

Anti-Inflammatory Activity of Quantum Energy Living Body on Lipopolysaccharide-Induced Murine RAW 264.7 Macrophage Cell Line

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Abstract

Recent developments from science and medical science show a growing interest in the anti-inflammatory activity of natural materials. Inflammation is the body's physiologic response to injurious stimulation and is known to be mediated by various pro-inflammatory cytokines (e.g. TNF- α , IL-1 β , IL-6 etc) and iNOS (inducible nitric oxide synthase). Quantum energy living body (QELBY) powder is a fusion of a special ceramic powder with natural clay mineral classified as quantum energy radiating material (QERM). The powder, composed mostly of silicon dioxide, is known to radiate reductive radiant energy. This study was designed to evaluate the anti-inflammatory activities of QELBY powder on RAW 264.7 mouse macrophage cells. QELBY powder was mixed with DMEM media and was allowed to stand for 48 hours. Afterwards, the supernatant was taken and diluted to various concentrations (0.5, 10, 20, 40 μ g/ml) prior to use. CCK-8 assay was done to determine the effects on cell viability. In addition, NO assay performed to elucidate the effect of QELBY on the NO production of LPS-stimulated macrophages. Lastly, RT-PCR and Western blot analysis for the detection of the mRNA and protein expressions, respectively, of pro-inflammatory cytokines and iNOS was made. Results demonstrated that QELBY powder causes both an increase in cell proliferation and a concentration-dependent decrease in NO production. Moreover, the mRNA and protein expressions of pro-inflammatory cytokines and iNOS were also inhibited. Taken together, these show that QELBY powder has anti-inflammatory activity and could therefore be used further in the development of materials that induce such kinds of benefits.

Keywords: Quantum energy radiating material (QERM); Quantum energy living body (QELBY); Quantum energy; RAW 264.7 murine macrophage cell line; Anti-inflammation

Introduction

Quantum energy living body (QELBY) powder is a fusion of a special ceramic powder with natural clay mineral classified as quantum energy radiating material (QERM). The powder, composed mostly of silicon dioxide, is stable in temperatures as high as 1000°C and is known to radiate reductive radiant energy [1]. Several tests have already been done to assess the effect of QERM. QERM is starting to be applied in different fields including improvements in the biologic processes of plants and animals. It has been shown that it has antioxidant, antibacterial, immune enhancing and anticancer properties. Aside from the aforementioned biological effects, it has also been shown to absorb electromagnetic waves, prevent corrosion and neutralize toxicity. QERM is said to exert its biological functions through the emission of reductive radiant energy which is said to activate both plant and animal cells. This kind of energy is said to be able to penetrate glass, chemicals and even the human body making the structure of water molecules hexagonal in shape. In addition, reductive radiant energy also generates negative electrons which allows for the exertion of its antioxidant, immune enhancing and cancer suppressing properties. Inflammation is the primary and physiologic response of the immune system to injury which is aimed at removing pathological noxae and restoring homeostasis [2,3]. The 5 hallmarks of inflammation are redness, swelling, heat, pain and loss of function. The descriptions are due to an increase in the blood flow into the site of injury due to the release of so-called inflammatory mediators (eicosanoids and cytokines) by the sentinel cells (e.g. dendritic cells). Inflammation has garnered tremendous amount of attention due to established links with several pathologies including cancer, metabolic and degenerative diseases [3-6]. Macrophages are classified under the mononuclear phagocytes of the immune system. These cells are derived

from blood monocytes, which originated from bone marrow precursor cells. Macrophages are important in innate immunity for they serve as Sentinel cells that have vital functions in pathogen clearance and, more importantly, antigen presentation. Macrophages are known to be able to phagocytose a wide variety of pathogens through countless years of evolution. This function is due to the presence of pattern recognizing receptors (PRRs), which serve as receptors for a wide array of pathogenic organisms', mostly bacterial, conserved surface antigens [7]. The monocytes-macrophage system exists in at least two distinct phenotypes: classical/pro-inflammatory (M1) and alternative/anti-inflammatory (M2) [8]. The activated state of each distinct phenotype largely depends on the variety of cytokines and microbial products that switch on distinct transcription networks [9,10]. Upon inflammatory stimuli, such as lipopolysaccharide, macrophages are triggered to produce and release a diverse set of inflammatory mediators such as tumor necrosis factor- α (TNF- α), interleukin-1, interferons, prostaglandins and nitric oxide (NO) [11,12]. These so-called mediators are part of the host defense system against the potential etiologic agent or cause of injury. However, excessive production of these inflammatory products, for instance TNF- α and NO, could

