

Antioxidant Capacity and Immune Regulation Properties of a Herbal Mixture Including *Sparassis crispa*, *Aureobasidium pullulan*, and *Ganoderma lucidum*

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Abstract Many Chinese herbal medicines have been proven to have anti-inflammatory effects. Among them, edible and medicinal mushrooms are rich in a variety of physiologically active ingredients, which can enhance immunity and are good materials for the development of health foods. This article aims to investigate the beneficial effects of SOD Light, a herbal formula consisting of *Sparassis crispa*, *Aureobasidium pullulan*, and *Ganoderma lucidum*, on anti-inflammation, antioxidant, and immune-modulation. SOD Light was able to inhibit ROS expression level and boost the SOD activity in PBMCs by 11% and 43.8%, respectively, as compared with the control group, and the significant improvement effect of the phagocytic activity of neutrophils was exhibited. Moreover, SOD Light also significantly enhanced the expression of the lymphocyte development-associated and adaptive immune-related genes (i.e., *CD134*, *CD26*, *CD96*, *CD247*, and *IL-16*). This study successfully unveils that the combination of *S. crispa*, *A. pullulans*, and *G. lucidum* was available for anti-oxidation, cytokines production, enhancement of T cell activation, and lymphocyte development in PBMCs, which implies that SOD Light are beneficial for immune modulation.

Keywords: antioxidant, *Aureobasidium pullulan*, *Ganoderma lucidum*, *Sparassis crispa*, immune modulation

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1. Introduction

Cancer is the leading cause of death worldwide, and the death toll continues to rise. Especially the immune response is the main cause of cancer development [1]. Several studies have proven that the expression of pro-inflammatory cytokines and chemokines are associated with the onset and progression of cancer [2]. Moreover, oxidative stress (i.e., reactive oxygen species, ROS) is imperative to affect immune modulation [3]. For example, certain low levels of reactive oxygen species (ROS) are beneficial for innate immune activities against viral and bacterial infections and cancer growth [4]. High levels of ROS in cancer microenvironment may suppress T cell activation and stymie its differentiation [5]. In recent years, several methodologies of immune improvement and cancer prevention have been proposed. Among of them, dietary supplements are recognized as a potent strategy [6]. Natural supplements/herbal extracts possess anti-oxidant, anti-inflammatory, and anti-cancer effects [7].

The medicinal mushroom-based extract (called SOD Light), mainly composed of *S. crista*, *A. pullulans*, and *G. lucidum* for the influence of immune modulation via *in vitro* methodology [8]. These fungi are commonly used in Asian regions and have been discovered with diverse immune-associated advantages, but, to the best of our knowledge, there are few studies to discuss their synergistic effect on the possible immune modulation. *S. crista* is an edible medicinal mushroom and composed of polysaccharide $\beta(1-3)$ -glucan, which has anti-oxidant and anti-inflammatory effects [9]. *S. crista* regulated the secretion of inflammatory cytokines by regulating the differentiation or activity of immune cells [10]. *S. crista* can affect the activity of macrophages, dendritic cells, neutrophils and natural killer (NK) cells through the expression of nitric oxide (NO) and related inflammatory cytokines, and, in turn, inhibit the growth of cancer cells [11]. *A. pullulans*, a black-yeast-like fungus, is a common organism discovered in the phyllosphere and carposphere of fruits and vegetables crops [12]. *A. pullulans* consists of β -glucans with the main chain of β -(1, 3)-D-glycosidic linkages and the branch of β -(1, 6)-D-glycosidic linkages [13]. *A. pullulans* can increase the differentiation of Th1

