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Combination Therapy with Glucan and Coenzyme Q_{10} in Murine Experimental Autoimmune Disease and Cancer

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Abstract. *Background/Aim: Coenzyme Q_{10} is a well-accepted anti-oxidant agent known to play a protective role in various physiological and disease processes. Recently, Coenzyme Q_{10} is gaining attention as a substance with significant anti-inflammatory properties. β-Glucan is the most studied immunomodulator with significant synergetic effects with numerous bioactive molecules. We aimed to evaluate the possible synergistic effects of simultaneous use of coenzyme Q_{10} with the well-established immune modulator, β-glucan, on immune reactions and cancer development. Materials and Methods: Coenzyme Q_{10} and β-glucan were used, both in vivo and in vitro, and their effects were evaluated using phagocytosis and cytokine secretion. Results: Our study confirmed the strong anti-inflammatory effects of coenzyme Q_{10} and showed that these effects were further potentiated with the addition of β-glucan. The anticancer effects of coenzyme Q_{10} were less pronounced, but stronger, with the addition of β-glucan. Conclusion: There is significant synergy between coenzyme Q_{10} and β-glucan.*

Coenzyme Q_{10} (CoQ_{10}), also known as ubiquinone or ubidecarenone, is an essential substance for electron transport in oxidative phosphorylation, and forms an important component in the respiratory chain at the mitochondrial level. It is a lipophilic molecule present in the inner side of mitochondrial membrane. Commercially, it usually exists in the form of ubiquinone, but for the real biological effects, its reduced form, ubiquinol, should be utilized.

In addition to functioning as an electron carrier, CoQ_{10} also serves as an important antioxidant. Numerous reports evaluating the biological effects of CoQ_{10} have found decreased LPS-induced release of TNFα (1). Other studies have revealed significant anti-inflammatory, antiangiogenic, and anti-nociceptive activities probably *via* suppressing the level of nitric oxide (2). In rodents, CoQ_{10} inhibited the oxidative damage in blood, heart, kidney, and liver (3). Additional studies have shown prevention of atherosclerosis by attenuation of LDL oxidation and endothelial lesions (4), improving quality of life in patients with end-stage heart failure (5) and antiaging effects (6). In addition to the effects on various aspects of immune system, CoQ_{10} was also found to positively affect fatigue (7), have positive effects on type 1 and type 2 diabetes mellitus (8), and serve as a preventive agent against microcystin-LR–induced toxicity acting *via* modulation of oxidative stress (9).

Further studies have shown suppression of TNFα and IL-2 secretion by human blood cells after CoQ_{10} supplementation, suggesting possible mechanisms affecting immune functions (10). In addition to anti-inflammatory effects, CoQ_{10} also induced Treg (11) in graft *versus* host disease (12); some studies have suggested a possible role in the inhibition of carcinogenesis (13, 14).

A detailed study of the mechanisms of action revealed that actions of CoQ_{10} are mediated *via* inhibition of NFκB/AP-1 activation and induction of Nrf2/ARE signaling (15). Most of the studies are based on supplementation with CoQ_{10}. One study, however, described a case of a girl with immune dysregulation and CoQ_{10} deficiency. All conditions significantly improved after immunoglobulin and CoQ_{10} replacement therapy (16).

β-Glucans are structurally complex homopolymers of glucose, isolated from various sources including yeast, fungi, and wheat. Their role as biologically active immunomodulators has been well documented for more than 50 years (17-20). The positive effects of β-glucan treatment have been repeatedly confirmed in clinical trials (21).

