International Journal of Immunology
2016; 4(5): 35-45
http://www.sciencepublishinggroup.com/j/iji
doi: 10.11648/j.iji.20160405.12
ISSN: 2329-177X (Print); ISSN: 2329-1753 (Online)

Impact of Biofield Energy Treated Herbomineral Formulation (The Trivedi Effect®) on Mouse Dendritic and Splenocyte Cells for Modulation of Pro-inflammatory Cytokines

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To cite this article:

Mahendra Kumar Trivedi, Alice Branton, Dahryn Trivedi, Gopal Nayak, Aileen Carol Lee, Aksana Hancharuk, Carola Marina Sand, Debra Jane Schnitzer, Rudina Thanasi, Eileen Mary Meagher, Faith Ann Pyka, Gary Richard Gerber, Johanna Catharina Stromsnas, Judith Marian Shapiro, Laura Nelson Streicher, Lorraine Marie Hachfeld, Matthew Charles Hornung, Patricia M. Rowe, Sally Jean Henderson, Sheila Maureen Benson, Shirley Theresa Holmlund, Stephen P. Salters, Mayank Gangwar, Snehasis Jana. Impact of Biofield Energy Treated Herbomineral Formulation (The Trivedi Effect®) on Mouse Dendritic and Splenocyte Cells for Modulation of Pro-inflammatory Cytokines. *International Journal of Immunology*. Vol. 4, No. 5, 2016, pp. 35-45. doi: 10.11648/j.iji.20160405.12

Received: November 7, 2016; **Accepted**: November 22, 2016; **Published**: December 8, 2016

Abstract: The use of herbomineral formulation in the healthcare sector for different chronic diseases is gaining popularity due to its fewer side effects, high safety profile, and cost effectiveness. A new proprietary herbomineral formulation was formulated, consisting of four essential ingredients *viz.* herbal root extract (ashwagandha), and minerals (zinc, magnesium, and selenium). The study aims to evaluate the *in vitro* effect of Biofield Energy Healing (The Trivedi Effect®) on the test formulation using murine dendritic (DCs) and splenocyte cells. The herbomineral formulation was divided into two parts; one was represented as control, while the other part was treated with the Biofield Energy Healing Treatment remotely by eighteen renowned Biofield Energy Healers (The Trivedi Effect®) and defined as the Biofield Treated formulation. The effect of the test formulation on these cells were monitored by an estimation of pro-inflammatory cytokines level such as tumor necrosis factor (TNF-α), macrophage inflammatory protein (MIP-1α), and interleukin (IL-1β) in cell culture supernatants at the non-cytotoxic concentrations of the test formulation using MTT assay. The DCs were treated with the Biofield Energy Treated test formulation at different concentrations (*i.e.* 1.05 to 1052.5 µg/mL) for 24 hours, and the results showed significant ($p \leq 0.001$) suppression of TNF-α levels at all the tested concentrations with a maximum percentage decrease by 43.64% at 5.2 µg/mL concentration in the Biofield Treated formulation as compared with the untreated test formulation. Further, the Biofield Treated formulation also demonstrated inhibition of MIP-1α and IL-1β at a concentration range of 0.0000105 to 10.5 µg/mL in LPS stimulated splenocyte cells. There was a significant ($p \leq 0.001$) inhibition of MIP-1α (26.52%) and IL-1β (35.28%) in the Biofield Treated test formulation at concentration 0.000105 µg/mL and 0.0000105 µg/mL, respectively in comparison to the untreated test formulation. Overall, these results suggest that the Biofield Energy Healing based herbomineral formulation (The Trivedi Effect®) significantly down-regulated the tested cytokines expression in DCs and splenocyte cells as compared to the untreated formulation. Therefore, the Biofield Healing based formulation might be useful as a better anti-inflammatory product for many chronic and acute inflammatory disease conditions and autoimmune disorders. The Biofield Energy Treatment based

formulation can also be effectively applied in cases of organ transplants, stress management and anti-aging by improving overall health.

Keywords: Biofield Energy Healing Treatment, Biofield Energy Healers, The Trivedi Effect®, Herbomineral Formulation, Immune-Modulation, Pro-Inflammatory Cytokines, Dendritic Cells, Splenocytes

1. Introduction

In the last few years, there has been exponential growth reported in the herbal medicine sector. In developing and developed countries alike, medicinal plant-derived drugs are gaining popularity due to their natural origin and less side effects. Many traditional and complementary medicines currently in use are derived from medicinal plants, minerals, and organic matter, which are commonly used for the prevention and treatment of many diseases [1]. However, the use of traditional remedies has gained importance in cases when conventional medicine is ineffective for certain diseases, due to being widely perceived as natural, safe, and non-toxic. Herbal and traditional medicine are suitable candidates for new therapeutics due to their vast chemical diversity and various biological effects [2]. *Withania somnifera* (ashwagandha) is an important medicinal plant that belongs to the family *Solanaceae*. It is commonly known as Indian ginseng and is used for various treatments in alternative therapy [3]. Withanolides have been reported as major active constituents that are isolated from the root and leaves of the ashwagandha plant for biological activity [4]. Apart from its important antibacterial activity, several reports have demonstrated its potent immunomodulatory and anti-tumor activity [5]. Preclinical and clinical studies report that each of the active constituents of ashwagandha have shown immunomodulatory effects in various inflammatory diseases [6], but the mechanisms of anti-inflammatory/immunomodulation remain unknown. However, reports have shown that the role of trace elements is significant in immunomodulation due to their strong interaction with the immune system. Selenium, zinc, copper, and magnesium, *etc.* are highly recommended trace elements because of their immunomodulatory impact [7]. The coordinated interactions of these molecules with the immune cells may evoke an appropriate immune response.