helper cells and the activity of cytotoxic T lymphocyte, and next affect the secretion related inflammatory cytokines: IL-6, IL-6, IL-12 and TNF- α [14]. In addition, *A. pullulans* increased the apoptosis ability by activating the TNF-related apoptosis-inducing ligand (*TRAIL*) in cancer [15]. *G. lucidum*, commonly known as Lingzhi in China, is a uniquely oriental fungus and embraces the capabilities of anti-cancer, anti-oxidation and the modulation of immune responses [16]. The main bioactive compounds of *G. lucidum* include polysaccharides (β -(1, 3)-glucans) and triterpenes [17]. *G. lucidum* can regulate the expression of immune-related factors between macrophages and T cells, and can also increase the phagocytic ability of macrophages [18]. Triterpenes in *G. lucidum* inhibited the growth of cancer cells by regulating cell arrest and apoptosis [19]. *G. lucidum* can also reduce the expression of MMP-2 and MMP-9 through the NF- κ B signaling pathway, which incurs cancer metastasis [20]. Thus, this study want to explore whether SOD Light (an herbal formula composed of *S. crispa*, *A. pullulan* and *G. lucidum*) has antioxidant capacity and immune regulation function.

2. Materials and Methods

2.1. The Herbal Formula and Cell Culture

SOD light major ingredients: 3% *S. crispa*, 10% *A. pullulan*, 6% *G. lucidum*, water, apple juice, noni juice, sugar. Minor ingredients: Citric acid monohydrate, pectin, peach flavor, malic acid. Peripheral blood mononuclear cells (PBMCs; ATCC[®] PCS-800-011[™]), culture media [X-VIVO[™] 10(Lonza) medium 10% fetal bovine serum, 1 mM sodium pyruvate, and 1% penicillin/streptomycin] [21].

2.2. SOD Activity Analysis

We added 1×10^5 PBMCs in 2 mL of growth media to each well in 6-well plates, and incubated the cells for 24 hours. Then, we replaced the media with the fresh media/the media with 0.25%/0.5% SOD Light solution. Following 24 hours incubation, we treated the cells with radioimmunoprecipitation assay (RIPA) buffers to lyse cells and collected the cell lysate for SOD activity analysis. The measurement of SOD activity of the cells was referenced by the Cayman Chemical SOD Assay kit [22].

2.3. ROS Assay

1×10^5 PBMCs in 2 mL of growth media were placed on each well in 6-well plates and underwent 24 hours incubation. Afterwards, the media were replaced by the new media/the media with 1 mM H₂O₂/the media with 1 mM H₂O₂ and 0.25%/0.5% SOD Light solution followed by an hour incubation. After the oxidative stress induction, we removed the liquids and washed the cell with PBS buffers twice, and treated the cells with 10 μ g/mL 2,7-Dichlorofluorescein diacetate (DCFH-DA; Sigma) for 40 minutes. DCFH-DA interacted with H₂O₂ and formed a fluorescent compound. In the end, we detached the cells from wells with trypsin and put the suspending cells into a

cytometry (excitation wavelengths: 450-490 nm; emission wavelengths: 510-550 nm) [23].

2.4. Neutrophil Phagocytosis

We put 1×10^6 neutrophils in 2 mL X-VIVO[™] 10 (Lonza) into each well in 6-well plates. Then, we added the media containing 0.1% fluorescent particles and 0.25%/0.5% SOD Light solution and incubated the cells for 5 hours. Lastly, the phagocytic activity of the neutrophils was evaluated through a flow cytometry[24].

2.5. mRNA Expression Analysis

We dispersed 1×10^5 cells of PBMCs in 2 mL culture media with 0.5% SOD Light solution into each well of 6-well plates, and the cells were incubated for 24 hours. And we collected the treated PBMCs and extracted their total RNA by the RNA extraction kit (Genaid Biotech). The analysis of mRNA expression level was following the protocol of nCounter[®] platform (NanoString Technologies) [25].

2.6. Statistical Analysis

All the experimental results were leveraged Student's t-test for statistical analysis; $p < 0.05$ indicated statistically significant difference.

3. Results and Discussion

3.1. Evaluation of SOD Activity

In order to examine whether the SOD Light drink product had anti-oxidative effect, we used SOD Light to treat peripheral blood mononuclear cells (*PBMC*) and observe superoxide dismutase (*SOD*) activity. *SOD* is an enzyme that catalyzes the conversion of superoxide into oxygen and hydrogen peroxide through a dismutation reaction. It is widely present in various animals, plants, and microorganisms, and is an important antioxidant [26]. 0.5% SOD Light drink significantly increased the SOD activity compared with the control group (Figure 1). Whereas, 0.25% SOD light drink no significant change (Figure 1). This result showed that 0.5% SOD Light drink can increase SOD activity. Some studies also showed that the liquid culture of *S. crispa* had better antioxidant activity than water extract of mycelium, and increased the production of antioxidant protein SOD [27]. *A. pullulans* had antioxidant capacity by increasing *SOD* activity [28]. In addition, cancer cells had low activity of SOD [29].

