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Effect of the Consciousness Energy Healing Treatment on DMEM for the Proliferation and Differentiation of Human Bone Osteosarcoma Cells to Improve Bone Health

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Abstract The bone health is an important part of healthy-life and longevity in current situation due to huge toxins and contaminants in the environment and food chain. Considering the importance of bone health in the modern era, the present study was undertaken to investigate the effect of the Consciousness Energy Healing (The Trivedi Effect®) Treatment on Dulbecco's Modified Eagle Medium (DMEM) in which the human bone osteosarcoma cells - MG-63 (ATCC® CRL-1427™) was grown for the assessment of bone cell proliferation and differentiation *in vitro*. The study parameters were assessed using cell viability by MTT assay, alkaline phosphatase (ALP), and collagen synthesis on bone health using ELISA-based assay. The cell viability was significantly increased by 24% in the Biofield Energy Treated group supplemented with 10% charcoal-dextran with fetal bovine serum (CD-FBS) (G3) compared to the untreated cells group (G1). The level of ALP was significantly increased by 72% in the G3 group compared to the G1 group. Additionally, the level of collagen synthesis was significantly ($p \leq 0.001$) increased by 19% in the G3 group compared to the G1 group. The overall results demonstrated that the Biofield Energy Treated DMEM has the potential for bone mineralization and bone cells growth as evident *via* increased levels of collagen and ALP. Therefore, the Biofield Energy Healing (The Trivedi Effect®) Treatment could be useful as a bone health promoter for various bone-related disorders like low bone density, osteogenesis imperfecta, and osteoporosis, etc.

Keywords: *Biofield Energy Healing, The Trivedi Effect®, Alkaline phosphatase, Collagen, Osteosarcoma cells - MG-63, β-Estradiol, MTT, ELISA*

1. Introduction

The simplest medium has been routinely used in the cell culture is minimum essential media (MEM), that contains more than 20 different substance can be divided into four subgroups *viz.* inorganic salt, amino acid, vitamins, and carbohydrate. Apart from four basic substance related to cell growth, the medium also uses phenol-red as pH indicator. MEM is not common basic medium. The common medium has more than 30 kinds of component, such as RPMI1640, DMEM etc. These mediums generally contains some non-essential amino acids and vitamins, including serine, proline, biotin, vitamin B12, etc. [1]. Bone formation needs differentiated and active osteoblasts for the synthesis of extracellular matrix, which enhance the process of mineralization [2]. Osteosarcoma cells are derived from malignant bone tumors with abnormal cellular functions. The human osteosarcoma cell line, MG-63 has been used to study the production of the bone-specific protein, osteocalcin. In contrast, an increase in extracellular calcium, which stimulate osteocalcin release. The regulation

of osteocalcin secretion is mediated by the PTH and PGE2 in normal human bone cells, which can be produced in the human osteoblast-like cell line MG-63. Both cell cultures showed time- and dose-dependent stimulation of osteocalcin secretion in response to 1, 25-dihydroxyvitamin D3 (1, 25$(OH)_2$ D_3) active form of vitamin D3. Thus, human osteosarcoma cell line, MG-63 is useful as an alternative osteoblast-like cell model to study the regulation of osteocalcin secretion [3]. Alkaline phosphatase (ALP) has many different functions in different organisms and tissues that plays a major role in mineralization of bone tissues. More than 80 years ago, the high level of ALP expression in bone was observed indicating it's important for hard tissue formation. Scientific research demonstrated that ALP had increased the local concentration of inorganic phosphate (Pi), a concept known as the 'booster hypothesis' [4-6]. Apart from ALP and collagen, other cellular factors are also responsible for the growth and development of bone tissue. Platelet-derived growth factor (PDGF) is a polypeptide found in bone tissue, where it acts as a regulator of skeletal remodeling [7]. Tumor necrosis factor (TNF) alpha and basic fibroblast growth factor (bFGF) stimulates the synthesis of DNA, which causes an increased number of collagen-synthesizing cells [8,9]. Bone morphogenetic proteins (BMPs) also play a specific role in osteoblast differentiation *in vitro* and bone formation *in vivo* [10].

