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Preclinical and Clinical Evaluation of Nutritional Supplement Designed to Protect Against COVD-19

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Is this study a clinical trial?<hr><i>A clinical trial is defined by the World Health Organisation as 'any research study that prospectively assigns human participants or groups of humans to one or more health-related	No

interventions to evaluate the effects on health outcomes'.</i>	

Preclinical and Clinical Evaluation of Nutritional Supplement Designed to Protect Against COVD-19

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Abstract:

Currently there are no science-based, over the counter interventions available to address the coronavirus pandemic. Conventional drugs in development are not expected to reach the market for at least 18 months. Here we assess preclinical and clinical effects of QuadraMune™, a nutraceutical developed to address issues of susceptibility, inflammation, and viral immunity. The product is formulated from 4 main active ingredients: 1) Pterostilbene, which stimulates natural killer cells and reduces inflammation; 2) Epigallocatechin gallate (EGCG), an activator of T cells; 3) Sulforaphane, which protects lungs from pathology; and 4) Thymoquinone, chemically related to hydroxychloroquine, possessing antiviral effects. We demonstrate that the combination product is effective at suppressing inflammatory markers while upregulating NK cell activity in vitro and in healthy volunteers. These data serve as the basis of larger clinical trials in patients with COVID-19.

Introduction

SARS-CoV-2, the viral pathogen causative of COVID-19, is a novel coronavirus that is most phylogenetically similar to SARS. The virus is presumed to have initially been transmitted from an animal reservoir (bats) to humans, most likely via an amplifying host (pangolin) [1, 2]. It is a single strand positive sense RNA virus whose infectivity is mediated by the envelope spike (S) glycoprotein which binds to its cellular receptor angiotensin-converting enzyme 2 (ACE2) [3]. Interestingly, antibody responses have also been detected towards the S glycoprotein [3-6].

It is known that Coronaviruses (CoVs) belong to a subfamily of large and enveloped viruses containing a single strand of sense RNA. There are four genera, of CoVs, i.e., alpha, beta, gamma, and delta, of which alpha- and beta-CoVs are known to infect humans [7]. Infectivity of CoVs is mediated by the envelope spike (S) glycoprotein which binds to its cellular receptors angiotensin-converting enzyme 2 (ACE2) and dipeptidyl peptidase 4 (DPP4) for SARS-CoV and MERS-CoV, respectively, this facilitates fusion of the virus with the membrane [8, 9]. The viral RNA genome is released into the cytoplasm; after replication of the viral genome, genomic RNA accompanied by envelope glycoproteins and nucleocapsid proteins forms virion-containing vesicles, which then fuse with the plasma membrane to release the virus [10]. The viral cause of COVID-19 is attributed to the SARS-CoV-2 type of CoV, which was found to be a new type of beta-CoV with more than 99.98% genetic identity among 10 sequenced samples collected from the original site of the outbreak [11]. SARS-CoV-2 is genetically more similar to SARS-CoV than to MERS-CoV [12]. Subsequent to the virus binding to the target cells, which appear to be primarily type 2 pulmonary epithelial cells, the virus fuses with the cellular membrane, allowing viral entry into the cytoplasm [13]. Once the viral genome is unleashed in the cell, it replicates and the viral RNA accompanied by envelope glycoproteins and nucleocapsid proteins form virion-containing vesicles, which then fuse with the plasma membrane to release the virus [14].

Currently no interventions have demonstrated efficacy in double blind trials, however multiple approaches are in development. These can be categorized into a) antigen specific vaccines [15-18]; b) innate immune stimulators [19-21]; c) small molecule antivirals; d) small molecules which modulate viral interactions with host cells [22-25]; e) plasma/antibodies from patients who recovered [26-36]; f) small molecule blockers of cytokine signaling [37-40]; g) antibodies to inflammatory cytokines [41-49]; and h) cell based therapies [50-60].

At present there appears to be no clear consensus on which approaches are most promising, with various institutions utilizing differing protocols. It is expected that clinically significant signals will be reported in up to 18 months from now. In the absence of established pharmaceutical approaches, exploration of scientific based natural based treatments may have merit. Below we will describe the in vitro and in vivo activities of QuadraMune™ which we propose to investigate as a prophylactic to COVID-19.