3.2. Analysis of ROS Production

Next, in order to examine whether the SOD Light drink product decreased oxidative stress, we used hydrogen peroxide (H₂O₂) to treat *PBMC* to mimic oxidative stress condition, then examined reactive oxygen species (*ROS*). *ROS* are a common cause to oxidative damage and may lead to DNA damage, aging, and inflammation in cells [30]. 0.5% SOD Light drink significantly decreased the *ROS* production compared with the H₂O₂ group (Figure 2).

In addition, 0.25% SOD Light drink also significantly decreased the ROS production (Figure 2). This result suggested that 0.5% SOD Light can act as a useful antioxidant. *S. crispa* extract effectively inhibited ROS production [31], and *A. pullulans* can decrease ROS and free radicals [32]. *G. lucidum* protected non-malignant cells from the accumulation of reactive oxygen species [33]. ROS were not only mediators of oxidative stress but also involved immune regulation in cancer cells, suggesting ROS in tumor cells play critical role in anticancer therapy [34].

3.3. Investigation of Phagocytic Capacity of Neutrophils

To examine whether the SOD Light drink product had immune modulation, we used neutrophils to examine

phagocytic activity. The phagocytic activity is an important part of innate immunity, in which specialized phagocytes (macrophages, monocytes and neutrophils) perform various host defense functions that depend on the phagocytosis of pathogens, and are inducible by cytokines [35]. 0.5% SOD Light drink significantly increased phagocytic activity compared with control group (Figure 3), whereas 0.25% SOD Light drink no significantly change (Figure 3). This result suggested that 0.5% SOD Light drink can increase phagocytic activity of neutrophils. The extract from *S. crispa* stimulated the production of cytokines that activate phagocytes [36]. *A. pullulans* culture supernatant significantly stimulated phagocytosis of THP-1 macrophages [37]. *G. lucidum* can promote phagocytosis by macrophage from peripheral blood [38]. Moreover, macrophage phagocytic activity against cancer cells [39].

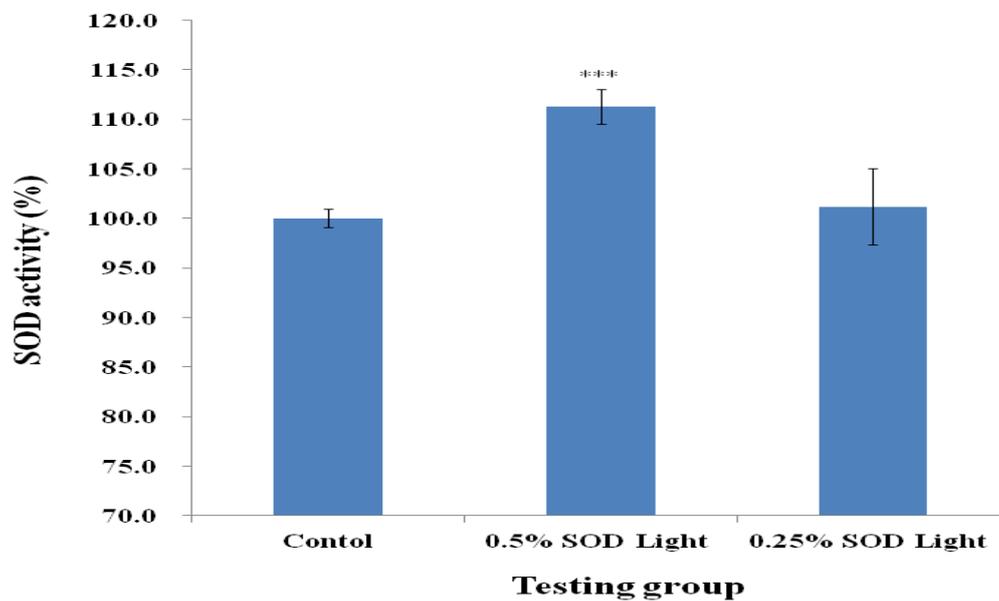


Figure 1. Evaluation of SOD activity for different concentrations of SOD Light drink. ($n = 3$, mean \pm S.D.; ***, $p < 0.001$)