In the current global scenario, Energy Therapy like Biofield Energy Healing has been widely used and recommended as an alternative method. It has an astounding impact on various properties of living organisms in a cost-effective manner [11]. The Trivedi Effect® - Biofield Energy Healing has been known to improve the potential beneficial effects in a broad spectrum field around the globe. It improved the productivity of crops in agriculture and livestock [12-15], positive impact on cancer [16,17], and altered characteristics features of microbes in the field of microbiology [19-21]. It also alters the structural, physical, and thermal properties of several metals and ceramics [22-24], causes genetic alteration in various microorganisms [25,26], and improves various nutraceutical compounds in the areas of nutraceuticals [27,28] and biotechnology [29-31]. Wide spectrum of treatment approaches are available for the proliferation and differentiation of bone cells using conventional medicines. However, due to its high cost, anticipated toxicity, render it undesirable for the patients for longer uses. Due to well impact of The Trivedi Effect® in multi-field, authors wish to investigate the Biofield Energy Healing on bone health in *in vitro* cellular model *i.e.*, human bone osteosarcoma cells - MG-63. Therefore, authors evaluated the effect of Biofield Energy Healing (The Trivedi Effect®) on the human bone osteosarcoma cells - MG-63 (ATCC® CRL-1427™) for the assessment of bone proliferation and differentiation.

2. Materials and Methods

2.1. Chemicals and Reagents

Antibiotics solution (penicillin-streptomycin) and Dulbecco's Modified Eagle Medium (DMEM) - (phenol-red free) were procured from HiMedia, India. DMEM was procured from GIBCO, USA. Direct Red 80, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium) (MTT), and β-Estradiol (positive control) were purchased from Sigma Chemical Co. St. Louis, MO, USA. All the other chemicals used in this experiment were analytical grade procured from India.

2.2. Cell Line: Culture and Maintenance

Human bone osteosarcoma cell line -MG-63 (ATCC® CRL-1427™) was used as test system in the present study. MG-63 cell line was maintained under DMEM growth medium for routine culture supplemented with 10% FBS. Growth conditions were maintained at 37 ℃, 5% CO_2, and 95% humidity and subcultured by trypsinisation followed by splitting the cell suspension into fresh flasks and supplementing with fresh cell growth medium. Three days before the start of the experiment (*i.e.*, day -3), the growth medium of near-confluent cells was replaced with fresh phenol-free DMEM, supplemented with 10% charcoal dextran stripped FBS (CD-FBS) and 1% penicillin-streptomycin [32].

2.3. Biofield Energy Healing Approach

An aliquot of DMEM culture medium was received Biofield Energy Treatment (The Trivedi Effect®) under laboratory conditions for ~3 minutes from a distance of ~25 cm. The energy transmission was done without touching the cells through

the healer's unique Energy Transmission process to the test samples under laboratory conditions. Further, the control group was treated with a sham healer for comparative purposes. The sham healer did not have any knowledge about the Biofield Energy Treatment. After that, the Biofield Energy Treated and untreated samples were kept in similar sealed conditions for experimental study for culture of osteosarcoma cells.

2.4. Experimental Design

Group 1 (G1) served as the untreated medium (200 µL of phenol-free DMEM supplemented with 10% CD-FBS). G2 served as the positive control (β-Estradiol + Biofield Treated medium supplemented with 10% CD-FBS). G3 contained Biofield Energy Treated phenol-free DMEM supplemented with 10% CD-FBS.

2.5. Assessment of Cell Proliferation/Viability

The MG-63 cells were trypsinized, counted, and plated in wells of flat bottom 96-well plates at the density corresponding to 5 X 10^3 cells/well/180 µL of Biofield Energy Treated growth medium. The cells in the above plate were incubated for 48 hours in a CO_2 incubator at 37 ℃, 5% CO_2, and 95% humidity. After 48 hours of incubation, the plate was taken out and 20 µL of 5 mg/mL of MTT solution was added to all the wells followed by additional incubation for 3 hours at 37 ℃. The supernatant was aspirated and 150 µL of DMSO was added to each well to dissolve formazan crystals. The absorbance of each well was read at 540 nm using Synergy HT microplate reader, BioTek, USA [33]. The percentage cell growth corresponding to each treatment was calculated using formula (1):

$$\% \text{ Cell growth} = \left[\left[\left(X - Tz\right)/\left(R - Tz\right)\right] \times 100\right] \quad (1)$$

Where, X = Absorbance of cells corresponding to positive control and test groups after 48 hours
R = Absorbance of cells corresponding to Baseline group after 48 hours
Tz = Absorbance of untreated cells at time 0 hour

Positive control was run in parallel to the sample. Concentrations were determined, and the experiment was done in triplicates.