Despite being the most studied immunomodulator, β-glucan is clearly not the only known immunomodulator. Scientists are increasingly experimenting with improvements of β-glucan action by adding additional immunomodulators. Therefore, combinations of various natural immunomodulating molecules

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are becoming more popular. The most common feature of these mixtures is β-glucan, which, 20 years ago, was found to have strong synergy with vitamin C (22). Later studies have found strong synergy between yeast-derived β-glucan and humic acid, with potentiated phagocytosis, cytokine release, and protection against hepatotoxicity (23). Recently, our group found that β-glucan combined with resveratrol and vitamin C showed significant improvements in stimulation of both cellular and humoral immunity, including anticancer activities (24). Additional findings indicated that adding *Withania somnifera* extract to the Maitake mushroom β-glucan significantly regulates stress-induced increase on corticosterone levels (25). The aim of this study was to evaluate the possible synergistic effects of glucan and CoQ_{10} on immune reactions and cancer development.

Materials and Methods

Materials. RPMI 1640 medium, HEPES, penicillin, streptomycin, carrageenan, cyclophosphamide, TNFα, and methotrexate were purchased from Sigma (St. Louis, MO). Fetal calf serum (FCS) was purchased from Hyclone Laboratories (Logan, UT, USA). Incomplete Freund's adjuvant was purchased from Difco Laboratories, Detroit, MI, USA). CoQ_{10} with ubiquinol content over 30% was purchased from Kaneka (Pasadena, TX, USA).

Cell lines. RAW264.7, Ptas64, and Lewis lung cancer cell lines were obtained from the ATCC (Manassas, VA, USA) and maintained in RPMI 1640 medium containing HEPES buffer supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, in plastic disposable tissue culture flasks at 37˚C in a 5% CO_2/95% air incubator.

Animals. Female, 8-week-old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by cervical dislocation. Adjuvant arthritis (AA) was induced by a single intradermal injection of heat-inactivated Mycobacterium butyricum in incomplete Freund's adjuvant. Methotrexate treatment consisted of oral doses of 0.3 mg/kg twice a week. CoQ_{10} was used at 1 mg/mouse dose, glucan at 100 μg/mouse.

Carrageenan-induced inflammation. A carrageenan-induced inflammation in the air pouch technique was used as described previously (2).

Immunological tests. Supernatants from cultured cells were tested by ELISA assay according to manufacturer's instructions using a Quantikine mouse IL-1α and IL-1β kit (R&D Systems, Minneapolis, MN). The technique employing phagocytosis of synthetic polymeric microspheres was described by Vetvicka *et al*. (26). For evaluation of possible inhibition of TNFα-mediated inflammatory reaction, Griess assay was performed as described (27). For evaluation of imultaneous phagocytosis and oxidative burst, the double fluorescence of FITC-labeled *Staphylococcus aureus* cells and hydrohyethidine oxidized to ethidium bromide was tested by flow cytometry as described (28).

Table I. *Effect of CoQ_{10} and glucan on carrageenan-induced inflammation in the air pouch model.*

Group	Volume of exudate (ml)	No. of total leukocytes ($\times 10^7$ cells)	Nitrite (μM)
PB S	2.47±0.03	4.76±0.43	22.77±0.36
CoQ_{10}	2.11±0.05*	3.29±0.26*	18.01±0.67*
Glucan	2.20±0.10*	3.32±0.28	19.91±0.57*
CoQ_{10} + Glucan	1.98±0.11*	1.65±0.07	6.11±0.23*

Results are expressed as mean±SD. *Results are significant at the $p<0.05$ level.

Breast cancer model. Mice were injected directly into the mammary fat pads with 1×10^6/mouse of Ptas64 cells in PBS. The experimental treatment was begun after palpable tumors were found (usually 14 days after injection of cells) and after mice were assigned to experimental groups. Experimental treatment was achieved by intraperitoneal injections of tested samples diluted in PBS (once/day for 14 days). After treatment, the mice were sacrificed, and tumors removed and weighed (29).