In order to design a traditional medicine therapy, it is important to understand how herbal medicine effectively impacts disease [8]. Recently, we prepared a new proprietary herbomineral formulation, which consists of a combination of the herbal root extract ashwagandha and minerals (zinc, magnesium, and selenium). These different ingredients of the test formulation possess significant anti-inflammatory, antioxidant, anti-infective, anti-viral and immune-modulating properties [5, 7, 9, 10], which plays a key role in protecting cells from oxidative stress. Based on recent literature, it was reported that the herbomineral formulation had exhibited the level of phagocytic index and improved antibody titre to suggest a significant immunomodulatory response. Further, it was reported that the effect was potentiated in the presence of minerals [11], which can be useful for immune-compromised patients, autoimmune disorders, and cancer; for promoting anti-stress and anti-aging; and in reducing the risk of cardiovascular diseases on a long term basis.

The different type of immune cells such as dendritic cells (DCs), macrophages, and spleen can play an important role in order to stop the acute/chronic inflammation and retrieve a steady state strategy through the secretion of immuno-modulating cytokines [12]. These immune cells have been reported to be useful as cellular models for *in vitro* studies. To initiate the adaptive immune response, DCs are reported to play an important role against different pathogens such as bacteria, viruses, and fungi [13]. DCs play an important role in regulating the immune system and are regarded as potent antigen-presenting cells (APC) to maintain and induce the primary immune response [14]. T helper (Th) cells regulate the immune function through the production of cytokines during infectious, allergic, or autoimmune diseases. These cells regulate the protective and pathological immune responses [15]. Using different types of cytokine production *in vitro* or *ex vivo*, the molecular mechanism of Th cells can be studied using murine splenocyte cells [16]. Here, murine bone marrow derived dendritic (BMDCs) and splenocyte cells were used to assess the effect of the Biofield Treated formulation on *in vitro* cell cultures.

In recent years, Biofield Energy Treatment (The Trivedi Effect®) has been reported worldwide as an alternative treatment method which has been known for its significant impact on various cancerous cells [17]. According to many scientific studies, Biofield Energy Healing has been reported to have significant outcomes that may prove to be a more cost effective alternative to other approaches [18]. Complementary and Alternative Medicines (CAM) are now rising 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. However, Biofield Energy can exist in different forms such as kinetic, magnetic, potential, electrical, and electromagnetic. The human body has the power to produce low intensity electromagnetic signals known as the Biofield. Thus, a human has the ability to harness energy from the environment and transmit it to any living or nonliving object (s) around the globe. The objects always receive the energy and respond in a useful way. This process is known as Biofield Energy Healing Treatment (The Trivedi Effect®). Based on the literature data, Biofield Energy Treatment in terms of a Complementary and Alternative Medicine (CAM) approach was practiced worldwide [19] in addition to herbal medicine. The National Center of Complementary and Integrative Health (NCCIH) has

recognized and accepted Biofield Energy Healing as a complementary and alternative medicine (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, cranial sacral therapy and applied prayer (as is common in all religions, like Christianity, Hinduism, Buddhism and Judaism). To this day, Biofield Energy Healing has had significant impact in the transformation of living organisms and nonliving materials including metals, polymers, ceramics, chemicals, and pharmaceutical compounds. Even further, Biofield Energy Healing Treatment (The Trivedi Effect®) has been published in numerous peer-reviewed science journals due to its significant impacts in the science fields of biotechnology, genetics, cancer, microbiology, materials science and agriculture.

In this study, the authors sought to explore the impact of Biofield Energy Healing (The Trivedi Effect®) on a herbomineral formulation and its immunomodulatory properties. Therefore, the study objective was to investigate the effect of a Biofield Treated and untreated test formulation on pro-inflammatory cytokines expression in *in vitro* cellular models (*i.e.*, mice dendritic and splenocyte cells) using modern scientific methods.

2. Material and Methods

2.1. Chemicals and Reagents

Lipopolysaccharide (LPS), 3-(4, 5-diamethyl-2-thiazolyl) 2, 5 diphenyl-2 H-tetrazolium) (MTT), RPMI-1640, L-glutamine, penicillin, streptomycin, HEPES, and 2-mercaptoethanol were purchased from Sigma Chemical Company (St. Louis, MO), a subsidiary of Sigma-Aldrich Corporation. ELISA assay kits (enzyme-link immunosorbent assay) for all cytokines TNF-α, MIP-1α, and IL-1β were purchased from R&D systems, USA. Fetal bovine serum (FBS) was purchased from GIBCO, USA. Ashwagandha (*Withania somnifera*) root extract powder (≥ 5% of total withanolides) was procured from Sanat Products Ltd., India. Zinc chloride and magnesium (II) gluconate hydrate was procured from Tokyo Chemical Industry Co., Ltd. (TCI, Japan). Sodium selenate was procured from Alfa Aesar, USA. Rapamycin, MTT, $NaHCO_3$, and EDTA were procured from Sigma, USA. All other chemicals used were of analytical grade available locally in India.

2.2. Experimental Design and Reference Standard

The herbomineral test formulation contained the combination of four ingredients including ashwagandha root powder extract, zinc chloride, sodium selenite, and magnesium gluconate. Different concentrations of the test formulation were used for the study *i.e.* concentration range of 1.05 to 1052.5 µg/mL for bone marrow DCs and 0.0000105 to 10.5 µg/mL with LPS stimulated splenocyte culture for cell viability assay. LPS was used as an immunostimulatory agent, while rapamycin was used as a reference standard (positive control) for immunosuppressive action in splenocyte cells assay. DCs were treated with 0.1% DMSO as a vehicle control group for the estimation of TNF-α. MIP-1α and IL-1β were estimated in splenocyte cells, which includes splenocyte cells treated with LPS (0.5 µg/mL) along with 0.005% DMSO, and was defined as the vehicle control group.

