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The Bone Regenerative Effects of the Biofield Energy Treated Vitamin D_3 in Human Bone Osteosarcoma Cells (MG-63)

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Abstract

The current research work was presented to evaluate impact of Biofield Energy Treated vitamin D_3 and DMEM on bone health in human bone osteosarcoma cells (MG-63). The Test Items (TI), were distributed into two parts. One part of each sample was received Consciousness Energy Healing Treatment by James Jeffery Peoples and labeled as Biofield Energy Treated (BT) samples, while other parts of each sample were denoted as Untreated Test Items (UT). Test samples were found as safe in tested concentrations by MTT assay. ALP was significantly increased by 54.43% and 111.24% at 10 and 100 μg/mL, respectively in BT-DMEM + UT-TI than UT-DMEM + UT-TI. Moreover, ALP was significantly elevated by 128.14%, 77.84%, and 62.28% in UT-DMEM + BT-TI, BT-DMEM + UT-TI, and BT-DMEM + BT-TI, respectively at 1 μg/mL compared to untreated. Collagen was significantly increased by 286.67% and 340% in BT-DMEM + UT-TI and BT-DMEM + BT-TI, respectively at 0.1 μg/mL, while increased by 134.08% in UT-DMEM + BT-TI at 10 μg/mL than untreated. Besides, percent of bone mineralization was remarkably increased by 140.94%, 113.72%, and 129.87% at 1 μg/mL in UT-DMEM + BT-TI, BT-DMEM + UT-TI, and BT-DMEM + BT-TI, respectively, while increased by 187.91% in BT-DMEM + UT-TI at 0.1 μg/mL than untreated. Altogether, Biofield Treated vitamin D_3 was significantly improved the bone growth and it could be able to fight against various bone-related disorders (osteoporosis, osteogenesis imperfecta, Paget's disease, rickets, osteomalacia), autoimmune and inflammatory diseases, stress management, and anti-aging by improving overall health.

Keywords: Biofield Energy Treatment; Bone Health; Osteosarcoma cells; Osteoporosis; The Trivedi Effect®

Abbreviations

ALP : Alkaline Phosphatase

BT : Biofield Energy Treated

CAM : Complementary and Alternative Medicine

DMEM : Dulbecco's Modified Eagle's Medium

FBS : Fetal Bovine Serum

NHIS : National Health Interview Survey

NCCIH : National Center of Complementary and Integrative Health

MG-63 : Human Bone Osteosarcoma Cells

TI : Test Item

UT : Untreated

Introduction

Vitamin D has multiple effects, which regulate the functions in different organs *viz.* kidneys, brain, skeletal, liver, lungs, heart, immune and reproductive systems. Moreover, it has significant Anti-Stress, Anti-Inflammatory, Anti-osteoporosis, Anti-Aging, Anti-Arthritic, Anti-Apoptotic, Anti-Cancer, Wound Healing, Anti-Fibrotic and Anti-Psychotic Actions [1]. Vitamin D Receptors (VDR) are present in different part of body organs *viz.* heart, brain, liver, kidney, lungs, large and small intestines, pancreas, muscles, nervous system, reproductive, etc. It influences cell-to-cell communication, normal cell growth, differentiation, cycling and proliferation. It can also regulate skin health, neurotransmission process, hormonal balance, immune and cardiovascular functions. Vitamin D naturally synthesize in the presence of sunlight in the skin and plays a vital role for the development and growth of skeletal system [2]. Due to several reasons like aging, indiscriminate uses of skin protecting medical preparations the production of vitamin D_3 has decreased [3,4]. Manifestation of various metabolic disorders like osteomalacia and exacerbate osteoporosis, etc. due to deficiency of vitamin D_3 [5]. Serum calcium and Alkaline Phosphatase (ALP) are the two principal biomarkers for bone metabolism especially in post-menopausal women, while bone-specific ALP is the most important marker for osteoblast differentiation [6,7]. To maintain a sufficient level of these two biomarkers vitamin D is very essential. Hence, vitamin D has a good impact for development and maintaining a strong bone [8,9]. Apart from calcium and ALP, collagen is also take part in the process of bone calcification [10,11]. Numerous scientific reports and clinical trials have exhibited the useful effects of Biofield Energy Treatment to enhance immune function in cancer patients *via* therapeutic touch [12], massage therapy [13], etc. Complementary and Alternative Medicine (CAM) therapies are now emerging as preferred models of treatment, among which Biofield Therapy (or Healing Modalities) is one approach that has been reported to have several benefits to enhance physical, mental and emotional human wellness. Biofield Therapy has been included under the class of a CAM system, recommended by National Center of Complementary and Integrative Health (NCCIH). Apart from conventional drug therapy various alternative therapies *viz.* deep breathing, natural products, yoga, Tai Chi, chiropractic/osteopathic manipulation, Qi Gong, massage, meditation, special diets, homeopathy, progressive relaxation, guided imagery, acupressure, acupuncture, relaxation techniques, healing touch, hypnotherapy, pilates, movement therapy, rolfing structural integration, Ayurvedic medicine, mindfulness, aromatherapy, traditional Chinese herbs and medicines, essential oils, naturopathy, Reiki, and cranial sacral therapy have been extensively used for the treatment of various disorders. Human Biofield has a subtle form of energy that has ability to work in an effective way [14]. CAM treatment approaches have been practiced worldwide with significant clinical benefits in various disease cases [15]. The impact of The Trivedi Effect® has been published in reputed peer-reviewed science journals with fruitful outcomes in the field of tumor research [16, 17], microbiology [18-20], biotechnology [21,22], pharmaceutical science [23-26], agricultural science [27-29], materials science [30-32], nutraceuticals [33,34], skin health [35,36], human health and wellness. Therefore, considering the perspective of vitamin D on bone cell development the authors designed this experiment to investigate the potential of Consciousness Energy Healing Treatment on the test samples (vitamin D_3 and DMEM) using estimation of ALP, collagen, and bone mineralization in MG-63 cells.

