Original Research Paper

A Novel Glucan-Sulforaphane Combination Stimulates Immune Response to Influenza in Mouse Model

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Article history
Received: 06-05-2016
Revised: 27-07-2016
Accepted: 28-07-2016

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Abstract: Influenza remains a serious health problem and causes approximately 500,000 deaths world-wide. With current available treatments offering neither dependable protection nor a rapid cure, many have focused their attention to alternative treatments. The aim of this study was to evaluate the possible effect of a novel maitake glucan-sulforaphane combination on immune response against influenza challenge in mice. We evaluated the effects of a glucan-sulforaphane combination on basic immune reactions, virus titre and overall survival after influenza infection. Results: We found 2 weeks supplementation with this glucan-sulforaphane combination significantly improved immunosuppression caused by the viral infection. Based on these results, we conclude that the significant immunostimulation caused by this combination helps to overcome virus-dependent suppression of defense reactions and that addition of sulforaphane to glucan can improve already established biological effects of glucan.

Keywords: Glucan, Sulforaphane, Influenza, Immune System, Virus

Introduction

The concept of curing or at least ameliorating individual diseases using natural remedies is as old as mankind. β-Glucans represent one of the most studied natural immunomodulators with over 10,000 studies published in peer-reviewed journals. Glucans, most of all 1,3/1,6-β-D-glucans (hereafter referred to as "glucans"), are complex carbohydrates forming structural parts of cell walls of mushroom, yeast and seaweed. Their biological effects are well established and include stimulation of anti-infectious and anti-cancer immunity, inhibition of stress, lowering cholesterol levels and inhibition of inflammation of the gastrointestinal tract (Vetvicka, 2013; Vannucci *et al*., 2013).

Interests in glucans are gaining amongst people focused on promoting and supporting human health with natural compounds. After establishing glucans as an official drug in Japan (Ina *et al*., 2013), a series of clinical studies are evaluating other effects of glucans, from stimulation of salivary immunity in children (Vetvicka *et al*., 2013; Richter *et al*., 2014) to using glucans as part of the vaccine against neuroblastoma (Kushner *et al*., 2014).

In addition to the use of various immunomodulators, studies have established that improved nutrition, specifically increasing consumption of fruit and vegetables, leads to a decrease in the incidence of many diseases. Aside from the vitamins and minerals contained in fruits and vegetables, these health benefits are usually resulting from the action of various phytochemicals. One such phytochemical is sulforaphane, derived from its precursor glucoraphanin found in broccoli and other cruciferous vegetables. Sulforaphane is an isothiocyanate that is created from the hydrolysis of the glucosinolate glucoraphanin. Sulforaphane has demonstrated anti-inflammatory effects (Checker *et al*., 2015), strong ability to induce apoptosis in adipocytes (Yao *et al*., 2015) and significant anti-cancer activities (Wang *et al*., 2015).

Influenza remains a serious health problem and causes approximately 500,000 deaths world-wide. With current available treatments offering neither dependable protection nor a rapid cure, many have focused their attention to alternative treatments. Some immunomodulators have been shown to reduce mortality (Zheng *et al*., 2008). Our own study found enhancement of immune response against influenza challenge by oral stimulation with glucans (Vetvicka and Vetvickova, 2015). The findings that sulforaphane can regulate susceptibility to influenza in human cells (Kesic *et al*., 2011) together with previous studies showing enhancements of immune response in influenza-challenged mice by glucan supplementation (Vetvicka and Vetvickova, 2015) led us to investigate potential efficacy against an influenza challenge using a novel glucan-sulforaphane combination.

Materials and Methods

Animals

Female, 8 week old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were sacrificed by CO_2 asphyxiation followed by cervical dislocation.

Material

The combination of phytochemicals used in this study consisted of Maitake Gold 404 (Tradeworks, Brattleboro, VT, USA), providing Maitake-derived glucans and sulforaphane, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The combination contained 1.54 mg kg^{-1} maitake extract and 0.308 μmol sulforaphane representing a mouse equivalent dose of the actives in Avmacol Immune™ (Nutramax Laboratories Consumer Care, Inc., Edgewood, MD, USA).