2.3. Biofield Energy Treatment Strategies

One part of the test formulation was treated with Biofield Energy by renowned Biofield Healers (also known as The Trivedi Effect®) and coded as the Biofield Energy Treated formulation, while the second part of the test formulation did not receive any sort of treatment and was defined as the untreated test formulation. This Biofield Energy Treatment was provided through a group of eighteen Biofield Energy Healers who participated in this study and performed the Biofield Energy Treatment remotely. Eleven Biofield Energy Healers were remotely located in the U. S. A, four were remotely located in Canada, two in Finland, and one of which was remotely located in Albania, while the test herbomineral formulation was located in the research laboratory of Dabur Research Foundation near New Delhi in Ghaziabad, India. This Biofield Treatment was administered for 5 minutes through the Healer's unique Energy Transmission process remotely to the test formulation under laboratory conditions. None of the Biofield Energy Healers in this study visited the laboratory in person, nor had any contact with the herbomineral samples. 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 and used for the *in vitro* study on DCs and splenocytes for cytokines estimation.

2.4. Experimental Animal

C57BL/6 male mice were purchased from Vivo Bio Tech Ltd., Hyderabad, India and acclimatized for one week prior to the experiments. Rodent laboratory diet and drinking tap water were provided *ad libitum* under controlled conditions with a temperature of 22 ± 3°C, humidity of 30% to 70% and a 12-hour light/12-hour dark cycle. All the procedures were in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Approval of the Institutional Animal Ethics Committee has been obtained prior to the animal experiments.

2.5. Mouse Bone Marrow-Derived Dendritic Cells (BMDCs) Cultures

C57BL/6 male mice were euthanized by CO_2

asphyxiation. Subsequent experimental steps were conducted in a laminar air flow. BMDCs were induced from bone marrow (BM) cells using the modified method of Inaba K, *et al.,* 1992 [20]. Briefly, a single cell suspension was prepared from BM obtained from femurs. After removing all muscle tissues with gauze from the femurs, the bones were placed in a 90 mm dish with 70% alcohol for 1 minute, washed twice with phosphate buffer saline (PBS), and transferred into a fresh dish with RPMI 1640. Both ends of the bones were cut with scissors in the dish, and flushed with 2 mL of RPMI 1640 using a syringe and 25-gauge needle. The tissue was suspended, passed through nylon mesh to remove the debris and small bone pieces, and red blood cells were lysed with ammonium chloride. After washing, the lymphocytes and various other cells were killed with the cocktail of monoclonal antibodies (mAbs), and rabbit complement for 60 min at 37°C. After that whole BM cells (2×10^6 cells/mL) were cultured in RPMI 1640 medium in 90 mM petri dishes (Sigma Aldrich, St. Louis, MO) at 37ºC, 5% CO_2, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin G, 100 mg/mL streptomycin, and 20 ng/mL recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (R & D systems Inc., USA). Cells were incubated for 24 hours. Petri plates were then gently swirled and the medium containing non-adherent cells was removed and replaced with nutrient medium with similar procedure. The supplemented medium was replaced at every three-day interval. On day 6, non-adherent and loosely adherent DCs were harvested by gentle pipetting for further assays. The cell counts were performed using a hemocytometer and cell viability was determined using the trypan-blue dye exclusion technique with the results showing ≥95% of viable cells. Cells were cultured in 96-well tissue culture plates with 5 x 10^3 cells per well. They were incubated at 37ºC in a humidified atmosphere of 5% CO_2 for the indicated period. The respective vehicle controls (DMSO) were kept in the assay for comparison.

2.6. Mouse Splenocyte Cultures

C57BL/6 male mice were sacrificed and their spleens were aseptically removed and grounded by passing through a sterile plastic strainer under aseptic conditions. After the cells were centrifuged twice at 1000 *g* for 5 minutes, erythrocytes were lysed by lysis buffer (0.15 M NH_4Cl, 0.01 M $NaHCO_3$, and 0.1 mM EDTA, pH 7.4) and then cell pellets were washed twice with RPMI-1640 medium. Further, the cells were resuspended in complete RPMI-1640 medium (RPMI 1640 medium plus 10% fetal bovine serum, 2 mM glutamine, 100 IU/mL of penicillin and streptomycin, 15 mM HEPES and 50 mM 2-mercaptoethanol). The cell counts were performed using a hemocytometer and cell viability was determined using the trypan-blue dye exclusion technique with the results showing ≥95% of viable cells. Cells were cultured in 96-well tissue culture plates with 0.2 x 10^6 cells per well. They were incubated at 37°C for 24 hours in a humidified atmosphere of 5% CO_2 for the indicated period [21]. The respective vehicle controls (DMSO) were kept in the assay for comparison.

2.7. Cytotoxicity by MTT Assay

The number of viable cells is determined by the ability of mitochondria to convert MTT to formazan dye. Both the cells were cultured overnight in 96-well plates, at a density of 5 x 10^3 cells per well for BDMCs and 0.2 x 10^6 cells per well for splenocyte cells. After the treatment and incubation period, the medium was removed, with 20 µL of 5 mg/mL MTT then added to each well, and incubated for 3 hours further, at 37ºC in a humidified 5% CO_2 atmosphere. The cells were centrifuged and supernatants were removed. The cell pellet in each well was resuspended in 150 µL of DMSO to dissolve formazan crystals. The optical density of each well was read at 540 nm using Biotek Reader (SIAFRT/Synergy HT multimode reader, US).

The effect of the test formulation on cell viability of DCs and splenocyte cells was determined as-

$$\%\ \text{Cell viability} = 100 - \%\ \text{cytotoxicity}$$

Where; % cytotoxicity=[(O. D. of control cells–O. D. of cells treated with the test formulation)/O. D. of control cells]*100.

The concentration that resulted in >75% viability was selected for subsequent cytokine estimation.