Materials 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 D_3 (denoted as test item) and L-ascorbic acid were obtained from Sigma-Aldrich, USA. Antibiotic solution (penicillin-streptomycin) was procured from HI Media, India, while 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium) (MTT), Direct Red 80, and Ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma, USA. All the other chemicals used were analytical grade obtained from India.

Cell Culture

The human bone osteosarcoma (MG-63) cell line was used as test system, maintained under the DMEM growth medium for routine culture and supplemented with 10% FBS. Growth conditions were maintained at 37°C, 5% CO_2 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 [37].

Experimental Design

The experimental groups consisted of cells in baseline control (untreated cells), vehicle control groups (0.05% DMSO with Biofield Energy Treated and untreated DMEM), a positive control group (Rutin hydrate) and experimental test groups. Experimental test groups included the combination of the Biofield Energy Treated and untreated vitamin D_3/DMEM. It consisted of four major treatment groups on specified cells with UT-DMEM + UT-Test item, UT-DMEM + Biofield Energy Treated test item (BT-Test item), BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item.

Biofield Treatment Strategies

The test item (i.e., vitamin D_3 and DMEM) was separated into two parts. One part each of the test item was treated with the Biofield Energy (The Trivedi Effect®) and defined as Biofield Treated items, while the second part did not receive any treatment referred as the untreated. Biofield Energy Treatment was given by James Jeffery Peoples, who participated in this study and performed the Biofield Energy Treatment remotely through a unique Energy Transmission process for ~5 minutes under laboratory conditions. Healer remotely located in the USA, while the test samples were located at Dabur Research Foundation, New Delhi, India. In this study, Healer never reached the research laboratory in person or nor had any contact with the test item and medium. 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. After that, the Biofield Energy Treated and untreated samples were kept in similar sealed conditions for experimental study.

Determination of Non-Cytotoxic Concentration

The cell viability was performed by MTT assay in MG-63 cells. The cells were counted and plated in a 96-well plate at 5 X 10^3 to 10 X 10^3 cells/well/180 µL of cell growth medium. The above cells were incubated overnight and subjected to serum stripping or starvation. The cells were treated with the test item, DMEM, and the positive control. The untreated cells were served as baseline control. The cells in the above plate(s) were incubated for a time point ranging from 24 to 72 hours in a CO_2 incubator at 37˚C, 5% CO_2 and 95% humidity. After incubation, the plates were taken out and 20 µL of 5 mg/mL of MTT solution was added to all the wells followed by an additional incubation for 3 hours at 37˚C. 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 a Synergy HT microplate reader, BioTek, USA. The percentage cytotoxicity at each tested concentration of the test substance was calculated using Equation (1):

$$\%\,Cytotoxicity = \{(1 - X)/R\} * 100 \quad \text{-----------------------} \; (1)$$

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

The percentage cell viability corresponding to each treatment was then be obtained using the following Equation (2):

$$\%\,Cell\ Viability = 100 - \%\,Cytotoxicity \quad \text{-----------------} \; (2)$$

The concentration found ≥70% cell viability was considered as non-cytotoxic [38].

