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RESEARCH ARTICLE

The effects of Spirulina on nitric oxide production in peritoneal macrophages of Balb/C mice with systemic candidiasis

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Abstract

Spirulina (*Spirulina platensis*) is a dietary supplement that is interesting for its immune-enhancing properties. In the present study, the effects of Spirulina on nitric oxide (NO) production in peritoneal macrophages of mice with systemic candidiasis were investigated**.**

Mice were divided into four groups (G1-G4), 5 mice per group; animals received a dose of 800 mg/kg of *S. platensis* for four days and then were inoculated intravenously with 1×10^6*Candida albicans* (*C. albicans*). After 24 hours, they were euthanized and their intraperitoneal lavage macrophages were collected. Peritoneal macrophages were stimulated by Spirulina (10µg/ml), LPS(2µg/ml), and LPS+ Spirulina (2µg/ml, 10µg/ml) *in vitro*. Dulbecco`s modified Eagle medium (DMEM) alone was used for control group. Macrophages were cultivated for 24 hours, then the levels of nitrite in culture supernatants were measured by reading the optical density at 540 nm. The results revealed that there was a significant difference in NO production among the understudy groups ($p<0.05$) as well as among the isolated cells that were cultured in media with different treatments(DMEM, LPS, Spi, and LPS+Spi) ($p<0.001$). The NO production was synergistically induced when the cells were treated with LPS + Spirulina (9.6±0.38) in comparison with control culture (7.39±0.3). It seems that applying Spirulina+LPS has more acceptable effect on NO production than Spirulina alone.

INTRODUCTION

Clinical significance of systemic candidiasis in immunocompromised patients with resistance to current antifungal therapies has increased in recent years [10, 16]. Candida albicans is a widespread opportunistic pathogen with different virulence factors which can invade deep organs in definite conditions [17]. Patients with defects in cell-mediated immunity are susceptible to mucocutaneous, but not to systemic candidiasis, however systemic infections are commonly associated with neutropenia and congenital defects. Both neutrophils and macrophages have been shown as the first line of defense against systemic Candida infections.Biological response modifiers can be useful in improving host immune responses [5,11].In previous studies, some substances such as bacterial lipopolysaccharide (LPS), fungal β-glucan, Carageenans, and Spirulina extracts have been used to activate macrophages [6,8,9,13,14,15].

Stimulation of macrophages by these substances enhances the production of inflammatory mediators such as nitric oxide (NO), tumor necrosis factor α, (TNF α), and interleukin -1β [4,23]. Nitric oxide is a highly reactive molecule produced from a guanidino nitrogen of arginine by NO synthase (NOS). Nitric oxide also reacts with molecular

oxygen or reactive oxygen species to yield peroxide nitrite or N_2O_3, which will broaden the spectrum of antimicrobial reactivity [3].

Spirulina is a member of the blue-green algae family that is attracting interests as a dietary supplement. Spirulina has a soft cell wall and can be digested easily [2]. In our previous study, it was demonstrated that prophylaxis with Spirulina increases the level of some serum cytokines (INF-γ and TNF-α) and also the survival rate of mice infected with systemic candidiasis [21]. In order to find out the stimulatory effects of Spirulina on macrophages against systemic candidiasis, our interest was to evaluate the *in vivo* and *in vitro* effects of Spirulina and Spirulina+ LPS on NO production of murine peritoneal macrophages.

2. Materials and methods

Twenty male Balb/C mice, ranging from 6 to 8 weeks old and the average weight of 27 g were obtained from Razi Institute (Karaj, Iran). Animals were housed at the animal facilities during the time of the experiments in the temperature of 24±2°C, humidity of 55±10%, and 12/12 h dark-light cycle and were provided with sterilized food and water. This study was performed according to the approved ethical protocol of Department of Pathobiology, Faculty of Veterinary Medicine, University of Tehran.

2.1. In vivo treatment with Spirulina

Spirulina platensis powder, was purchased from Aquatic Research in Iran, and it was suspended in physiological saline (0.85% NaCl). Of this suspension, 0.2 ml containing 800 mg/kg body weight was orally administrated to each mouse. Control group received 0.2ml normal saline in the same route.

2.2. Candida albicans inoculum

C. albicans (ATCC 10231) was cultured on Sabouraud glucose agar (SGA, *Merck Co., Darmadstat, Germany*) at 35°C for 20 h. Yeast colonies were washed twice with phosphate-buffered saline (PBS) and adjusted to 1×10^7 cells/ml. Animals were inoculated via the tail vein with 0.1 ml of yeast suspension.