Materials and Methods

Patients

32 healthy volunteers between 18-65 years of age, lacking chronic or active disease were made aware of the investigational nature of the project, potential risks and potential benefit to the “greater good” if they choose to enter the trial. Eligible and willing volunteers were recruited and signed informed

consent. The study was approved by the La Jolla Institutional Review Board, Protocol #2429. Patients were randomized into 4 groups, and administered QuadraMune™, or placebo. Group 1 was placebo control, Group 2, received 2 pills per day, group 3 received 4 pills per day, and group 4 received 8 pills per day. The trial lasted 7 days. QuadraMune™ was provided by Therapeutic Solutions International.

In Vitro Experiments

Individual ingredients of QuadraMune™ were purchased from Millipore Sigma and diluted to appropriate concentrations as described. Reagents were admixed with monocytes which were isolated from peripheral blood mononuclear cells (PBMC) by plastic adherence. Monocytes were plated in 96 well flat bottom plates at a concentration of 100,000 monocytes per well. LPS was added at a concentration of 100 ng/ml to induce cytokine production. Media comprised of Roswell Park Memorial Institute (RPMI)-1640 with 10% fetal calf serum. Cells were cultured in fully humidified environment with 5% carbon dioxide at 37 Celsius. Incubation was performed for 48 hours and cytokine levels were analyzed by enzyme linked immunosorbent assay (ELISA).

Blood Immune Analysis

Blood was taken on days 3, 5 and 7 in EDTA tubes and mononuclear cells were purified using Ficoll technique. Cells were stimulated in vitro with LPS as described above and cytokines analyzed after 48 hours by ELISA. Natural Killer (NK) activity was assessed using dual fluorescent staining of target cells (K562 cell line). The DIOC18 dye labeled K562 cells were incubated with whole blood and then counterstained with 7-AAD enabling the measurement of dead target cell and then percent cytotoxicity was calculated [61]. For assessment of T cell proliferation, 100,000 PBMC were plated in round bottom 96 well plates and stimulated with 5 micrograms per ml of phytohemmaglutinin (PHA) for 48 hours. Media comprised of Roswell Park Memorial Institute (RPMI)-1640 with 10% fetal calf serum. Cells were treated with 1 microCurie per well of tritiated thymidine and proliferation was measured by scintillation counting, expressed as counts per minute (CPM).

Results

QuadraMune™ Combination Yields Superior Inhibition of Inflammatory Cytokines to Individual Components

TNF-alpha is a macrophage derived fundamental driver of inflammation, which has been shown to be upregulated in patients with COVID-19 [62]. Some studies suggest that blockade of TNF-alpha possesses some efficacy at reducing COVID-19 progression [63]. Accordingly, we established an experimental system in which human monocytes were activated with the toll like receptor 4 agonist lipopolysaccharide (LPS) as an in vitro model of cytokine storm. We added various concentrations of the components of QuadraMune™ in vitro and assessed production of TNF-alpha. As seen in Figure 1, reduction of TNF-alpha production from LPS activated monocytes was noted with pterostilbene (Figure 1a), thymoquinone (Figure 1b), EGCG (Figure 1c) and sulforaphane (Figure 1d). Interestingly, the combination of the ingredients led to increased suppression of TNF-alpha production (Figure 2). These data suggest the possibility that the combination of natural ingredients in QuadraMune™ possess additive if not synergistic effects in suppressing this important mediator of inflammation.

Inteleukin-6 is a potent inflammatory cytokine whose expression correlates with severity of COVID-19 [64]. Currently several clinical trials have suggested potential efficacy of antibodies to interleukin-6 as a

means of treating COVID-19 [65-68]. Additionally, studies in classical acute respiratory distress syndrome (ARDS) show negative correlation between levels of interleukin-6 and poor prognosis [69, 70]. Similar to TNF-alpha, we observed using the same in vitro system that treatment with pterostilbene (Figure 3a), thymoquinone (Figure 3b), EGCG (Figure 3c) and sulforaphane (Figure 3d) all led to reduction of interleukin-6 production. Most interestingly, as with TNF-alpha, the post potent inhibition was observed when all four ingredients were combined (Figure 4).