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lead to pathologic consequences [13,14]. The aim of the study was to determine the effect of direct exposure with QELBY powder to the inflammation-related changes in LPS-induced RAW 264.7 murine macrophage cells.

Materials and Methods

Preparation of treatment stock from QELBY powder

QELBY powder was bought from Quantum Energy® and was sterilized by autoclaving. 0.3 g of QELBY powder was mixed with 30 ml DMEM with 10% FBS and 1% penicillin-streptomycin (P/S) for 24 hours. After soaking, the supernatant was filtered with Whatman Filter paper and the weight of the filtered powder obtained in order to compute for the total dissolved QELBY powder in the media. The stock solution was then diluted to certain concentrations (5,10,20,40 µg/ml) prior to use.

Cell line and cell culture

Murine RAW 264.7 was obtained from the Korean Cell line Bank (KCLB, Korea) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco®, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco®, USA) and Penicillin/Streptomycin (100 U/mL) (Lonza, USA) and 3.7 mg/mL of NaHCO₃ at 37°C in 5% CO₂.

Cell viability analysis

Cell counting kit-8 (CCK-8, Dojindo, Japan) was used to quantify viability of RAW 264.7 macrophage cells according to the manufacturer's instruction. Seeding at a density of 10⁴ cells per well was done and were incubated in DMEM at 37°C in 5% CO₂ for 24 hours. The cells were then pretreated with LPS (10 µg/mL). After 2 hours, treatment with media with increasing concentrations of dissolved QELBY was performed. After 24 hours of incubation, media in the wells were suctioned and 100 µL of fresh media with 10 µL CCK-8 was added per well which was incubated at 37°C for 2 hours. Absorbance at 450 nm was measured using an ELISA plate reader (TECAN, Switzerland). Viability of the treated cells was expressed as the percentage of control cells.

NO assay

Total NO produced by Lipopolysaccharide (LPS) (Sigma®, USA) stimulated RAW 264.7 macrophage cells was determined with the use of Griess reagent. 5 × 10⁴ cells seeded per well were incubated for 24 hours. Pretreatment with LPS (10 µg/mL) was done prior to treatment with increasing concentrations of media with QELBY. After 24 hours of incubation, 50 µL of the media collected from each well was transferred to a new well with an equal volume of Griess reagent (5% phosphoric acid (99.9%), 2% sulfacilamide (99.9%), 2% N-(1-Naphthyl) ethylenediamine dihydrochloride (98%)). The plate was covered in foil and incubated for 15 minutes. Absorbance at 540 nm was measured using an ELISA plate reader. The total NO content of each well was obtained by the formula below.

$$\text{NO content} = \text{OD}_{570} \text{ reading} / 0.0049$$

RT-PCR

Total RNA was obtained from LPS and QELBY treated RAW 264.7 cells using Trizol reagent (Takara, Korea) according to the manufacturer's instructions. 1 µg of RNA was used to acquire the complementary DNA (cDNA) using the protocol provided by M-MuLV reverse transcriptase (Fermentas, Lithuania). Specific

primers were used to amplify different genes. PCR products were then separated by electrophoresis using 1.5% agarose stained with ethidium bromide and UV transillumination was done afterwards. PCR analysis was done three times and the densitometry analysis carried out using Lane 1-D® software.