Phagocytosis

Phagocytosis of synthetic polymeric microspheres was described earlier (Vetvicka and Vetvickova, 2010). Briefly: 0.1 mL of peripheral blood from mice was incubated *in vitro* with 0.05 mL of 2-hydroxyethyl methacrylate particles (HEMA; 5×10^8/mL). The tubes were incubated at 37°C for 60 min., with intermittent shaking. Smears were stained with Wright stain (Sigma). The cells with three or more HEMA particles were considered positive. At least 300 cells were examined in each experiment.

Challenge the Virus to Mice

Mice were orally treated with the glucans and sulforaphane or PBS once a day for 14 days by gavage. At day 14, the same mice were intranasally challenged with the H5N1 A/HK/483 influenza virus (1,000 50% mouse infectious dose diluted in PBS to a 50 μL volume) as described previously (Szretter *et al*., 2007). Mice were monitored daily for an additional 14 days post influenza challenge. Samples ascertained during the study were immediately frozen and stored at -80°C for subsequent determination.

Cytokines

Lung homogenates were analyzed for the levels of IL-1β, TNF-α and IFN-γ by use of ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Antibody Titer

Anti-influenza hem agglutination-specific antibodies in serum were measured by ELISA following a previously described protocol (Wen *et al*., 2009). A purified hem agglutination protein was used for plate coating at 2 mg L^{-1} concentration.

In vitro Cytotoxicity Assay

Cell suspension of splenic cells was generated by pressing minced spleen against the bottom of a petri dish containing PBS. After elimination of erythrocytes by 10-sec incubation in distilled water and five washes in cold PBS, the cells were resuspended in PBS and counted. The viability was determined by try pan blue exclusion and only cells with viability better than 95% were used in subsequent experiments. Cells (10^6/mL; 0.1 mL/well) in V-shaped 96-well microplates were then washed three times with RPMI 1640 medium. After washing, 50 μL of target cell line K562 (ATCC, Manassas, VA, USA) was added. After spinning the plates at 250× g for 5 min., the plates were incubated for 4 hrs at 37°C. The cytotoxic activity of cells was determined by the use of CytoTox 96 Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions. Specific cell-mediated cytotoxicity was calculated using the formula: Percent-specific killing (% cytotoxicity) = 100×[(OD_{492} experimental -OD_{492} spontaneous) divided (OD_{492} maximum -OD_{492} spontaneous)] as described in the manufacturer's instructions where spontaneous release was target cells incubated with medium alone and maximum release was obtained from target cells lysed with the solution provided in the kit.

Virus Titer

Plaque assay for monitoring virus titers of homogenates of individual organs was performed as described previously (Takada *et al*., 2003). Briefly, 10% suspensions of the lung homogenates were examined. Serial dilutions of the samples were inoculated on Madin-Darby canine kidney cells, overlaid with RPMI 1640 medium containing 1% Bacto Agar, incubated for 48 hrs and enumerated.

Results

Evaluation of overall survival revealed that all mice in the control group died from influenza challenge by day 28 (14 days after the challenge), whereas the treated group showed 60% survival at the end of study (Fig. 1). Furthermore the treated mice started to gain weight with return to normal at the end of the study (Fig. 2).

For evaluation of phagocytic activity, we employed synthetic microspheres which, due to their hydrophilic properties, eliminate false positivity. Our results summarized in Fig. 3 showed that the glucan-sulforaphane combination not only restored the virus-suppressed phagocytosis of peripheral blood neutrophils, but increased this activity above control (PBS) values. Similar data were found for NK cell assay (Fig. 4).

