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Timing of Pulsed Electromagnetic Field Stimulation does not affect the Promotion of Bone Cell Development

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Abstract

Pulsed electromagnetic field (PEMF) devices have been used clinically to promote the healing of surgically resistant fractures in vivo. However, there is a sparsity of data on how the timing of an applied pulsed electromagnetic field effects the osteogenic cells that would be present within the fracture gap. The purpose of this study was to examine the response of osteoblast-like cells to a PEMF stimulus, mimicking that of a clinically available device, using four protocols for the timing of the stimulus. The PEMF signal consisted of a 5ms pulse burst (containing 20 pulses) repeated at 15Hz. Cultures of a human osteosarcoma cell line, SaOS-2, were exposed to the four timing protocols, each conducted over three days. Protocol one stimulated the cells for eight hours each day, protocol two stimulated the cells for 24 hours on the first day, protocol three stimulated the cells for 24 hours on the second day and protocol four stimulated the cells for 24 hours on the third day. Cells were seeded with either 25,000 or 50,000 cells/well (24 well cell culture plates). All assays showed reduced proliferation and increased differentiation (alkaline phosphatase activity) in the PEMF stimulated cultures compared with the control cultures, except for protocol four alkaline phosphatase measurements. No clear trend was observed between the four protocols, however this may be due to cell density. The results indicated that an osteoblast-like cell line is responsive to a 15Hz PEMF stimulus, which will stimulate the cell line to into an increasing state of maturity.

Key Words: osteoblast; PEMF; proliferation; bone fracture healing; alkaline phosphatase

Introduction

Commercial devices employing pulsed electromagnetic fields (PEMF) for treatment of fracture healing have been available for over 20 years. These devices are not restricted to long bone fractures [Frykman et al. 1986; Kahanovitz et al. 1994] and, among other pathologies, can be used in osteoarthritic joints [Trock et al. 1994] and osteoporotic bone [Chang and Chang 2003; Tabrah et al. 1990]. This method of stimulation has also been effective in reversing femoral head necrosis and augmenting spinal fusions [Aaron 1994; Bassett et al. 1989; Guizzardi et al. 1994; Linovitz et al. 2002]. Promotion of tibial bone fracture union with these devices has been shown to be at least as effective as surgical intervention, with an increased success rate for patients who have already undergone failed surgical intervention [Gossling et al. 1992].

Bone cell proliferation and differentiation are important factors during bone tissue healing and exogenously applied stimuli, that specifically promote one or the other, have great therapeutic potential. Clinical PEMF devices have been shown to affect proliferation and differentiation of bone cells in vitro [Chang et al. 2003; Fitzsimmons et al. 1995; Lohmann et al. 2000; Lohmann et al. 2003; McLeod et al. 1991; Sollazzo et al. 1997; Yonemori et al. 1996]. PEMFs stimulate many subcellular responses in living systems and appear to demonstrate exquisite specificity of action depending upon both the physical and biological factors involved [Bassett 1989]. The proposed principal target for PEMFs is the plasma membrane and transmembrane proteins rather than the cytoplasm [Adair 1998; Luben 1993]. Gap junctions, specialised

intercellular junctions, have been proposed as mediators of the PEMF related cellular responses [Lohmann et al. 2003; Vander Molen et al. 2000].

Although many in vitro studies have been performed, there has been no consistency with PEMF exposure dose and timing. The few studies that have focused upon the exposure timing [De Mattei et al. 1999; Cane et al. 1997] used differing doses of PEMF stimulation and varied exposure time. To the authors' knowledge, no study has been published to evaluate pure timing effects of PEMF stimulation on osteoblast-like cell cultures. It is very likely that cell culture confluence and cell division rate have an effect on PEMF stimulation effects as highlighted by a recent study [Diniz et al. 2002]. Cultures in differing states of cellular maturation, when exposed to PEMF stimulation, were found to have different reactions. Initial seeding density is also a potent mediator of cell cultures exposed to PEMF stimulation [McLeod et al. 1993; Noda et al. 1987; Pavlin et al. 2002]. Thus by evaluating the effects of PEMF stimulation at differing seeding density and times of cell growth, the mechanism of cellular responses to PEMFs may become clearer.

The aim of this study was to evaluate the effects of PEMF stimulation timing and cell density on the development of the osteoblast-like cell cultures. The PEMF stimuli tested mimicked a commercially available bone-healing device (EBI Incorporated, Parsippany, USA). As an experimental model we used an osteogenic human osteosarcoma cell line (SaOS-2) and evaluated the effects of PEMFs on cell proliferation, measured by ³H-Leucine incorporation and cell differentiation via alkaline phosphatase production.

