

## Combination of Coq10, Omega 3 and Zinc Improves the Severity of Experimental Arthritis by Inhibiting the Immune-inflammatory Response

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### Abstract

Omega 3, zinc and coenzyme Q10 (CoQ10) are used as dietary supplements revealing an anti-inflammatory function. Individually they have been recognized as important players in rheumatoid arthritis (RA). However, there is no evidence of the synergic effect of omega 3, zinc and CoQ10 in RA. This study aims to identify if omega 3, zinc and CoQ10 can improve the progression of zymosan-induced arthritis (ZIA) using a mouse model. Results showed that the combination of omega 3, zinc and CoQ10 decreased ZIA severity decreasing IgG levels, joint inflammation and cartilage damage. The combination of omega 3, zinc and CoQ10 reduced interleukin (IL)-6, -17, tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$  expression. There was also a decrease in osteoclastogenesis with the combination of omega 3, zinc and CoQ10. These observations demonstrate that the combination of omega 3, zinc and CoQ10 improved ZIA progression by reducing proinflammatory cytokines expression compared to CoQ10 alone. Thus, the combination of omega 3, zinc and CoQ10 presents as an important preventive measure in RA, which can aid in understanding in its pathogenesis.

**Keywords:** Rheumatoid arthritis; Coenzyme Q10; Omega 3; Zinc

### Background

Rheumatoid arthritis (RA) is a clinically and excessive inflammatory disease resulting in systemic inflammation causing chronic cartilage damage-inducing disability. Though the etiology of RA is not clear, the upregulation of proinflammatory cytokines may be related to the pathogenesis of RA. It has been demonstrated that various cell types in the inflamed hypertrophic synovium release proinflammatory cytokines perpetuating inflammation [1]. Thus, proinflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-17 have been suggested to be significant targets in RA therapy. For instance, IL-17- and IL-17-secreting CD4+ T cells conduct significant roles in pathogenesis of RA [2]. Previous reports suggest that the expression of IL-17 is abundantly present in the serum and synovial fluids of RA patients [3].

Osteoclasts are bone-resorbing macrophage polykaryons cells. As osteoclasts induce bone tissue destruction, osteoclastogenesis has a pivotal role in the pathogenesis of RA. It is well documented that osteoclastogenesis results in bone loss involved in chronic arthritis where there was an increase in the synovium among RA patients [4-6]. Additionally, it has been reported that proinflammatory cytokines play a role in osteoclastogenesis. Accumulating evidence demonstrates that TNF- $\alpha$  and IL-17 upregulate the creation of osteoclasts [7,8]. Specifically, it has been shown that exceedingly high levels of TNF- $\alpha$  is found in the serum and the arthritic synovium of RA patients [9].

Thus, detecting the inhibitor of osteoclastogenesis can be a potential therapeutic molecule for RA [10].

Various natural and synthetic molecules may be useful as scaffolds for several biological applications [11-13]. It is well documented that peptides and proteins such as self-assembling peptides have considerable capability in drug delivery and immune adjuvants [14,15]. In addition, a modified proteasome inhibitor revealing potentiality as a drug can be combined with chemotherapeutic agents producing important results in clinical trials [16,17].

Individually, Coenzyme Q (CoQ) 10, omega 3 and zinc are used as a dietary supplement revealing anti-inflammatory activity. Evidence suggests that CoQ10 downregulate oxidative stress as well as the inflammatory response [18-20]. Additionally, omega 3 and zinc have shown to downregulate proinflammatory cytokines [21,22]. As a diet supplement, it has been suggested that there is a significant decrease in zinc production among patients with RA compared to normal controls [23]. Notably, CoQ10 revealed a therapeutic effect in ZIA through downregulating the inflammatory response and osteoclastogenesis [24]. Omega 3 has shown to decrease inflammatory mediators and several clinical parameters involved in RA [25]. However, the combination of omega 3, zinc and CoQ10 has not been implicated in the treatment of RA.