2.8. Cytokines Assays Using ELISA Method

The effect of the Biofield Treated and untreated test formulations on the production of TNF-α, MIP-1α, and IL-1β was measured by an enzyme-linked immunosorbent assay (ELISA) using culture supernatants collected from treated cells. Briefly, ELISA plates were coated overnight at 4ºC with an antibody in coating buffer at the recommended concentration. After washing with PBS-T (PBS with 0.05% Tween 20), the plates were blocked with assay diluent for at least 2 hours at room temperature. A total of 100 µL culture supernatant from different experimental samples and standards were incubated overnight at 4ºC and, after three washes, biotinylated anti-mouse cytokine (TNF-α, MIP-1α, and IL-1β) antibodies at the recommended concentrations were incubated for 1 hour at room temperature. The plate was incubated for 45 minutes at room temperature with gentle shaking. The plates were again washed 3 times and then 100 µL of horseradish per-oxidase (HRP)–streptavidin conjugate solution was added and the plate was incubated for 45 minutes at room temperature with gentle shaking. Next, the plate wells were washed 3 times as previous and 100 µL of 3, 3, 5, 5'-tetramethylbenzidine (TMB) one-step substrate reagent was added followed by 30 minutes incubation at room temperature in the dark. Then 50 µL of 0.2 mole/L sulphuric acid was added to each well to stop the reaction and the plates were read for absorbance at 450 nm using a Biotek reader (SIAFRT/Synergy HT multimode reader). Standards were run in parallel to the samples, and the concentrations were determined in triplicates.

2.8.1. TNF-alpha Level in Dendritic Cells (DCs)

DCs were seeded in a 24-well plate at a density of 0.5 x 106 cells per well with 1 mL of culture medium and co-incubated for 24 hours with different non-cytotoxic concentrations ranging from 1.05 to 105.2 µg/mL of the Biofield Treated and untreated test formulations. DCs treated with 0.1% DMSO were included as a vehicle control. After 24 hours of incubation, cell-free culture supernatants were collected and analyzed for the TNF-α using ELISA as per the manufacturer's instructions [22]. The concentrations were determined for two wells in each sample in triplicate.

2.8.2. MIP-1α and IL-1β in Splenocyte Cells

For the estimation of MIP-1α and IL-1β in LPS induced splenocyte, the cells (2 x 10^6 cells/well in 24-well culture plates) were treated with the Biofield Energy Treated and untreated test formulations at selected non-toxic concentrations (0.0000105 to 1.05 µg/mL) in triplicate. Splenocyte cells were treated with LPS (0.5 µg/mL) along with 0.005% DMSO were included as control cells. Rapamycin was included as a positive control. After 48 hours of incubation, supernatants were analyzed for the secreted levels of MIP-1α and IL-1β using ELISA as per the manufacturer's instructions [22].

2.9. Statistical Analysis

Data were expressed as mean ± SEM and were subjected to Student's *t*-test for two group comparison. Statistical significance was considered at $p \leq 0.05$.

3. Results

3.1. In Vitro Immune Cells Viability Assay by MTT Assay

The effect of the Biofield Energy Treated and untreated test formulations on the proliferation of DCs and splenocyte cells were examined using MTT cell viability assay. The effect of the Biofield Treated and untreated test formulations on viabilities of DCs and splenocyte cells are shown in Figure 1 and 2, respectively. The untreated test formulation at concentrations 1.05 µg/mL and 5.2 µg/mL showed 120.8% and 141% cell viability, respectively in DCs. The increased cell viability with respect to the vehicle control might be due to the proliferation in cell cultures. Further concentration dependent increase in cell viability was observed till 5.2 µg/mL of the untreated test formulation. However, further increase in concentration resulted in reduced viability up to 76.3% at concentration 105.2 µg/mL. Therefore, the concentration range from 1.05 to 105.2 µg/mL were selected for cytokines estimation with cell viability more than 75%. Similarly, the Biofield Energy Treated test formulation showed increased percentage of cell viability pattern with higher concentrations as compared with the untreated test formulation at 51.2 and 105.2 µg/mL. It was suggested that the decreased trend of cell viability in DCs with respect to the increased concentration of the test formulation after 24 hours as compared with the vehicle control.

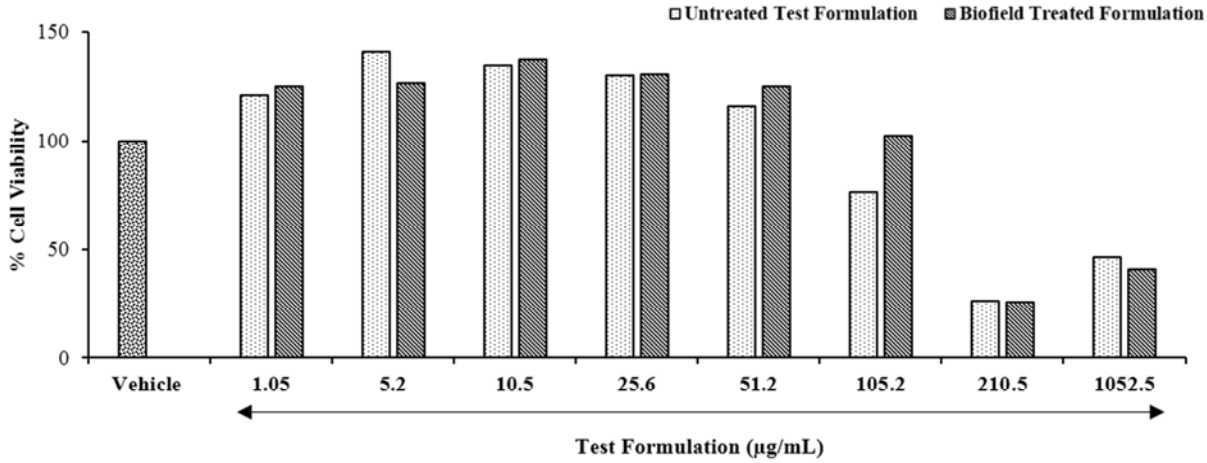

Figure 1. *MTT assay in dendritic cells after 24 hours of treatment with different concentrations of the test formulation. The absorbance of the MTT formazan was determined at 540 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of the formulation treated cells relative to the untreated vehicle cells.*

