



In Vitro Effects of Biofield Energy Treated Vitamin D3 Supplementation on Bone Formation by Osteoblast Cell Line (MG-63)

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Abstract

Inadequate intake of vitamin D leads to hormonal imbalance, aging, decreased calcium absorption, and bone loss. Present study aimed to evaluate potential of The Trivedi Effect[®]- Biofield Energy Healing Treatment on vitamin D₃ as Test Item (TI) and DMEM to improve bone health in MG-63 cells. One part of each samples was received Consciousness Energy Healing Treatment by Dahryn Trivedi and those samples were labeled as Biofield Energy Treated (BT) samples, while other parts of each sample were denoted as untreated TI (UT-TI). Cell viability assay (MTT) found test items were safe and nontoxic in the tested concentrations. ALP was considerably improved by 753.3%, 1173.3%, and 424.4% in UT-DMEM+BT-TI, BT-DMEM+UT-TI, and BT-DMEM+BT-TI, respectively at 0.1 µg/mL compared to untreated. Collagen was significantly increased by 454.7% and 96.2% in BT-DMEM+UT-TI and BT-DMEM+BT-TI, respectively at 1 µg/mL, while 202.4% (at 50 µg/mL) increased collagen in UT-DMEM+BT-TI compared to untreated. Moreover, percent of bone mineralization was significantly increased in UT-DMEM+BT-TI, BT-DMEM+UT-TI, and BT-DMEM+BT-TI by 301.4% (at 50 µg/mL), 143.3% (at 50 µg/mL), and 178.0% (at 100 µg/mL) respectively, compared to untreated. Thus, Biofield Energy Treated vitamin D₃ and Biofield Energy Treated DMEM were found safe and to have remarkably improved the bone health parameters, which could be a powerful alternative nutraceutical supplement to combat against various bone-related diseases including low bone density and osteoporosis, osteogenesis imperfecta, Paget's disease of bone, rickets, osteomalacia, bone and/or joint pain, increased frequency of fractures, deformed bones, osteoma, chondrodystrophia fetalis, hormonal imbalance, stress, aging, bone loss and fractures.

Keywords: ALP; Alizarin Red S Staining; Bone mineralization; Collagen; Consciousness Energy Healing; Osteosarcoma cells (MG-63); The Trivedi Effect[®]

Abbreviations

MG-63	:	Human Bone Osteosarcoma Cells
ALP	:	Alkaline Phosphatase
CAM	:	Complementary and Alternative Medicine
NHIS	:	National Health Interview Survey
NCCIH	:	National Center of Complementary and Integrative Health
DMEM	:	Dulbecco's Modified Eagle's Medium
FBS	:	Fetal Bovine Serum
UT	:	Untreated
BT	:	Biofield Energy Treated

TI	:	Test Item
FBS	:	Fetal Bovine Serum
EDTA	:	Ethylenediaminetetraacetic Acid

Introduction

Vitamin D has various functions in various organs such as kidneys, lungs, brain, liver, heart, etc. It has significant anti-aging, wound healing, anti-stress, anti-inflammatory, anti-osteoporosis, anti-psychotic, and anti-cancer activities. Vitamin D Receptors (VDRs) are widely present in most of the body organs like brain, heart, lungs, kidney, liver, pancreas, large and small intestines, muscles, reproductive, nervous system, etc. [1]. VDRs contributes neurotransmission, hormonal balance, cell differentiation, cell-to-cell communication, and cell cycling and proliferation. In addition, vitamin D plays a vital role in preserving a healthy mineralized skeleton of most of the vertebrates including humans. Some major sources such as sunlight, irradiation of other foods including plants, cod liver oil, etc. are found to be effective against bone-related disorders and in inflammatory diseases [1]. The role of vitamin D has been well defined not only for improving the bone mineralization but also helped in bone resorption, inflammation and overall quality of life. Vitamin D₃ is synthesized by sunlight in the skin and sequentially metabolized in liver and kidney to form 1, 25-dihydroxyvitamin D (calcitriol) [2]. Calcitriol play an important role in maintaining level of calcium and phosphorus, promotes bone mineralization, and inhibit hypertension [3]. Vitamin D insufficiency is the major health problem, which causes metabolic bone disease in young and elderly populations [4]. Most of the foods even in fortified foods contain very less amount of vitamin D. In order to avoid the bone-related disorders calcium (1000-1500 mg/day) and vitamin D (400 IU/day) is very important for maintaining a good bone health [5]. The response of MG-63 cells to 1, 25-dihydroxyvitamin D₃ administration has been studied in normal human osteoblast cells [6]. Hence, MG-63 cell line is widely used for studying the potential of any test compounds to improve the bone health [7]. ALP is a phenotypic marker for the early differentiation and maturation of osteoblasts [8]. In similar way, active osteoblasts synthesize and extrude collagen, which plays an important role in the formation of bone extracellular matrix by providing strength and flexibility [9]. Likewise, calcium phosphate is deposited in the Osteoid and gets mineralized (combination of calcium phosphate and hydroxyapatite) and provides rigidity to the bone [10]. Authors evaluated the *in vitro* effect of the Biofield Energy Treated vitamin D₃ as a test item, a Complementary and Alternative Medicine (CAM) on bone health using MG-63 cells.