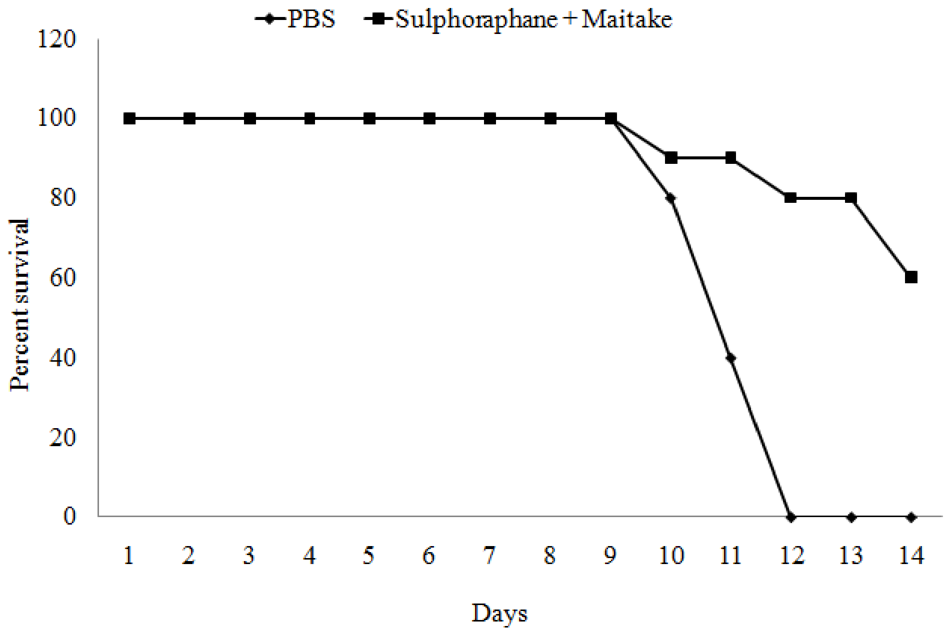

Fig. 1. The oral administration of a glucan-sulforaphane combination protects mice from lethal dose of virus. All mice were infected with influenza. Treated group was fed with a glucan-sulforaphane mixture. Control group was fed with PBS. Ten mice/group

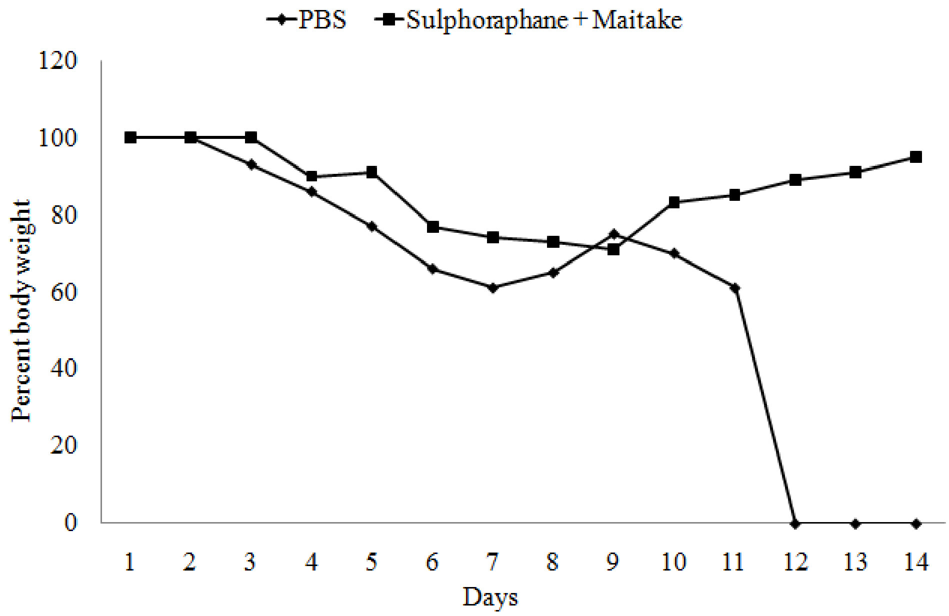

Fig. 2. Effects of a glucan-sulforaphane combination on body weight post influenza challenge. All mice were infected with influenza. Treated group was fed with a glucan-sulforaphane combination. Control group was fed with PBS. Ten mice/group

Next, we determined release of pro-inflammatory cytokines in lungs of infected animals. Lungs were collected on day 1, 3 and 5 after the treatment and homogenates were evaluated for IFN-γ, IL-1 and TNF-α using an ELISA assay. In some cases (IL-1 and TNF-α, influenza challenge raised significant amounts of cytokines, however the treatment in all cases and intervals increased the cytokine release. In the case of IFN-γ, this increase was statistically significant in all tested intervals. For TNF-α the increase was significant only on day 3. In the case of IL-1 the increase was significant on days 3 and 5 (Fig. 5). In untreated mice, the levels of cytokines were very low (data not shown).

