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Evaluation of Vitamin D_3 metabolite (25-OH Vit D_3), Neurotransmitter (ACh), and the Expression of Proinflammatory Cytokines (IL-6 and TNF-α) in Tissue Homogenate after Administration of Biofield Energy Healing-based Novel Proprietary Test Formulation and Biofield Treatment *per se* to the Animals in Vitamin D_3 Deficiency Diet (VDD)-induced Sprague Dawley Rats

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Abstract

A novel proprietary test formulation was designed which included minerals, vitamins, β-carotene, cannabidiol isolate, and *Panax ginseng* extract. This present study was evaluated the impact of the Trivedi Effect® on novel proprietary test formulation in male Sprague Dawley rats, fed with vitamin D_3 deficiency diet (VDD). The novel test formulation was divided into two parts; one part was defined as untreated test formulation, while the other part was defined as the Biofield Energy Treated sample, which received the Biofield Energy Healing Treatment by renowned Biofield Energy Healer, Mr. Mahendra Kumar Trivedi. The level of 25-OH Vit. D_3 was measured in brain homogenate, which was found to be increased by 20.13%, 24.12%, 45.86%, 14.79%, and 29.96% in the G5 group treated with Biofield Treated test formulation, Biofield Energy Treatment *per se* to the animals (G6), 15 days pre-treatment of Biofield Energy Treated test formulation (G7), Biofield Energy Treatment *per se* plus Biofield Energy Treated test formulation from day -15 (G8), and untreated test formulation to the Biofield Energy Treated animals (G9) groups respectively, as compared with the disease control (G2) group. Brain acetylcholine (ACh) level was increased by 61.33% in the G7 group as compared with the untreated test formulation (G4) group. The expression of interleukin-6 (IL-6) was significantly reduced by 43.44% ($p \leq 0.01$), 30.93%, 21.42%, 45.99% ($p \leq 0.01$), and 60.85% ($p \leq 0.01$), respectively as compared with the G4. Lung pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) level was significantly reduced in the G5, G6, G7, and G8 by 24.86%, 32.55% ($p \leq 0.01$), 30.12% ($p \leq 0.01$), and 42.69% ($p \leq 0.01$), respectively, as compared with the G4 group. Altogether, the Biofield Treated test formulation and/or *per se* treatment to the animals significantly improved the levels of active form of vitamin D_3 metabolite (25-OH Vit D_3) and neurotransmitter (ACh); consequently significantly lowered the expression of proinflammatory cytokines (IL-6 and TNF-α). Therefore, the energized test formulation or *per se* treatment could be effectively useful against neuronal damage and inflammation for the management of brain disorders such as Alzheimer's disease, dementias, brain cancer, epilepsy and other seizure disorders, mental disorders, and Parkinson's. Thus, the results showed a significant slowdown of disease progression and all other disease-related complications/ symptoms in the preventive Biofield Energy Treatment group *per se* and the Biofield Energy Treated Test formulation groups (*viz.* G6, G7, G8, and G9) as compared to the disease control group.

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Introduction

The inflammation has been considered as the basis of the etiology and pathophysiology of several brain-related malfunctions such as depression [1], Alzheimer's disease [2], cognitive aging [3] post-stroke depression [4], and mortality [5]. Such neuropsychiatric disorders could be identified by the specific markers such as cytokines that act as an important mediators of systemic inflammation. Such cytokines include interleukin 1 beta, tumor necrosis factor alpha, and interleukin 6, *etc.* that play vital role in physiological processes of central nervous system [6]. The biomarkers are considered as the useful tools in the field of medicine, toxicology, environmental health, basic scientific research, and developmental biology. Among these biomarkers, interleukin 6 (IL-6) could help in demonstrating both the neurodegenerative [2] as well as neuroprotective [7] properties. For example, the neuropsychiatric conditions such as Alzheimer's disease and depression [8] showed an increased levels of IL-6; while it also shows various immunosuppressive and anti-inflammatory activities in inflammatory conditions that may help in down-regulation of those processes [9]. Another biomarker *i.e.,* AChE is a type of cholinesterases (ChEs), which acts by hydrolyzing the acetylcholine neurotransmitter. It is essential for the normal functioning of the CNS and PNS as it helps in terminating the synaptic transmission and thereby preventing the continuous nerve firings at the nerve endings [10]. Its expression at the neuromuscular synapse has been used as a marker for nerve–muscle interactions [11], and therefore, the measurement of AChE activity is mostly used as the biomarker of neurotoxicity [12].