The splenocyte cells were treated with the Biofield Treated and untreated test formulations in the concentration range from 0.0000105 to 10.5 µg/mL in the presence of LPS (0.5 µg/mL) for 48 hours. The effect of the Biofield Energy Treated and untreated test formulations on the viability of splenocyte cells showed an alteration with respect to different concentrations of the test formulations after 48 hours as compared with the vehicle control. The result of the percent cell viability is presented in Figure 2. Further, the cytokines analysis was conducted using these concentration ranges. The percentage viability of LPS alone at 0.5 µg/mL was 122.8%, while rapamycin (reference standard of immunosuppressive agent) showed 74.2% and 81.6% cell viability at 1 and 10 nM, respectively. Both the Biofield Treated and untreated test formulations showed more than 82% cell viability up to concentration at 1.05 µg/mL. So, the concentration range of the test formulation from 0.0000105 to 1.05 µg/mL was selected for cytokines (MIP-1α and IL-1β) estimation.

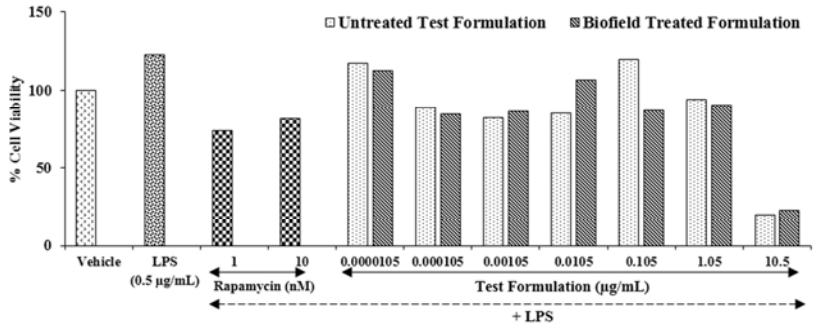

Figure 2. *MTT assay in splenocyte cells after 48 hours of treatment with various concentrations of the test formulation in the presence of 0.5 µg/mL LPS. The absorbance of the MTT formazan was determined at 540 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of formulation treated cells relative to untreated vehicle cells.*

3.2. Effect of Herbomineral Formulation on the Expression of Pro-inflammatory Cytokines TNF-α, MIP-1α, and IL-1β in Immune Cells

Lymphocyte proliferation and the activation of NK cells are cytokine dependent [23]. The type of cytokine up/down regulation affects the course of immune response and the whole network of immune regulation. The pro-inflammatory cytokines such as TNF-α, MIP-1α, and IL-1β play a pivotal role in inflammation, immune modulation, and lymphocyte activation. Various herbomineral formulations have the potential to modulate the expression and activation of cytokines. Therefore, an experiment was conducted to observe the expression TNF-α, MIP-1α, and IL-1β at two different immune cells, such as dendritic and spleen cells, after exposure to the Biofield Treated and untreated test formulations. To examine the effect of the test formulation on the production of the pro-inflammatory cytokines, TNF-α, MIP-1α, and IL-1β, mice bone marrow derived dendritic cells and spleen cells were incubated with various concentrations of the Biofield Energy Treated and untreated test formulations in DCs and splenocytes for 24 hours and 48 hours, respectively. The production of the cytokines by these immune cells was tested in the culture supernatants using commercial ELISA kits.

3.3. Modulation of TNF-α Expression in Mouse Dendritic Cells (DCs)

The effect of the Biofield Treated test formulation on TNF-α secretion in DCs is represented in Figure 3. Both the untreated and Biofield Energy Treated test formulation groups demonstrated significant decrease in the level of TNF-α secretion at different tested concentrations *i.e.* at 5.2, 10.5, 25.6, 51.2, and 105.2 µg/mL, which indicated decrease in the level of pro-inflammatory cytokine expression in the presence of the test formulation as compared to the vehicle control group. The maximum fall in TNF-α concentration was reported at 5.2 µg/mL ($p \leq 0.001$) and showed a statistically significant difference. The untreated test formulation showed 110.7 pg/mL, while the Biofield Energy Treated test formulation showed 63.5 pg/mL of TNF-α at 5.2 µg/mL, which suggests that the Biofield Treated test formulation showed a better effect and decreased the TNF-α level by 43.64% as compared to the untreated test formulation. Further, the level of TNF-α was decreased from 60.3 ± 1.07 (untreated test formulation) to 38.7 ± 5.35 (Biofield Treated test formulation) and showed statistical significance at 10.5 µg/mL ($p \leq 0.05$) of both the treated and untreated test formulations. The Biofield Treatment enhanced the down-regulation of pro-inflammatory cytokines of TNF-α expression in DCs as compared to the untreated test formulation at different concentrations (Figure 3).

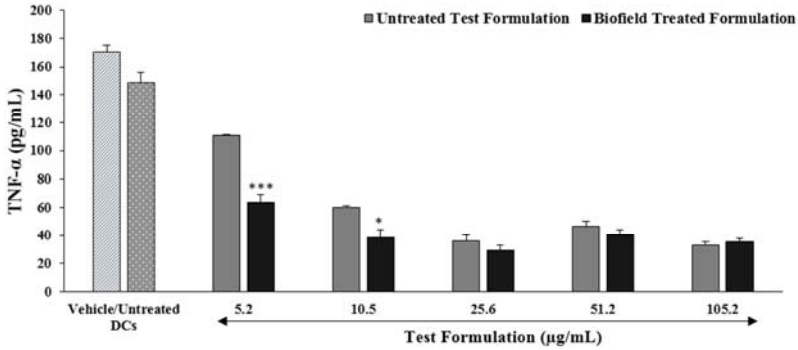

Figure 3. *Concentration-dependent inhibition of TNF-α by the Biofield Treated formulation in mice dendritic cells. For each concentration treatment, the level of TNF-α release was measured after 24 hours of treatment. The values are represented in pg/mL as mean ± SEM (***$p \leq 0.001$ and *$p \leq 0.05$ as compared with the untreated test formulation).*