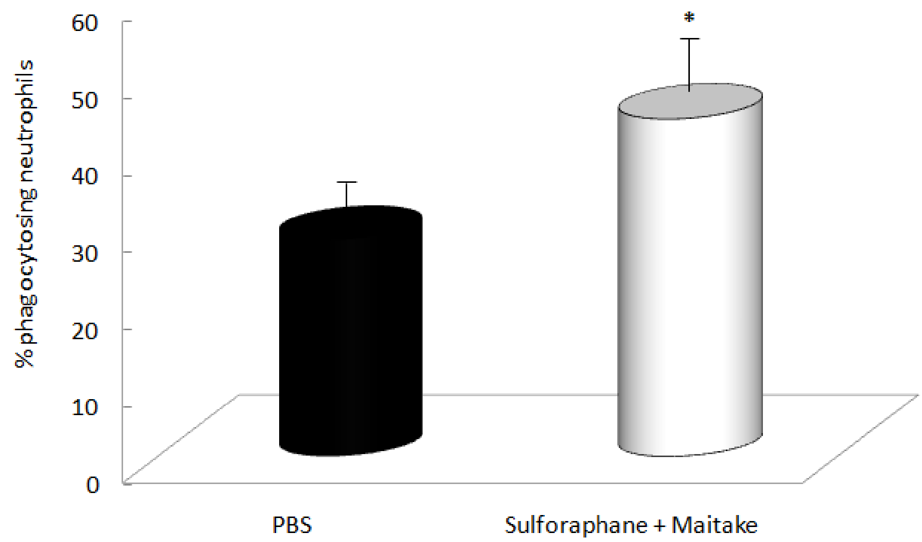

Fig. 3. Effects of the glucan-sulforaphane mixture on phagocytosis of peripheral blood neutrophils. Influenza-treated (Influenza), negative control (PBS) and glucan-sulforaphane mixture groups. Data represents mean ± SD. *Significant differences between control and experimental group at $p<0.05$ level