The role of vitamin D in bone calcium homeostasis has been clearly established however, it also plays an important role in other biologic targets such as the cardiovascular system, the nervous system, and the endocrine system [13]. 25-OH Vit. D_3, one of the active form of vitamin D in body, could conduct signal through the vitamin D receptor (VDR) that is structurally similar with the broader nuclear steroid receptor family [14]. The research studies reported that the VDR are widespread in various regions of the human brain such as, prefrontal cortex, substantia nigra, cingulate gyrus, caudate/putamen, hypothalamus, basal forebrain, lateral geniculate nuclei, thalamus, cerebellum, and hippocampus, which is particularly affected by neurodegenerative disorders [15]. Therefore, it could act as biomarker in such type of brain disorders. Similarly, another biomarker is tumor necrosis factor α (TNF-α), which is also known as cachectin. TNF-α is produced by activated T and B lymphocytes, neutrophils, LAK cells, NK cells, endothelial cells, astrocytes, smooth muscle cells, and some transformed cells. It plays vital role in normal host resistance against various types of infections and the growth of malignant tumors, by acting as the immune-stimulants and mediating the inflammatory response; and therefore its dysregulation has been implicated in a variety of human diseases [16, 17]. Thus, a novel test formulation was designed for estimation of various brain and lungs biomarkers. The test formulation was the combination of various minerals (selenium, zinc, iron, calcium, copper, and magnesium), vitamins (ascorbic acid, cyanocobalamin, alpha tocopherol, cholecalciferol,

and pyridoxine HCl), cannabidiol isolate, and *Panax ginseng* extract. All the minerals and vitamins used in the test formulation have significant functional role to provide vital physiological role [18-20]. Besides, biological importance of cannabidiol has been widely reported [21, 22], while ginseng extract is regarded as the one of the best immune booster for overall immunity [23].

NCCAM recommended Biofield Energy healing approach against many disorders, which are accepted worldwide by more than 80% of the population as one of the Complementary and Alternative Medicine (CAM) treatment [24-26]. Complementary and Alternative Medicine (CAM) has several advantages instead of the current preferred treatment approach [27]. 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 deep breathing, natural products, Tai Chi, yoga, therapeutic touch, Qi Gong, Johrei, pranic healing, Reiki, polarity therapy, hypnotherapy, guided imagery, chiropractic/ osteopathic manipulation, massage, meditation, homeopathy, progressive relaxation, special diets, relaxation techniques, mindfulness, pilates, movement therapy, traditional Chinese herbs Ayurvedic medicine, and medicines in biological systems [28, 29]. The Trivedi Effect®-Consciousness Energy Healing have been accepted worldwide, which has been scientifically studies on various models in the materials science [30, 31], agriculture science [32], microbiology [33, 34], biotechnology [35], and improved bioavailability of various compounds [36, 37], skin health [38, 39], nutraceuticals [40], cancer research [41], bone health [42, 43], overall human health and wellness. The present study was planned to evaluate different brain and lung biomarkers in male Sprague Dawley rats fed with VDD diet and Biofield Treated test formulation/*per se* to the animals by a renowned Biofield Energy Healer.

Materials and Methods

Chemicals and Reagents

Calcitriol, pyridoxine hydrochloride (vitamin B_6), magnesium (II) gluconate, β-carotene (retinol, provit A), and zinc chloride were obtained from TCI, Japan. Cyanocobalamin (vitamin B_{12}), copper chloride, vitamin E (alpha-tocopherol), cholecalciferol (vitamin D_3), calcium chloride, iron (II) sulfate, and sodium carboxymethyl cellulose (Na-CMC) were purchased from Sigma-Aldrich, USA. Sodium selenate and ascorbic acid (vitamin C) were obtained from Alfa Aesar, India. *Panax ginseng* extract and cannabidiol isolate were procured from Panacea Phytoextracts, India and Standard Hemp Company, USA, respectively. For the estimation of brain biomarkers, specific ELISA kits were used such as for detection of 25-Hydroxy Vit D_3, TNF-α, and IL-6 kit were procured from CUSABIO, USA while acetylcholine (ACh) level was estimated using MyBioSource, USA.