Assessment of Alkaline Phosphatase (ALP) Activity

Hemocytometer was used for the estimation of required cell density. At the density to 1 X 10^4 cells/well was plated in a 24-well plate in phenol-free DMEM with 10% CD-FBS and incubate for 48 hours in a CO_2 incubator at 37˚C, 5% CO_2 and 95% humidity. The cells were washed with 1X PBS and lysed by freeze-thaw method *i.e.,* incubation at -80°C for 20 minutes followed by incubation at 37˚C for 10 minutes. To the lysed cells, 50 µL of substrate solution *i.e.,* 5 mM of *p*-nitro phenyl 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˚C. The absorbance of the above solution was read at 405 nm using Synergy HT microplate reader (Biotek, USA). The absorbance values obtained were normalized with substrate blank (*p*NPP solution alone) absorbance values. The percentage increase in ALP activity than untreated cells (baseline group) was calculated using Equation (3):

$$\%\,Increase\ in\ ALP = \{(X - R)/R\} * 100 \quad \text{---------------------} \; (3)$$

Where,

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

R = Absorbance of cells corresponding to baseline group (untreated cells)

Evaluation of Collagen Synthesis

With the help of a Hemocytometer the MG-63 cells were counted and plated in a 24-well plate at 10 X 10^3 cells/well in phenol-free DMEM supplemented with 10% CD-FBS, incubate for 48 hours in a CO_2 incubator at 37°C, 5% CO_2 and 95% humidity. The amount of collagen accumulated in MG-63 cells respective to each treatment was measured using Direct Sirius red dye binding assay. 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 percentage increase in collagen level with respect to the untreated cells (baseline group) was calculated using Equation (4):

$$\%\,Increase\ in\ collagen\ levels = \{(X - R)/R\} * 100 \quad \text{-----} \; (4)$$

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

R = Collagen levels in cells corresponding to baseline group (untreated cells)

Assessment of Bone Mineralization by Alizarin Red S Staining

Using a Hemocytometer MG-63 cells were counted and plated in a 24-well plate at 10 X 10^3 cells/well in phenol-free DMEM containing 10% CD-FBS. The cells were incubated for 48 hours in a CO_2 incubator at 37˚C, 5% CO_2 and 95% humidity to allow cell recovery and exponential growth. The cells were subjected to serum stripping for 24 hours. Then, the cells were treated with non-cytotoxic concentrations of the test samples and positive control and incubate for 3 days. After which, the cells were fixed in 70% ethanol for 1 hour and Alizarin Red solution (40 µm; pH 4.2) was added to the samples for 20 minutes with shaking. The cells were washed with distilled water (DW) to remove unbound dye. For quantitative analysis by absorbance evaluation, nodules were solubilized with 10% cetylpyridinium chloride for 15 minutes with shaking. Absorbance was measured at 562 nm using Biotek

Synergy HT microplate reader. The percentage increase in bone mineralization with respect to the untreated cells (baseline group) was calculated using Equation (5):

$$\% \; Increase = \{(X - R)/R\} * 100 \text{------------------------} (5)$$

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

Statistical Analysis

All the values were represented as percentage of respective parameters. For multiple group comparison, one-way Analysis of Variance (ANOVA) was used followed by post-hoc analysis by Dunnett's test. Statistically significant values were set at the level of $p \leq 0.05$.

Results and Discussion

MTT Assay

The results of the MTT cell viability assay of the Biofield Energy Treated vitamin D_3 and DMEM in MG-63 cells are shown in (Figure 1). The data showed that the test samples in combination found as nontoxic and safe (as evidence of cell viability approximately greater than 71%) across all the tested concentrations up to 100 µg/mL. Hence, the same concentrations were used for the evaluation of Alkaline Phosphatase (ALP) activity, collagen synthesis, and bone mineralization in MG-63 cells.