We hypothesized that the synergistic effects of omega 3, zinc and CoQ10 can have beneficial anti-inflammatory effects in RA. The aim of the present investigation was to identify whether the combination of omega 3, zinc and CoQ10 shows anti-arthritic as well as a therapeutic

capabilities of reducing the expression of proinflammatory cytokines including TNF- $\alpha$  and IL-17 in a mice model of RA. Thus, *in vivo* and *in vitro* tests were conducted to demonstrate the preventive activity of this combination.

## Materials and Methods

### Animals

SKG mice (BALB/c background; 8 weeks old) were obtained from Professor Shimon Sakaguchi (Department of Experimental Immunology, World Premier International Immunology Frontier Research Center, Osaka University). All mice were maintained in a specific pathogen-free environment under climate-controlled conditions with a 12 hour light/dark cycle at the Catholic University of Korea. The mice had free access to water and food throughout the study. After 1 week of adaptation, they were divided into three groups (n = 5 per each group): vehicle, CoQ10, and the combination of CoQ10, omega 3 and zinc. Surgeries were performed under isoflurane anesthesia with all efforts to minimize suffering. Experimental procedures were approved by the Institutional Animal Care and Use Committee at the School of Medicine, Animal Research Ethics Committee of The Catholic University of Korea. They were performed in accordance with the Laboratory Animals Welfare Act, Guide for the Care and Use of Laboratory Animals.

### Zymosan induced arthritis mouse model

Zymosan (Sigma-Aldrich) is a polysaccharide prepared from the cell wall of *Saccharomyces cerevisiae*. Arthritis was induced in all SKG mice by a daily i.p. injection of 100 mg/ml, and orally fed Coenzyme Q10, Coenzyme Q10 + omega 3 + zinc or vehicle control for 8 weeks.

### Clinical observation of arthritis

Clinical scores for limbs were recorded in accordance with a scale of 0-4 as described previously [26]. Joint swelling was monitored by inspection and scored as follows: 0 = Normal, 1 = Mild swelling of the ankle to the mid foot or erythema, 2 = Erythema and moderate swelling of the finger joint, 3 = Severe redness and swelling of the entire paw, including the digits, and 4 = Maximally inflamed limb with involvement of multiple joints. The scores for all fingers of forepaws and hind paws, wrists, and ankles were then totaled for each mouse.

### Measurement of IgG (total), IgG1, IgG2a in serum

The production of autoantibodies in SKG mice serum samples was measured by ELISA. The concentrations of IgG (total), IgG1, and IgG2a cytokines were measured by ELISA (BD Biosciences, MA, USA), according to the manufacturer's instructions. Briefly, the Coster ELISA plates were coated overnight at 4°C with a capture antibody for a specific cytokine, followed by washing and blocking the plate. Subsequently, the serum levels of IgG (total), IgG1, and IgG2a were measured using an IgG enzyme-linked immunosorbent assay (ELISA) kit for mice according to the manufacturer's instructions. The OD values at 550 nm were subtracted from those at 450 nm, and the values of samples were determined.

### Isolation of mouse splenocytes cell culture and ELISA assay

Splenocytes were isolated from the spleen of SKG mice. After splenocytes isolation,  $5 \times 10^5$  cells/well in 48-well plates containing 100

ng/ml lipopolysaccharide (LPS) in the presence or stimulated with plate-bound anti-CD3 (0.5  $\mu$ g/mL) and treated with Coenzyme Q10, omega 3 and zinc. Culture supernatant and cells were collected three days post treatment. The concentrations of IL-6, TNF- $\alpha$ , IFN- $\gamma$ , and IL-17 cytokines were measured by ELISA (BD Biosciences, MA, USA), according to the manufacturer's instructions. The optical density (OD) value was measured with a VersaMax Microplate Reader.

### Histological assessment of arthritis

Samples of the joints from each SKG mouse were fixed in 10% formalin, decalcified in EDTA, and embedded in paraffin wax. The embedded joints were sliced to a thickness of 4  $\mu$ m for histological analysis. H, E and safranin O stained sections were scored for inflammation and cartilage damage [27].