Maintenance of Animal

Randomly breed male *Sprague Dawley* (SD) rats with body weight ranges from 200 to 300 gm were used in this study. The animals were purchased from M/s. Vivo Bio Tech, Hyderabad, India. Animals were randomly divided into nine groups based on their body weights consist of 6 animals of each group. They were kept individually in sterilized polypropylene cages with stainless steel top grill having provision for holding pellet feed and drinking water bottle fitted with stainless steel sipper tube. The animals were maintained as per standard protocol throughout the experiment.

Consciousness Energy Healing Strategies

The test formulation was consisted of zinc chloride, iron (II) sulfate, copper chloride, vitamin B_6, vitamin B_{12}, vitamin D_3, sodium selenate, calcium chloride, ascorbic acid, vitamin E, beta carotene, *Panax ginseng* extract, cannabidiol isolate and magnesium (II) gluconate. Each ingredient of the novel test formulation was divided into two parts. The test formulation was divided into two parts, one part of the test compound was not received any sort of treatment and were defined as the untreated or control sample. The second part of the test formulation was treated with the Trivedi Effect® - Energy of Consciousness Healing Treatment (Biofield Energy Treatment) by a renowned Biofield Energy Healer, Mr. Mahendra Kumar Trivedi under laboratory conditions for ~3 minutes. Besides, three group of animals also received Biofield Energy Healing Treatment (known as the Trivedi Effect®) by Mr. Trivedi under similar laboratory conditions for ~3 minutes. The blessing/treatment was given to the test items remotely without touching in the laboratory of Dabur Research

Foundation, near New Delhi, India. After that, the Biofield Energy Treated samples was kept in the similar sealed condition and used as per the study plan. Similarly, the control test formulation was subjected to "sham" healer for ~3 minutes, under the same laboratory conditions. The "sham" healer did not have any knowledge about the Biofield Energy Treatment. The Biofield Energy Treated animals were also taken back to experimental room for further proceedings.

Experimental Procedure

Seven days after acclimatization, animals were randomized and grouped based on the body weight. Dosing for groups G7 and G8 were initiated on day -15 and continued till end of the experiment. However, G1 to G6 and G9 groups were dosed from day 1 till the end of experiment. All the animals except G1 group received vitamin D_3 deficient diet (VDD) daily to the end of the experiment. Three weeks after the initiation of induction of VDD, all the groups were dose with the respective formulations.

Preparation of Tissue Homogenate

About 100 mg of the brain and lung tissue was rinsed with 1X PBS, homogenized in 1 mL of 1X PBS and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000*g*, at 2 to 8°C. The supernatant was removed and assayed immediately. Alternatively, aliquot and store samples at -20°C or -80°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles.

Estimation of Brain Biomarkers (Acetylcholine, 25 (OH) D_3, IL-6), and Lung Homogenate-TNF alpha

Brain homogenate was subjected for the estimation of acetylcholine, 25 (OH) D_3, and IL-6; while lung homogenate was subjected for the estimation of TNF-α level. All the brain biomarkers estimation was performed using ELISA method as per manufacturer's recommended standard procedure.

Statistical Analysis

The data were represented as mean ± standard error of mean (SEM) and subjected to statistical analysis using Sigma-Plot statistical software (Version 11.0). For multiple comparison One-way analysis of variance (ANOVA) followed by post-hoc analysis by Dunnett's test and for between two groups comparison Student's *t*-test was performed. The $p \leq 0.05$ was considered as statistically significant.