Within the burgeoning ground of CAM therapies, Biofield Treatment or energy medicine, is emerging with significant benefits in various scientific fields. The effects of the CAM therapies have great potential, which include external Qi Gong, Tai Chi, Johrei, Reiki, meditation, yoga, relaxation techniques, Ayurvedic medicine, therapeutic touch, polarity therapy, pranic healing, deep breathing, chiropractic/osteopathic manipulation, massage, special diets, homeopathy, progressive relaxation, mindfulness, Rolwing

structural integration, guided imagery, acupressure, acupuncture, hypnotherapy, healing touch, movement therapy, pilates, traditional Chinese herbs and medicines in biological systems [11]. The human body can emit the electromagnetic waves in the form of bio-photons, that surround the body and it is commonly known as "Biofield". Therefore, the Biofield consists of electromagnetic field, being generated by moving electrically charged particles (ions, cell, molecule etc.) inside the human body. Biofield Energy contains a putative bioenergy, which can be channeled by a renowned Biofield Energy Healing practitioner from a distance. Thus, human has the ability to harness the energy from environment or universe and can transmit into any living or nonliving object(s) around the Globe. The objects always receive the energy and responding into useful way that is called biofield energy and the process is known as biofield treatment. Biofield Energy Healing as a CAM therapy showed a significant result in biological studies [12]. However, the National Center for Complementary and Alternative Medicine (NCCAM), well-defined Biofield therapies in the subcategory of Energy Therapies [13]. The Trivedi Effect®- Consciousness Energy Healing Treatment has been reported with significant revolution in the physicochemical properties of metals, chemicals, ceramics and polymers [14-17], improved agricultural crop yield, productivity, and quality [18-20], transformed antimicrobial characteristics at genetic level [21-23], biotechnology [24-26], skin health [27,28], nutraceuticals [29,30], cancer research [31,32], and human health and wellness. Based on the significant outcomes of Biofield Energy Treatment and vital role of vitamin D₃ on bone health, authors performed this experiment to evaluate the impact of the Biofield Energy Treatment (The Trivedi Effect®) on vitamin D₃ as test sample for bone health activity with respect to the assessment of different bone health parameters like collagen content, ALP, and bone mineralization using standard *in vitro* assays in MG-63 cells.

Material and Methods

Chemicals and Reagents

Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Life Technology, USA. Rutin hydrate was purchased from TCI, Japan, while vitamin D₃ (denoted as test item) and L-ascorbic acid were obtained from Sigma-Aldrich, USA. Antibiotics solution (penicillin-streptomycin) was procured from HiMedia, India, while 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium) (MTT), Direct Red 80, and Ethylene Diamine Tetra Acetic Acid (EDTA) were purchased from Sigma, USA. All the other chemicals used in this experiment were analytical grade procured from India.

Cell Culture

The human bone osteosarcoma cell line, MG-63, was used as the test system in the present study. The MG-63 cell line was maintained under the DMEM growth medium for routine culture and supplemented with 10% FBS. Growth conditions were

maintained at 37°C, 5%CO₂, and 95% humidity and sub-cultured 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 [33].