### Osteoclast culture and differentiation assay

The osteoblast-enriched bone cell population was obtained from the tibiae and femurs where it was isolated using a similar protocol as described by Bakker AD et al. [28]. Cultures were maintained in  $\alpha$ -MEM in the presence of 10 ng/ml M-CSF for 3 days osteoclast precursor cells. After the osteoclast precursors were cultured with in the presence of 10 ng/ml M-CSF and 50 ng/ml RANKL (R&D Systems Inc., Minneapolis, MN, USA) for 4 days to generate osteoclasts. Upon completion of forming mature osteoclasts, TRAP staining was then performed. The osteoclast areas (with  $\geq 3$  nuclei) were identified with microscopy.

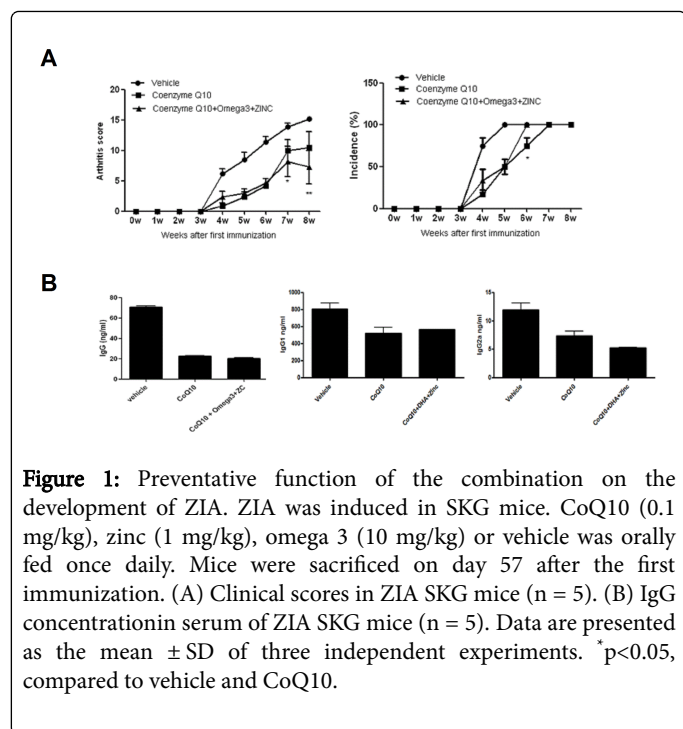
### Statistical analysis

Data are presented as means  $\pm$  standard deviations (SD). Statistical analysis was conducted with the unpaired Student's t-test using the GraphPad Prism (version 5.01, GraphPad Software, San Diego, CA). Values of  $P < 0.05$  were considered of statistical significance.