Materials and Methods

PEMF Device

The PEMF device (Figure 1) consisted of two separate coils connected together in series and placed 20mm apart, with dimensions 150 X 100 mm, designed for use with 24-well cell culture plates. Each coil was made up of 50 turns of 0.51mm diameter acrylic coated copper wire, producing a resistance of 2.3Ω . Two identical devices were built, characterised and tested for conformity. Coils were wired to a PEMF pulse generator (purpose built) that produced a pulsed magnetic field perpendicular to the cell monolayer (Figure 2a, measured with a Tesla meter, Model 5080, FW Bell, Orlando, FL, U.S.A.), inducing a parallel-aligned electric field (Figure 2b). A direct current supply powered the pulse generator.

The PEMF signal consisted of a 5ms pulse burst (each containing 20 pulses) repeated at 15Hz, creating an asymmetrical 'quasi-square wave' voltage trace during each burst at a frequency of $\sim 4\text{kHz}$. This pulse mimics the FDA approved clinical bone-healing device manufactured by EBI[®] (EBI Incorporated, Parsippany, USA).

Peak coil current duration lasted for $204\mu\text{s}$, producing a maximum magnetic flux of 1.3mT. Measurements of the flux at each seeded well location showed a $\pm 0.05\text{mT}$ fluctuation around this maximum value. The induced electrical field measured by a coil probe (62 turns of 0.07mm nominal diameter wire coiled with 5mm internal diameter and voltage measured across a 470Ω resistor) contained a 15mV positive amplitude and 100mV negative amplitude. Although the magnetic field is uniform

across each of the seeded wells, the electrical field will not be because of the conductive growth medium (assumed to have a conductivity of 1410mS/m). The electrical field strength induced at any position inside the well is dependant on radial distance from the centre. It has been theoretically and experimentally validated by others that the current density follows a sinh function decay, with the maximum of $2\mu\text{A}/\text{cm}^2$ at the outer well boundary decaying to zero at the centre of the well [McLeod et al. 1983].

Cell cultures

A human osteosarcoma cell line, SaOS-2 (ATCC No: HTB-85, Rockville, U.S.A.), was used for these experiments. The cells were cultured in minimum essential medium alpha (α MEM) supplemented with 10% fetal bovine serum, 1% penicillin - streptomycin diluted from stock solution [both 5,000 U/ml] and 0.01% gentamicin [10mg/ml] (All from Gibco, Grand Island, U.S.A.). The cells were less than 10 passages from original cell stock. For experimental procedures the cells were seeded (with 1ml of growth media) at densities of either 50,000 or 25,000 cells per well into the centre 8 wells of 24 well cell culture plates. Each well has an effective growth area of 1.9cm^2 (Nunc, Roskilde, Denmark). Only the centre 8 wells of the plates were seeded due to inhomogeneity of the PEMF stimulant at the edges of culture plates. For each PEMF protocol, after seeding, two 24 well plates were assigned to the PEMF exposed and two to the control group. The cells were allowed to attach for 2 hours before experimentation. All experimental procedures were conducted within a CO_2 incubator at a temperature of 37°C in an atmosphere of 95% air/5% CO_2 and 100%

relative humidity. This was performed 3 times for each protocol except for Protocol 1 that was repeated twice due to technical difficulties. Control cell culture plates were prepared in an identical manner and placed within the same incubator. When coils were activated the measured magnetic flux at the control plate showed no increase above the 0.1mT background field.

Measurements of temperature within the PEMF exposed cell culture wells during operation of the coils (with door of incubator closed) were conducted with a thermocouple. These results demonstrated a consistent controlled temperature of $37 \pm 0.5^{\circ}\text{C}$ indicating that heating effects did not contribute to changes in the cell cultures.

Experiments

The cell cultures were subject to four protocols, each with a different period of PEMF stimulation. All protocols had a total of 24 hours PEMF exposure, eliminating dose response as a variable and allowing PEMF timing to be studied over the three-day period (Figure 3). After 72 hours, cellular proliferation and differentiation were assayed.