Experimental Design

The experimental groups consisted of cells in baseline control, vehicle control groups (0.05% DMSO with Biofield Energy Treated and untreated DMEM), a positive control group (rutin hydrate) and experimental test groups. The experimental groups included the combination of the Biofield Energy Treated and untreated vitamin D₃/DMEM. It consisted of four major treatment groups on specified cells with Untreated-DMEM + Untreated-Test item (UT-TI), UT-DMEM + Biofield Energy Treated Test Item (BT-TI), BT-DMEM + UT-TI, and BT-DMEM + BT-TI.

Consciousness Energy Healing Treatment Strategies

The test item (vitamin D₃ and DMEM) was divided into two parts. One part each of the test item were treated with the Biofield Energy (also known as The Trivedi Effect®) by Dahryn Trivedi’s unique Energy Transmission process remotely for ~5 minutes to the test samples under laboratory conditions and coded as the Biofield Energy Treated items. While the second part did not receive any sort of treatment and was defined as the untreated samples. Further, the untreated group was treated with a “sham” healer for comparative purposes. The sham healer did not have any knowledge about the Biofield Energy Treatment. Biofield Energy Healer, Dahryn Trivedi was remotely located in the USA, while the test items were located in the research laboratory of Dabur Research Foundation, New Delhi, India, never visited the laboratory in person, nor had any contact with the test item and medium. After that, the Biofield Energy Treated and untreated samples were kept in similar sealed conditions for experimental study.

Determination of Non-Cytotoxic Concentration

MTT assay was used for the evaluation of viable cells in MG-63 cells after treatment with Biofield Energy Treated and untreated test samples. The details procedure of cell viability assay was followed by Lauree, et al. (2018) with slight modification [34]. The cytotoxicity of each tested concentration of the test items was calculated with the help of Equation (1):

$$\% \text{ Cytotoxicity} = \left\{ \frac{1 - X}{R} \right\} * 100 \dots \dots \dots (1)$$

Where X = Absorbance of treated cells; R = Absorbance of untreated cells

The percentage of cell viability corresponding to each treatment group was calculated by Equation (2):

$$\% \text{ Cell Viability} = (100 - \% \text{ Cytotoxicity}) \dots \dots \dots (2)$$

The concentration exhibiting ≥70% cell viability was considered as non-cytotoxic [35].

Assessment of Alkaline Phosphatase (ALP) Activity

The level of ALP enzyme activity was performed after treatment with the Biofield Energy Treated test items in MG-63 cells. The procedure of cell counting, plating, and treatment was followed as per Krista et al. [36]. The percent increase in ALP activity with respect to the untreated cells was calculated using Equation (3):

$$\% \text{ Increase in ALP} = \left\{ \frac{X - R}{R} \right\} * 100 \dots \dots \dots (3)$$

Where X = Absorbance of cells corresponding to positive control and test groups,

R = Absorbance of cells corresponding to untreated cells.

Assessment of Collagen Synthesis

The level of collagen in MG-63 cells, standard methods were used for the evaluation of the potential of Biofield Treated test items and the procedure in details was as per Lorraine et al. with few modifications [37]. Briefly, the MG-63 cells were counted using a hemocytometer and plated in 24-well plates at the density corresponding to 10 X 10³ cells/well in phenol-free DMEM supplemented with 10% CD-FBS. Following the respective treatments, the cells in the above plate were incubated for 48 hours in a CO₂ incubator at 37°C, 5% CO₂ 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 room temperature 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 Biotek Synergy HT microplate reader. The level of collagen was extrapolated using standard curve obtained from purified Calf Collagen Bornstein and Traub Type I (Sigma Type III). The percent increase collagen level with respect to the untreated cells was calculated using Equation (4):

$$\% \text{ Increase in collagen levels} = \left\{ \frac{X - R}{R} \right\} * 100 \dots \dots \dots (4)$$

Where X = Collagen levels in cells corresponding to positive control and test groups,

R = Collagen levels in cells corresponding to untreated cells.

Assessment of Bone Mineralization by Alizarin Red S Staining

For the evaluation of the percent alteration in bone mineralization after treatment with the Biofield Energy Treated test items in MG-63 cells, and the details steps were followed according to Balmer, et al. [38]. The percentage increase in bone mineralization compared

to the untreated cells was calculated using Equation (5):

$$\% \text{ Increase} = \left\{ \frac{X - R}{R} \right\} * 100 \dots \dots \dots (5)$$

Where X = Absorbance in cells corresponding to positive control or test groups; R = Absorbance in cells corresponding to untreated group.