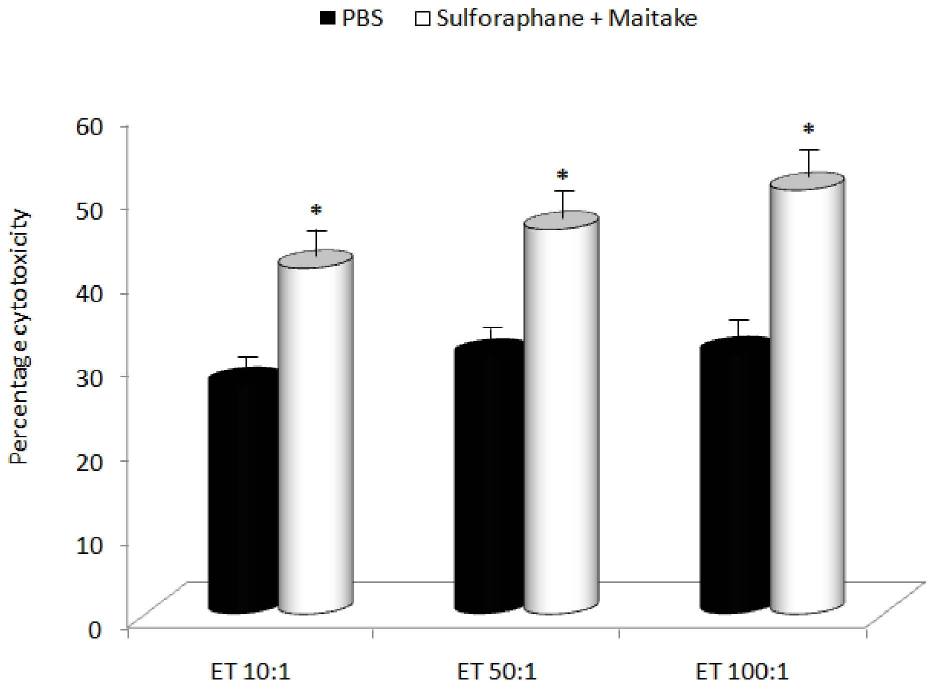

Fig. 4. Effects of the glucan treatment on NK cell activity of mouse splenocytes. Influenza-treated and glucan-sulforaphane mixture groups. Data represents mean ± SD. *Significant differences between control group and experimental groups at $p<0.05$ level

The glucan-sulforaphane treatment significantly potentiated the formation of anti-virus antibodies (Fig. 6). The total titers of virus were insignificantly lowered in heart and spleen, but the decrease in thymus was highly significant (Fig. 7). When we did detailed examination of the viral load in lungs, we found significant decrease from day one after the viral challenge (Fig. 8).