Proliferation

De novo protein synthesis was assayed from ^3H -leucine incorporation into acid precipitable proteins (^3H -leucine, Amersham International, Little Chelford, UK)[Freshney 2000]. This measure gives an indication of growth in cell number. At the beginning of each experiment, the radiolabelled amino acid was added to the

growth media (1 μ Ci per millilitre of growth media) and was thus available for protein incorporation throughout the entire 72-hour experimentation protocol. Cells were rinsed with Hanks Balanced Salt Solution (Gibco, Grand Island, NY, U.S.A.) and treated with 5% trichloroacetic acid. Cell precipitates were then washed with sterile distilled water and solubilized in 0.5M NaOH / 0.1% Triton X-100 for 12 hours on a shaker table. Samples were manually triturated prior to sampling to ensure homogeneity.

Triplicate samples of 100 μ L from each well were then counted in a liquid scintillation counter (Wallac MicroBeta TriLux, Boston, MA, U.S.A.) with 150 μ L of scintillation fluid (Optiphase SuperMix, Perkin Elmer, Boston, MA, U.S.A.).

Differentiation

Levels of alkaline phosphatase in the culture medium were measured for an indication of early stage osteoblastic differentiation. 20 μ L samples of conditioned culture medium were admixed to 20mM of p-nitrophenol phosphate (Sigma-Aldrich, St. Louis, MO, U.S.A.). Phosphatase activity was determined by measuring light absorption at a wavelength of 405nm using a spectrophotometer (Beckman Coulter, Fullerton, CA, U.S.A.). Repeat measurements were obtained immediately, at one minute and at two minutes after addition of the cultured medium (Sigma Diagnostics Alkaline Phosphatase Procedure No 245). These three measurements were then used to calculate the rate of increase in light absorption. The rate was checked for linearity, and used to calculate alkaline phosphatase volume by means of the following equation:

$$\text{Alkaline Phosphatase (Units/Litre)} = (\Delta A \text{ per min} * TV * 1000) / (18.45 * SV * LP)$$

Where TV represents total volume, SV is sample volume, LP is length of light path, 18.45 is millimolar absorptivity of p-nitrophenol at 405nm and ΔA per min is the change in absorbance per minute. One unit of alkaline phosphatase activity is defined as the amount of enzyme that will produce one micromole of p-nitrophenol per minute.

Statistical Analysis

To facilitate comparisons, all PEMF exposed cultures in each protocol were normalised against control values, which were considered as 100% percent.

All experimental data for each protocol and cell density were pooled and averaged to produce each proliferation measurement. Error bars are expressed as +/- Standard Error of the Mean (SEM) on each graphed result.

Alkaline phosphatase error bars were calculated as:

$(\text{SEM of light absorbance rate} / \text{Average light absorbance rate}) * \text{Alkaline Phosphatase volume}$.

A non-parametric analysis of variance test, Kruskal-Wallis, was performed between results for all four protocols and seeding densities in both the proliferation and alkaline phosphatase data sets. Student's t tests were performed for all protocols and seeding densities (that were normally distributed) to compare PEMF exposed and

control cultures. When data sets did not satisfy normality calculations, the non-parametric Mann Whitney U Test was applied, which in the case of proliferation was both seeding densities in Protocol 1, 25,000cells/well seeding density in Protocol 3 and 50,000cells/well seeding density in Protocol 4. P values of 0.05 or less were considered significant. Alkaline phosphatase measurements were limited to low replicate numbers (between n=4 and n=8) due to technical difficulties resulting in the necessity to use non-parametric statistical analysis. Even through standard errors of the mean were small and consistent trends were seen in the data, statistical significance with the non-parametric analysis was not achieved.

Results

Results are reported as a percentage of the control for each exposure protocol. Linear regression of light absorbance rates, used to calculate alkaline phosphatase volume, ranged from an R^2 value of 0.8508 to 0.9976. Observation of the data reveals no statistical difference of either proliferation or differentiation with the timing of the PEMF stimulation.

Protocol 1

Eight hours of PEMF stimulation each day for three days was conducted. Compared to the control cultures, PEMF exposed cultures showed a significant 9% and 14% average reduction in *de novo* protein incorporation of ^3H -leucine (Figure 4).

Assays for alkaline phosphatase activity exhibited an increase in PEMF exposed cultures with respect to control cell cultures at both seeding densities (16% for 25,000 cells/well and 12% for 50,000 cells/well, Figure 5).

Protocol 2

Stimulating cultures with PEMFs for 24 hours on the first day followed by no stimulation for the next two days produced a reduction in proliferation with respect to control cultures. Variability was observed between cultures seeded at 25,000 and 50,000 cells/well. In cultures seeded at 50,000 cells/well, no significant reduction in PEMF exposed proliferation relative to controls was observed. In contrast, a significant 11% reduction was observed in cultures seeded at 25,000 cells/well (Figure 4).