2.6. Assessment of Alkaline Phosphatase (ALP) Activity

The MG-63 cells were trypsinized, counted and plated in wells of flat bottom 96-well plates at the density corresponding to 10 X 10^3 cells/well/180 µL of the Biofield Energy Treated growth medium. The cells in the above plate were incubated for 48 hours in a CO_2 incubator at 37 ℃, 5% CO_2, and 95% humidity. After 48 hours of incubation, the plate was taken out and processed for measurement of ALP enzyme activity. The cells were washed with 1X PBS and lysed by freeze-thaw method *i.e.*, incubation at -80 ℃ for 20 minutes followed by incubation at 37 ℃ for 10 minutes. To the lysed cells, 50 µL of substrate solution *i.e.*, 5 mM of *p*-nitrophenyl phosphate (*p*NPP) in 1M diethanolamine and 0.24 mM magnesium chloride ($MgCl_2$) solution (pH 10.4) was added to all the wells followed by incubation for 1 hour at 37 ℃. The absorbance of the above solution was read at 405 nm using Synergy HT microplate reader. The absorbance values obtained in the above step were normalized with substrate blank (*p*NPP solution alone) absorbance values. The percentage increase in ALP enzyme activity with respect to the untreated cells (baseline group) was calculated using formula (2):

$$\% \text{ Increase} = \left[\left(X - R\right)/R\right] * 100 \quad (2)$$

Where, X = Absorbance of cells corresponding to positive control and test groups
R = Absorbance of cells corresponding to baseline group (untreated cells)

2.7. Assessment of Collagen Synthesis

The MG-63 cells were trypsinized, counted and plated in wells of 48-well plates at the density corresponding to 10 X 10^3 cells/well/0.5 mL of the Biofield Energy Treated growth medium. Following respective treatments, the cells in the above plate were incubated for 48 hours in a CO_2 incubator at 37 ℃, 5% CO_2, and 95% humidity. After 48 hours of incubation, the plate was taken out and the amount of collagen accumulated in MG-63 cells corresponding to each treatment

was measured by Direct Sirius red dye binding assay. In brief, the cell layers were washed with PBS and fixed in Bouin's solution (5% acetic acid, 9% formaldehyde and 0.9% picric acid) for 1 hour at room temperature (RT). After 1 hour of incubation, the above wells were washed with milliQ water and air dried. The cells were then stained with Sirius red dye solution for 1 hour at RT followed by washing in 0.01 N HCl to remove unbound dye. The collagen dye complex obtained in the above step was dissolved in 0.1 N NaOH and absorbance was read at 540 nm using Synergy HT microplate reader, BioTek, USA. The level of collagen was extrapolated using standard curve obtained from purified Calf Collagen Bornstein and Traub Type I (Sigma Type III). The percentage increase in collagen level with respect to the untreated cells (baseline group) was calculated using formula (3):

$$\%\,\text{Increase} = \left(\left(X - R\right) / R\right) * 100 \tag{3}$$

Where, X = Collagen levels in cells corresponding to positive control and test groups
R = Collagen levels in cells corresponding to Baseline group (untreated cells)

2.8. Statistical Analysis

Data analysis was performed with SigmaPlot Statistical Software (Version 11.0). Differences between means (in triplicates) were assessed for statistical differences using one-way analysis of variance (ANOVA) and *post-hoc* analysis was done by Dunnett's test. $p \leq 0.05$ was statistically significant. The results are shown as mean ± standard deviation (SD).

3. Results

3.1. Assessment of Cell Viability by MTT Assay

The effect of the Biofield Energy Treatment on DMEM for the assessment of human bone osteosarcoma cell proliferation was examined after 48 hours using MTT cell proliferation test is shown in Table 1. The cell viability was significantly increased by 24% in the Biofield Energy Treated group that was supplemented with 10% CD-FBS (G3) compared to the untreated medium group (G1). The cell viability in the positive control group (G2) was significantly increased by 19%, 68%, and 53% at the concentration of 1, 10, and 100 nM, respectively compared to the G1 group. Results showed that cell proliferation was higher in the G3 group compared to the G1 group. This increased cell proliferation and invasion might be due to stimulation of bone marrow-derived stem cells (BMSCs), which are localized just adjacent to the tumor tissues and may interact with tumor cells directly [34]. Apart from BMSC stimulation, other factors such as matrix metalloproteinase-9 (MMP-9), MMP-2, and vascular endothelial growth factor (VEGF) may also be involved in promoting the proliferation and invasion of osteosarcoma cells [35,36]. Based on Table 1, it was demonstrated that the Biofield Energy Treatment on DMEM culture significantly promotes the proliferation of osteosarcoma cell compared to the untreated medium. Besides, Wagner *et al.* 2011 reported that the development of osteosarcoma cells may be associated with the overexpression of Runx2 and due to defects in osteogenic differentiation [37]. Satoshi *et al.* 2014 reported an interaction between osteosarcoma cell growth and platelets. They described that due to interaction between osteosarcoma cells ultimately induce the release of platelet-derived growth factor (PDGF) from platelets, which promotes the proliferation of osteosarcoma cells. In this experiment, it is assuming that the Biofield Energy Healing based DMEM culture medium boost-up the growth and development of osteosarcoma cells.