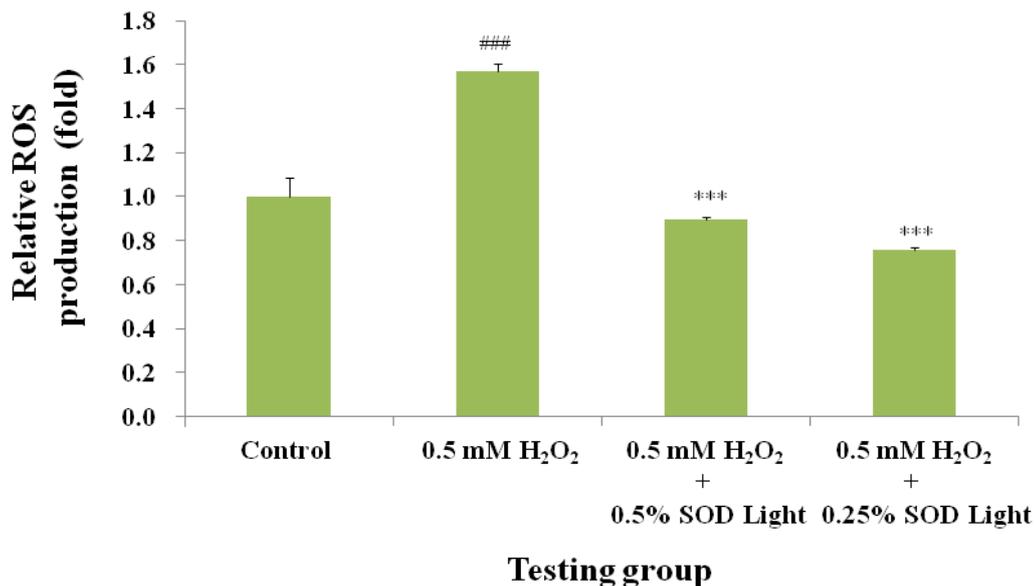


Figure 2. Analysis of ROS production in different concentrations of SOD Light drink. ($n = 3$, mean \pm S.D.) (Comparison with the control group: ###, $p < 0.001$) (Comparison with the H₂O₂ group: ***, $p < 0.001$)

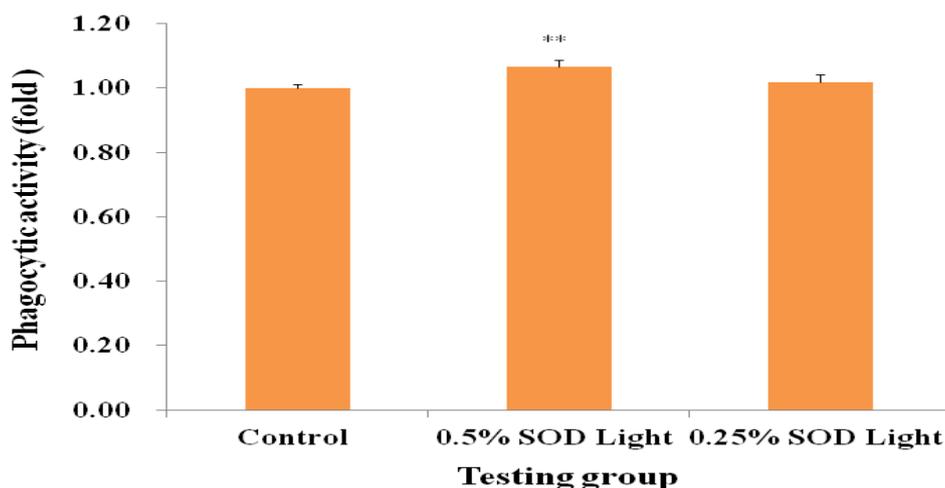


Figure 3. Investigation of phagocytic capacity of neutrophils for different concentrations of SOD Light drink. ($n = 3$, mean \pm S.D.; **, $p < 0.01$)

Table 1. Evaluation of the mRNA expression levels of immune-related gene in PBMCs treated with the 0.5% of SOD Light drink. ($n = 3$, mean value \pm S.D.)