Alkaline phosphatase activity was increased in PEMF exposed cultures with respect to the controls (20% for 25,000 cells/well and 14% for 50,000 cells/well, Figure 5). Greater increases in alkaline phosphatase production were observed at the lower seeding density than the higher density and inversely corresponded with the decrease in proliferation.

Protocol 3

Cells exposed to protocol 3 did not exhibit a significant down-regulation of proliferation in PEMF exposed cultures (Figure 4).

Cultures exposed to protocol 3 showed increases of alkaline phosphatase activity. There was a large increase of 38% for 50,000 cells/well, while 25,000 cells/well cultures exhibited a 19% increase, consistent with other protocols at that seeding density (Figure 5).

Protocol 4

Although there was a consistent trend for decreased proliferation from both seeding densities, this protocol was only significant for the 50,000 cells/well result (17% decrease, Figure 4).

The 50,000 cells/well result for this protocol was the only one that did not show the trend for increased alkaline phosphatase production. However, the 25,000 cells/well seeding density showed an increase in enzyme production of 22% over control cultures (Figure 5).

Discussion

In the study presented here, we have investigated the effects of pulsed electromagnetic field stimulation timing and cell density upon the development of the osteoblast-like cell cultures.

The data indicate that cells exposed to PEMFs exhibit reduced proliferation and suggest that they also exhibit a more differentiated phenotype due to the increased alkaline phosphatase production. These results are consistent with other studies using osteoblast-like cells, which have also shown decreased proliferation and increased differentiation of cultures exposed to PEMFs [Lohmann et al. 2000; McLeod et al. 1993; Vander Molen et al. 2000]. The study of Lohmann et al.[2000] exposed MG63 cells to an 8 hour period of PEMF stimulation over 1, 2 or 4 days. However, in contrast to this study, the cells were grown to confluence before PEMF stimulation.

The phenotypic state of the cells has been shown to influence exogenous PEMF stimulation effects on cell development, specifically, the greater a cell's differentiation as measured by means of alkaline phosphatase, the less proliferation is achieved [Diniz et al. 2002]. MG63 cells have very low basal levels of alkaline phosphatase activity compared to the human derived SaOS-2 cells [Rodan et al. 1987] and this may explain the discrepancy between our study and that of others who have used immature osteoblast-like cell lines and found proliferation increases from PEMF exposure [Chang et al. 2004; De Mattei et al. 1999; Sollazzo et al. 1997]. It also may explain why we have achieved similar results to that of Lohmann et al. (2000) when our PEMF stimulation methods are different.

Important regulators of an osteoblast's ability to communicate and respond to exogenous stimuli, such as PEMFs, are gap junctions. These are specialized intercellular channels for movement of small molecules and ions between adjacent cells and directly affect electrical conductance (induced from an exogenous PEMF stimulant) within the cell monolayer [Sreedharan and Zhang 2003]. This electrical conductance is amplified via cell coupling and is a proposed regulator of PEMF stimulation effects [Muehsam and Pilla 1999; Pilla 2002]. Recent studies show that the PEMF stimulated decrease in proliferation is independent of gap junctional coupling, while increased enzyme activity (alkaline phosphatase) levels are still dependant on the electrical communication achieved through gap junctions [Vander Molen et al. 2000]. Studies on gap junctional expression have concluded that PEMF exposure decreases the amount of gap junctional communication via a decrease in the mRNA expression of the gap junction protein connexin 43 in well-differentiated osteoblasts and osteocyte-like cells [Lohmann et al. 2003]. However, Yamaguchi et

al. [2002] reported that the decreased intercellular communication observed in immature osteoblasts from PEMF stimuli was nullified when using originally well differentiated cells. This suggests that communication through gap junctions between adjacent SaOS-2 cells used in this study may not have been affected by the PEMF stimulant. It has also been noted that these cells naturally show very little gap junction communication [Donahue et al. 1995]. Therefore, it is possible that the SaOS-2 cell line is not as sensitive to the PEMF stimulus as a cell line that expresses a more pre-osteoblast or greater gap junctional signalling phenotype.