3.2. Assessment of Alkaline Phosphatase (ALP) Activity

Effect of the Biofield Energy Treatment on DMEM in which the human bone osteosarcoma cells were grown and measured the level of alkaline phosphatase (ALP) is presented in Table 2. The absorbance level of ALP in the untreated cells (G1) group was 0.212 ± 0.031. Besides, the level of ALP was significantly increased by 72% in the Biofield Energy Treated group (G3) supplement with 10% charcoal-dextran treated fetal bovine serum (CD-FBS), compared to the G1 group. The positive control (β-estradiol) group (G2) showed a significantly increased the level of ALP by 38%, 35%, and 29% at the concentrations of 1, 10, and 100 nM, respectively compared to the G1 group (Table 2). Overall, the Biofield Energy Treated group (G3) that are supplemented with CD-FBS showed an improved the level of ALP in the Biofield

Treated medium containing human osteosarcoma cells with respect to the untreated group (G1). ALP is an enzyme responsible for physiological and pathological biomineralization. For calcification process ALP plays a vital role. ALP belongs to a group of enzymes, which individually promotes or inhibits the process of mineralization [38-40]. It is a cell membrane-associated enzyme that hydrolyzes to produce an inorganic pyrophosphate (PPi), which is a potent suppressor of hydroxyapatite (HA) for crystal growth. Further, inorganic phosphate (Pi) is formed after hydrolysis of PPi, which is a substrate for HA mineral [41-43], and thus contributes for the regulation of normal bone formation as well as in pathological extraosseous mineralization. Hypophosphatasia (HPP) is a genetic disease, which arises due to inborn error of metabolism characterized by defective mineralization of bone and tooth. Reduced level of serum ALP activity are a biochemical hallmark of this disease. It is well characterized that development of this genetic disease due to various mutations in the tissue-nonspecific alkaline phosphatase (TNS-ALP) gene [44-47]. Based on the numerous literatures, it was proven that ALP plays a vital role for bone health, osteogenesis, and calcification process. In this experiment it was also evident that the Biofield Energy Treated group supplemented with 10% CD-FBS (G3) significantly ($p \leq 0.001$) increased the level of ALP expression, which might be helpful in bone disorder patients.

Table 1. Effect of the Biofield Energy Treatment on DMEM in which the human bone osteosarcoma cell was grown for the assessment of cell proliferation by MTT assay measured at 540 nm.

Group	G1		G2			G3
	Time (T_0)	Time (72h)	1 nM	10 nM	100 nM	
Absorbance	0.527 ± 0.054	0.910 ± 0.034	0.984 ± 0.041	1.170 ± 0.101	1.114 ± 0.033	1.002 ± 0.018
% Cell Proliferation w.r.t. T_0	0	100	119	168***	153***	124*

G1: Untreated medium supplemented with 10% CD-FBS; G2: Positive control (β-Estradiol) at the concentrations of 1, 10 and 100 nM. G3: Biofield Energy Treated phenol-free DMEM supplemented with 10% CD-FBS. Values are represented as mean ± SD and the experiment was carried out in triplicates. ***$p \leq 0.001$ *vs* G1 (using one-way ANOVA) and *$p \leq 0.05$ *vs* G1 (using paired *t*-test).

Table 2. Effect of the Biofield Energy Treatment on human bone osteosarcoma cell for the assessment of alkaline phosphatase enzyme activity measured at 405 nm.

Group	G1	G2			G3
		1 nM	10 nM	100 nM	
Absorbance	0.212 ± 0.031	0.293 ± 0.028	0.287 ± 0.040	0.275 ± 0.045	0.366 ± 0.031
% Cell Proliferation w.r.t. G1	100	138***	135	129	172***

G1: Untreated phenol-free DMEM supplemented with 10% CD-FBS; G2: Positive control (β-Estradiol) at the concentrations of 1, 10, and 100 nM. G3: Biofield Energy Treated phenol-free DMEM supplemented with 10% CD-FBS. Values are represented as mean ± SD and the experiment was carried out in triplicates. ***$p \leq 0.001$ *vs* G1 (using one-way ANOVA).