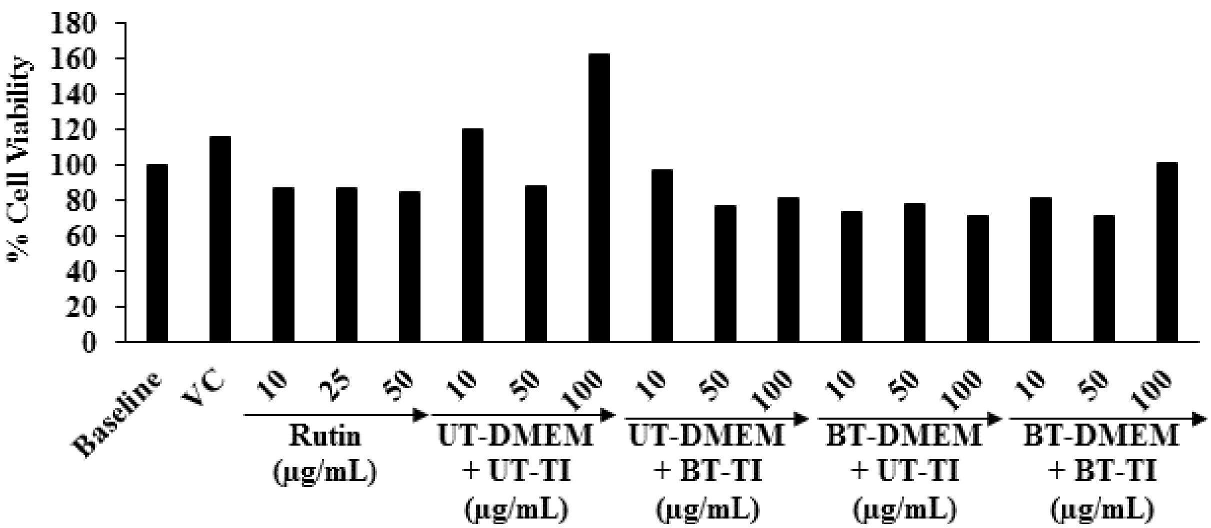

Figure 1: The effect of the test items (vitamin D_3 and DMEM medium) on cell viability in MG-63 cells after 72 hours of treatment; VC: Vehicle control (0.05% DMSO); UT: Untreated; BT: Biofield Energy Treated; TI: Test item.

Alkaline Phosphatase (ALP) Activity

The effect of the test samples on ALP activity in MG-63 cells is shown in (Figure 2). The vehicle control group showed 23.6% level of ALP activity as compared to the untreated cells group. The ALP activity was significantly increased by 43.44%, 53.55%, and 83.33% in the positive control (Rutin) group in a dose-dependent manner at the concentration of 0.01, 0.1, and 1 µg/mL, respectively compared to the untreated cells group. The level of ALP was significantly increased by 23.53%, 47.06%, and 75.63% in the UT-DMEM + BT-Test item, BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item groups, respectively at 0.1 µg/mL with respect to the UT-DMEM + UT-Test item group. Further, the level of ALP was significantly increased by 128.14%, 77.84%, and 62.28% in the UT-DMEM + BT-Test item, BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item groups, respectively at 1 µg/mL compared to the UT-DMEM + UT-Test item group. Moreover, at 10 µg/mL ALP level was significantly increased by 17.72%, 54.43%, and 30.48% in the UT-DMEM + BT-Test item, BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item groups, respectively compared to the UT-DMEM + UT-Test item group. Synthesis of ALP was significantly elevated by 12.35%, 16.47%, and 38.82% in the UT-DMEM + BT-Test item, BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item groups, respectively at 50 µg/mL compared to the UT-DMEM + UT-Test item group.

However, at higher concentration (at 100 µg/mL) showed the ALP level was significantly increased by 111.24% and 11.65% in the BT-DMEM + UT-Test item and BT-DMEM + BT-Test item groups, respectively than the untreated group (Figure 2).

According to Franceschi, RT et al. reported that for the induction of osteoblast marker, ALP coupled to collagen matrix synthesis and accumulation [39]. Thus, for complete development of bone cells ALP plays a vital role. Overall, the Consciousness Energy Treated (The Trivedi Effect®) vitamin D_3 showed an improved synthesis of ALP in the human osteosarcoma cells with respect to the untreated item items group, which might be advantageous to maintain a healthy skeletal structure for the patients suffering from various bone-related disorders.