Genes	Net change (%) (Subtracting from the control)	p-value
TNFRSF4(CD134)	21.8	0.02538
DPP4(CD26)	21.8	0.01618
CD7	18.9	0.01416
TCF7	8.3	0.00113
CD3D	7.6	0.00177
CD96	5.8	0.00005
IL16	5.7	0.00544
CD247	5.1	0.00546
IL7R	4.2	0.00014
MS4A1(CD20)	3.2	0.01844
IL1B-mRNA	2.4	NS
CD7	18.9	NS
IL8	1.2	NS
IL32	5.2	NS
CCL3(MIP-1 α)	1.4	0.01416
CCL4(MIP-1 β)	0.9	NS

NS: not significant.

3.4. Analysis of mRNA Expression Levels of Immune-related Genes

Finally, to explore whether the SOD Light drink product regulated immune and inflammatory gene expression, we used PBMC to examine immune-related gene expression by nCounter® platform (Table 1). The expression levels were normalized in accordance with the control result. 0.5% SOD Light drink significantly increased the T-cell activation associated genes (*CD134*, *CD26*, *CD96*, *CD247*, and *IL-16*), and significantly increased lymphocyte associated and adaptive immune-related genes (*CD7*, *TCF7*, *IL-7R*, *CD2*, and *CD3D*). However, *IL-8*, *IL-1 β* , *MIP-1*, and *IL-32* only slightly increased, but no significantly change. Above this result, we find that 0.5% SOD light drink can increased the T-cell activation associated genes and immune-related genes. *S. crispa* decreased inflammatory genes and suppressed the tumor growth and metastasis [40]. *A. pullulans* was involved in the inhibition of tumor angiogenesis by regulating inflammatory cytokines [41]. *G. lucidum* suppressed

growth and proliferation of tumors by the T cell receptor signaling and PI3K-Akt signaling pathway, but also regulated the immune cytokines [42]. Inflammation is a series of very complex processes. It requires the body to secrete many cytokines, pro-inflammatory cytokines, prostaglandins, various white blood cells and vasodilators released by platelets to control the operation of this inflammatory response system, and eliminate foreign pathogens and help the body repair [43]. Those inflammatory genes (*IL-8*, *IL-1 β* , *MIP-1*, and *IL-32*) that have not changed significantly need more research to confirm. In this study, we revealed that the SOD light drink product including *S. crispa*, *A. pullulans*, and *G. lucidum* had anti-oxidation, inflammatory cytokines production, enhancement of T cell activation, and lymphocyte development in PBMCs, which suggested that SOD Light can be used as a supplement to enhance immunity, and because prevention is more important than treatment, SOD Light has the opportunity to be used as an anti-cancer supplement in the future.

4. Conclusions

In this study, we successfully demonstrated the utility of SOD Light for the immune modulation. SOD Light effectively lowered oxidative stress in PBMCs via the inhibition of ROS expression level and the increase of SOD activity, suggesting SOD Light had antioxidant activity. Moreover, SOD Light could also significantly enhance the expression of the lymphocyte development-associated and adaptive immune-related genes (i.e., *CD134*, *CD26*, *CD96*, *CD247*, and *IL-16*) and remarkably reinforced the phagocytic activity of neutrophils. Nevertheless, further investigation is required for the substantial immune benefits in humans. In brief, SOD Light has potential in modulating immune system and boosting cytokine expression, and could be used as a supplement for individuals and cancer patients for the purpose of anti-oxidative, anti-inflammatory.

Statement of Competing Interests

The authors have no competing interests.

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