Statistical Analysis

All the values were represented as percentage of the respective parameters.

Results and Discussion

MTT Assay- Non-Cytotoxic Effect of the Test Item

The effect of the Biofield Energy Treated test item and DMEM was tested for its cytotoxic on MG-63 cells, and the results are presented in (Figure 1) along with positive control, rutin at various concentrations. The results of percentage cell viability in the tested cell line showed the cell viability range of 70% to 141% in different test item groups with DMEM, while for rutin group showed more than 85% cell viability. These data suggest that the Biofield Energy Treated test item along with DMEM groups were found safe at all the tested concentrations range from 0.1 to 100 µg/mL against the tested MG-63 cells.

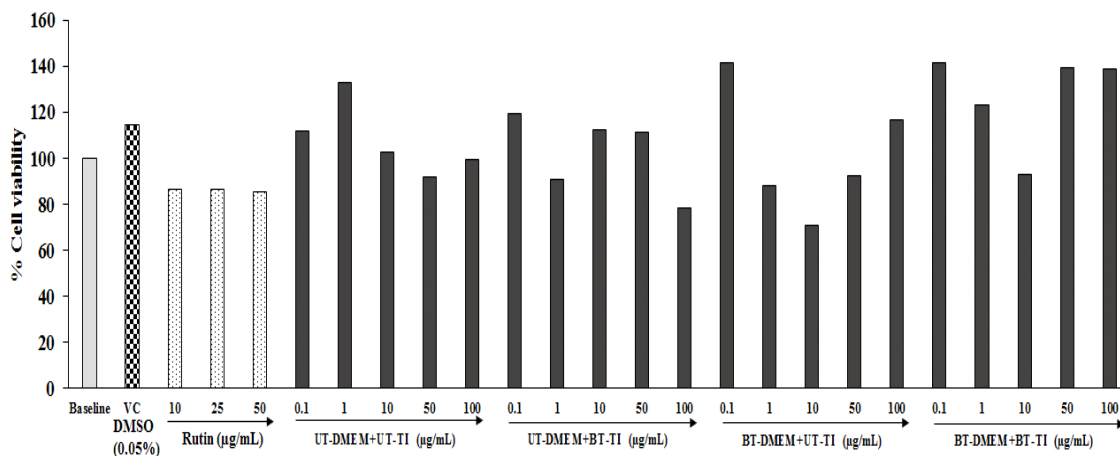


Figure 1: Effect of the test item on MG-63 cell line for cell viability after 72 hours using the MTT assays. VC: Vehicle control (DMSO-0.05%), UT: Untreated; BT: Biofield Treated; TI: Test Item.

Assessment of Test Items for Alkaline Phosphatase (ALP) Enzyme Activity

The effect on the ALP level after treatment with the Biofield Energy Treated test item and DMEM showed a significant increased at various experimental test item concentrations on MG-63 cell line (Figure 2). The positive control, rutin showed a significant increased value of ALP by 43.60%, 80.90%, and 35.80% at 0.1, 1, and 10 µg/mL, respectively as compared with the untreated cells. After Biofield Energy Healing Treatment, the experimental test group's viz. untreated medium and Biofield Treated Test item (UT-DMEM+BT-TI) showed a significant increased ALP

level by 753.3%, 67.0%, and 46.8% at 0.1, 1, and 10 µg/mL, respectively while Biofield Treated medium and untreated Test item (BT-DMEM+UT-TI) showed a significant increased ALP level by 1173.3%, 175.0%, and 148.9% at 0.1, 1, and 10 µg/mL, respectively as compared with the untreated test item and DMEM group (UT-DMEM+UT-TI). However, the Biofield Energy Treated DMEM and Biofield Energy Treated Test Item (BT-DMEM+BT-TI) showed a significant increased ALP level by 424.4% at 0.1 µg/mL as compared with the UT-DMEM+UT-TI group. Overall, all the experimental test groups showed a significant improved level of ALP at the tested concentrations.