3.4. Modulation of MIP-1α and IL-1β Expression in Mouse Splenocytes

Splenocytes were isolated from the male mice and were cultured *in vitro* in RPMI-FBS (10%) with LPS and the levels of MIP-1α and IL-1β cytokines were measured by ELISA in the culture supernatants. The effect of the Biofield Treated formulation on cytokine production was observed at various tested concentrations of the Biofield Treated and untreated samples. Rapamycin was used as a positive control. As shown in Figures 4 and 5, the effect for both cytokine MIP-1α and IL-1β production after LPS stimulation was increased and the major effect was observed after the culture period of 48 hours.

3.4.1. MIP-1α Activity

The effect of the Biofield Energy Treated and untreated test formulation on MIP-1α secretion is shown in Figure 4, which demonstrates the comparative effect of the Biofield Energy Treated and untreated test formulation on MIP-1α secretion in splenocyte cells. Both the untreated and treated test formulations demonstrated inhibition of MIP-1α expression as compared to the LPS stimulated cells. However, the Biofield Energy Treated test formulation improved the down-regulation of MIP-1α. At concentration 0.000105 µg/mL, maximum decreased level of MIP-1α was reported *i.e.* 26.52% in the Biofield Treated formulation ($p \leq 0.05$), while at 0.105 µg/mL it showed 21.05% decreased ($p \leq 0.001$), which exhibited better immunosuppressive activity compared with the untreated test formulation.

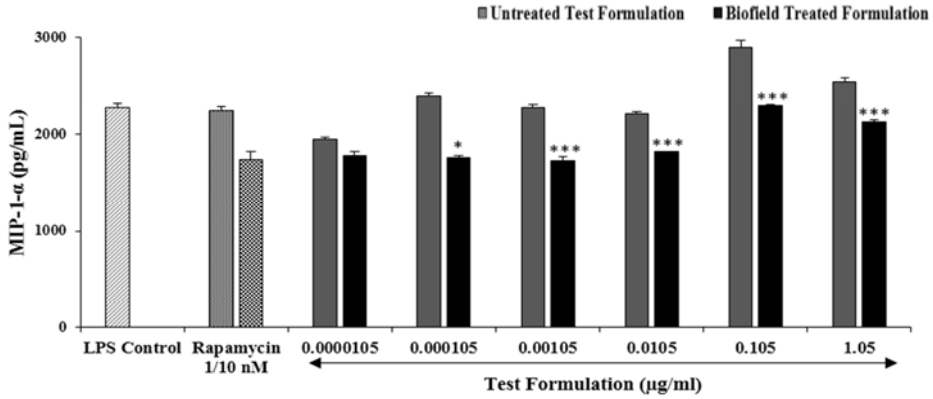

Figure 4. *Concentration-dependent inhibition of LPS mediated production of MIP-1α by the Biofield Treated formulation in mice splenocyte cells. For each concentration treatment, the level of MIP-1α release was measured after 48 hours of treatment. The values are represented in pg/mL as mean ± SEM (*$p \leq 0.05$ and ***$p \leq 0.001$ as compared with the untreated test formulation).*

3.4.2. IL-1β Activity

The overall effect of the test formulation on IL-1β secretion is represented in Figure 5. The figure demonstrates the comparative effect of the Biofield Energy Treated and untreated test formulations on IL-1β secretion. Both the untreated and Biofield Treated test formulations demonstrated inhibition of IL-1β as compared to the LPS stimulated cells. At all the tested concentrations, the Biofield Energy Treated test formulation exhibited better inhibition of IL-1β secretion compared with the untreated formulation. The maximum suppression (35.28%) of IL-1β was reported at 0.0000105 µg/mL ($p \leq 0.001$), while all the tested concentrations were statistically significant with respect to the untreated formulation.

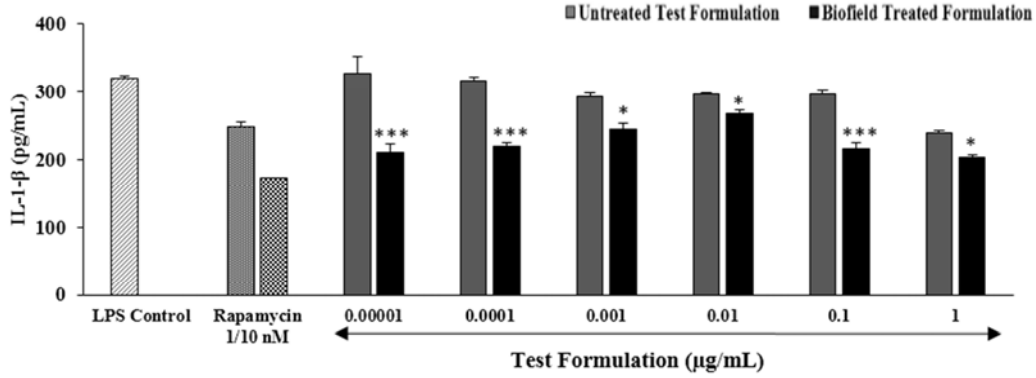

Figure 5. *Concentration-dependent inhibition of LPS mediated production of IL-1β by the Biofield Treated formulation in mice splenocyte cells. For each concentration treatment, the level of IL-1β release was measured in cell supernatant after 48 hours of treatment. The values are represented in pg/mL as mean ± SEM (*$p \leq 0.05$ and ***$p \leq 0.001$ as compared with the untreated test formulation).*

4. Discussion

The use of herbomineral products to maintain or improve health has gradually increased across the globe. Moreover, the formulation of new products that have the ability to improve the overall health by reducing inflammation is essential because of the potential for long-term effectiveness, decreased toxicities, and lower costs. Herbal extracts have been shown to modulate immune responses during inflammation. A new proprietary natural herbomineral formulation was designed in this study, which might be better utilized to regulate immune function as it contains the essential minerals required to modulate immunity.