Results and Discussion

Effect of the Test Formulation on Brain Biomarkers: 25-Hydroxy Vitamin D_3

Vitamin D deficiency is a worldwide problem as it associates with an increased mortality for all causes. In the global population, more than 70% is vitamin D deficient and it is a key factor for maintenance of calcium and phosphate metabolism and bone homeostasis [44, 45]. 25 OH vitamin D_3 level in the brain of rats fed with vitamin D_3 deficient diet (G2) was found to be 603.24 ± 90.82 µg/L, which was significantly ($p \leq 0.05$) decreased (847.53 ± 35.65 µg/L) by 28.82% as compared to the normal control (G1) group. However, positive control, calcitriol (G3) treatment showed significantly ($p \leq 0.001$) increased level of 25 OH vitamin D_3 level (1136.41 ± 64.79 µg/L) by 88.39% as compared to the G2 group. Similarly, the untreated test formulation given to the untreated rats (G4) showed increased the brain 25 OH vitamin D_3 level (713.01 ± 90.64 µg/L) by 18.20% as compared to the G2. However, G5 group (Biofield Energy Treated test formulation to the untreated rats) showed an increased brain 25 OH vitamin D_3 level (724.66 ± 82.11 µg/L) by 20.13% as compared to G2, while 1.63% increased level as compared to the untreated test formulation group (G4). Biofield Energy Treatment *per se* to the animals (G6) showed increased the brain 25 OH vitamin D_3 level (748.73 ± 78.38 µg/L) by 24.12% and 5.01% as compared to the G2 and G4 group, respectively. 15 days pre-treatment of Biofield Energy Treated test formulation (G7) showed significantly increased brain 25 OH vitamin D_3 level (879.90 ± 90.69 µg/L) by 45.86% and 23.41% in the G2 and G4 groups, respectively. 15 days pre-treatment of Biofield Energy Treated test formulation to the Biofield Energy Treated rats (G8) group showed significantly increased the brain 25 OH vitamin D_3 level (692.49 ± 37.07 µg/L) by 14.79% as compared to the G2. Untreated test formulation to the Biofield Energy Treated animals (G9) showed significantly increased the brain 25 OH vitamin D_3 level (783.97 ± 41.00 µg/L) by 29.96% and 9.95% as

compared to the G2 and G4 groups, respectively. Thus, the test formulation can be used against cardiovascular diseases, type 2 diabetes mellitus, autoimmune disorders, cancer, mental disorders, and infectious diseases in vitamin D deficient states. Fig 1.

Effect of the Test Formulation on Brain Biomarkers: Acetylcholine (ACh)

Acetylcholine (ACh) is an organic chemical, which plays a vital role in the brain and body of many types of animals, including humans, as a neurotransmitter, a chemical message released by nerve cells to send signals to other cells (neurons, muscle cells, and gland cells). In the brain, acetylcholine acts as a neurotransmitter and as a neuromodulator [46]. The effect of the test formulation on the level of brain ACh was determined and the data are presented in Figure 2. Brain ACh level in vitamin D_3 Deficient diet (G2) group was 126.84 ± 10.26 pg/mL, which showed slight decreased value as compared to the normal control (G1, 127.06 ± 14.18 pg/mL) group. Calcitriol treatment (G3), showed an increased ACh level (139.23 ± 8.47 pg/mL) by 9.77% as compared to the G2. G4 group animals showed decreased brain ACh level (89.41 ± 6.32 pg/mL) by 29.51% as compared to G2. However, G5 and G6 groups showed decreased level of ACh by 15.8% and 4.2%, respectively as compared with the G4. However, 15 days pre-treatment of Biofield Energy Treated test formulation (G7) group showed an increased the brain ACh level (144.25 ± 45.58 pg/mL) by 61.33% as compared with the G4 group. In addition, G8 and G9 groups were reported with reduced level of ACh by 16.29% and 20.23% respectively as compared with the G4 group.