Lung cancer model. For Lewis lung carcinoma therapy, mice were injected IM with 1×10^5 of Lewis lung carcinoma cells. Cyclophosphamide (30 mg/kg) was used IP at day 8 after tumor application (positive control), individual substances were used from day 0 to day 14 after tumor application. The control group of mice (negative control) received IP PBS daily. Each group held a minimum of five mice. At the conclusion of the experiment (day 14), mice were euthanized, their lungs were removed and fixed in 10% formalin, and the number of hematogenic metastases in lung tissue was estimated using a binocular lens at 8x magnification.

Results

Glucan is a natural immunomodulator, originally supposed to influence natural immunity only. Not surprisingly, phagocytosis remains to be one of the most commonly studied reactions. For the glucan administration, we used a dose of 100 μg/mouse, which is the most commonly used dose for glucan experiments in mice. CoQ_{10} was used at 1 mg/mouse dose, based on our preliminary experiments using a 500 mg/mouse to 5,000 mg/mouse range (data not shown). Data summarized in Figure 1 show that a glucan–CoQ_{10} combination improves the effects of glucan.

In the air pouch model, CoQ_{10} significantly reduced the exudate volume, total number of leukocytes, and nitrite formation. Glucan alone had similar, but less pronounced effects. A glucan–CoQ_{10} combination had stronger effects, particularly on lowering the nitrite production and total number of leukocytes (Table I).

To examine the potential therapeutic function of CoQ_{10} and glucan in inflammatory reaction of macrophages, we used

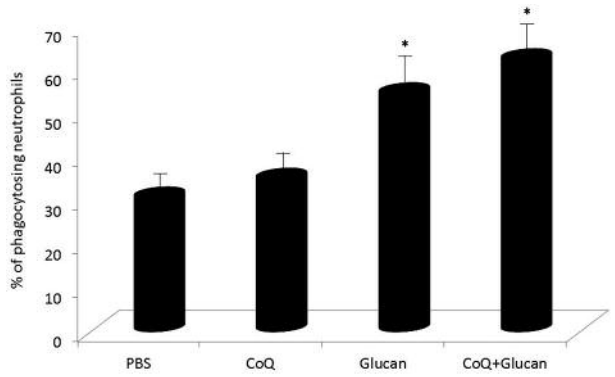

Figure 1. *Effect of oral administration of individual samples on phagocytosis by peripheral blood monocytes. Each value represents the mean±SD. *Represents significant differences between control (PBS) and tested samples at p≤0.05 level. All experiments were performed in triplicate.*

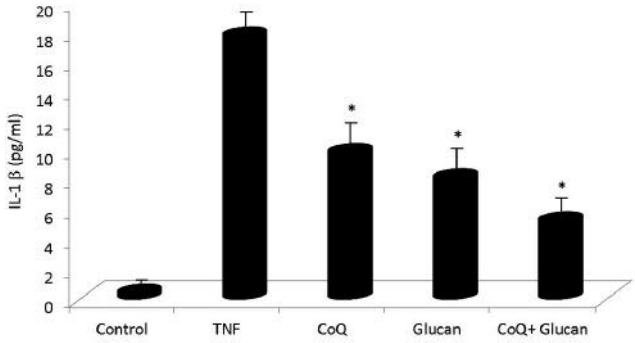

Figure 3. *Effect of CoQ_{10} and/or glucan addition on TNFα-mediated IL-1β synthesis by RAW264.7 cell line. Each value represents the mean±SD. *Represents significant differences between control (PBS) and tested samples at p≤0.05 level. All experiments were performed in triplicate.*

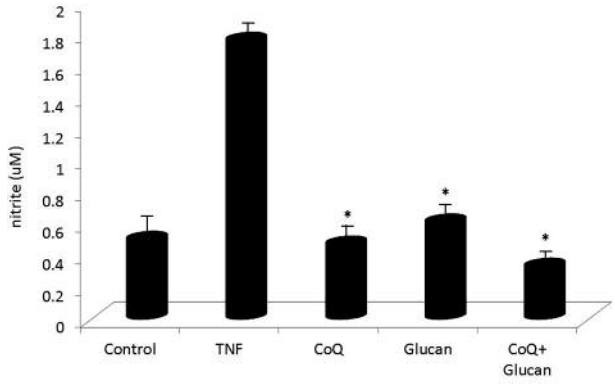

Figure 2. *Effect of CoQ_{10} and/or glucan addition on TNF-α-mediated NO synthesis by RAW264.7 cell line. Each value represents the mean±SD. *Represents significant differences between control (PBS) and tested samples at p≤0.05 level. All experiments were performed in triplicate.*

