

Effect of Shark Liver Oil on Peritoneal Murine Macrophages in Responses to Killed-*Candida albicans*

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Abstract

Objective(s)

Shark Liver Oil (SLO) is an immunomodulator. Macrophages play a key role in host defense against pathogens like fungi. *Candida albicans* have mechanisms to escape immune system. We determined the effect of killed-*Candida* on the *in vitro* viability of macrophages and the effect of SLO on augmentation of this potency.

Materials and Methods

Peritoneal macrophages were separated and cultured (3×10^5 /well). At first, the effect of killed-*Candida* (200 cells/well) on macrophage viability was evaluated, using MTT test. Then, MTT was performed on macrophages stimulated with killed-*Candida* in the presence of SLO.

Results

Killed-*Candida* suppressed the ability of MTT reduction and hence macrophages viability ($P=0.026$), but addition of SLO (100 mg/ml) significantly enhanced cell viability ($P=0.00$). So, SLO could neutralize the inhibitory effect of *Candida*.

Conclusion

Simultaneous with cytotoxic effect of killed-*Candida* cells on macrophages viability, SLO augment macrophages viability. So, it can be applied in candidiasis as a complement.

Keywords: *Candida albicans*, Macrophage, Shark, Liver Oil

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Introduction

Candida albicans is the most frequently isolated fungal pathogen in humans. The fungus *C. albicans* behaves as a common as well as a true pathogen of areas such as skin and mucosal surfaces. This organism forms part of the normal microflora in the gastrointestinal tract and vagina even in individuals who do not have an apparent immunological dysfunction. In general, superficial mucocutaneous candidiasis is frequent in patients with T-cell deficiencies, such as AIDS patients. The more serious, life-threatening, deep-seated or disseminated candidiasis is normally found in a spectrum of severely immunocompromised patients (1).

Fungal antigens may stimulate specific cell-mediated and humoral immune responses. It has been well documented that the host defense mechanism against mucosal infection with *C. albicans* is mediated mainly by cellular immunity and most invasive fungal infection occur in patients with defective cellular immunity. Th₁ cells mediate phagocyte-dependent protection and are the principal mediators of acquired protective immunity. In contrast, production of inhibitory cytokines such as IL-4 and IL-10 by Th₂ cells and high levels of IgE are associated with disease progression. Th₂-like reactivity is frequently observed in patients with *C. albicans* related pathology, such as in symptomatic infections and allergy. Th₁-type responses may thus characterize the carriage of saprophytic yeast and the resistance to disease seen in healthy humans, whereas Th₂ responses associate predominantly with pathology (2).

Phagocytic cells such as neutrophils and macrophages are potential components of the immune defense that protects mammals against *C. albicans* infection (1).

Investigations (3) have demonstrated that IFN- γ -activated macrophages required reactive nitrogen intermediates to exhibit effective fungicidal activity against yeast and hyphal forms of *C. albicans*. Nitric oxide (NO) generated by the inducible isoform of NO-synthase (iNOS or NOS₂) is thought to contribute to candidacidal effector functions by activated macrophages (4). NO, as a

critical effector molecule for macrophage activity can be released upon stimulation of macrophages with a variety of stimuli such as bacterial products or cytokines (5).

There are certain mechanisms that result in evasion of *C. albicans* from the host defense system (6). For example, live *C. albicans* suppressed the release of H₂O₂ by neutrophils, superoxide anion production and release of azurophilic and specific granule components by activated neutrophils. Inhibition of NO production is another mechanism that allows *C. albicans* to resist macrophage fungicidal activity (7).

SLO has been found to be useful in the treatment of conditions resulted from inadequate immune response, and also in the adjunctive treatment of several types of cancer (8). It is useful in prevention of radiation side effects from cancer x-ray therapy, by increase in leukocyte and thrombocyte counts, as well as in the treatment of infectious diseases (9).

SLO contains great amounts of alkyl glycerol and squalene (40% or more), and moderate amount of N-3 polyunsaturated fatty acids (N-3 PUFA) which are modulators of immunity to infections and cancer (10).

The present study was undertaken to understand whether SLO has positive effect on macrophages proliferation ability against *C. albicans*.

Materials and Methods

Animals

Female inbred BALB/c mice (8 to 10 weeks old) were purchased from Pasteur Institute, Tehran, Iran. They were kept in animal house of Tarbiat Modares University, given sterilized water and autoclaved standard mouse pellet throughout the study. The animal study was approved by a local Ethics Committee.

Organism and culture condition

C. albicans strain ATCC10321 was grown on Glucose-Yeast Extract Peptone (GYEP) medium at 37 °C for 48 hr in shaker incubator. Hydrophilic yeast cells were collected from the broth culture, washed in cold (0-4 °C) sterile PBS (Phosphate buffered saline) and

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centrifugated at 800 g for 20 min. *C. albicans* cells were killed by heating at 56 °C for 1 hr.

Shark liver oil

Shark liver oil capsules were purchased from Kraftsatim ehf Company (Iceland). Each capsule has 100% pure natural arctic shark liver oil (350 mg/ capsule), extracted from the liver of the Greenland Shark (*Somniosus microcephalus*), which contains natural vitamins A, D and E (minor amount). Included in this oil are alkyl glycerols (35 mg/capsule) and omega-3 polyunsaturated fatty acids (42 mg/capsule). The SLO obtained from the capsules, was diluted, using Tyrode buffer to prepare 100 mg/ml of SLO. This concentration of SLO selected for the present study, with respect to our previous research on the effect of SLO on peritoneal macrophages from BALB/c mice.