Effect of the Test Formulation on Brain Biomarkers: Interleukin-6 (IL-6)

IL-6 is a soluble mediator with a pleiotropic effect on inflammation, immune response, and hematopoiesis [47]. The present study estimated the level of IL-6 in brain homogenate in various experimental groups (Figure 3). IL-6 level with vitamin D_3 deficient diet (G2) was found to be 1.31 ± 0.19 pg/mL, which showed significant ($p \leq 0.05$) increased value by 91.4% as compared to the normal control (G1, 0.68 ± 0.14 pg/mL). Calcitriol, positive control (G3) showed an increased the brain IL-6 level (1.48 ± 0.15 pg/mL) by 13.0% as compared to the G2. However, G4 group showed an increased the brain IL-6 level (1.65 ± 0.26 pg/mL) by 25.68% as compared to G2. In addition, G5 (0.93 ± 0.21 pg/mL), G6 (1.14 ± 0.10 pg/mL), G7 (1.30 ± 0.17 pg/mL), G8 (0.89 ± 0.14 pg/mL), and G9 (0.65 ± 0.11 pg/mL) groups showed significant decreased values of IL-6 by 43.44% ($p \leq 0.01$), 30.93%, 21.42%, 45.99% ($p \leq 0.01$), and 60.85% ($p \leq 0.01$), respectively as compared with the untreated test formulation group (G4). However, similar decreased IL-6 pattern were reported in all the experimental test groups as compared with the G2 group. Overall, our results revealed significant decreased values of IL-6 level in brain in all the experimental test groups as compared to G4.

Effect of the Test Formulation on Lung Tumor Necrosis Factor Alpha (TNF-α)

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine and has a major role in airway inflammation and airway remodeling in asthma [48]. It plays a central role in inflammation, immune modulation, and lymphocyte activation in various immune-mediated disorders. TNF-α level in the lung of rats fed with vitamin D_3 Deficient diet (G2) was 30.92 ± 1.40 pg/mL, which was significantly ($p \leq 0.01$) increased by 44.4% as compared to normal control (G1, 21.41 ± 2.56 pg/mL). Calcitriol, positive control (G3) showed increased lung TNF-α level (46.02 ± 8.60 pg/mL) by 48.85% as compared to the G2. Untreated test formulation to the animals (G4), increased TNF-α level (52.63 ± 2.99 pg/mL) by 70.2% as compared to the G2. However, other experimental test groups such as G5, G6, G7, G8, and G9 showed significantly reduced level of pro-inflammatory cytokine TNF-α level by 24.86%, 32.55%, 30.12%, 42.69%, and 4.7% respectively, as compared with the untreated test formulation group (G4). Fig 4.

In this research plan, four groups were considered as preventive maintenance groups. These groups were G6 (Biofield Energy Treatment *per se* to animals at -15 days), G7 (Biofield Energy Treated test formulation from day -15), G8 (Biofield Energy Treatment *per se* to animals along with Biofield Treated test formulation from day -15), and G9 (Biofield treatment *per se* at -15 days to animals with untreated test formulation). The results showed a significant