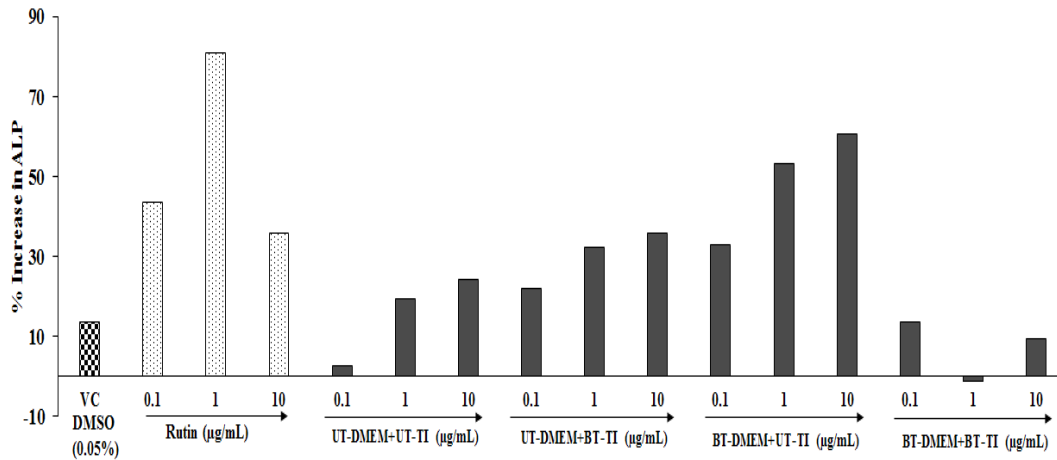


Figure 2: Effect of the test items on MG-63 cell line for the level of alkaline phosphatase (ALP) enzyme activity. VC: Vehicle control (DMSO-0.05%), UT: Untreated; BT: Biofield Treated; TI: Test Item.

ALP plays an important role for bone differentiation, maturation, osteogenesis, and calcification process. The ALP is a membrane-bound ectoenzymes and defined as the phenotypic marker for the early differentiation and maturation of osteoblasts. In addition, ALP is an important hard tissue formation component that enhanced the local concentration of inorganic phosphate for bone mineralization and hence is an important marker for osteogenic activity. ALP is reported to improve the local concentration of inorganic phosphate, a mineralization promoter along with inhibition of extracellular pyrophosphate concentrations, an inhibitor of mineral formation. Beside bone mineralization, ALP has also been implicated in cardiovascular calcification. Thus, it might be expected that Biofield Energy Treated vitamin D₃ and DMEM improved the enzyme expression, which is a good predictor of neotissue mineralization, which might be very advantageous to maintain a healthy skeletal structure to the patients suffering from various bone related disorders [39]. The Trivedi Effect[®]-Energy of Consciousness Healing based vitamin D₃ could provide a therapeutic prospect for the treatment of bone diseases, which might boost the ability to create useful bone biomaterials.

Effect of Test Items on Collagen Synthesis

The effect of the Biofield Energy Treated test item and DMEM on the collagen level showed a significant increased in the collagen level at various experimental tested concentrations on MG-63 cell line. The results of collagen synthesis are presented in (Figure 3). The positive control, rutin showed a significant increased the value of collagen by 51.9%, 42.5%, and 9.4% at 1, 10 and 25 µg/mL, respectively as compared with untreated cells. The experimental test group's viz. UT-DMEM+BT-TI showed a significant increased collagen level by 202.4% at 50 µg/mL, while BT-DMEM+UT-TI group showed a significant increased collagen level by 454.7%, 398.3%, and 280.7% at 1, 10, and 50 µg/mL, respectively as compared with the UT-DMEM+UT-TI group. However, the BT-DMEM+BT-TI group showed a significant increased collagen level by 96.2%, 90.0%, and 62.7% at 1, 10, and 50 µg/mL, respectively as compared with the UT-DMEM+UT-TI group. Overall, all the experimental Biofield Energy Treated test item and DMEM groups showed a significant improved level of collagen at all the tested concentrations.

