June 26, 2018

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International Journal of Bacteriology, Virology and Immunology : ISSN-2384-5066, Vol. 4(1): pp 053-062,June, 2018.

Full Length Research Paper

Investigation of Biofield Treated Vitamin D_3 to Improve Immunity of Bone Cells in MG-63 Cell Line.

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Accepted 29th June, 2018.

The study was aimed to evaluate the effect of Consciousness Energy Healing-based vitamin D_3 and DMEM on bone health in human bone osteosarcoma cells (MG-63). Test items (vitamin D_3 and DMEM), were divided into two parts. One part of each sample was received the Biofield Energy Treatment by Inthirani Arul and those samples were denoted as Biofield Treated (BT) samples, while other parts of each sample were referred as untreated test items (UT). Cell viability revealed that test samples were found as safe in tested concentrations. ALP was significantly increased by 260% in BT-DMEM + BT-TI group at 0.1 µg/mL compared to UT-DMEM + UT-TI group. Moreover, collagen level was significantly increased by 102.94% and 52.94% in UT-DMEM + BT-TI and BT-DMEM + BT-TI groups, respectively at 0.1µg/mL than UT-DMEM + UT-TI group. Further, collagen level was significantly increased by 114.94%, 114.03%, and 152.33% in UT-DMEM + BT-TI, BT-DMEM + UT-TI, and BT-DMEM + BT-TI groups, respectively at 1µg/mL than untreated. Besides, percent of bone mineralization was significantly enhanced by 82.95% and 119.55% in UT-DMEM + BT-TI and BT-DMEM + BT-TI groups, respectively at 10µg/mL than untreated. Moreover, it was significantly increased by 166.44% and 212.49% in BT-DMEM + UT-TI and BT-DMEM + BT-TI groups, respectively at 50µg/mL than untreated. It was also significantly increased by 104.44%, 100%, and 230.22% in UT-DMEM + BT-TI, BT-DMEM + UT-TI, and BT-DMEM + BT-TI groups, respectively at 100µg/mL than untreated. Among three combination regimens, the Biofield Treated vitamin D_3 and DMEM group was comparatively more improved the bone-related parameters and might be useful against various bone-related disorders (rickets, low bone density, osteomalacia, osteoporosis, Paget's disease, chondrodystrophia fetalis), stress management, anti-aging, autoimmune and inflammatory disorders near future.

Keywords: The Trivedi Effect®, Bone health, Biofield Energy Healing Treatment, Vitamin D_3, Osteosarcoma cells