Pilot Clinical Trial in Healthy Volunteers

Healthy volunteers (8 per group) were treated with increasing concentrations of QuadraMune™ or placebo control. Dose 1 was equal to the recommended dose of QuadraMune™, which is 250 mg of Pterostilbene, 500 mg of black cumin seed (Nigella Sativa), 500 mg of Green Tea Extract, and 500 mg of Broccoli Sprout Extract, these being take twice a day. Dose 2 was double, and Dose 3 was 4 times the concentration of Dose 1.

Peripheral blood mononuclear cells (PBMC) were extracted and stimulated in vitro with LPS at days 3, 5, and 7 after initiation of therapy. Interestingly, there was a negative dose response between concentration of QuadraMune™ and suppression of IL-6 (Figure 5). Regardless, at the recommended dose, QuadraMune™ seemed to suppress induced IL-6 production by more than 85%.

Blood samples were also analyzed for cytotoxic activity of natural killer cells against target cell like K562. In contrast to suppression of IL-6, treatment with QuadraMune™ increased activity of these innate immune cells (Figure 6). In order to assess whether any impact on adaptive immunity was attained after administration of QuadraMune™, we also assessed T cell proliferative activity in response to mitogenic stimulation with phytohemagglutinin (PHA). As seen in Figure 7, a more than double augmentation of proliferative activity was observed in patients treated with QuadraMune™. In line with other parameters, no dose response was observed.

Discussion

To date there are no FDA cleared means of preventing COVID-19. Despite work being performed at a "Warp-Speed" in vaccine development, these efforts are still believed to be months if not years away. We aimed to perform a series of experiments to assess preclinical and pilot clinical feasibility of QuadraMune™, an over the counter available "nutritional supplement" which was developed to address the issue of immune stimulation while inhibiting inflammation.

The current data suggest that the combination of ingredients in QuadraMune™, individually possess anti-inflammatory properties, from the data demonstrating suppression of TNF-alpha and interleukin-6. Furthermore, the combination of the ingredients was demonstrated to be more potent than the ingredients in isolation.

In vivo administration of QuadraMune™ was successful in suppressing both TNF-alpha and IL-6. Interestingly, the suppression was not dose dependent and was not cumulative over time. Future studies are needed to assess whether lower doses may be utilized without losing efficacy. Importantly, even volunteers at 4 times the dose commercially used did not experience any adverse effects. One interesting observation is that although no medical claims are made for QuadraMune™, case reports of remission of rheumatoid arthritis have been conveyed to the authors by patients taking this supplement. Given that patients with rheumatoid arthritis respond to FDA approved therapy based on

suppressing TNF-alpha or interleukin-6 [71, 72], it is interesting to speculate that QuadraMune™ may have activity against these conditions.

Although suppression of inflammation can be achieved with numerous agents available today, one of the concerns is that approaches, such as glucocorticoids, sometimes block the immune system. Accordingly, we assessed NK activity in healthy volunteers. More than two-fold stimulation of NK cell activity was observed. The increased natural killer cell activity we observed is interesting. Other studies have reported that components in QuadraMune, in various systems, upregulate NK activity. One speculation, of relevance to COVID-19, is that interleukin-6 may act as an inhibitor of NK activity. In fact, in a recent paper, reduced cytotoxic potential was identified in COVID-19 patients, particularly in those that required intensive care. The latter group of patients showed also increased serum IL-6 levels, that correlated to the frequency of granzyme-expressing NK cells. Off-label treatment with tocilizumab, which blocks IL-6, restored the cytotoxic potential of NK cells [73].

Furthermore, lymphopenia is often observed in patients with COVID-19 [74, 75], which is believed to lead to unrestrained innate immune activation and cytokine storm [76]. It is also known that in numerous situations, COVID-19 is associated with suppression of T cells to proliferate. Accordingly, we assessed propensity of T cells to multiply after stimulation with a known T cell activator, PHA. An increase in proliferation was similar to that observed with the increase in NK cytotoxicity.