Western blot analysis

The isolation and determination of protein concentration was carried out using Bradford assay. Cells that had undergone treatment for 24 hours were lysed using a protein extraction solution (Intron Biotechnology, Korea) and the protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, USA). 30 µg of the extracted protein was separated in 10% SDS-PAGE. Afterwards it was transferred to a nitrocellulose membrane (Schleicher and Schuell, Germany) and blocked for 2 hours with 5% skim milk in TBST buffer (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20). The membrane was washed four times with TBST and incubated overnight in 2% skim milk containing the primary antibodies (monoclonal anti-TNFα, anti-iNOS, anti-NF-κB, anti-IL-1β, anti-IL-4 and anti-IL-6) at a 1:1000 dilution. Washing with TBST buffer was done for four times then the membranes were incubated with horse radish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology, USA) diluted to 1:2000 for another 2 hours. Lastly, the membranes were washed four times with TBST buffer and detection of bands was done with the use of the enhanced chemiluminescence system (ECL, Western Blot Analysis System Kit, Amersham Biosciences, Korea). The experiment was carried out three times and the resulting blot was quantified using densitometry analysis with Lane 1-D® software.

Statistical analysis

All experiments were done in triplicates and data were expressed as means ±SD. The difference between control and treated cells were evaluated using one-way ANOVA followed by Duncan Multiple Range Test. P values less than 0.05 were considered statistically significant.

Results

Cell viability analysis

CCK-8 assay is a widely-used method to quantify cells to determine a compound's mitogenic or toxic properties to cells. Cell viability analysis has shown a dose-dependent increase in the quantity of LPS-stimulated cells after direct treatment with QELBY powder for 24 hours. It can be seen in Figure 1 that even at very low concentrations of the said mineral, a statistically significant increase in the proliferation of the RAW 264.7 macrophage cells occurs with as much as 43.95% increase in the quantity of cells at 40 µg/mL of exposure (p<0.05). This data also shows that QELBY powder has no cytotoxic effects with respect to the concentrations used in this study Figure 1.

NO assay

NO determination by Griess reaction has shown a statistically significant decrease (p<0.05) in the NO production by the LPS-induced RAW 264.7 macrophage cells after treatment with QELBY powder for 24 hours. Similar to the cell viability assay, a dose-dependent effect was observed even after treatment with a small concentration of the powder with as much as 10.87%, 15.0%, 22.83% and 31.85% inhibition for 5, 10, 20 and 40 µg/mL respectively Figure 2.

RT-PCR analysis

Exposure of the macrophage cells used in the study showed a dose-

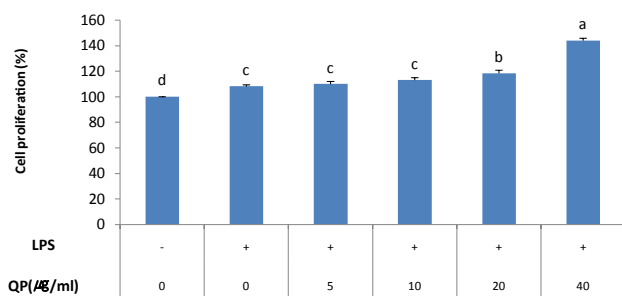


Figure 1: Effect of treatment with QELBY powder (QP) on the viability of murine LPS-induced RAW 264.7 macrophage cells after incubation for 24 hours. Data are means \pm SEM (n=4). Means with different superscripts are significantly different at $p < 0.05$.

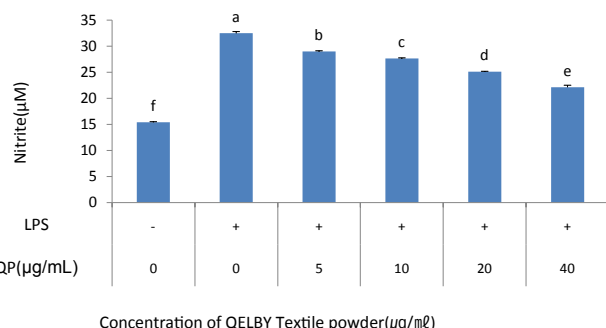


Figure 2: Effect of treatment with QELBY powder (QP) on the NO production of LPS-stimulated murine RAW 264.7 macrophage cells after incubation for 24 hours. Data are means \pm SEM (n=4). Means with different superscripts are significantly different at $p < 0.05$.

dependent decrease in the mRNA expression of all inflammation-related genes using RT-PCR assay. Marked decrease in the band intensities was seen for NF- κ B, TNF α , IL-4 and iNOS with increasing concentration of QELBY powder while a partial decrease was seen in IL-1 β and IL-6 as shown in Figure 3.