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

INTRODUCTION

Bone and the immune system are connected together to regulate the skeleton functions and the body's response to invading microbes. It is necessary for the development of immune cells in the bone marrow and for the function of bone cells in health. Vitamin D has multiple effects, which regulate the functions in different target organs such as brain, liver, lungs, heart, kidneys, skeletal, and reproductive, immune systems. Additionally, it has a significant anti-inflammatory, anti-aging, anti-stress, anti-arthritic, anti-osteoporosis, anti-apoptotic, wound healing, anti-cancer, anti-psychotic and anti-fibrotic actions (Holick et al. 2004). Vitamin D receptors (VDRs) are widely distributed in most of the body organs *viz.* brain, liver, heart, lungs, kidney, pancreas, small and large intestines, muscles, reproductive, nervous system, etc. VDR scan influence cell-to-cell communication, normal cell growth, cell differentiation, cell cycling and proliferation, hormonal balance, neurotransmission process, skin health, immune and cardiovascular functions. In any living vertebrates, vitamin D plays an important role in maintaining a healthy skeletal structure and is essential for bone health. Hence, authors intended to study on vitamin D_3. Naturally, it is synthesized in presence of sunlight in the skin (Holick et al. 1996). Most of the foods do not contain vitamin D, additionally now-a-days due to aging, use of sunscreen, and change of zenith angle of the sun, the production of vitamin D_3 has been decreased (Matsuoka et al. 1987). Increasing age is not only related to a decrease in bone marrow depression and muscle strength but is also associated with marked changes in the immune and inflammatory responses (Barnes et al. 2006). Various cytokines that directs the terminal differentiation of osteoclast precursor cells and stimulates and maintains resorption activity in mature bone cells. Deficiency of vitamin D_3 causes metabolic bone diseases like osteomalacia and exacerbate osteoporosis, etc. (Laird et al. 2010). The quality of life (QoL) for menopausal women is one of the most critical health problems in the modern world. Metabolic bone disorders like osteoporosis are mainly prevalent in post-menopausal women. Hormonal factors and rapid bone loss in post-menopausal women leads to an increased risk of fractures (Bhattarai et al. 2014). Hence, the serum calcium and alkaline phosphatase (ALP) levels in post-menopausal women are the main two vital biochemical markers of bone metabolism. However, bone-specific ALP is the most important marker for osteoblast differentiation (Iba et al. 2004). Further, it is generally accepted that an increased calcium intake along with an adequate source of vitamin D is important for maintaining good bone health. Vitamin D also plays an important role in maintaining an adequate level of serum calcium and phosphorus. Therefore, vitamin D has a great impact on forming and maintaining strong bones (Holick et al. 2006; DeLuca 2006). Bone strength depends on the quality, geometry, shape, micro architecture, turnover, mineral content, and the collagen content. Collagen is the major structural protein responsible for bone calcification. In the aging state, the mechanical properties of the bones become impaired, and the bones get fragile, that causes various clinical disorders associated with bone collagen abnormalities and bone fragility, such as osteogenesis imperfect a and osteoporosis (Viguet-Carrin et al. 2006; Sroga et al. 2012).

In recent years, numerous scientific reports and clinical trials have revealedthat the useful effects of Biofield Energy Treatment, which have shown to enhanced immune function in case of cervical cancer patients *via* therapeutic touch (Lutgendorf et al. 2010), massage therapy (Ironson et al. 1996), etc. Complementary and Alternative Medicine (CAM) therapies are now rising as preferred models of treatment, among which Biofield Energy Therapy (or Healing Modalities) is one approach that has been reported to have several benefits to enhance physical, mental and emotional human wellness. However, as per the data of 2012 from the National Health Interview Survey (NHIS), which indicated that the highest percentage (~17%) of the Americans used dietary supplements as a complementary health approach as compared with other practices in the past years. The National Center of Complementary and Integrative Health (NCCIH) has recognized and accepted Biofield Energy Healing as a CAM health care approach in addition to other therapies, medicines and practices such as natural products, deep breathing, yoga, Tai Chi, Qi Gong, chiropractic/osteopathic manipulation, meditation, massage, special diets, homeopathy, progressive relaxation, guided imagery, acupressure, acupuncture, relaxation techniques, hypnotherapy, healing touch, movement therapy, pilates, rolfing structural integration, mindfulness, Ayurvedic medicine, traditional Chinese herbs and medicines, naturopathy, essential oils, aromatherapy, Reiki, and cranial sacral therapy. Human Biofield Energy has subtle energy that has the capacity to work in an effective manner (Jain et al. 2015). CAM therapies have been practiced worldwide with reported clinical benefits in different health disease profiles (Rubik 2002). This energy can be harnessed and transmitted by the experts into living and non-living things *via* the process of Biofield Energy Healing. Biofield Energy Treatment (The Trivedi Effect®) has been published in numerous peer-reviewed science journals with significant outcomes in many scientific

fields such as cancer research (Trivedi et al. 2015a,b), microbiology (Trivediet al. 2015c,d,e,f), biotechnology (Trivedi et al. 2015g,h), pharmaceutical science (Trivedi et al.2015i,j,k,l), agricultural science (Trivedi et al. 2015m,n,o,p), materials science (Trivedi et al. 2015q,r,s,t), nutraceuticals (Trivedi et al. 2015u,v), skin health (Trivedi et al. 2015w,x), human health and wellness.