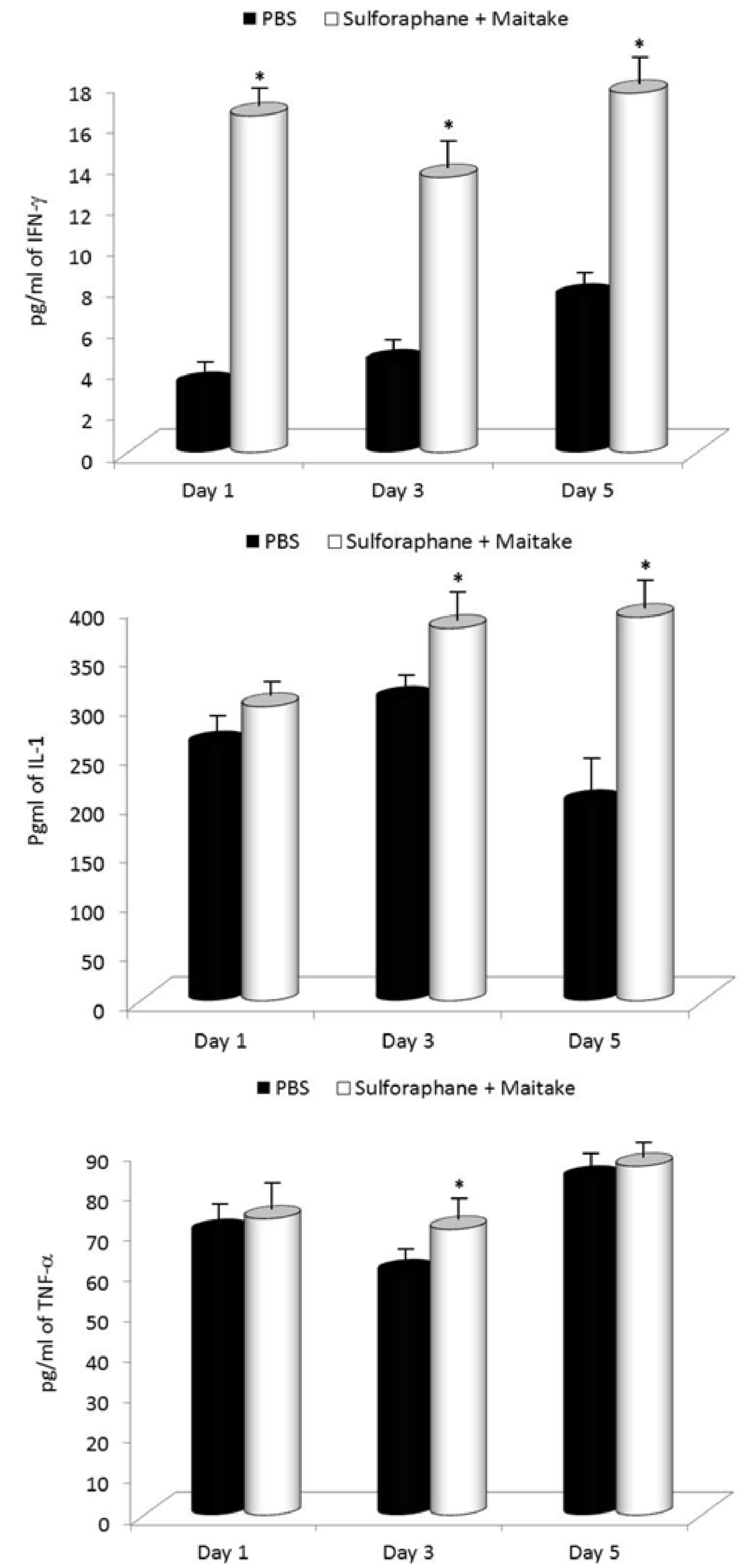

Fig. 5. Evaluation of proinflammatory cytokine levels in lungs. Influenza-treated and glucan-sulforaphane mixture groups. Data represents mean ± SD. *Significant differences between groups at $p<0.05$ level. A) IFN-γ B) IL-1β, C) TNF-α

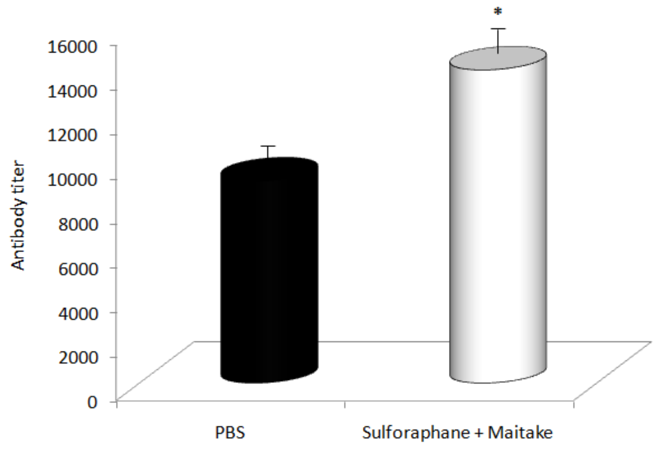

Fig. 6. Effects of glucan-sulforaphane mixture on the antibody response induced by influenza challenge. Ten mice/group. Data represents mean ± SD. *Significant differences between mixture group and Influenza group at $p<0.05$ level

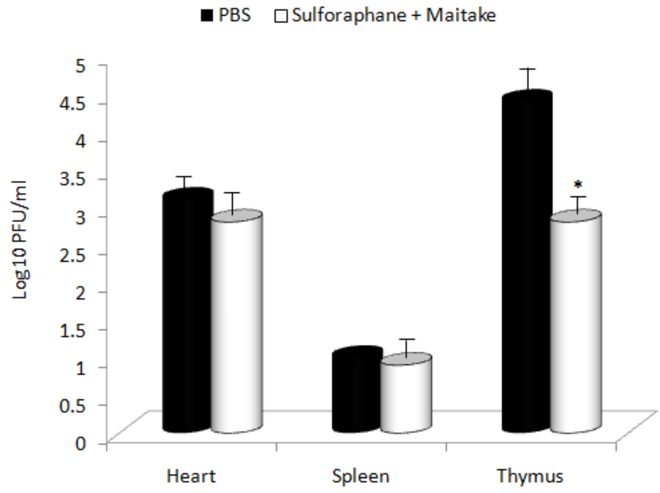

Fig. 7. Effects of glucan-sulforaphane mixture on virus titers in thymus, heart and spleen measured at day 5 after infection. Ten mice/group. Data represents mean ± SD. *Significant differences between glucan-sulforaphane mixture group and group with Influenza (PBS) at $p<0.05$ level

