-COLLAGEN PROTEIN (NCP) IN FETAL RAT CALVARIA: PERCENT COLLAGEN SYNTHESIS (PCS) IS CORRECTED FOR THE RELATIVE ABUNDANCE OF PROLINE CDP AND NCP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CDP</th>
<th>NCP</th>
<th>PCS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/μg dry weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>40.3 ± 6.5</td>
<td>84.5 ± 9.7</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>PEMF</td>
<td>25.4 ± 3.8</td>
<td>56.7 ± 5.2*</td>
<td>7.5 ± 0.7</td>
</tr>
<tr>
<td><strong>Experiment B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>33.0 ± 6.5</td>
<td>84.9 ± 6.8</td>
<td>6.6 ± 1.0</td>
</tr>
<tr>
<td>PEMF</td>
<td>32.1 ± 6.4</td>
<td>92.5 ± 7.2</td>
<td>5.9 ± 0.8</td>
</tr>
</tbody>
</table>

Values are means ±SE for 5-6 half calvaria incubated for 48 h and pulsed for the last 2 h with $[^{3}H]$ proline.

*Significantly different from control  P < .05
### TABLE III
EFFECT OF AN INITIAL 24 H PULSE OF PEMF ON THE INCORPORATION OF $[^{3}\text{H}]$ PROLINE INTO COLLAGENASE-DIGESTIBLE (CDP) AND NON-COLLAGEN PROTEIN (NCP) IN FETAL RAT CALVARIA: PERCENT COLLAGEN SYNTHESIS (PCS) IS CORRECTED FOR THE RELATIVE ABUNDANCE OF PROLINE IN CDP AND NCP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment A</th>
<th></th>
<th></th>
<th>Experiment B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDP</td>
<td>NCP</td>
<td>PCS</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dpm/µg dry weight</td>
<td></td>
<td></td>
<td>dpm/µg dry weight</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>34.1 ± 5.7</td>
<td>73.8 ± 7.6</td>
<td>7.8 ± 0.6</td>
<td>25.3 ± 4.8</td>
<td>58.2 ± 3.6</td>
</tr>
<tr>
<td>PEMF</td>
<td>40.3 ± 7.8</td>
<td>74.4 ± 5.7</td>
<td>8.9 ± 1.0</td>
<td>28.8 ± 4.9</td>
<td>57.2 ± 7.4</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 half calvaria incubated for 48 h and pulsed for the last 2 h with $[^{3}\text{H}]$ proline.
TABLE IV. EFFECT OF AN INITIAL 24 H INTERMITTENT PEMF (12 H ON, 12 H OFF, 12 H ON, 12 H OFF) ON THE INCORPORATION OF [$^3$H] PROLINE INTO COLLAGENASE-DIGESTIBLE (CDP) AND NON-COLLAGEN PROTEIN (NCP) IN FETAL RAT CALVARIA: PERCENT COLLAGEN SYNTHESIS (PCS) IS CORRECTED FOR THE RELATIVE ABUNDANCE OF PROLINE IN CDP AND NCP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CDP dpm/μg dry weight</th>
<th>NCP dpm/μg dry weight</th>
<th>PCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>52.2 ± 11.0</td>
<td>88.7 ± 6.0</td>
<td>9.4 ± 1.2</td>
</tr>
<tr>
<td>PEMF</td>
<td>50.2 ± 5.0</td>
<td>93.4 ± 6.9</td>
<td>9.0 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6 half calvaria incubated for 48 h and pulsed for the last 2 h with [$^3$H] proline.
TABLE V.  EFFECT OF AN INITIAL 12 H PULSE OF PEMF ON THE INCORPORATION OF \[^{3}\text{H}]\ \text{PROLINE}\) INTO COLLAGENASE-DIGESTIBLE (CDP) AND NON-COLLAGEN PROTEIN (NCP) IN FETAL RAT CALVARIA: PERCENT COLLAGEN SYNTHESIS (PCS) IS CORRECTED FOR THE RELATIVE ABUNDANCE OF PROLINE IN CDP AND NCP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CDP dpm/\mu g dry weight</th>
<th>NCP dpm/\mu g dry weight</th>
<th>PCS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.4 ± 1.5</td>
<td>42.1 ± 2.0</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>PEMF</td>
<td>16.8 ± 3.0</td>
<td>52.9 ± 4.9</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td><strong>Experiment B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.7 ± 0.4*</td>
<td>42.3 ± 1.8</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>PEMF</td>
<td>11.9 ± 1.2</td>
<td>38.3 ± 3.6</td>
<td>5.4 ± 0.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4-6 half calvaria incubated for 48 h and pulsed for the last 2 h with \[^{3}\text{H}]\ \text{proline}.

*Significantly different from control P < .02

†Significantly different from control P < .002
TABLE VI. EFFECT OF MAGNETIC FLUX DENSITY, INTERMITTENT (12 H ON, 12 H OFF, 12 H ON, 12 H OFF) PEMF, INSULIN (10-8 M) AND PARATHYROID HORMONE (10-8 M) ON THE INCORPORATION OF [3H] PROLINE INTO COLLAGENASE DIGESTIBLE (CDP) AND NON-COLLAGEN PROTEIN (NCP) IN FETAL RAT CALVARIA: PERCENT COLLAGEN SYNTHESIS IS CORRECTED FOR THE RELATIVE ABUNDANCE OF PROLINE IN CDP AND NCP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CDP</th>
<th>NCP</th>
<th>PCS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/µg dry weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>40.0 ± 10.4</td>
<td>57.9 ± 14.1</td>
<td>10.6 ± 0.6</td>
</tr>
<tr>
<td>PEMF</td>
<td>64.1 ± 13.2</td>
<td>67.8 ± 13.8</td>
<td>14.7 ± 0.5‡</td>
</tr>
<tr>
<td>Insulin (10-8 M)</td>
<td>58.1 ± 2.4</td>
<td>65.5 ± 1.8</td>
<td>13.9 ± 0.6</td>
</tr>
<tr>
<td>PTH (10-8 M)</td>
<td>29.3 ± 4.1</td>
<td>61.7 ± 4.3</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>PEMF + Insulin (10-8 M)</td>
<td>79.7 ± 12.8§</td>
<td>75.6 ± 8.0</td>
<td>16.3 ± 1.7*</td>
</tr>
<tr>
<td>PEMF + PTH (10-8 M)</td>
<td>34.7 ± 2.7</td>
<td>65.4 ± 3.6</td>
<td>9.0 ± 0.6^</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 half calvaria incubated for 48 h and pulsed for the last 2 h with [3H] proline.

‡Significantly different than control P < .001
*Significantly different than control P < .02
§Significantly different than control P < .05
^Significantly different than PEMF P < .001
FIGURE 1

Signal Parameters for D Field

Positive width = 220 µsec
Positive amplitude = 15 mV
Negative width = 20-30 µsec
Negative amplitude = 140 mV
Burst width = 5.2 msec
Repetition rate = 15 Hz
IX. Bibliography


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