Based on the importance of vitamin D_3 on bone growth and development, the authors designed the experiment to evaluate the impact of the Biofield Energy Treatment (The Trivedi Effect®) on the test samples (vitamin D_3 and DMEM medium) for bone health activity with respect to the assessment of different bone parameters like ALP, collagen content, and bone mineralization using standard assay 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 obtained from TCI, Japan, while vitamin D_3, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium) (MTT), Direct Red 80, ethylenediaminetetraacetic acid (EDTA) and L-ascorbic acid were obtained from Sigma-Aldrich, USA. Antibiotic solution (penicillin-streptomycin) was procured from HiMedia, India. All the other chemicals used in this experiment were analytical grade procured from India.

Cell Culture

The human bone osteosarcoma cells (MG-63) were used as the test system in this experiment. The MG-63 cells supplemented with 10% FBS were maintained under the DMEM growth medium for routine culture. 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 prior 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 (Czekanska et al. 2012).

Experimental Design

The experimental groups consisted of cells in baseline control (untreated cells), vehicle control group (0.05% DMSO), a positive control group (rutin hydrate) and experimental test groups. The experimental 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.

Preparation of Test items

Vitamin D_3 was weighed and dissolved in suitable solvent at 10 mM - 50 mM (based on the requirement of the assay). Stock solution was further diluted in SFM to treat cells. Besides, commercially supplied DMEM was dissolved in 800 mL of distilled water. Added calculated amount of $NaHCO_3$, adjusted pH (7.2-7.4), and 10 mL of penicillin/streptomycin were added to make final volume 1 L. Then, filtered into sterile flasks using 0.2 µm filter using peristaltic pump and checked for sterility by incubating in a CO_2 incubator for 24 hours. Then, stored the content at 2-8 ºC till used. Here, concentration (µg/mL) of vitamin D_3 was used as specific amount and mixed in DMEM during experiment.

Consciousness Energy Healing Treatment Strategies

The test item (vitamin D_3) and DMEM were divided into two parts. One part each of the test item and DMEM were treated with the Biofield Energy (also known as The Trivedi Effect®) 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. This Biofield Energy Healing Treatment was provided by Inthirani Arul, who participated in this study and performed the Biofield Energy Healing Treatment remotely for ~5 minutes. Biofield Energy Healer was remotely located in the Canada, while the test samples were located in the research laboratory of Dabur Research Foundation, New Delhi, India. The Biofield Energy Treatment was administered for 5 minutes through the healer's unique Energy Transmission process remotely to the test samples under laboratory conditions. Inthirani Arul in this

study, never visited the laboratory in person, nor had any contact with the test item and medium. 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 until end of the experiment.

MTT Assay

The cell viability was performed using MTT assay in MG-63 cells. The details procedure of cell viability assay was followed by Karen BA *et al.* (2018) with slight modification [38]. The cytotoxicity of each tested concentration of the test items was calculated with the help of Equation (1):

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

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):

$$\%\ Cell\ Viability = (100 - \%\ Cytotoxicity) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

The concentration exhibiting ≥70% cell viability was appraise as non-cytotoxic [39].

Alkaline Phosphatase (ALP) Activity

The effect of the Biofield Energy Treatment on the test items for the evaluation of ALP activity in MG-63 cells. The procedure of cell counting, plating, and treatment was followed as per Liu SC et al. [40]. The percent increase in ALP activity with respect to the untreated cells was calculated using Equation (3):

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

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

R = Absorbance of cells corresponding to untreated cells

Collagen Activity

The MG-63 cells were used for the evaluation of the potential of Biofield Treated test items and the procedure in details was as per Parulkar VR et al. with few modifications [41]. The increase collagen level with respect to the untreated cells was calculated using Equation (4):

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

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

R = Collagen levels in cells corresponding to untreated cells

Bone Mineralization Activity

Evaluation of the percent increased of mineralization after treatment of the Biofield Treated test items in MG-63 cells, and the details steps were followed according to Slade TC et al. [42]. The percentage increase in bone mineralization compared to the untreated cells was calculated using Equation (5):

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

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

Statistical Analysis

The obtained data were expressed as percentage (%) of the respective study parameters. Sigma-Plot (version 11.0) was used as a statistical tool for data interpretation. Statistically significant values were set at the level of $p \leq 0.05$.