Macrophage culture and stimulation

Peritoneal cells obtained from 8-10 weeks old male BALB/c mice were washed twice in cold PBS, resuspended in cell culture medium (RPMI) supplemented with 10% FCS and added to 96-well microplates (3×10^5 cells/well) followed by incubation at 37 °C for four hr under 5% CO₂. The non-adhering cells were then removed by washing with PBS, pre-warmed to 37 °C (11). More than 95% of adherent cells were macrophages. The adherent cells were incubated for 48 hr with either RPMI medium (supplemented with 10% FCS) or alone (only macrophages as negative control group), medium containing 200 killed-*Candida*/well, and 100 mg/ml of SLO plus 200 killed-*Candida*/well. The samples were assayed in triplicated wells. Final volume of each well of microplates reached to 200 µl.

MTT assay

Macrophage viability was evaluated by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) assay. After 48 hr of macrophage culture, 20 µl of MTT (5 mg/ml in PBS) was added to wells and the plates were incubated for 4 hr. Formazan crystals, developed from MTT reduction by living cells. The supernatants

were then gently removed, 100 µl of isopropanol in 0.04% HCl (Sigma, USA) was added in order to dissolve the formazan crystals. The plates were incubated overnight and the absorbance was read at 540 nm in a microplate Labsystem Multiskan MS reader. Stimulation Index (SI) was determined by the formula (12):

Absorbance at 540 nm of control group / absorbance at 540 nm of test group

Statistical analysis

Data are presented as mean±SD. Results of MTT test were analyzed, using one-way analysis of variance (ANOVA) followed by Tukey tests, with a value of $P < 0.05$ indicating significant.

Results

The effect of killed *Candida* on MQ proliferation

In order to examine the effect of killed *C. albicans* on MTT reduction and macrophage proliferation, 200 killed organisms were added to each well of macrophage culture microplate. MTT test was performed after 48 hr and SI were calculated. Our findings expressed in Figure 1, indicated that SI, thus the macrophage proliferation in the 200 killed cell/well group was significantly ($P=0.026$) lower than the control group which was culture of macrophage cells without any stimulation.

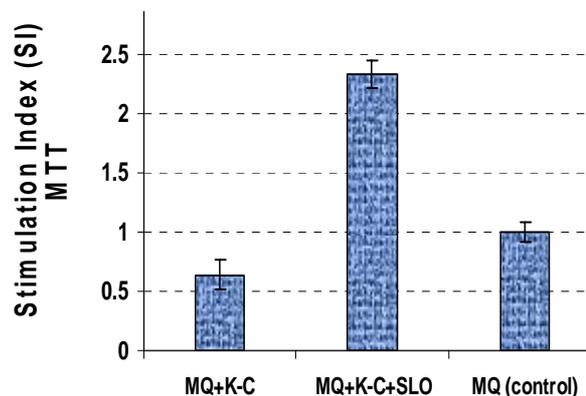


Figure 1. The values (mean±SD) of SI obtained from MTT test by mouse peritoneal macrophages. SI, so MQ viability in the MQ+K-C group is lower ($P=0.026$) than the control group, While SLO treatment augment MQ viability ($P=0.00$). MQ: macrophages, K-C: Killed-*C. albicans*, SLO: Shark Liver Oil, SD: Standard Deviation.

The simultaneous effect of SLO and killed C. albicans on MQ proliferation

To evaluate the effect of SLO on macrophage viability, 100 mg/ml of Shark Liver Oil (SLO) was applied for macrophage stimulation when cultured with killed-*C. albicans*. Macrophage activity was evaluated by MTT assay, after 48 hr. As shown in Figure 1, SLO treatment significantly ($P=0.00$) increases SI and augments the macrophages viability as compared with other groups (only macrophages culture and killed-*C. albicans* exposed macrophages).

Discussion

Although the candidial infection process is complex and involves interactions between the pathogen and many host cell types, *in vitro* studies involving specific cultured immune system cells can permit the analysis of interactions under controlled conditions.

Macrophages are important components of natural immunity involved in inhibition of infections and destruction of tumor cells. It is known that these cells can be activated by lymphokines and bacterial products (13). *C. albicans* represent mechanisms of immune evasion that contributes to the virulence. Celia Murciano *et al* showed that killed *C. albicans* inhibit IFN- γ release by murine natural killer cells (14).

IFN- γ production activates phagocytic cells (15), and up-regulates the fungicidal activity of these cells.

The present work assessed the ability of SLO

to stimulate macrophage proliferation in response to *C. albicans*. This idea obtained from our recent findings that showed SLO augments some functions of murine macrophages.

The proliferation activity was evaluated by MTT assay. The cell proliferation data showed that killed *C. albicans* suppresses macrophages from male BALB/c mice.

However, co-culture of macrophages with SLO and killed *Candida* for 48 hr results in elevated cell proliferation.

Thus, SLO is able to induce macrophage multiplication activity and could be used as a supplement and complementary agent in immune system boosting in candidiasis. Moreover, elucidation of SLO effect on other aspects of immune responses to fungi is promising.

Results of this study and our recent finding (shifting cytokine pattern to Th₁ in mice by SLO) accompanied with other parallel effects on immune system such as leucocytes proliferation and function, support the hypothesis that SLO has immunostimulatory effects that may improve immune responses in immunocompromised hosts and could be used for immunotherapy in fungal infections.

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