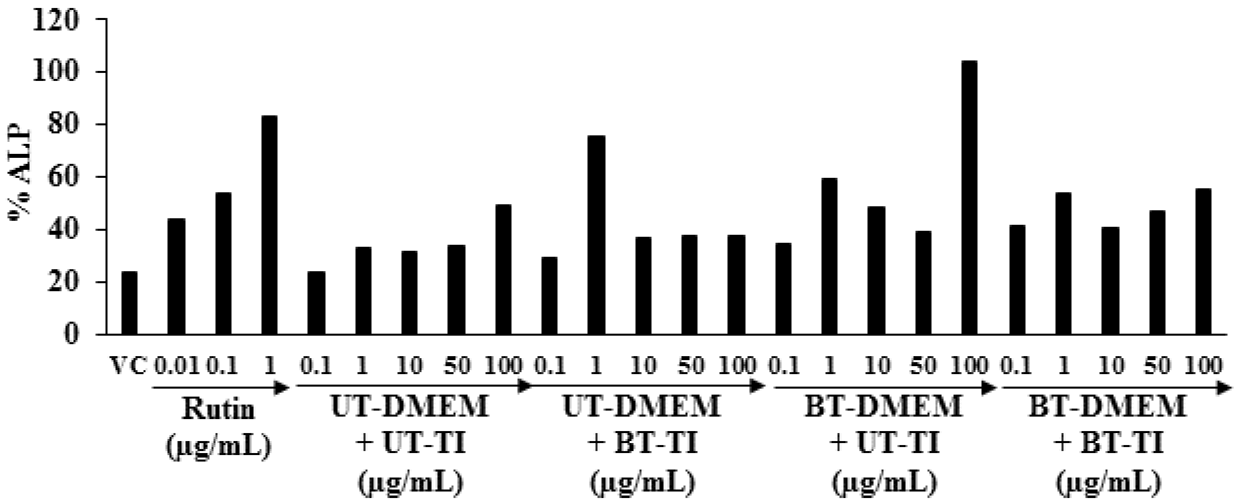

Figure 2: The effect of the test items (vitamin D_3 and DMEM medium) on alkaline phosphatase enzyme activity was assessed in human bone osteosarcoma cell after treatment with the Biofield Energy Treated test samples; VC: Vehicle control (0.05% DMSO), UT: Untreated; BT: Biofield Energy Treated; TI: Test item.

Assessment of Collagen Activity

The effect of the test substances on the collagen activity in MG-63 cells is shown in (Figure 3). Vehicle control group showed 4.9% increased the level of collagen as compared to the untreated cells (normal control) group. The level of collagen synthesis was significantly increased by 46.59%, 51.97%, and 65.41% at 0.01, 0.1, and 1 µg/mL, respectively in the positive control (Rutin) group compared to the untreated cells group. The collagen synthesis was significantly increased by 286.67% and 340% in the BT-DMEM + UT-Test item and BT-DMEM + BT-Test item groups, respectively at 0.1 µg/mL compared to the UT-DMEM + UT-Test item group. Moreover, the collagen level was significantly increased by 11.43% in the UT-DMEM + BT-Test item group at 1 µg/mL compared to the UT-DMEM + UT-Test item group. Additionally, at 10 µg/mL the level of collagen was also significantly increased by 134.08% in the UT-DMEM + BT-Test item group with respect to the UT-DMEM + UT-Test item group (Figure 3). Bone defects, including bone loss and fractures have great socioeconomic impact in disability. Type I collagen is one of the vital structural proteins in hard tissues, responsible for various functions on osteoblast such as initial attachment, proliferation, and differentiation etc. [40-41]. The extracellular matrix component collagen, plays a vital role in the maintenance of bone structure. The turnover of the bone matrix is influenced by collagen synthesis and degrading metalloprotease enzymes increase with the mechanical loading [42]. Overall, the Consciousness Energy treated vitamin D_3 had significantly improved the synthesis of collagen fibers in the human osteosarcoma cells with respect to all the treatment groups. Hence, it is assumed that The Trivedi Effect® has the significant potential to improve the bone health in various skeletal disorders.