RESULTS AND DISCUSSION

MTT Assay

The cell viability using MTT assay of the test samples *i.e.,* vitamin D3 and DMEM medium in MG-63 cells is shown in Figure 1. The cell viability results are expressed as percentage. The percentage of cell viability was lies in the ranges of 85.20% to 105.82% in the tested concentrations of all the groups and did not show any cytotoxicity (as evidence of cell viability approximately greater than 85%) across all the tested concentrations up to 100 µg/mL. Hence, the safe concentrations were used in this experiment to see the effect of the test samples on the levels of alkaline

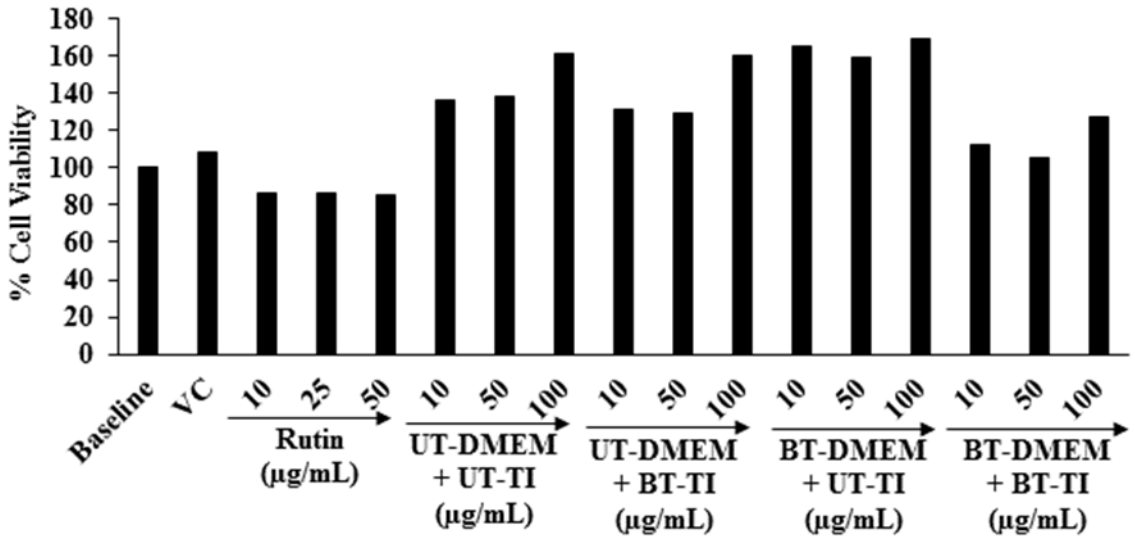

Figure 1: The cell viability activity of the test items (vitamin D3 and DMEM medium) after Biofield Energy Treatment in different tested concentrations in MG-63 cells. VC: Vehicle control (0.05% DMSO); UT: Untreated; BT: Biofield Energy Treated; TI: Test item

Assessment of Alkaline Phosphatase (ALP) Enzyme Activity

The effect of the Biofield Energy Treated test samples on ALP activity in MG-63 cells is shown in Figure 2.