These data are preliminary and long term effects of QuadraMune™ administration on the immune system are unknown. Despite an excellent safety record, and the product being used commercially, it is difficult to state whether the immune stimulatory and anti-inflammatory effects observed will maintain throughout a long term period of use.

In conclusion, we present data which supports further clinical development QuadraMune in trials of COVID-19 patients and patients at risk of developing COVID-19.

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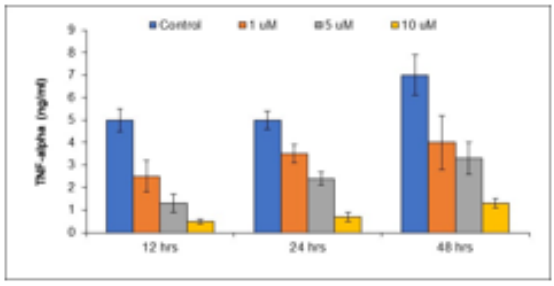
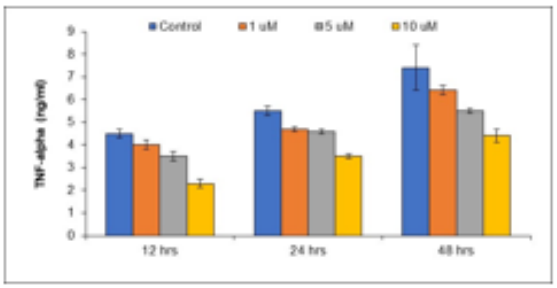
Figure 1. Suppression of TNF-alpha Production by Individual Ingredients of QuadraMune™

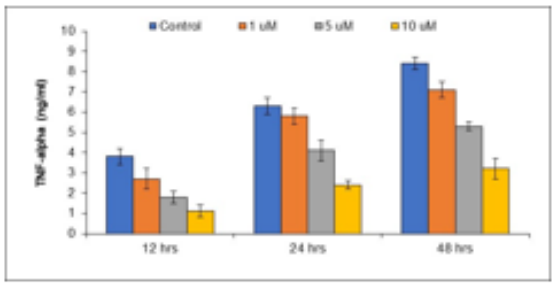
a) Pterostilbene



b) Thymoquinone



c) EGCG



d) Sulforaphane

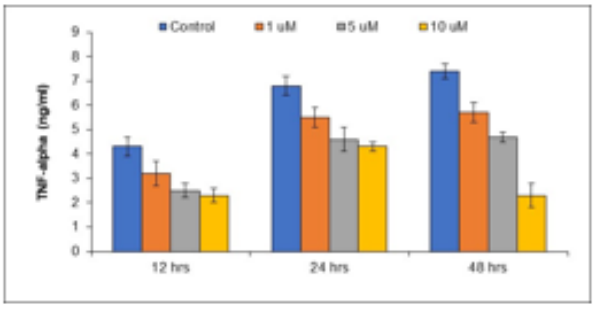


Figure 2. QuadraMune™ is Superior to its Individual Components in Suppression of TNF-alpha