There was no obvious difference between the protocols and this suggests that the timing of PEMF stimulation may not be a critical feature. It has been reported that as little as 30 minutes of PEMF stimulus provides significant increases in proliferation for *in vitro* cultures of osteoblast-like cells, while the effects of stimulation taper off after 24 hours [De Mattei et al. 1999]. Thus a shorter period of stimulation may have a greater influence over cellular development and could explain why protocol 1 with its repeated stimulation periods of 8 hours per day over the three days is the only consistently significant protocol for both seeding densities, while the longer exposure protocols (protocol 2, 3, 4) do not show as much consistency.

McLeod et al. [1993], using a protocol similar to our protocol 4, demonstrated that a 'window' effect occurs such that *in vitro* cultures of ROS 17/2.8 osteoblast-like cells with high (50,000 cells/cm²) or low (6,000 cells/cm²) seeding densities exhibited an apparent reversal in the general trend of increased PEMF induced alkaline phosphatase. ROS 17/2.8 osteoblast-like cells have been matched as closely resembling SaOS-2 cells in osteoblastic qualities [Rodan et. al 1987] and may explain

the differences seen in the differentiation result from protocol 4 compared with the other protocols.

Some limitations of this study include the very small but consistent magnetic flux experienced by the control cultures. This could potentially mask or diminish the results seen from the PEMF exposed cultures when making comparisons with the controls. However, controls from each protocol underwent the same small exposure, removing any influence it may have had on the PEMF timing results. The number of repeat measurements of alkaline phosphatase volume stifled statistical significance from these experiments but consistent trends were still apparent in the data. The resolution of the temperature measurements with the thermocouple was $\pm 0.5^{\circ}\text{C}$ but no demonstrable heating effects were seen. Another small confounding factor may have been due to vibration of the cultures from mechanical vibration of the coil during operation. However, both test and control cultures would have experienced the vibration and so the differences seen cannot be attributed to this.

While care was taken to ensure the experiments were controlled as well as possible, confounding artefacts may have been produced. However due to the experimental design these would tend to mask differences and lend credence to the differences actually seen.

Conclusions

Our results indicate that a 15Hz PEMF stimulus on monolayers of an osteoblast-like cell line leads to a depression in proliferation with a concomitant increase in alkaline phosphatase production. Since alkaline phosphatase is related to bone cell

differentiation and bone mineralisation, these results support the hypothesis that a commercially available PEMF device will stimulate an osteoblast-like cell line into an increasing state of maturity. Applying the stimulus at different times following culture seeding did not appear to affect the response of the cells (although there is evidence that this may be due in part to the density of the cell cultures during PEMF exposure and/or sensitivity to PEMFs in the cell line studied). These results provide more evidence to help explain the mechanism by which clinical PEMF stimuli alter *in vitro* cultures of bone cells.

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Fig. 1. The device used to expose osteoblast-like cells cultured in 24 well cell culture plates to a 15Hz pulsed electromagnetic field. Shown are the two coil apparatuses, pulse generator and voltage power supply. Plates are placed within each Helmholtz coil unit with centre 8 wells seeded.

Fig. 2. a) 15Hz magnetic field waveform produced by pulsed electromagnetic field device that is aligned perpendicularly to cell monolayer as measured from a handheld Tesla meter. b) Induced voltage waveform in an inductive coil probe placed within the magnetic field waveform. This waveform mimics the electric field experienced by the cells.

Fig. 3. Pulsed electromagnetic field exposure protocols used on osteoblast-like SaOS-2 cells to quantify effects on cellular proliferation and differentiation. Shaded sections denote PEMF exposure while clear sections denote normal cell culture conditions (absence of PEMF stimulation).

Fig. 4. Effect of pulsed electromagnetic field stimulation, using 4 different protocols, on ³H-leucine incorporation by SaOS-2 osteoblast-like cells seeded at low density (25,000cells/well) and high density (50,000cells/well) using a 24 well culture plate. Values of PEMF exposed cultures are normalised with reference to control values (100%). Graphs show +/- SEM. # Indicates statistically significant difference from control (P < 0.05).

Fig. 5. Effect of pulsed electromagnetic field stimulation, using 4 different protocols, on alkaline phosphatase activity by SaOS-2 osteoblast-like cells seeded at low density (25,000cells/well) and high density (50,000cells/well) using a 24 well culture plate. Values of PEMF exposed cultures are normalised with reference to control values (100%). Graphs show +/- SEM.