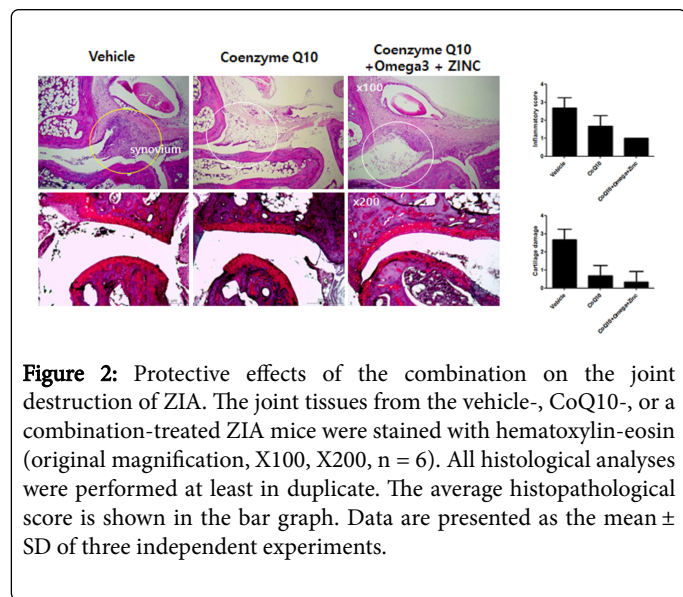
## Results

### The combination inhibited the development of zymosan induced arthritis

To examine the effect of omega 3, zinc and CoQ10 on arthritis development, we administered the combination once a day starting from day 7 after the first immunization. The arthritis index was significantly lower in the combination-treated ZIA mice than in the vehicle-treated ZIA mice during the entire observation period. Moreover, treatment with the combination attenuated the severity of arthritis compared with mice receiving CoQ10 alone (Figure 1A). Results also showed that combination-treated ZIA mice had lower concentrations of IgG, IgG1 and IgG2a than the vehicle and CoQ10-treated ZIA mice (Figure 1B). Consistent with the arthritis score, histological analyses revealed severe immune cell infiltration and destroyed cartilage among the vehicle and CoQ10-treated ZIA mice, whereas there were minimal signs of inflammation and cartilage destruction detected in the combination-treated ZIA mice (Figure 2). These results suggest that the combination of omega 3, zinc and CoQ10 shows preventative effect in the suppression of arthritis development and joint inflammation.



**Figure 1:** Preventative function of the combination on the development of ZIA. ZIA was induced in SKG mice. CoQ10 (0.1 mg/kg), zinc (1 mg/kg), omega 3 (10 mg/kg) or vehicle was orally fed once daily. Mice were sacrificed on day 57 after the first immunization. (A) Clinical scores in ZIA SKG mice (n = 5). (B) IgG concentration in serum of ZIA SKG mice (n = 5). Data are presented as the mean ± SD of three independent experiments. \*p<0.05, compared to vehicle and CoQ10.



**Figure 2:** Protective effects of the combination on the joint destruction of ZIA. The joint tissues from the vehicle-, CoQ10-, or a combination-treated ZIA mice were stained with hematoxylin-eosin (original magnification, X100, X200, n = 6). All histological analyses were performed at least in duplicate. The average histopathological score is shown in the bar graph. Data are presented as the mean ± SD of three independent experiments.

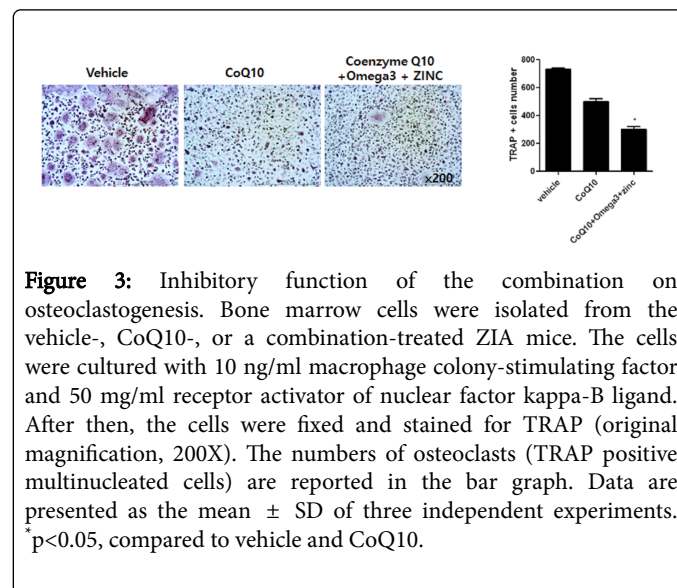
### The combination exhibited downregulated osteoclastic differentiation of purified bone marrow cells

Because we found that the combination of omega 3, zinc and CoQ10 suppressed joint inflammation and destruction, we next studied whether the combination reduces osteoclast differentiation.