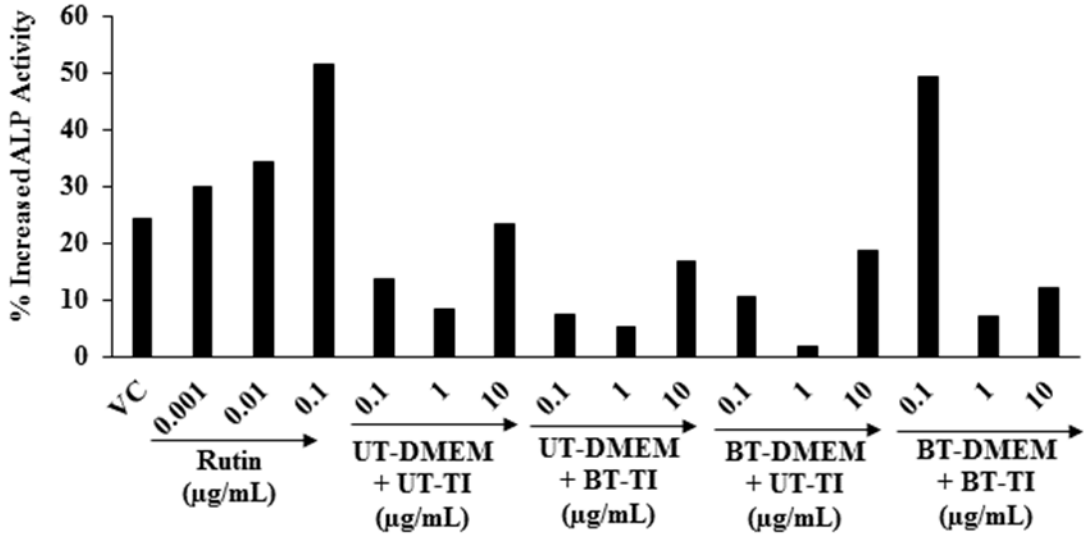

Figure 2: The effect of the test substances on alkaline phosphatase (ALP) enzyme activity in human bone osteosarcoma cell (MG-63). VC: Vehicle control (0.05% DMSO), UT: Untreated; BT: Biofield Energy Treated; TI: Test item

phosphatase (ALP) activity, collagen synthesis, and bone mineralization in MG-63 cells.

The vehicle control (VC) group showed 24.4% increased level of ALP as compared to the untreated cells group. The reference standard was used in this experiment (rutin hydrate) showed 30.02%, 34.31%, and 51.47% increased the level of ALP at the concentration of 0.001, 0.01, and 0.1 µg/mL, respectively compared to the untreated cells group in a concentration-dependent manner. The results of the test items showed that the level of ALP was significantly enhanced by 260% in the BT-DMEM + BT-TI group at 0.1µg/Ml as compared to the UT-DMEM + UT-Test item group. According to Prins *et al.,* reported that an increased levels of ALP in an *in vitro* experiment can predicts *in vivo* bone forming capacity (Prins Henk-Jan et al. 2014). Overall, the Biofield Energy Treated (The Trivedi Effect®) test item group (*i.e.,* vitamin D_3) showed an improved synthesis of ALP enzyme in the MG-63 cells with respect to the untreated test item group, which might be advantageous to the patients those are suffering from various bone-related disorders.

Assessment of Collagen Activity

The collagen activity of the test items in human bone osteosarcoma cells is shown in Figure 3. Collagen level in the VC group was found as 2.8% increased as compared to the untreated cells group. The positive control (rutin hydrate) showed 17.76%, 28.27%, and 46.50% increased the level of collagen in a concentration-dependent manner at 0.001, 0.01, and 0.1 µg/mL, respectively compared to the untreated cells group. The collagen synthesis was significantly increased by 102.94%, 29.39%, and 52.94% in the UT-DMEM + BT-Test item, BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item groups, respectively at 0.1µg/mL compared to the UT-DMEM + UT-Test item group. Moreover, the collagen level was significantly increased by 114.94%, 114.03%, and 152.33% in the UT-DMEM + BT-Test item, BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item groups, respectively at 1µg/mL compared to the UT-DMEM + UT-Test item group. Additionally, at 10 µg/mL the level of collagen was also increased by 1.76% in the BT-DMEM + UT-Test item group with respect to the UT-DMEM + UT-Test item group (Figure 3). Vitamin D deficiency affects approximately 1 billion people worldwide and is responsible for bone cells growth and development (Angeline et al. 2014). Apart from vitamin D, the fiber material collagen also plays an important role for the regulation of osteoblast phenotypic expression (Shi et al. 1996). Overall, The Trivedi Effect® - Consciousness Energy Healing Treatment modality showed a significant improvement of the collagen level in human osteosarcoma cells. Thus, it is assumed that The Trivedi Effect® has the significant potential to improve the bone health in various skeletal disorders.