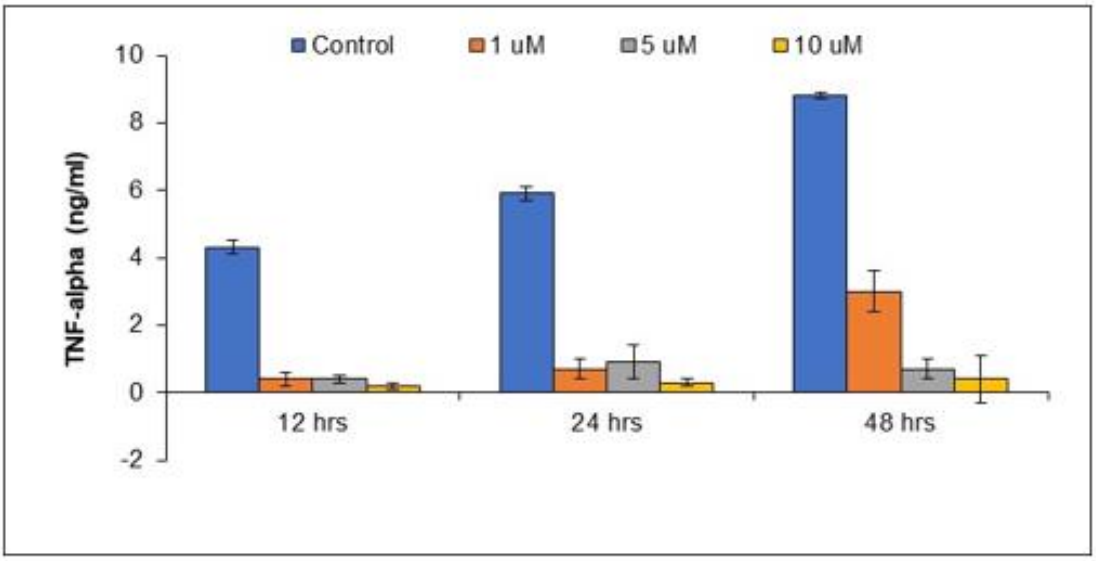
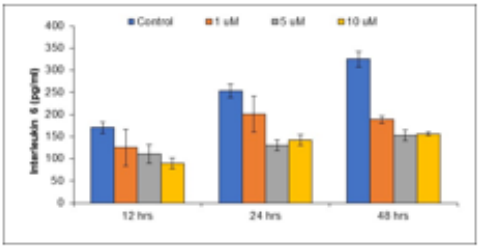
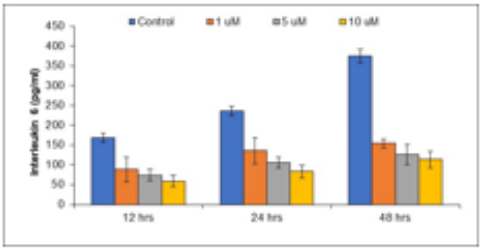


Figure 3. Suppression of Interleukin-6 Production by Individual Ingredients of QuadraMune™

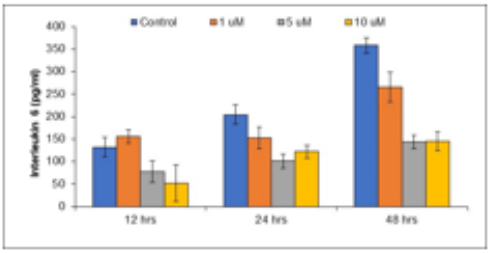
a) Pterostilbene



b) Thymoquinone



c) EGCG



d) Sulforaphane

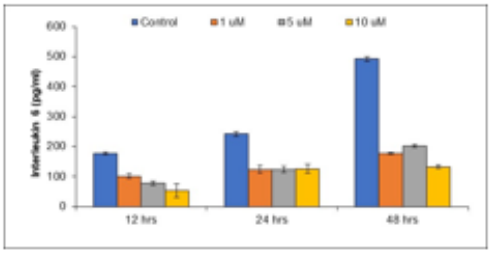


Figure 4. QuadraMune™ is Superior to its Individual Components in Suppression of IL-6