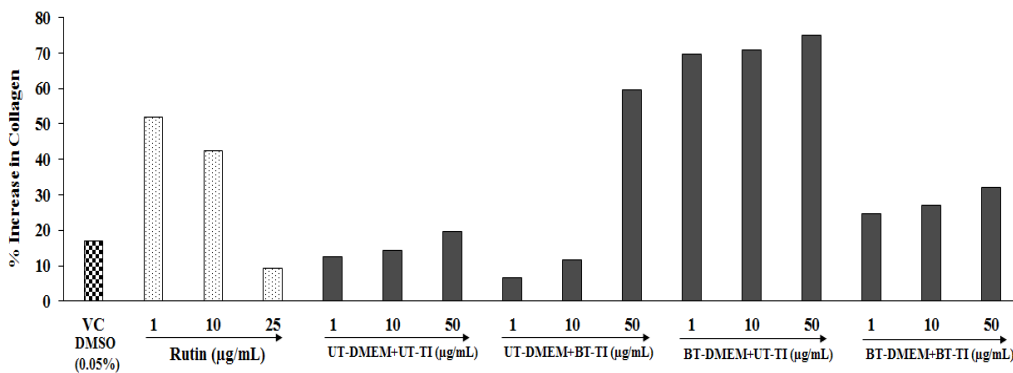


Figure 3: Effect of the test item on MG-63 cell line for collagen level. VC: Vehicle control (DMSO-0.05%), UT: Untreated; BT: Biofield Treated; TI: Test Item.

Collagen crosslink has been well defined in scientific literatures that it provides one of the earliest and most sensitive indications of a bone disturbance due to vitamin D deficiency. However, vitamin D specifically acts to increase the rate of maturation of bone collagen [40]. It is the most abundant and complex protein present in various parts such as cornea, skin, tendon, cartilage, and bone. The complexity of bone is composed of collagen fibrils, which form a scaffold for a highly organized arrangement of uniaxially oriented apatite crystals. The molecule of collagen enhances the infiltration of the fibrils with Amorphous Calcium Phosphate (ACP), which results in enhanced bone apatite formation [41]. Thus, it can be concluded that the Biofield Energy (The Trivedi Effect[®]) Treated vitamin D₃ and DMEM would be an important source to improve collagen level and bone mineralization against different orthopedic and skeletal diseases.

Effect of Test Items on Bone Mineralization by Alizarin Red S Staining

The Biofield Energy Treated test item and DMEM were

studied for bone mineralization and data showed a significant increased bone mineralization process at various experimental tested concentrations on MG-63 cell line. The results of bone mineralization among different groups are presented in (Figure 4). The positive control, rutin showed a significant increased value of bone mineralization by 84.7%, 108.7%, and 137.9% at 10, 50 and 100 µg/mL, respectively. The Biofield Energy Treated experimental test group's *viz.* UT-DMEM+BT-TI showed a significant increase in bone mineralization by 301.4% and 285.3% at 50 and 100 µg/mL, respectively while the BT-DMEM+UT-TI group showed a significant increased bone mineralization by 143.3% and 127.5% at 50 and 100 µg/mL, respectively as compared with the UT-DMEM+UT-TI group. However, the BT-DMEM+BT-TI group showed a significant increased bone mineralization by 136.2% and 178.0% at 50 and 100 µg/mL, respectively as compared with the UT-DMEM+UT-TI group. Overall, all the experimental groups *i.e.* the combination of Biofield Energy Treated test item and DMEM groups showed a significant improved level of bone mineralization at the tested concentrations.

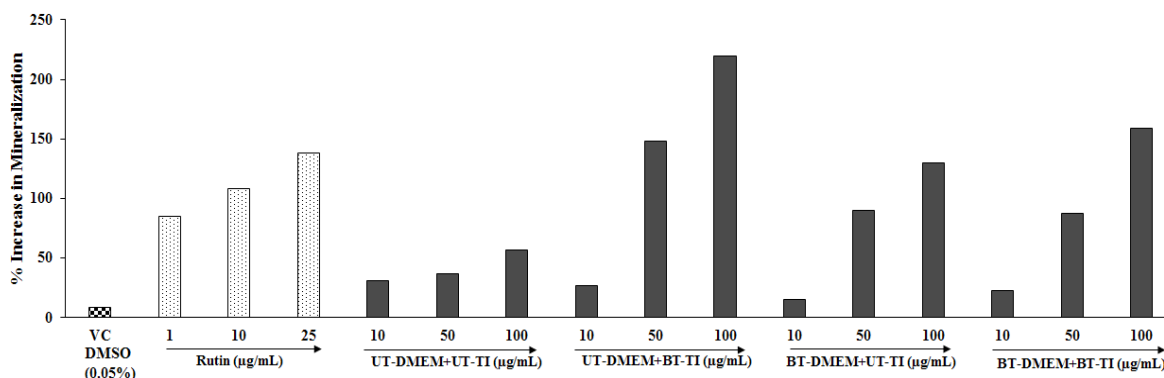


Figure 4: Effect of the test items on MG-63 cell line for bone mineralization. VC: Vehicle control (DMSO-0.05%), UT: Untreated; BT: Biofield Treated; TI: Test Item.