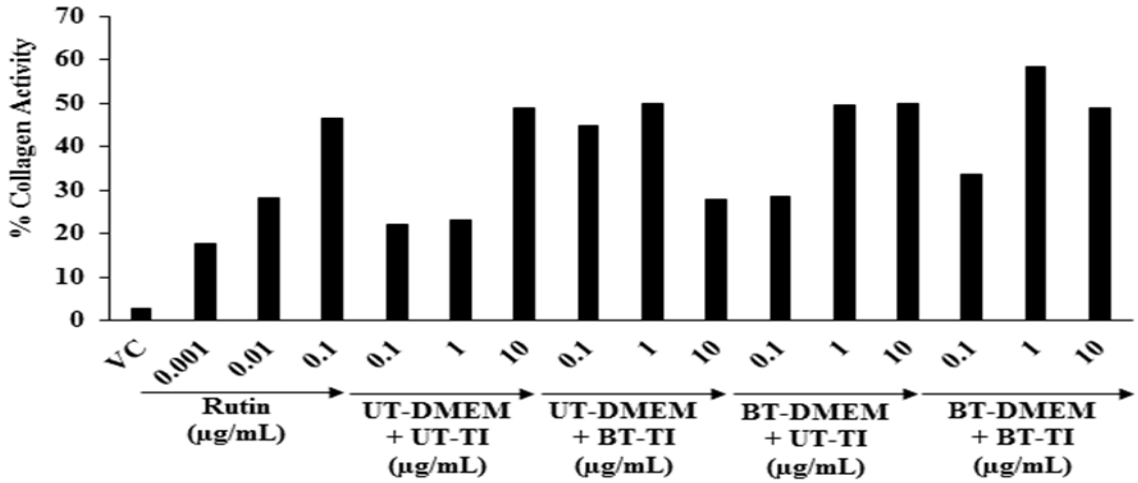

Figure 3: The effect of the test substance on the estimation of the collagen level in human bone osteosarcoma cells after exposure with the Biofield Energy Treated test samples. VC: Vehicle control (0.05% DMSO), UT: Untreated; BT: Biofield Energy Treated; TI: Test item

Bone Mineralization

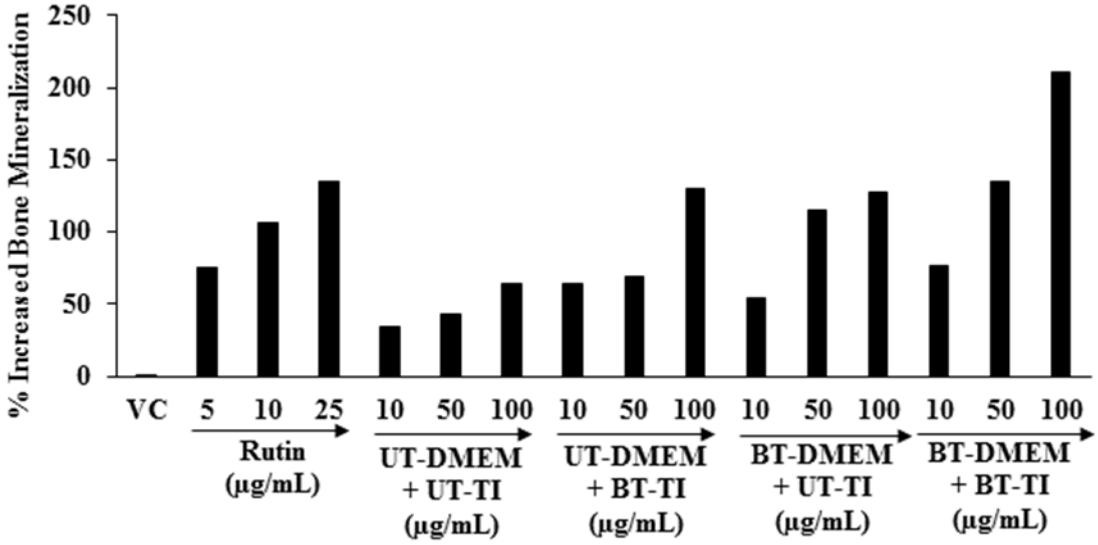

Figure 4: The effect of the bone mineralization activity of Biofield Energy Treated test samples in human bone osteosarcoma cells. VC: Vehicle control (0.05% DMSO), UT: Untreated; BT: Biofield Energy Treated, TI: Test item