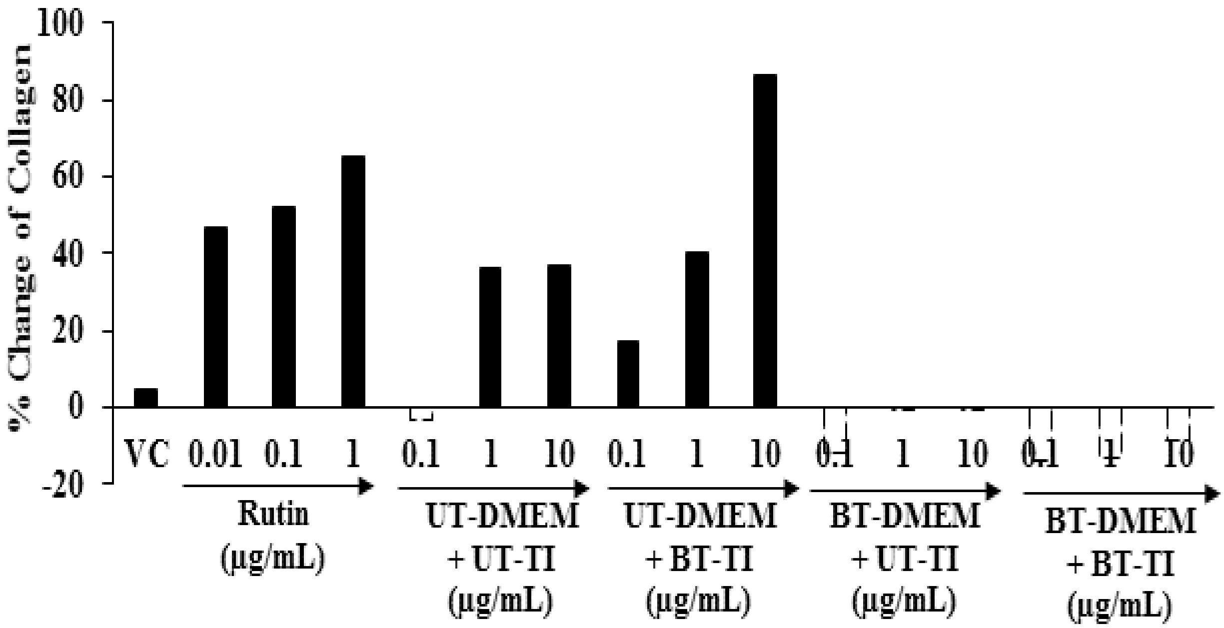

Figure 3: The effect of the test items (vitamin D_3 and DMEM medium) on collagen activity in MG-63 cells**;** VC: Vehicle control (0.05% DMSO), UT: Untreated; BT: Biofield Energy Treated, TI: Test item.

Assessment of Bone Mineralization by Alizarin Red S (ARS) Staining

Staining with alizarin red S is an appropriate technique for the assessment of skeletal mineralization during skeletal development, maintenance, and regeneration process [43]. ARS staining has been used for the evaluation of calcium-rich deposits by cells in culture [44]. The bone mineralization is essential for the growth and development of overall health. Alteration of bone mineralization process can leads to a variety of medical difficulties [45]. The effect of the test items on mineralization of bone in MG-63 cells is shown in (Figure 4). The vehicle control (VC) group showed 8.9% increased bone mineralization as compared to the untreated cells (normal control) group. The percentage of bone mineralization was significantly increased in a concentration-dependent manner by 47.98%, 59.73%, and 139.02% at 5, 10, and 25 µg/mL, respectively in the positive control group compared to the untreated cells group. The percent of bone mineralization was remarkably increased by 19.32% and 187.91% in the UT-DMEM + BT-Test item and BT-DMEM + UT-Test item group at 0.1 µg/mL compared to the UT-DMEM + UT-Test item group. Further, a noticeably increased percentage of bone mineralization by 140.94%, 113.72%, and 129.87% in the UT-DMEM + BT-Test item, BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item groups, respectively was found at 1 µg/mL with respect to the UT-DMEM + UT-Test item group. In addition to, the data showed a significant increased of percent bone mineralization by 5.48%, 36.75%, and 28.89% in the UT-DMEM + BT-Test item, BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item groups, respectively than the UT-DMEM + UT-Test item group (Figure 4) at 10 µg/mL. Thus, results envisaged that the Biofield Energy Treated vitamin D_3 remarkably improved bone mineralization by *in vitro* in MG-63 cells compared to all others treatment groups.