3.3. Assessment of Collagen Synthesis

The DMEM treated with Biofield Energy in which human bone osteosarcoma cell was grown and the level of collagen is presented in Table 3. The level of collagen in the untreated medium (G1) group was 163.9 ± 2.31 µg/mL. Besides, the level of collagen synthesis was significantly ($p \leq 0.001$) increased by 19% in the Biofield Energy Treated group (G3)

supplemented with 10% charcoal-dextran treated fetal bovine serum (CD-FBS), compared to the G1 group. The positive control (β-estradiol) group (G2) showed a significantly increased in the synthesis of collagen by 19.3%, 10.5%, and 3.0% at the concentrations of 1, 10, and 100 nM, respectively compared to the G1 group (Table 3). Overall, the Biofield Energy Treated group (G3) that are supplemented with CD-FBS showed an improved synthesis of collagen in the human osteosarcoma cells with respect to all the groups except G2 (1 nM). Bone is a complex material in which collagen fibers formed a scaffold oriented apatite crystals. It is generally considered as a passive scaffold and template for mineral formation [48]. Due to complex nature of biological systems the study of collagen mineralization in *in vivo* models are very challenging [49,50]. Recently, collagen mineralization was achieved by substituting the non-collagenous proteins (NCPs) with either polyaspartic acid (pAsp) or fetuin, *in vitro* [51,52]. Scientific investigation is continued to find out the ways that can improve collagen synthesis in patients with skeletal disorders. As Biofield Energy had felt under the umbrella of complementary and alternative therapy and already been recommended by National Center for Complementary and Alternative Medicine (NCCAM) and about 30% US population are regularly used this therapy for the prevention and treatment of various diseases [53]. Besides, in this experiment the Biofield Energy Treatment modality showed significant improvement of collagen level in the human osteosarcoma cells. It is assumed that The Trivedi effect® has the potential to improve bone health in various skeletal disorders.

Table 3. Effect of the Biofield Energy Treatment on DMEM in which human bone osteosarcoma cell was grown for the assessment of collagen synthesis measured at 540 nm.

Group	G1	G2			G3
		1 nM	10 nM	100 nM	
Collagen (µg/mL)	163.9 ± 2.31	195.7 ± 9.83	181.2 ± 4.75	168.9 ± 9.01	195.1 ± 4.75
Collagen (%) w.r.t. G1	100	119.3***	110.5***	103.0	119.0***

G1: Untreated medium; G2: Positive control (β-Estradiol) at the concentrations of 1, 10, and 100 nM. G3: Biofield Energy Treated phenol-free DMEM medium supplemented with 10% CD-FBS. Values are represented as mean ± SD and the experiment was carried out in triplicates. ***$p \leq 0.001$ *vs* G1 (using one-way ANOVA).

4. Conclusions

The cell viability was assessed using MTT assay of human bone osteosarcoma cells, which was grown in the Biofield Energy Treated DMEM culture. The MTT assay data found that Biofield Energy Healing showed a significantly increased the levels of proliferation in osteosarcoma cells compared to the untreated cells (G1) group. Besides, the level of ALP was significantly increased by 72% in the Biofield Energy Treated osteosarcoma cells group supplemented with 10% charcoal-dextran treated fetal bovine serum (CD-FBS) group (G3) compared to the G1 group. Besides, the level of collagen synthesis was also significantly increased by 19% in the G3 group compared to the G1 group. Overall, the Biofield Energy Treatment on DMEM culture medium enhanced bone mineralization and bone cells differentiation compared to the untreated cells group on human bone osteosarcoma cells. In conclusion, the Biofield Energy Healing might act as an effective bone health enhancer and it can be used as a complementary and alternative treatment for the prevention of various types of skeletal abnormality such as acromesomelic dysplasia, arthritis, bone erosion, bone metastasis, brachymetatarsia, osteoarthritis, caudal regression syndrome, chandler's disease, chondrodystrophy, opsismodysplasia, wrist osteoarthritis, zimmermann–laband syndrome, mastoiditis, ollier disease, rickets, oncogenic osteomalacia, handigodu syndrome, metachondromatosis, pacman dysplasia, osteochondrodysplasia, *etc*.

5. Abbreviations

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS: Phosphate buffer saline; ELISA: Enzyme-linked immunosorbent assay; CD-FBS: Charcoal-dextran treated fetal bovine serum; ALP: Alkaline phosphatase; NCCAM: National Center for Complementary and Alternative Medicine

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