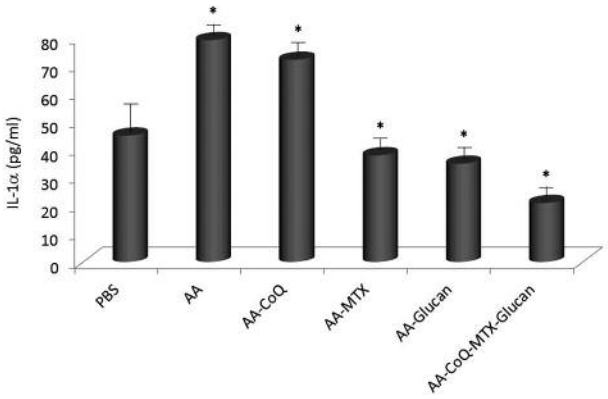

Figure 4. *Effect of CoQ_{10} and/or glucan addition on IL-1α in plasma in the model of adjuvant arthritis on day 28. Each value represents the mean±SD. *Represents significant differences between control (PBS) and tested samples at p≤0.05 level. All experiments were performed in triplicate.*

macrophage cell line, RAW264.7. We incubated these cells with 10 ng/ml TNFα, in the presence or absence of 20 μM CoQ_{10} and/or 10 μg glucan. As shown in Figure 2, both agents alone or in combination significantly abolished the levels of nitrite formation caused by TNFα. Similar results were found in case of IL-1β production (Figure 3). In both cases, the glucan–CoQ_{10} combination had stronger effects.

For further evaluation of the possible synergy between glucan and CoQ_{10}, we measured the level of IL-1α in plasma. In animals with AA, CoQ_{10} had only limited effects, compared to methotrexate monotherapy. Surprisingly, glucan treatment showed the same effects as methotrexate alone. The glucan–methotrexate–CoQ_{10} combination was the most active treatment (Figure 4).

Next, we evaluated the functionality of blood neutrophils by testing the phagocytosis, oxidative burst, and metabolic activity (Figure 5). Both phagocytosis and oxidative burst were increased due to the AA. Metabolic activity of neutrophils is the percentage of double positive cells – simultaneously phagocytosing and positive for oxidative burst. The immunosuppressive effects of methotrexate were found in all tested parameters, not only when compared to AA, but even to controls. Individual addition of CoQ_{10} or glucan increased phagocytosis and oxidative burst.

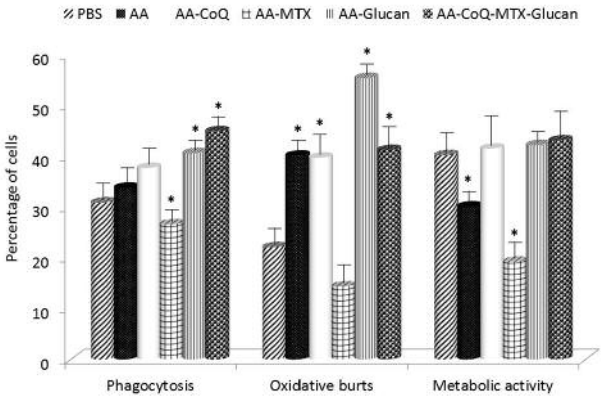

Figure 5. *Functionality of peripheral blood neutrophils determined on day 7 of induced adjuvant arthritis. Each value represents the mean±SD. *Represents significant differences between control (PBS) and tested samples at p≤0.05 level. All experiments were performed in triplicate.*