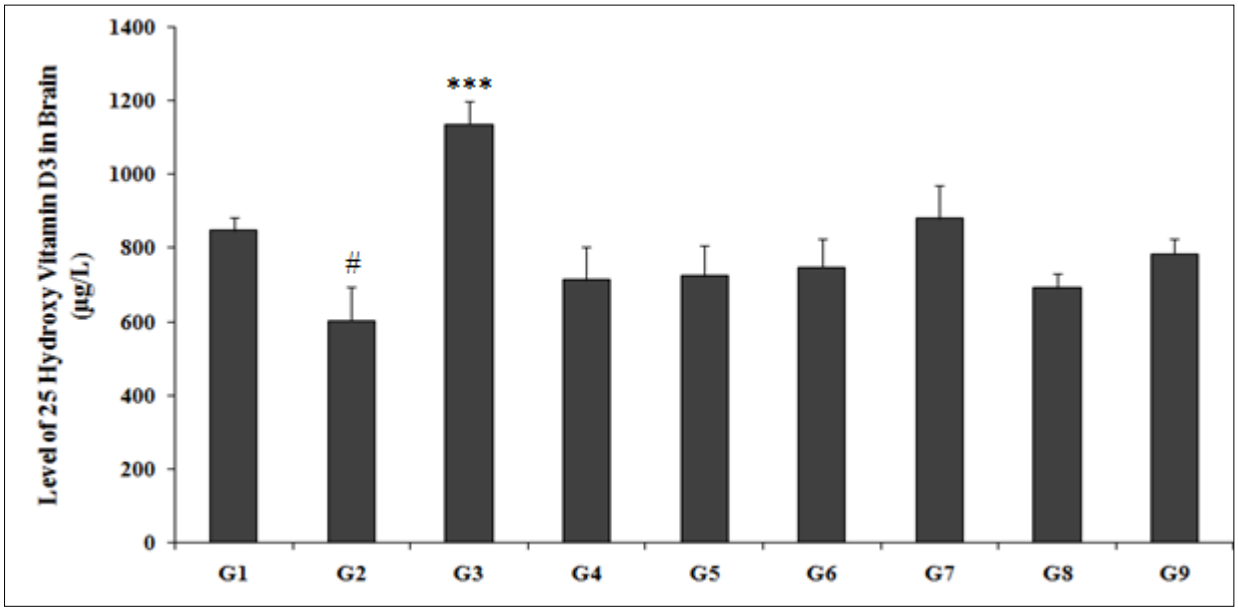

Figure 1. Effect of the test formulation on the level of 25-Hydroxy vitamin D_3 in brain homogenate of Sprague Dawley rats. G: Group; G1: Normal control (0.5% CMC); G2: Disease control (VDD: Vitamin D_3 deficient diet + 0.5% CMC); G3: Reference item (VDD + Calcitriol); G4: (VDD + Untreated test formulation); G5: (VDD + Biofield Energy Treated test formulation); G6: (VDD + Biofield Energy Treatment *per se* to animals from day -15; G7: (VDD + Biofield Energy Treated test formulation from day -15); G8: (VDD + Biofield Energy Treatment *per se* plus Biofield Energy Treated test formulation from day -15), and G9: (VDD + Biofield Energy Treatment *per se* animals plus untreated test formulation). Values are presented as mean ± SEM (n=6). $^{\#}p \leq 0.05$ *vs.* G1 and $^{***}p \leq 0.001$ *vs.* G2.

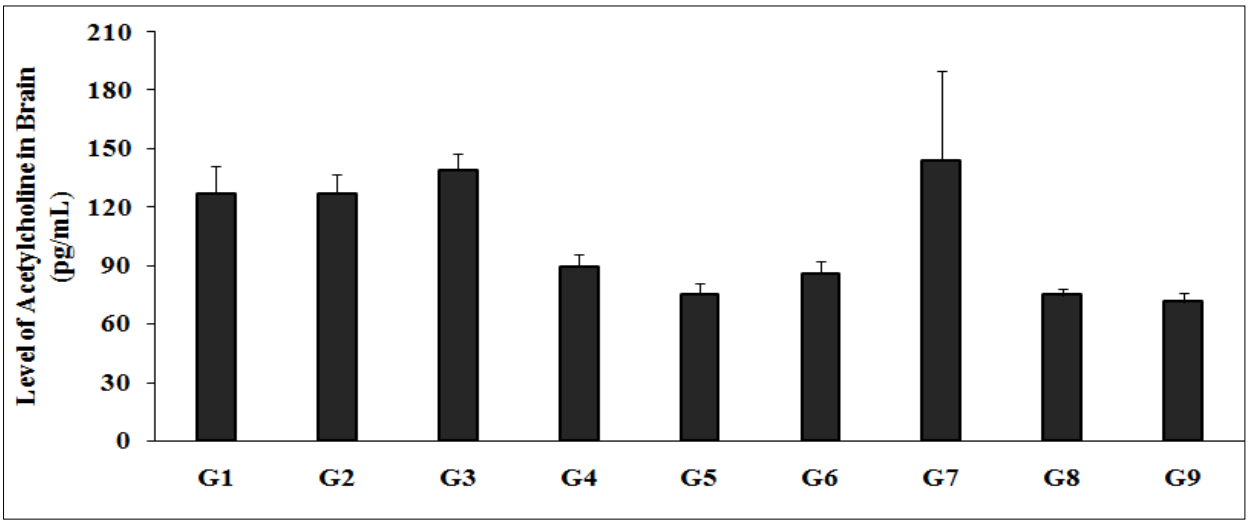

Figure 2. Effect of the test formulation on the level of acetylcholine (ACh) in brain homogenate of Sprague Dawley rats.