The effect of Biofield Energy Treatment on bone mineralization in MG-63 cells is shown in Figure 4. The percentage of bone mineralization was significantly increased by 75%, 105.94%, and 135.15% at 5, 10, and 50 µg/mL, respectively in the positive control group compared to the untreated cells group. The percent of bone mineralization was distinctly increased by 82.95%, 55.31%, and 119.55% in the UT-DMEM + BT-Test item, BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item groups, respectively at 10µg/mL compared to the UT-DMEM + UT-Test item group. Further, an increased percentage of bone mineralization was observed by 59.87%, 166.44%, and 212.49% in the UT-DMEM + BT-Test item, BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item groups, respectively at 50µg/mL with respect to the UT-DMEM + UT-Test item group. Moreover, the percentage of bone mineralization was significantly increased by 104.44%, 100%, and 230.22% in the UT-DMEM + BT-Test item, BT-DMEM + UT-Test item, and BT-DMEM + BT-Test item groups, respectively at 100µg/mL compared to the UT-DMEM + UT-Test item group (Figure 4). Bone mineral composition, crystallinity, and content of osteoporotic patients are different from normal subjects. According to Jagielska et al. reported that bone mineralization disorders as a complication of anorexia nervosa and supplementation of adequate amount of vitamin D and calcium from diet are required for the maintenance of bone mineral density (Jagielska et al. 2016). Thus, supplementation with calcium and vitamin D_3 increased the degree of bone mineralization (Boivin et al. 2003). In this study, authors have found that the Biofield Energy Treated vitamin D_3 significantly enhanced the level of bone mineralization in the form of calcium, assessed by Alizarin Red S Staining technique. Therefore, based on the above findings it is hypothesized that the Consciousness Energy Healing Treatment (The Trivedi Effect®) based test item groups (*i.e.,* vitamin D_3) showed a remarkable improvement of bone mineralization content assessed by *in vitro* in the human osteosarcoma cells (MG-63).

CONCLUSIONS

Based on the cytotoxicity data it was observed that the tested test items were nontoxic at a selected concentrations, and were used further for the analysis of others bone-related parameters. Results showed that alkaline phosphatase (ALP) level was significantly (260%) increased in the Biofield Treated vitamin D_3 and DMEM group as compared with the untreated group. Structural protein of bone like collagen was significantly increased (more than 100%) in all three groups even at lower concentration (1 µg/mL). Moreover, Biofield Treatment was also significantly increased (upto 230%)

bone mineralization compared to the untreated group. In conclusion, the Biofield Energy Treated (The Trivedi Effect®) test samples demonstrated an impact on bone parameters like ALT, collagen, and bone mineralization. Therefore, it might be suitable for the development of an alternative supplement for vitamin D_3 deficiency than naïve vitamin D_3, for the management of various bone-related disorders *viz.* osteoporosis, rickets, osteomalacia, etc. Besides, as it also improved the structural protein (collagen) of connective tissues, could be utilized in organ transplants, autoimmune disorders (addison disease, rheumatoid arthritis, multiple sclerosis, myasthenia gravis, pernicious anemia, type 1 diabetes, crohn's disease, etc.), inflammatory disorders (ulcerative colitis, irritable bowel syndrome, etc.), anti-stress, wound healing, anti-cancer, anti-aging, anti-psychotic, etc. to modulate the immune system by improving Quality of Life (QoL).

ACKNOWLEDGEMENTS

Authors are grateful to Trivedi Global, Inc., Trivedi Science, Trivedi Testimonials, Trivedi Master Wellness, and Dabur Research Foundation.

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