Western blot analysis

Similar to the results seen in RT-PCR, exposure of the LPS-stimulated macrophages to QELBY powder resulted in decreased protein expression of the molecules for the study. A decrease in the protein expression of TNF- α , iNOS, IL-1 β , NF- κ B, IL-4 and IL-6 was shown by Western blotting with β -actin serving as the house-keeping protein. The densitometry analysis shows a more detailed view of the results of obtained Figure 4.

Discussion

Macrophages are essential cells of the innate immune system due to their diverse functions and integral roles in pathogen clearance and inflammatory processes [7]. Cell viability analysis has shown that exposure to QELBY causes an increase in the quantity of cells even at low concentrations. An increase in the number of macrophages may be of importance since an increased quantity of these cells can be directly correlated to an increased capability of the innate immune system for pathogen clearance. Moreover, several studies [15-18] have already shown that macrophages are also beneficial in tissue homeostasis, tissue repair and cell proliferation after injury. Inflammation is a physiologic response to the presence of injurious stimuli in an animal's body. However, excessive inflammation leads to the development of several pathologies. Inflammation is already known to be involved in many important pathological processes of several diseases. This is due to the toxic effects of several inflammatory mediators produced by cells at the site of inflammation [19,20]. Several researches have already strongly

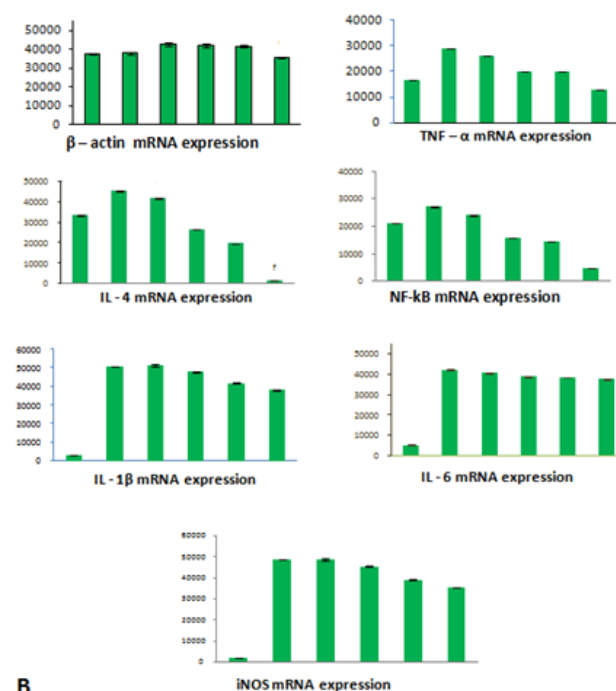
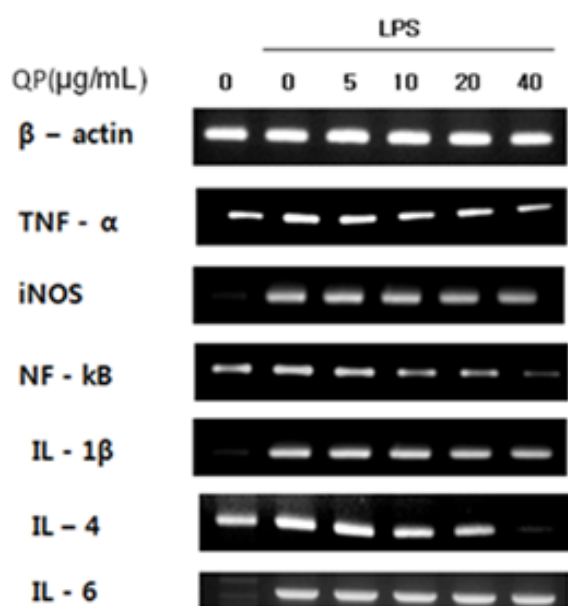