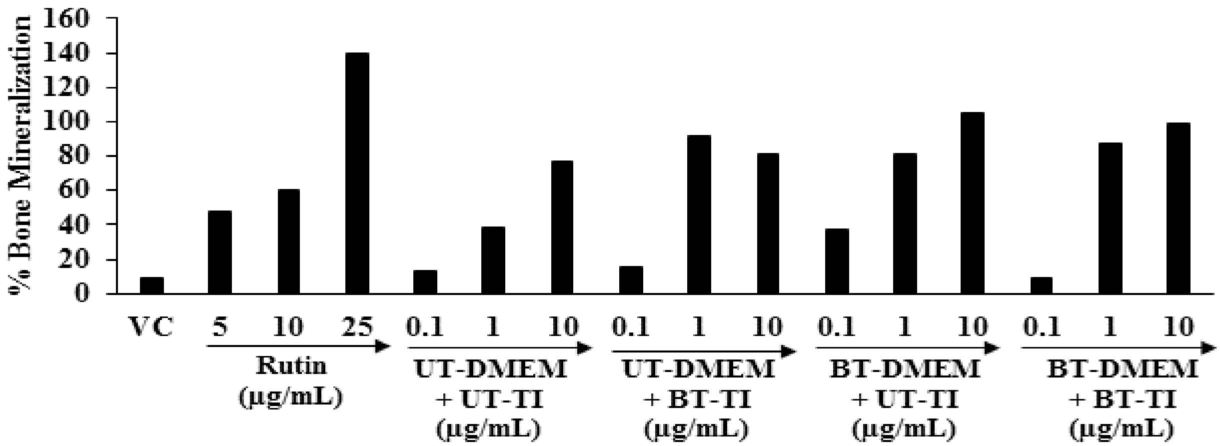

Figure 4: The effect of the test items (vitamin D_3 and DMEM medium) on bone mineralization activity in human bone osteosarcoma cells; VC: Vehicle control (0.05% DMSO), UT: Untreated; BT: Biofield Energy Treated, TI: Test item.

Conclusions

The MTT cell viability assay data showed greater than 71% cells were viable, which indicated that the test samples were safe and nontoxic in the selected concentrations. The UT-DMEM + BT-Test item group exhibited 128.14% increased the level of ALP at 1 µg/mL than untreated. Moreover, the BT-DMEM + UT-Test item group revealed the percent increased of ALP by 77.84%, 54.43%, and 111.24% at 1, 10, and 100 µg/mL, respectively as compared to the untreated group. Other parameter like collagen was significantly increased by 286.67% and 340% in the BT-DMEM + UT-TI and BT-DMEM + BT-TI, respectively at 0.1 µg/mL, while increased by 134.08% in the UT-DMEM + BT-Test item group at 10 µg/mL compared to the untreated group. Besides, the percent of bone mineralization was remarkably increased by 140.94%, 113.72%, and 129.87% at 1 µg/mL in the UT-DMEM + BT-TI, BT-DMEM + UT-TI, and BT-DMEM + BT-TI groups, respectively, while increased by 187.91% in the BT-DMEM + UT-Test item group at 0.1 µg/mL compared to the untreated group. In conclusion, Biofield Treated (The Trivedi Effect®) test samples demonstrated a significant impact on bone health parameters. Therefore, the Consciousness Energy Healing-based vitamin D_3 might be use for the management of bone-related disorders *viz.* low bone density and osteoporosis, rickets, osteogenesis imperfecta, bone and joint pain, osteoma, osteomalacia, bone fractures, etc. Further, it can used in organ transplants (kidney, liver, and heart transplants), autoimmune disorders (Systemic Lupus Erythromatosus, Addison Disease, Celiac Disease or gluten-sensitive enteropathy, Graves' Disease, Hashimoto Thyroiditis, Multiple Sclerosis, Myasthenia Gravis, Pernicious Anemia, Aplastic Anemia, Rheumatoid Arthritis, Type 1 Diabetes, Alopecia Areata, Crohn's Disease, Fibromyalgia, Vitiligo, Psoriasis, Scleroderma, Chronic Fatigue Syndrome and Vasculitis), and inflammatory disorders (Asthma, Ulcerative Colitis, Alzheimer's Disease, Atherosclerosis, Dermatitis, Diverticulitis, Hepatitis, Irritable Bowel Syndrome). Further, it could be useful for the management of stress, cancer, psychosis, fibrosis, aging, neurodegenerative disorders (Parkinson's), wound repair and improve Quality of Life.

Acknowledgements

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Conflict of Interest

Authors declare no conflict of action.

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