Mouse bone marrow (BM) cells isolated from vehicle, CoQ10 alone or the combination-treated SKG mice were cultured with M-CSF and RANKL, and then the cells were stained with TRAP. We observed that the TRAP positive cells were significantly reduced with the combination compared with vehicle and CoQ10 alone (Figure 3). The data demonstrated that the combination reduced OC differentiation, thereby inhibiting ZIA progression.

### The combination decreased the expression of IL-6, IL-17, TNF-α and IFN-γ cytokines

IL-6, -17, TNF-α and IFN-γ are representative proinflammatory cytokines that results in systemic inflammatory response. The combination significantly reduced the concentrations of IL-6 and TNF-α with SKG mice splenocytes induced by LPS (Figure 4A).



**Figure 3:** Inhibitory function of the combination on osteoclastogenesis. Bone marrow cells were isolated from the vehicle-, CoQ10-, or a combination-treated ZIA mice. The cells were cultured with 10 ng/ml macrophage colony-stimulating factor and 50 mg/ml receptor activator of nuclear factor kappa-B ligand. After then, the cells were fixed and stained for TRAP (original magnification, 200X). The numbers of osteoclasts (TRAP positive multinucleated cells) are reported in the bar graph. Data are presented as the mean ± SD of three independent experiments. \*p<0.05, compared to vehicle and CoQ10.

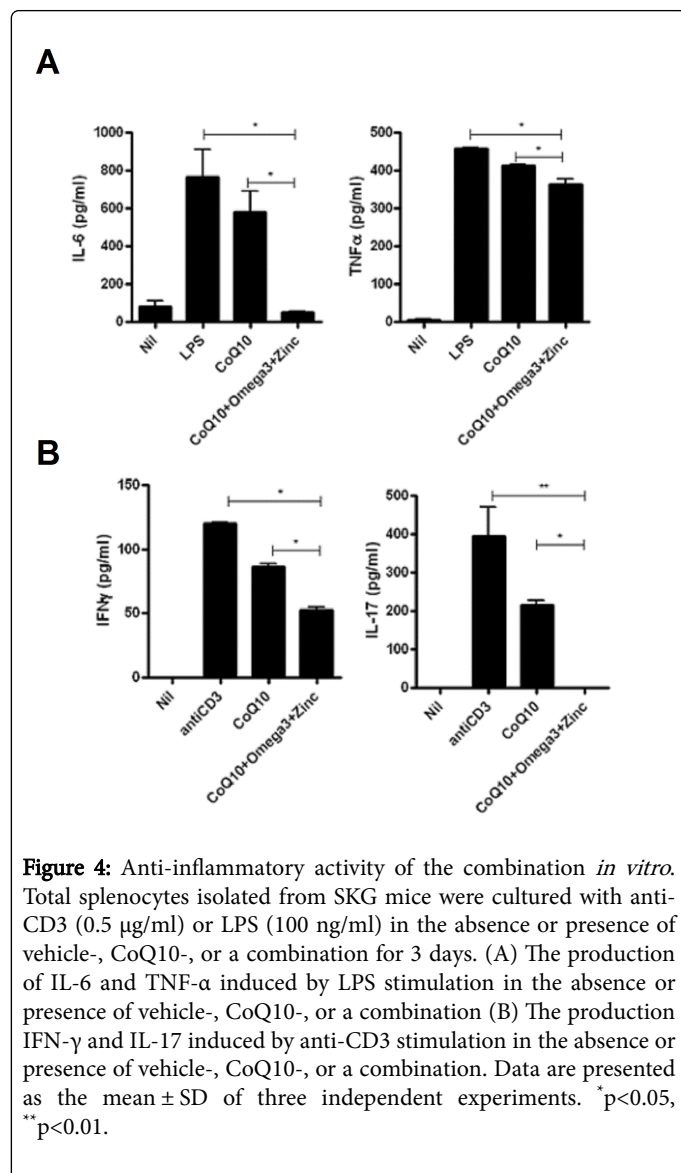
Additionally, the expression of IL-17 and IFN-γ stimulated by anti-CD3 significantly decreased with the combination using the SKG mice splenocytes (Figure 4B). These data demonstrate that the combination of omega 3, zinc and CoQ10 downregulated pro inflammatory cytokine expression.