Figure 3: The effect of treatment with QELBY powder (QP) on the mRNA expression of inflammation-related genes in RAW 264.7 murine macrophage cells stimulated with LPS. The PCR products after electrophoresis (A) and the graphs showing the comparative densities of the bands (B) are shown (n=3).

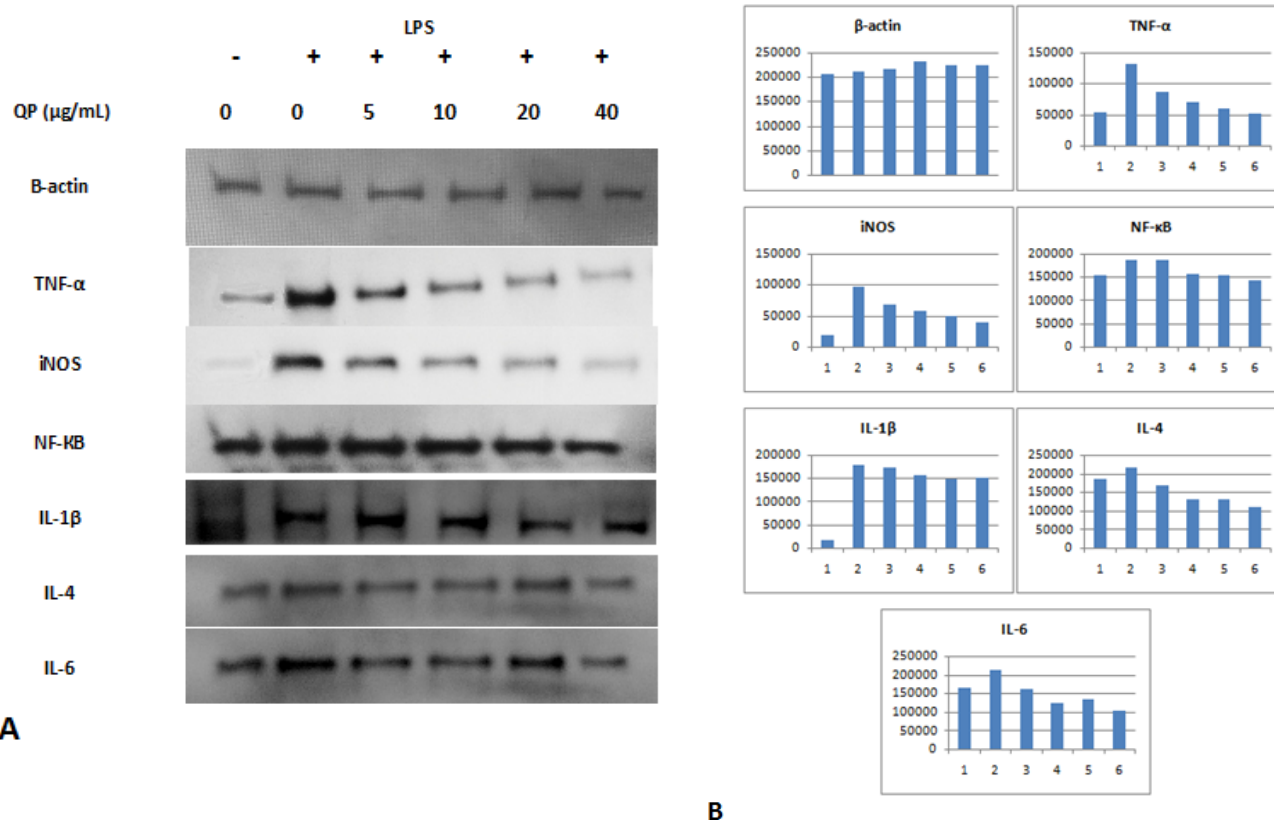


Figure 4: Effect of treatment with QELBY powder (QP) on the protein expression of inflammation-related genes in murine RAW 264.7 macrophage cells. The relative protein expression (A) and the quantitative protein expression obtained through densitometry analysis (B) are shown.