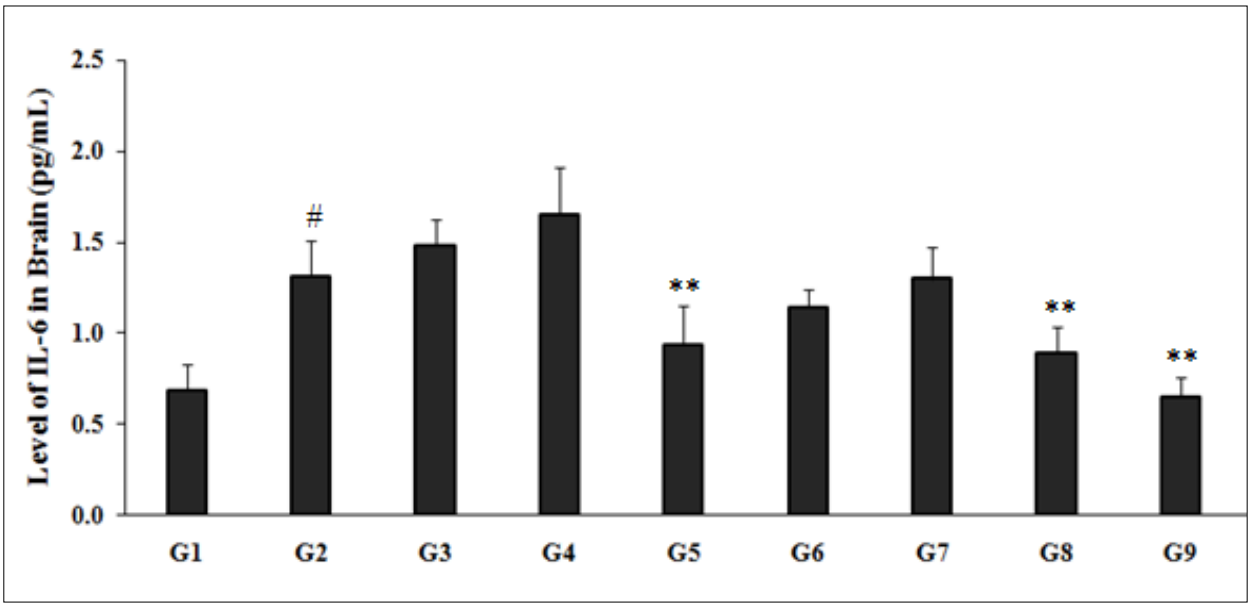

Figure 3. Effect of the test formulation on the level of IL-6 in brain homogenate of Sprague Dawley rats. $^{\#}p \leq 0.05$ *vs.* G1 and $^{**}p \leq 0.01$ *vs.* G4.