### Discussion

Though omega 3, zinc and CoQ10 are used as anti-inflammatory dietary supplements, little is known regarding their additive activity on autoimmune arthritis. To date, there is no proof that the combination could be used for inflammatory diseases. In this study, the anti-inflammatory effects of omega 3, zinc and CoQ10 was demonstrated in an autoimmune arthritis mouse model.

This study showed that the combination of omega 3, zinc and CoQ10 decreased the development of ZIA by reducing the inflammatory response and osteoclastogenesis. Several proinflammatory cytokines are involved in RA pathogenesis where the expression of IL-17 and TNF-α is highly presented in the synovium and synovial fluid of patients with RA [29]. IL-6 has been shown to be excessively produced in the synovial fluid and blood among RA patients and correlated with the RA progression and joint damage [30,31]. As bone erosion is a significant characteristic feature in RA, the inhibition of osteoclastogenesis is an important target for RA therapy. Indeed, osteoclasts play a major role in bone damage and destruction [32]. Additionally, osteoclastogenesis is found to be upregulated in RA patients compared to normal controls. It has been well reported for bone destruction to occur and increased with osteoclasts differentiation [4]. Previously, CoQ10 revealed anti-arthritis activity decreasing osteoclastogenesis and inflammation [24]. In this study, the combination inhibited the expression of proinflammatory cytokines such as IL-17 and TNF-α. Moreover, the

combination of omega 3, zinc and CoQ10 reduced osteoclastogenesis. Thus, the combination displays a major preventive role in autoimmune arthritis via suppressing inflammation and osteoclastogenesis.



Autoantibody production is an important factor in the development of autoimmune disease. It has been demonstrated that the presence of autoantibodies is a common character of autoimmune disease [33]. Since RA is systemic autoimmune disease, the expression of autoantibodies is an essential characterization of RA. It is well documented that IgG levels was correlated negatively with ZIA severity [34]. Recently, B cell depletion therapy reducing autoantibody expression induced positive clinical results in RA patients [35]. In this study, the combination of omega 3, zinc and CoQ10 decreased the expression of IgG, IgG1 and IgG2a improving experimental autoimmune arthritis. Therefore, the combination could have therapeutic activity in autoimmune arthritis through the downregulation of autoantibody production.

The SKG strain originating from BALB/c mice can spontaneously have chronic inflammation and arthritis [36]. ZIA on SKG mice show desperate infiltration of immune cells, excessive bone erosion and

cartilage destruction [19]. In this study, the combination of omega 3, zinc and CoQ10 decreased immune cells infiltration and cartilage damage of SKG joint reducing ZIA development. These results demonstrate the potential use in treating RA.

Though notable therapeutic improvements in biologics show remarkable efficacy and adequate safety, medical need has been unsatisfactory with RA treatment [37]. For instance, a subset of cases using biologics has only observed incomplete remission [38-41]. It has been suggested that disease-modifying anti-rheumatic drugs (DMARDs) dual therapy combinations produced effective results in randomized controlled trials [42-44]. Currently, various DMARDs in combination, a widely used therapeutic alternative, can show good responses in clinical course of RA [45]. Therefore, combination of various biologics can be a potential therapy for RA. Here, it was shown that the combination of omega 3, zinc and CoQ10 attenuates ZIA development, inflammation and osteoclasts differentiation. The combination might be a therapeutic agent for RA.

## Conclusion

Our findings of the additive activity of omega 3, zinc and CoQ10 indicate the potential use in treating RA. Previously, CoQ10 treatment showed therapeutic effect in experimental arthritis [24] thus, the combination might improve autoimmune arthritis. This investigation suggests that the combination of omega 3, zinc and CoQ10 inhibits inflammatory response and osteoclastogenesis in ZIA and is a potential candidate for the RA therapy.

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