The individual constituents of this test formulation have been shown to modulate the inflammatory parameters specifically using cytokine alteration [5, 7, 9, 10]. For example, ashwagandha was shown to inhibit NF-κB and AP-1 transcription factors in human peripheral blood and synovial fluid mononuclear cells in a study conducted by Singh *et al.,* 2007 [24]. Zinc deficiency influences the generation of cytokines, including IL-2, IL-6, IL-1β, and TNF-α. Further, zinc supplementation results in changed plasma cytokines that exhibit a concentration-dependent response. The mechanism through which zinc showed this ability either induces or inhibits the activation of NF-κB. The wide involvement of zinc in the immune system includes an ability to influence the production and signaling of numerous inflammatory cytokines in a variety of cell types [25].

Another important mineral of the test formulation was the magnesium gluconate, which was reported to reduce the cytokine production in the cases of intrapartum women and term and preterm neonates, suggesting the effectiveness in those at risk for inflammation-associated adverse perinatal outcomes, whereby magnesium plays a critical regulatory role in NF-κB activation, cytokine production, and disease pathogenesis [26]. Recently, Chen Y *et al.,* 2009 reported that the supplementation of MC3T3-E1 with methylseleninic acid (MSA) decreased the activation of NF-kB results in decreased level of cytokines such as IL-6, MCP-1, COX-2 and iNOS in response to MDA-MB-231 conditioned medium [27].

The authors of this study are trying to develop a herbomineral formulation, which can attribute to the immunomodulating effects and maintenance of the immune system. This may prevent the progression of acute or chronic infection. As infection starts, inflammation is the first response by the immune system. It is mainly caused by the release of cytokines for example TNF-α, IL-1, and IL-6, and eicosanoid such as PGE2. Thus, inhibitors of these cytokines have been considered as candidates for anti-inflammatory drugs. The evaluation on immune cells, especially in the BM cells such as dendritic and splenocyte cells, are always used as a standard to screen the potential immunomodulatory effect of a substance/product. This is the reason the thymus is regarded as the major primary lymphoid organ for the development of T cells, while bone marrow is for the matured B and NK cells. These immune cells then migrate to the spleen, which is the secondary lymphoid tissue, and respond to the antigens. Therefore, thymus and spleen cell proliferation are mainly regulated to maintain immune homeostasis, which might be considered an important marker for the control of the immune response [28]. Lymphocytes/monocytes/macrophages are mainly regarded as key mediators in inflammation and are widely distributed in the body. Therefore, mouse BM derived DCs and splenocyte cells, which represent appropriate model systems to study immune responses, were utilized to investigate the anti-inflammatory effects of a newly developed proprietary herbomineral formulation.

It has been assumed that the Biofield Energy Healing results might be due to the electromagnetic/energy fields [29] that were emitted from the Biofield Healers during the Biofield Energy Treatment. The Trivedi Effect® (Biofield Energy Treatment) has been reported worldwide with significant outcomes in many reasearch fields. Widespread studies were reported in case of cancer research [30, 31], altered antimicrobial sensitivity against pathogenic microbes was reported in microbiology [32-35], genetics [36, 37], altered physiochemical properties of important compounds in pharmaceutical science [38-42], improved crop productivity and quality of crops in agricultural science [43-45], and in changing the structure of the atom in relation to various polymers, ceramics, metals and chemicals in materials science [46-48], and much more.

Biofield Energy Treatment, as an evidence-based integrative medicine therapy, has been reported to reduce the metastatis in cancer cell lines, and also showed significant reduction the level of cytokines such as IL-1α, monokine-induced by gamma interferon (MIG), IL-1β, and macrophage inflammatory protein-2-alpha (MIP-2) with respect to the control/vehicle levels. Additionally, the specific splenic lymphocytes were reported to be reduced after the treatment. Cancer results in increased levels of cytokines, which were was siginificantly reduced by the human Biofield Therapy [49]. As per the U. S. Department of Health and Human Services and Centers for Disease Control and Prevention (CDC), 34% of adults in the U. S. A. depend upon some kind of complementary or alternative health care approach, such as energy medicine. Complementary and alternate medicine has several advantages over the current allopathic model of treatment. These energy therapies are well defined by National Center for Complementary and Integrative Health (NCCIH), a subdivision of the National Institutes of Health (NIH), and come under the category of energy medicine [50].

In this study, the anti-inflammatory effect of the Biofield Treated formulation was screened by assessing the metabolic activity of immune cells using MTT assay. The Biofield Energy Treatment is regarded as more rapid, less costly, and less time consuming because it was reported to show similar results as the bromodeoxyuridine (BrdU) assay, such as cell proliferation, cell growth, and metabolic activity as the reports suggest [51]. The anti-inflammatory effect of both the

Biofield Energy Treated and untreated test formulations were evaluated in two different immune cells from the mice. MTT assay was carried out in order to evaluate non-cytotoxic concentrations of the test formulation. The metabolic activity is evaluated using the MTT test by measuring the activity of succinate dehydrogenase, a mitochondrial enzyme. This test is widely used in the *in vitro* evaluation of the toxicity of any test item. Both DCs and splenocytes (immune cells) were exposed at various concentrations of the Biofield Treated and untreated test formulations for 24 hours and 48 hours, respectively. No sign of negative effects was observed after treatment with the tested concentrations (Figure 1 and 2). The concentrations higher than 105.2 µg/mL caused a significant reduction in the DCs viability and splenocyte cells were showed cytotoxic at 10.5 µg/mL.