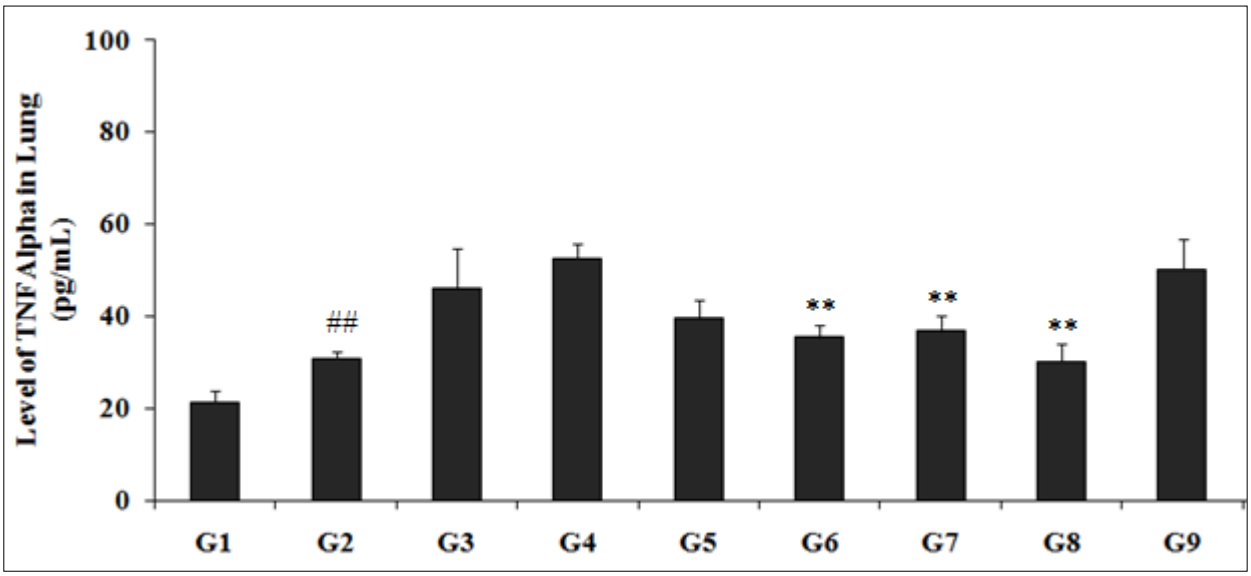

Figure 4. Effect of the test formulation on the level of TNF-α in lungs homogenate of Sprague Dawley rats. $^{\#\#}p \leq 0.01$ *vs.* G1 and $^{**}p \leq 0.01$ *vs.* G4.

slowdown of disease progression and all other disease-related symptoms/complications and also reduced the chances of disease susceptibility in these groups. Based on the overall data, it suggests that the Biofield Therapy was found to be most effective and beneficial to prevent and protect from the occurrence of any type of disease in the rat model. The data indicated that this therapy could act as a preventive maintenance therapy to prevent the occurrence of disease, slowdown the disease progression, when disease-related complications are present which will ultimately improve the overall health and quality of life.

Conclusions

The study results based on the estimation of brain biomarkers suggested that Biofield Energy Treated test formulation and Biofield Energy *per se* showed significant improved brain biomarkers level, which have significant clinical role in different brain disorders. 25 OH vitamin D_3 level in brain homogenate was increased by 20.13%, 24.12%, 45.86%, 14.79%, and 29.96% in the G5, G6, G7, G8, and G9 groups, respectively as compared with the disease control (G2) group. The data of ACh was increased by 61.33% in the G7 group as compared to the untreated test formulation group (G4). Similarly, IL-6 level was reported to be significantly decreased in the G5, G6, G7, G8, and G9 groups by 43.44%, 30.93%, 21.42%, 45.99%, and 60.85% respectively, as compared with the G4. In addition, pro-inflammatory cytokine TNF-α level was significantly reduced in the G5, G6, G7, and G8 by 24.86%, 32.55%, 30.12%, and 42.69%, respectively, as compared with the G4 group. This study reports significantly higher concentration of active metabolites of vitamin D_3, neurotransmitter (acetylcholine) and significantly reduce the levels of proinflammatory cytokines IL-6 and TNF-α in the treatment group as compared to the disease control and/or untreated test formulation group. The Biofield Energy Healing Treatment also helped to slowdown the disease progression and disease-related complications impacting the overall animals' health. These data suggested that Biofield Energy Treatment *per se* and Biofield Energy Treated Test formulation in combination would be the best treatment strategy to prevent and protect from the occurrence of any type of disease. Therefore, the Biofield Energy Healing Treatment (the Trivedi Effect®) *per se* might be effective in healthy humans, when used as a preventive maintenance therapy to sustain good health, to boost overall health, promote healthy aging and increase quality of life. This test formulation can also be used against other disorders such as systemic lupus erythematosus, fibromyalgia, Addison disease, multiple sclerosis, myasthenia gravis, pernicious anemia, aplastic anemia, psoriasis, rheumatoid arthritis, Crohn's disease, vitiligo, chronic fatigue syndrome and alopecia areata, as well as inflammatory disorders such as ulcerative colitis, atherosclerosis, dermatitis, hepatitis, and diverticulitis. However, Biofield Energy Healing Treated test formulation and Biofield Energy Healing Treatment *per se* can also be used in the prevention of brain disorders such as Alzheimer's disease, dementias, brain cancer, epilepsy and other seizure disorders, mental disorders, Parkinson's and other movement disorders, stroke and transient ischemic attack (TIA), and in the improvement of overall health and quality of life.

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