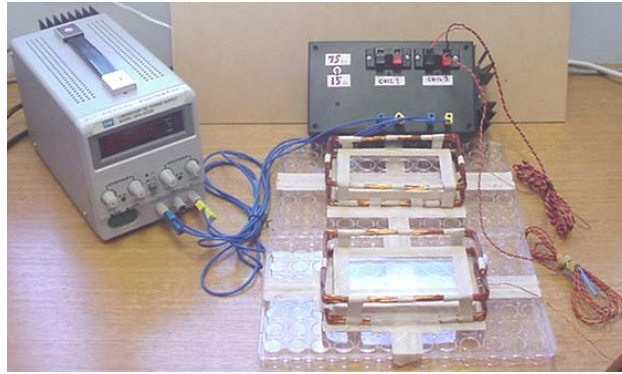


FIGURE 1

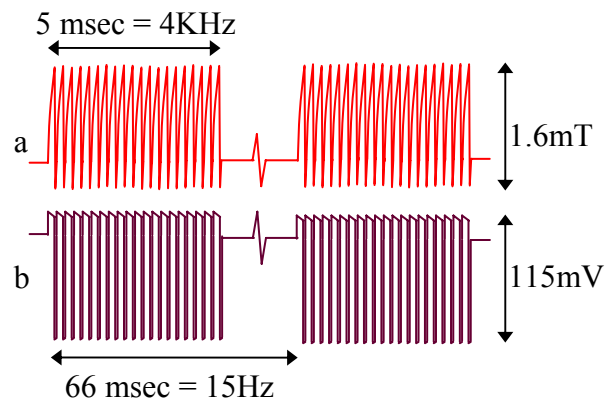


FIGURE 2

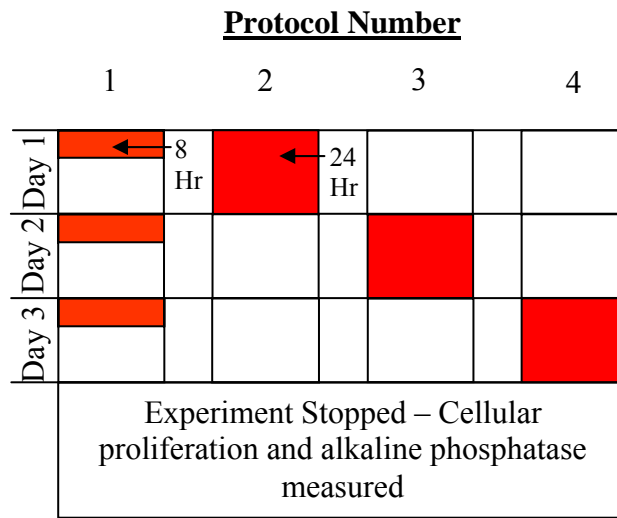


FIGURE 3

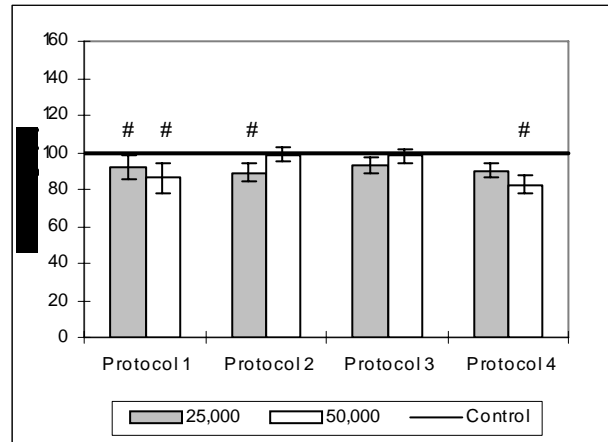


FIGURE 4

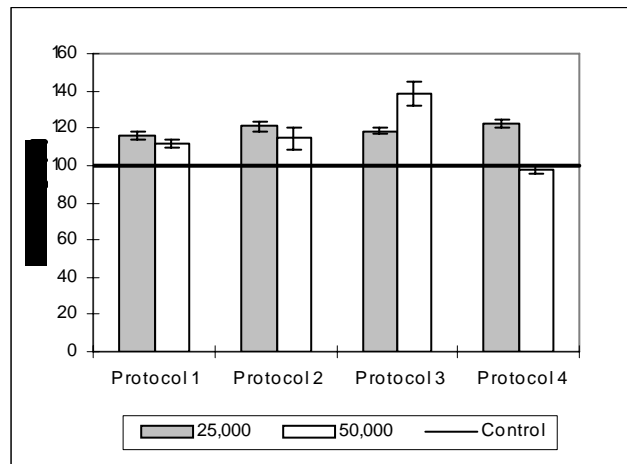


FIGURE 5