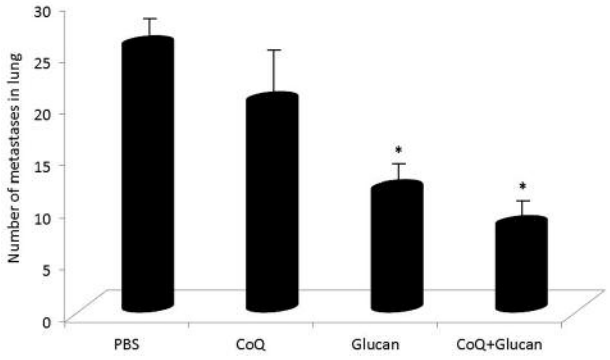

Figure 6. *Effect of tested substances on lung cancer growth in cyclophosphamide treated mice. Cyclophosphamide was injected into mice on day 8 of the inoculation of 1×10^5 tumor cells, followed by the daily oral doses of individual substances starting 48 hrs after injection of cyclophosphamide. Each value represents the mean±SD. *Represents significant differences between glucan alone and glucan/Se combination at p≤0.05 level.*

After evaluation of the effects of tested substances on individual activities, we also tested the effects on cancer. To be sure the effects of these samples truly reflected their potential anticancer properties, we used two different experimental models. With the first, using a well-defined Lewis lung carcinoma model, we found that whereas CoQ_{10} had only insignificant effects, glucan alone strongly lowered the number of lung metastases. Similarly, a glucan–CoQ_{10} combination was highly active, but the differences between a combination and glucan alone were not statistically significant (Figure 6). With the second, using a breast cancer model, we monitored the changes in tumor weight. Our results showed identical results (Figure 7).

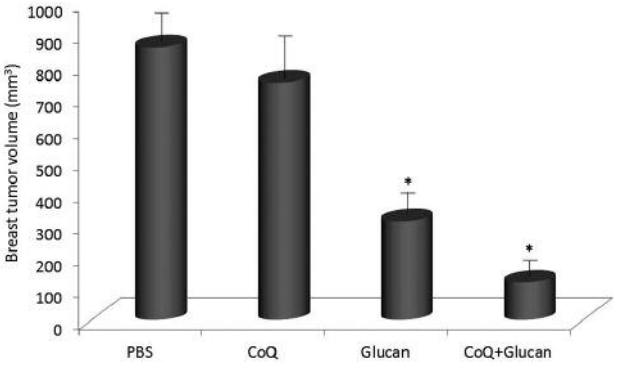

Figure 7. *Therapy of Balb/c mice with Ptas64 mammary carcinoma. Data from three independent experiments are shown. For each experiment, groups of three mice were tested for a response to therapy as indicated by the weight of tumors after two weeks of supplementation. Each value represents the mean±SD. *Represents significant differences between control and experimental samples at p≤0.05 level.*

Discussion

Immunomodulators usually offer systemic effects and the mechanisms of their effects are often unknown. We focused on the hypothesis that glucan and CoQ_{10} might together offer higher biological effects than individual molecules.

Polysaccharides, such as glucans, have been studied for almost a century. Almost 80 years ago, Shear and coworkers isolated a substance causing tumor necrosis from the culture of *Serratia marcescens* (30). From this pioneering research, subsequent scientific interest has resulted in over 16,000 studies of glucan activities, making glucan the most studied natural immunomodulator; several in-depth reviews are available (20, 31-34). Most of the glucan studies focused on cancer treatment (35). At the same time, the activities of glucans were found to increase when used together with various substances such as monoclonal antibodies (36) or vitamin C (37, 38). Our own previous research focused on basic immunostimulating capacity of a combination of glucan and resveratrol and showed the significant synergy of these two compounds (39).