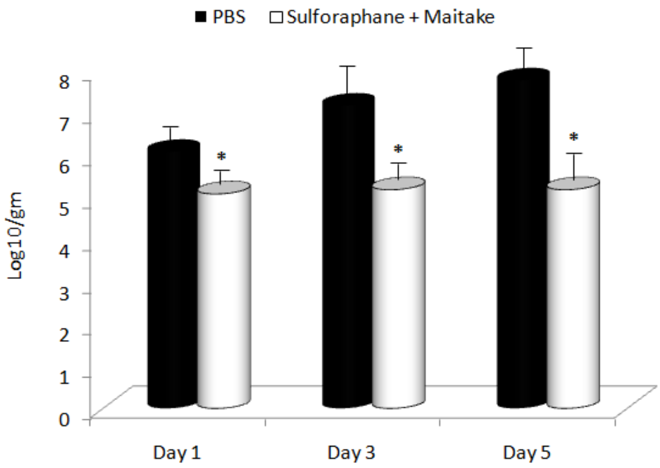

Fig. 8. Effects of glucan-sulforaphane mixture on virus titers in lung. Ten mice/group. Data represents mean ± SD. *Significant differences between groups at $p<0.05$ level

Discussion

Natural remedies have been used for centuries to treat a variety of maladies. Herbal remedies are being used throughout the world, sometimes as the only available treatment, or as an alternative or complementary medicine. Our study was based on previous evaluations of separate actions of glucans and sulforaphane on influenza infection (Kesic *et al*., 2011; Vetvicka and Vetvickova, 2015). With numerous studies showing that glucans can benefit from the addition of additional bioactive molecules such as resveratrol (Vetvicka and Vetvickova, 2012; Del Giudice *et al*., 2014) or humic acid (Vetvicka *et al*., 2015), we focused on uncovering the effects of a novel glucan-sulforaphane combination on a known lethal viral challenge.

This combination consisted of maitake-derived glucans, previously shown to significantly stimulate anti-infectious immunity (Kodama *et al*., 2001) and evaluated in clinical trials (Wesa *et al*., 2014) and sulforaphane with known anti-infectious and anti-viral effects (Chang *et al*., 2015; Schachtele *et al*., 2012) including suppression of inflammation via Nrf2-dependent pathway (Lin *et al*., 2008). As both components have anti-infectious effect, we hypothesized that this mixture might have complementary effects.

We designed our study as a direct comparison of the previous report showing significant enhancement of immune response in influenza-challenged mice by glucan supplementation (Vetvicka and Vetvickova, 2015). Data show significant improvements of basic glucan activities-effects on phagocytosis and NK cell activities. Similarly, the glucan-sulforaphane combination significantly potentiated release of important cytokines. In the case of phagocytosis, the mixture not only reversed the virus-caused suppression, but significantly improved this activity.

These results were followed up with an evaluation of effects on overall survival rate and changes in body weight. The survival rate in the treated group was significantly better with 60% of animals living at day 14 post viral challenge (vs. 0% in the control group). Similar data were obtained with loss of body weight.

Influenza infection results in significant changes in immune reactions, including phagocytosis, antibody production and cytokine release. Changes in cytokine production have been found in numerous organs including spleen and lungs (Hoeve *et al*., 2013; Han and Meydani, 2000). Levels of TNF-α, IL-1β, IL-6, IL-8 and IFN-α were changed. In addition, significant decreases of CD-4 lymphocytes and B lymphocytes and an increase of T-regulatory lymphocytes were observed (Giamarellos-Bourboulis *et al*., 2009). IFN-γ (Perry *et al*., 2005), IL-1 (Vogels *et al*., 1995) and TNF-α (Winthrop, 2006) offer some protection against infection.

Our data showed that the glucan-sulforaphane combination significantly increased levels of all three cytokines tested. When we evaluated the effects on antibody response, we found significant increases consistent with established effects of glucan on antibody production (Talbott and Talbott, 2013).

Conclusion

Current observations clearly showed that the glucan-sulforaphane combination stimulates the immune system more than glucan alone (Vetvicka and Vetvickova, 2015). These effects are manifested on both the cellular and humoral branch of immune responses, leading to lower viral load in some organs and resulting in higher overall survival rate. Increasing glucan's effects by adding sulforaphane might provide better natural treatment of influenza and other infections.

Acknowledgement

Authors confirmed no conflict of interest.

Author's Contributions

All authors equally contributed to this study.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues were involved.

Conflict of Interest

Authors declare no conflict of interest.

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