2.3. Experimental design

The mice were divided into 4 groups, 5 per group, including groups 1, 2, 3 and 4 as follows:

- Group 1: mice were orally given normal saline (0.2 ml) via cannula for 4 consecutive days and 0.1 ml saline injection.
- Group 2: mice were orally given *S. platensis* suspension (800 mg/kg body weight, 0.2 ml) via cannula for 4 consecutive days and 0.1 ml saline injection.
- Group 3: mice were orally given normal saline (0.2 ml) via cannula for 4 consecutive days and inoculated intravenously with *C. albicans* (1×10^6 CFU/mouse, ATCC=10231) through the tail vein.
- Group 4: mice were orally given *S. platensis* suspension (800 mg/kg body weight, 0.2 ml) by cannula for 4 consecutive days and were inoculated intravenously with C. *albicans* (1×10^6 CFU/mouse, ATCC=10231).

2.4. Macrophage cultureand Nitrite quantification

Nitrite accumulation was used as an indicator of NO production in the medium as previously described [7].

Mice were sacrificed using CO_2 euthanation 24h after inoculation. Peritoneal cells werecollectedfromperitonealcavity with 10 ml ice-cold sterile phosphate-buffered saline (PBS), pH: 7.4. The suspension was centrifuged and the cell concentration was adjusted to 10^5cells/well.

The cells of each group were cultured in duplicate in a row of 96-well flat bottom microtiter plates containing DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand island, NY, USA), L-glutamine (2mM), Penicillin (100 U/ml) and streptomycin (100 µg/ml).

Cultures were incubated for 2 hours at 37ºC and 5% CO_2 in humidified chamber to allow macrophages toadhere. Non adherent cells were removed by repeated washing by PBS. Adherent cells (>97% macrophages as determined by morphological examination) were cultured at 37ºC and 5% CO_2 and were stimulated with Spirulina (10 µg/ml), LPS (2 µg/ml), and LPS + Spirulina (2 µg/ml, 10 µg/ml). DMEM alone was used as control group. After 24h, cell-free supernatants were collected and transferred to -70ºC to assay the nitrite levels. Nitrite levels were assayed by Griess method [23]. Aliquots (100 µl) were removed from -70ºC and were mixed with an equal volume of Griess reagent (1% sulphanide amide and 0.1% naphthylethylenediamine hydrochloride in 2.5% H3PO4) and NaNO2 was used togenerate a standard curve. The concentration of NaNO2 was calculated according to a standard curve that was obtained bynitrite (1-200 µM in culture medium).

Statistical analysis

Statistical analysis of nitrite concentration was performed by two way ANOVA and Tukey post tests by SPSS version16.0 software. Values were expressed as the mean± SE of duplicates from two independent experiments (*P* value less than 0.05 was considered significant.).

Results

Concurrent effects of in vivo and in vitro factors on NO production levels were evaluated using two way analysis of variance tests. The test indicated that there was a significant difference in NO production among the understudy groups (1, 2, 3 and 4) ($p<0.05$) as well as among the isolated cells that were cultured in media with different treatments (DMEM, LPS, Spi and LPS+Spi) ($p<0.001$). Nitrite levels were increased by 18% in group 4 (9.52±0.33) in comparison with group 2 (8.06±0.36) and 22% in comparison with group1 (7.79±0.35) ($p< 0.05$). Moreover, nitrite levels were increased significantly in LPS+Spi-containing cultures (9.6±0.38) more than cultures containing onlyDMEM (7.39±0.3) ($p<0.001$). Also the level of nitrite in Spi cultures was higher (8.7±0.38) in comparison with control group (7.39±0.3) but the difference was not significant ($p=0.059$). A relationship was observed between animal groups and cultures. Infected mice groups stimulated *in vivo* by Spirulina produced more NO levels in LPS+Spi culture media than control groups (Fig.1).