Inflammation is one of the leading causes of mortality in the western world. Dietary supplementation with antioxidants is one of the commonly thought solutions, supported by numerous studies (40). CoQ_{10} is one of the molecules found to have antiinflammatory and antirheumatic properties (41). In addition, significant changes in the levels

of CoQ_{10} were found in a variety of diseases in both animal and human models. Currently, it is not clear if these changes are due to the excessive utilization of CoQ_{10} or by impairment in biosynthesis (42).

Despite numerous studies showing positive effects of CoQ_{10} on various biological reactions, the search is on for potential improvement of these effects. One possibility is a combination of CoQ_{10} with another bioactive molecule. A combination therapy with CoQ_{10} and additional bioactive substances, such as metformin, showed strong effects against autoimmune diseases including arthritis (43).

We tested peripheral blood neutrophils for changes in phagocytosis. Using synthetic microspheres based on 2-hydroxyethyl methacrylate, known for an extremely low spontaneous adhesion to the cell membrane and therefore minimal false negativity, we found that both glucan and glucan–CoQ_{10} combination caused a significant increase in phagocytosis, whereas CoQ_{10} had no effect. The data shown reflect the effects of a 3-day daily oral supplementation with tested substances.

The entire process of phagocytosis and subsequent production and often release of reactive oxygen metabolites are extremely important parts of the defense mechanisms. Neutrophils are heavily involved in most processes of recognition and elimination of invading pathogens. In a model of AA, we found that already seven days after AA induction, AA is accompanied by an increased number of peripheral blood neutrophils. These findings are in agreement with similar effects of CoQ_{10} found earlier (28).

Anti-inflammatory activity of tested substances was measured using an *in vivo* experimental model of air pouch, which measures the acute inflammatory response as an increase of cellular infiltration (44). Since nitric oxide represents an important intracellular pro-inflammatory mediator, changes in its level in air pouches were determined after treatment with tested substances. Changes in nitric oxide level production are considered to be linked with anti-inflammatory actions. Both individual substances decreased accumulated nitrite, an index of nitric oxide, but the combination of glucan–CoQ_{10} was three times more active. From these experiments, we can conclude that both substances possessed significant anti-inflammatory activity, with the highest activity seen with the glucan–CoQ_{10} combination. In addition, our results showed that incubation with both agents (and their combination in particular) strongly inhibited the levels of inflammatory biomarkers in the presence of TNFα. We presume that these effects were mediated by their antioxidant activity (45, 46).

In the last part of our study, we focused on potential role of CoQ_{10} and glucan on cancer. Whereas cancer-suppressive effects of glucan are well established in both animal and human models (see reviews by Vannucci *et al*. (20) and Sima *et al*. (47)), the possible role of CoQ_{10} is much less known. From the original study suggesting that it may have a potential for the cancer treatment (48), subsequent studies have focused more on the levels of CoQ_{10} in cancer patients (49 50) than on the therapeutic use (13). Some studies found progress on breast cancer therapy after food supplementation with CoQ_{10} (51). A combination of CoQ_{10}, nutritional antioxidants, and essential fatty acids resulted in partial remission of breast cancer (52), which led us to the study of a CoQ_{10}–glucan combination. Using two different cancer models, we found that whereas CoQ_{10} alone has only limited effects on cancer development, the combination with glucan further improves glucan's effects.

To answer the question of possible mechanism/s of action of the combination used in our study is difficult. Glucan works *via* binding to specific receptors, subsequent activation of several intracellular pathways leading to activation of cells able to kill tumors either directly or *via* release of bioactive molecules. Much less is known about the action of CoQ_{10}. Experiments focused on evaluation of the mechanism of action are in progress.

In conclusion, CoQ_{10} has strong anti-inflammatory effects in all experimental models, both *in vivo* and *in vitro*. These effects are more pronounced when the CoQ_{10}–glucan combination is used, suggesting that this combination has a potential for further development in anti-inflammatory and anticancer treatment.

Conflicts of Interests

No conflicts of interests exist for the authors.

Acknowledgements

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