The Biofield Energy Treated formulation demonstrated the greatest potential for modulating the inflammatory response of DC and splenocyte cells in *in vitro* assays. There was a significant reduction of TNF-α, MIP-1α and IL-1β levels in the cell supernatants of all tested concentrations. Importantly, these effects were not attributable to decrease in cellular viability indicating that this product was not adversely affecting the cultured murine DCs and splenocyte cells. Therefore, these results suggest that Biofield Energy Treated formulation may inhibit the inflammatory responsiveness of DCs and splenocyte cells by modulating signal transduction pathways involved in the generation of NO, IL-6 and TNF-α. This effect may be the result of specific inhibition of NF-κB, a transcription factor involved in the activation of many inflammatory mediator genes. It is worthwhile to note that the anti-inflammatory effects of the Biofield Energy Treated formulation were not limited to only DCs, on the other hand the Biofield Treated formulation also elicited suppressive effects in LPS stimulated mice splenocyte cells on pro-inflammatory cytokines (TNF-α, MIP-1α and IL-1β) production, which is another important population of innate immune cells that function as antigen presenting cells (APCs). Several cytokines are deeply associated with inflammatory diseases, so LPS was used in the study to induce the immune system, as it creates the disease condition. In particular, TNF-α and IL-1β are prominent contributors to chronic inflammatory disorders. TNF-α and IL-1 β receptor antagonists have been clinically successful to improve the symptoms of rheumatoid arthritis patients. Steroids, such as prednisolone and dexamethasone, are known to reduce the production of these cytokines, but have many associated side effects.

Collectively, our data suggest that the Biofield Energy Treated formulation may act as an effective anti-inflammatory product with little-to-no toxicity. The results of the Biofield Energy Treated formulation suggest better inhibition of T and B lymphocytes, which can be used in several applications related to immune-mediated disorders such as autoimmune disorder, stress, and asthma [52]. Because of its promising anti-inflammatory effects *in vitro*, the Biofield Treated herbomineral formulation would also be considered for different chronic inflammation and infectious diseases.

5. Conclusions

Based on these results, it was concluded that the Biofield Energy Treated formulation modulates the DCs and splenocyte cells function. Further, the Biofield Treated formulation significantly inhibited the activity of pro-inflammatory cytokines such as TNF-α, MIP-1α, and IL-1β in both the tested immune cells as compared to the untreated test formulation. The results of MTT assay in DCs after 24 hours of treatment incubation with different concentrations of the Biofield Treated formulation suggest a safe concentration range of 1.05 to 105.2 µg/mL, which was tested for estimation of TNF-α. Similarly, MTT assay in splenocyte cells after 48 hours of treatment incubation with the different concentrations of the Biofield Treated formulation in the presence of 0.5 µg/mL LPS, suggest that the concentration range of 0.0000105 to 1.05 µg/mL was found safe with more than 80% cell viability, which were selected for MIP-1α and IL-1β estimation. The Biofield Treated formulation showed significant ($p \leq 0.001$) suppression of TNF-α level in DCs cells at all the tested concentration ranges (*i.e.* 1.05 to 1052.5 µg/mL), while maximum suppression was reported at 5.2 µg/mL by 43.64% as compared with the untreated test formulation. However, the levels of MIP-1α and IL-1β at the tested concentration range (0.0000105 to 10.5 µg/mL) in LPS stimulated splenocyte cells were significantly inhibited. Further, data suggests that inhibition of MIP-1α (26.52%) and IL-1β (35.28%) in the Biofield Treated formulation at 0.000105 and 0.0000105 µg/mL, respectively as compared with the untreated test formulation.

Overall, the inhibition of pro-inflammatory cytokines might prevent the over activation of the human immune system. Thus, it may strongly counteract the pro-inflammatory responses, which might prevent immune-mediated tissue damage. Through insight into immunomodulatory cytokines, the Biofield Energy Treated test formulation can provide the innovative strategy to affect the immune system, which could help combat various acute and chronic inflamatory diseases as a better complementary and alternative medicine. So, it can be concluded that The Trivedi Effect®-Biofield Energy Healing (TEBEH) has the capacity to modulate the immunoinflammatory activity of herbomineral formulations with a safe therapeutic index and a faster absorption rate that can enhance the human well-being in the biomedical health care system. This can be ulitized against many acute and chronic inflammatory diseases like Asthma, Chronic peptic ulcer, Tuberculosis, Hepatitis, Irritable Bowel Syndrome, Parkinson's Disease, Rheumatoid arthritis, Chronic periodontitis, Ulcerative colitis, Crohn's disease, Chronic sinusitis, Systemic Lupus Erythematosus, Type 1 Diabetes and Chronic active hepatitis. Furthermore, the Biofield Energy Treatment Therapy applied to the herbomineral formulation can also be utilized for various autoimmune disorders such as Graves' Disease, Lupus, Celiac Disease (gluten-sensitive enteropathy), Dermatomyositis, Hashimoto Thyroiditis, Addison Disease, Multiple Sclerosis (MS), Myasthenia Gravis, Aplastic

Anemia, Reactive Arthritis, Sjogren Syndrome, Alopecia Areata, Fibromyalgia, Vitiligo, Pernicious Anemia, Psoriasis, Scleroderma, Chronic Fatigue Syndrome and Vasculitis, as well as to prevent the immune-mediated tissue damage in cases of organ transplants (for example kidney transplants, liver transplants and heart transplants) because of its safe therapeutic index and potential to modulate the immune system and improve overall health and quality of life. Overall, the Biofield Energy Healing Treated test formulation can also be used for anti-aging, stress prevention and management, and in the improvement of overall health and quality of life.

Acknowledgements

The authors are grateful to Dabur Research Foundation, Trivedi Science, Trivedi Global, Inc., and Trivedi Master Wellness for their support throughout the work.

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