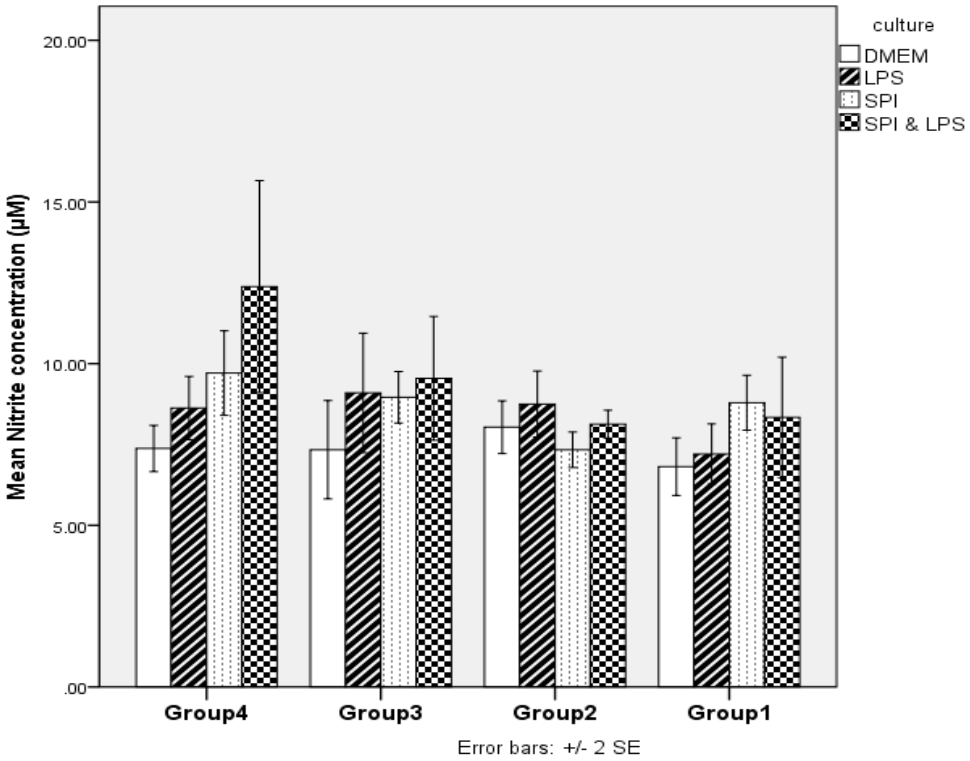

Fig.1.Effects of *in vivo*&*invitro* administration of Spirulina on nitrite concentration in peritoneal macrophage cell culture soup of mice groups (1×10^5 cell/well). Group1 received 0.2 ml and 0.1 ml normal saline for prophylaxis and inoculation, respectively. Group 2 received 800 mg/kg b.w. of *S. platensis* for prophylaxis and then were inoculated with 0.1 ml normal saline. Group 3 received 0.2 ml normal saline for prophylaxis and then were intravenously inoculated with 1×10^6*C. albicans*. Group 4 received 800 mg/kg b.w. of *S. platensis* for prophylaxis and then were intravenously inoculated with 1×10^6*C. albicans*. Cell cultures of each group were stimulated with Spirulina (10µg/ml), LPS(2µg/ml), LPS+ Spirulina (2µg/ml, 10µg/ml),and DMEM alone was used as control group. Nitrite levels were assayed by Griess method. Data were expressed as Mean±SE. (n = 5 mice/group, ANOVA; Tukey's post-test).

Spi: Spirulina

DMEM: Dulbecco`s modified Eagle medium.

LPS:lipopolysaccharide

Discussion

Nitric oxide is a highly reactive molecule which is produced by activated macrophage. Nitric oxide deficiency results in susceptibility to systemic opportunistic infections such as Candidiasis. In the present study, NO production was examined in peritoneal macrophages treated with Spirulina for 4 days before inducing candida infection. Obtained cells were stimulated by LPS (2µg), Spirulina (10µg) and LPS+Spi. According to the results, there was a significant difference in NO production between mouse groups and *in vitro* cultures and there was a relationship between *in vivo* and *in vitro* tests.

Son K et al (1995) showed that NO production increased in macrophages when the animals were challenged intraperitoneally with 10mg/ml Cisplatin and cultured in media containing LPS+INFγ [21].

Saito et al (2008) demonstrated that docetaxel, a taxane-derivative anti-cancer drug, has an enhancing action in vivo on LPS-induced iNOS expression and subsequent NO production through stimulation of p38 activity in alveolar macrophages isolated from rats injected intraperitoneally (IP) with docetaxel (4 mg/kg B.W., per day for 5 consecutive days) in comparison with those in macrophages from control rats which were administrated a vehicle. In vitro treatment of macrophages with docetaxel (5 and 10 µg/ml) inhibited LPS-induced NO production and iNOS expression [19].

Vaillier et al (1996) measured the NO production in spleen macrophage cultures which were stimulated by LPS, IL-2, and LPS+IL-2. With increasing NO production, in combination media , synergistic effects of LPS+IL-2 on INFγ production were shown [22].

We observed similar results in cultures containing LPS and Spirulina. It is clear that only LPS and IFNγ are stimulating factors of NO production by macrophages. The NO induced in the Spirulina-stimulated macrophage cultures was probably attributable to IFNγ, induced by Spirulina *in vivo* and *in vitro*. In the present study, the production of NO was significantly increased when LPS and *Spirulina* were used simultaneously in the cultures. *In vitro* stimulation of cells with Spirulina caused more potent NO production in cells from infected mice given Spirulina in comparison with the cells without pre-exposure to Spirulina. There was a relation between *in vivo* and *in vitro* stimulation. In our previous study, synergistic effects on the increase of serum IFNγ levels were observed in candida- infected- mice treated with *Spirulina* [20].

Saeki et al reported that hot-water extract of Spirulina enhances NK activation by IFNγ production in adult human and in mice by oral administration. So Spirulina acts as an inducer of IFNγ[18].

Pugh et al isolated a high molecular weight polysaccharide preparation from *Spirulina platensis*, refered to as Immolina with active potent to immunostimulatoryeffects [14].

According to the findings of the our study, it seems that *Spirulina* is able to induce some cytokine pathways leading to NO production and subsequently stimulates the innate immunity, and then it may be helpful to prevent systemic opportunistic infections such as invasive candidiasis in susceptible high risk patients. Regarding the present study, in order to illuminate the role of Spirulina to induce NO production in different immune cells, this study should be continued in the future.

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