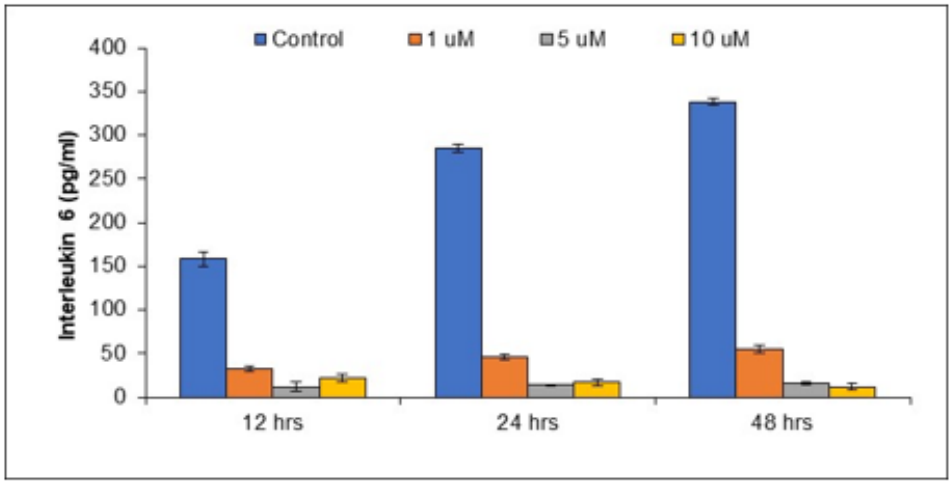


Figure 5. QuadraMune™ Inhibits Interleukin-6 in Healthy Volunteers

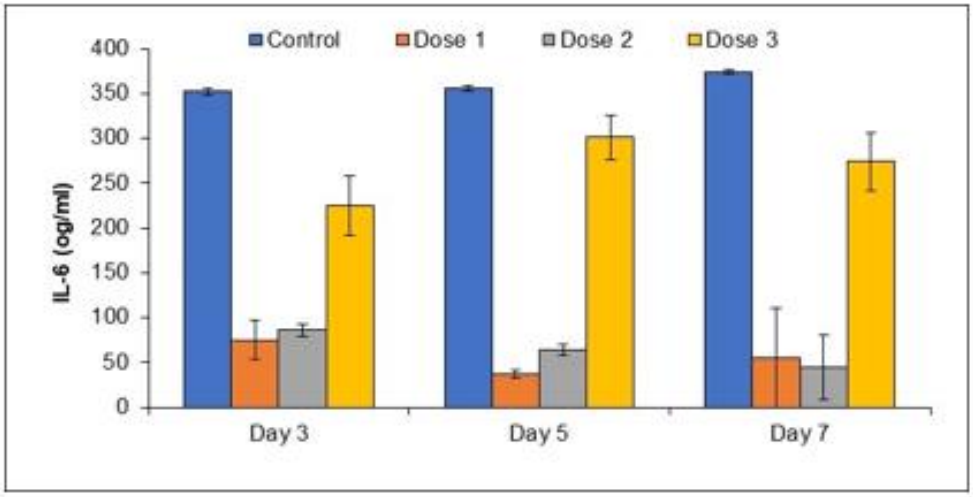


Figure 6. QuadraMune™ Increases NK Activity in Healthy Volunteers

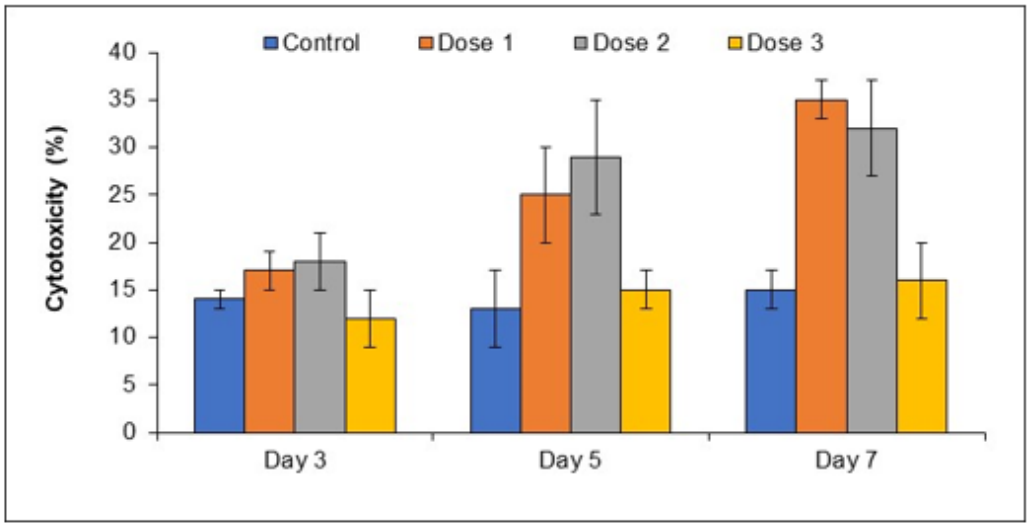


Figure 7. QuadraMune™ Increases T Cell Mitogenic Activity