In order to study the calcium-rich deposits or calcium crystals in cell culture study, Alizarin Red S (ARS) staining is widely used semi-quantitative method due to high sensitivity and versatility [42]. This assay detects bone mineralization with respect to calcium pyrophosphate crystals-based sensitivity [43]. Vitamin D supplements along with calcium and phosphorus are preferred approach to maintain calcium deficiency and its related disorders. Bone mineralization might be disturbed through vitamin D endocrine system, while Vitamin D Receptors (VDR) also play a major role in the bone mineralization. Based on the numerous scientific literature, the important relationship between the intracellular calcium phosphate in osteoblasts and their role in Extra Cellular Matrix (ECM), on which apatite crystals subsequently form. Calcium phosphate is deposited in the osteoid and gets mineralized (combination of calcium phosphate and hydroxyapatite) and provides rigidity to the bone. Various bone disorders can be overcome with the calcium supplementation, which may restore the bone mineralization [44,45].

Conclusions

The present *in vitro* experiment showed a significant effect of the Biofield Energy Treatment on vitamin D₃ and DMEM for various bone health parameters. MTT assay was used for cell viability at the various test concentrations that showed a significant improved cell viability greater than 80%, while Biofield Energy Treated test items improved the cell viability as compared with the untreated group. ALP level was significantly increased by 753.3%, 1173.3%, and 424.4% in the UT-DMEM+BT-TI, BT-DMEM+UT-TI, and BT-DMEM+BT-TI groups, respectively at 0.1 µg/mL, compared to the untreated group. In addition to, the collagen level was significantly increased by 454.7% and 96.2% in the BT-DMEM+UT-TI and BT-DMEM+BT-TI groups, respectively at 1 µg/mL, while 202.4% increased in collagen was also reported at 50 µg/mL in the UT-DMEM+BT-TI compared to the untreated group. Likewise, the percent of bone mineralization was significantly increased in the UT-DMEM+BT-TI and BT-

DMEM+UT-TI groups by 301.4% and 143.3% respectively, at 50 µg/mL, while 178.0% increased in bone mineralization in BT-DMEM+BT-TI group at 100 µg/mL as compared to the untreated test item and DMEM group. Overall, the Biofield Energy Treated (The Trivedi Effect®) test samples showed a significant impact on various bone health parameters *viz.* collagen, calcium, and ALP, which play a vital role in maintaining bone-related disorders. Thus, Biofield Treated vitamin D₃ might be an alternative nutritional supplement, which could be useful for the management of various bone-related disorders *viz.* chondrodystrophia fetalis, rickets, low bone density, osteoporosis, osteogenesis imperfecta, Paget's disease, osteomalacia, bone fractures, deformed bones, osteoma, and other bone disorders. Biofield Treated vitamin D₃ can be useful as anti-stress, anti-aging, anti-inflammatory, wound healing, anti-cancer, anti-psychotic and anti-fibrotic roles. It also influences normal cell growth, cell-to-cell communication, cell differentiation, cell cycling and proliferation, hormonal balance, and neurotransmission. Moreover, it can be used in organ transplants like liver, heart, and kidney transplants and various immune-related disease conditions such as Myasthenia Gravis, Parkinson's Disease, Ulcerative Colitis, Dermatitis, Alzheimer's Disease, Diverticulitis, Atherosclerosis, Dermatomyositis, Graves' Disease, Hashimoto Thyroiditis, Multiple Sclerosis, Pernicious Anemia, Aplastic Anemia, Sjogren Syndrome, Systemic Lupus Erythematosus, Diabetes, Hepatitis, Irritable Bowel Syndrome, etc. with a safe therapeutic index to improve overall health and Quality of Life (QoL).

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Conflict of Interest

The authors declare no competing interests.

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