emphasized and have shown compelling evidence on the importance of blocking inflammation for the prevention of many disease states both in animals and humans [19,21-23]. Results of the study have shown that exposure of RAW 264.7 murine macrophage cells, a prototype cell line for studies on inflammation, leads to a decrease in the inflammatory response through the decrease in the production of NO as well as other important pro-inflammatory products such as cytokines. Nitric oxide, a gaseous substance acting as a signaling molecule, is produced in large amounts from the amino acid L-arginine by the enzyme inducible nitric oxide synthase (iNOS) [24]. Many inflammatory cells, most especially macrophages, express iNOS upon stimulation with bacterial LPS, viruses or even other cytokines [11]. Nitric oxide exerts its effect in host defense owing to its antibacterial and virustatic properties. However, excessive production of NO could lead to detrimental effects due to its cytotoxic potential on normal host cells [11]. Results of the study have shown that exposure of the cells to QELBY caused a marked decrease in the NO produced by the LPS-stimulated cells as well as in the mRNA and protein expression of iNOS. This data shows that even at low concentrations of QELBY powder, a significant anti-inflammatory effect through the inhibition of NO production was achieved. Tumor necrosis alpha (TNF-α) and interleukin 1 beta (IL-1β) represent the archetypal pro-inflammatory cytokines rapidly released after the occurrence of tissue injury or infection [25]. TNF is mainly produced by macrophages and plays a major role as a pro-inflammatory mediator [26]. It is known as a central regulator of inflammation and antagonists could be effective in the prevention of inflammatory conditions or

in the treatment of inflammatory disorders in which TNF-α plays a significant role [27,28]. The interleukins comprise a group of molecules involved in the signaling between the cells of the immune system. Similar to tumor necrosis factor-alpha, some interleukins, most notably interleukin 1 secreted by monocytes/macrophages, are known to cause pathologic disturbances when produced excessively and continuously [29-31]. Our experiment has shown that a decrease in the pro-inflammatory interleukins (IL-1β, IL-4 and IL-6) as well as TNF-α dose-dependently. The decrease in the expression, both mRNA and protein, of the aforementioned cytokines entails an anti-inflammatory role of QELBY powder beyond just the suppression of NO production. Nuclear factor-kappa B (NF-κB) has long been considered as a prototypical pro-inflammatory signaling pathway. This pathway is activated upon stimulation of pro-inflammatory cytokines such as IL-1 and TNF-α. This transcription factor is known to play a role in the expression of pro-inflammatory genes including cytokines, chemokines and adhesion molecules [25,32]. Several chronic diseases have already been associated with NF-κB activation some of which includes asthma, inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis and several cancers [4,25,33]. It is for these, and several more compelling reasons, that NF-κB is considered the "holy grail" as a target for inflammatory suppressive agents. RT-PCR and Western blot analysis of cells treated with the powder resulted in a significant decrease in the expression of NF-κB. This result proves to be of immense significance for it provides strong evidence for the anti-inflammatory properties of QELBY powder even at very low

concentrations (40 µg/mL). These results could also serve as a basis for a decrease in other inflammatory mediators not used in the study for the role NF-KB plays as a pro-inflammatory transcription factor.

Conclusion

Results of the study have demonstrated that QELBY powder have anti-inflammatory properties through the suppression of pro-inflammatory mediators such as iNOS, TNF- α , IL-1 β , IL-4, and IL-6. In addition, it also causes a decrease in the expression of the master transcription factor of inflammation NF-KB. Taken together, these results show that QELBY Powder could therefore be used further in the development of materials which could be used for anti-inflammation.

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