Iranian Journal of Basic Medical Sciences
Vol. 12, No. 2, Summer 2009, 86-92
Received: Mar 15, 2009; Accepted: Apr 22, 2009

IJBMS

Original article

Effect of Cell Wall, Cytoplasmic Fraction and Killed-*Candida albicans* on Nitric Oxide Production by Peritoneal Macrophages from BALB/c Mice

[1]Monire Hajimoradi, [1]Saeed Daneshmandi, [2]Maryam Roudbary, [2]Shahla Roudbar Mohammadi, *[1]Zuhair Mohammad Hassan, [2]Rozita Heidari, [1]Hasan Namdar Ahmadabad

Abstract

Objective(s)
The fractions of *Candida albicans* have been used as an immunomodulator. The present work assessed the effect of different fractions of *C. albicans* on nitric oxide (NO) production by mice peritoneal macrophages.
Materials and Methods
Cell wall and cytoplasmic fractions of *C. albicans* ATCC 10321 strain were extracted. Mice peritoneal macrophages were purified and cultured. Different concentrations of both fractions and also killed *C. albicans* cells were used for macrophages stimulation and evaluation of NO production. NO amount was detected in culture supernatants of macrophages by Griess reagent. Also, MTT assay was performed to assess the viability of macrophages.
Results
The results elucidated that suppressive effect of cell wall proteins on NO release was significant at the dose of 100 µg/ml (P=0.01), while cytoplasmic fraction increased NO amount at the dose of 1 µg/ml compared to the control group (P=0.003). Augmentation of NO production was statistically significant at 200 killed *C. albicans* per well (P=0.006).
Conclusion
According to our findings, cytoplasmic fractions and killed *C. albicans* have a positive effect on NO production by peritoneal macrophages, while cell wall fractions did not. Therefore, it is proposed that *C. albicans* fractions can be studied more as inflammation modulators.

Keywords: *Candida albicans*, Macrophages, Nitric Oxide

1- Department of Immunology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran
*Corresponding author: Tel: +98-21- 8288-3565; Fax: +9821- 88006544; email: hasan_zm@modares.ac.ir
2- Department of Mycology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Introduction

Candida albicans is a component of the normal microflora of the alimentary tract and mucocutaneous membranes of the healthy host. However, when immune defenses are compromised or the normal microflora balance is disrupted, *Candida* transforms itself into an opportunistic pathogenic fungus. Indeed, dissemination of *Candida* is the leading cause of invasive fungal disease in premature infants, diabetics and surgical patients and of oropharyngeal disease in AIDS patients (1, 2).

As the outer most cellular structure, the cell wall plays an essential role in the interaction with the host, including the triggering and modulation of the anti-candidal host immune responses, which appear to rely on a complex interplay between natural and adaptive immunity (3).

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 (4-6). The importance of cellular defense mechanisms for protection against fungal infections is supported by the clinical observation that most invasive fungal infections occur in patients with defective cellular immunity (7, 8).

Activated macrophages play a central role in infections by eliminating microbial pathogens through the generation of nitric oxide (NO) (9). Recent investigations (10) have demonstrated that IFN-γ-activated macrophages required reactive nitrogen intermediates to exhibit effective fungicidal activity against yeast and hyphal forms of *C. albicans*.

Some microorganisms possess a variety of mechanisms that allow them evade the host defense systems and grow within the area of infection. *Cryptococcus neoformans*, another fungal agent, resists host defense mechanisms by inhibiting NO-mediated fungicidal activity of macrophages (11). In the present study, we examined the *in vitro* effect of different fractions of *C. albicans* on NO production by peritoneal macrophages.

Materials and Methods

Mice

Female inbred BALB/c mice (8 to 10 weeks old) were purchased from "Karaj Complex Production Center, Pasteur Institute of Iran", Tehran, Iran. They were kept in animal house of Tarbiat Modares University, given sterilized water and autoclaved standard mouse pellets throughout the study.

Organism and culture condition

In order to obtain cell wall and cytoplasmic fractions, *C. albicans* strain ATCC 10321 was obtained from Pasteur Institute, Tehran, Iran. *C. albicans* 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 and centrifuged at 800×g for 20 min.

Cell wall protein extraction

Yeast cell wall was disrupted with glass bead in lysis buffer containing 2ME (2-Mercaptoethanol 0.3M), SDS (2.3 g), PBS (pH=7.2), the supernatant material was collected, following centrifugation at 2000×g for 15 min, as cell wall fraction (12). This fraction dialyzed against cold-d-H_2O to remove salts (13).

Cytoplasmic protein extraction

In order to obtain cytoplasmic fraction, washed yeast cells disrupted with glass beads in sterile PBS buffer. The supernatant material was centrifuged at 3000 ×g for 10 min as cytoplasmic fraction, followed by ultracentrifugation at 100,000 ×g for 1 hr. Then it was purified by gel filtration on G200-sephadex column. The Amount of protein assayed by Bradford method.

Electrophoresis of cell wall and cytoplasmic fraction

Both cell wall and cytoplasmic fraction were evaluated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Then gel stained by comasi blue G250. For elimination of some of low molecular weight protein bands, ultrafilteration (cut off 50 kD, Millipore) was

performed. SDS-PAGE electrophoresis pattern of cytoplasmic fraction, cell wall fraction and ultrafilterated cell wall fraction are shown in Figures 1, 2 and 3, respectively.

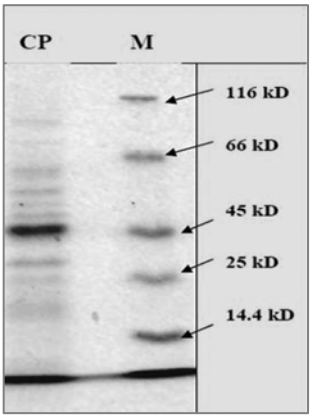

Figure 1. SDS-PAGE electrophoresis pattern of cytoplasmic protein of *Candida Albicans*. M: Marker, CP: Cytoplasmic.

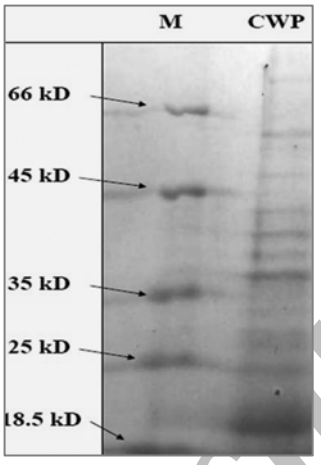

Figure 2. SDS-PAGE electrophoresis pattern of cell wall proteins of *Candida albicans*. M: Marker, CWP: Cell Wall Protein.

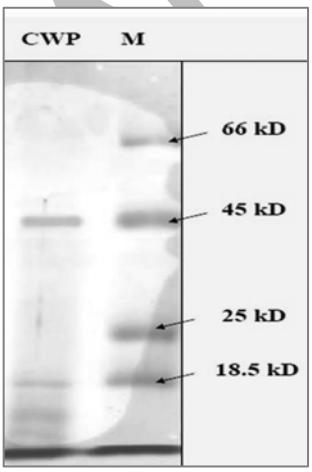

Figure 3. SDS-PAGE electrophoresis pattern of cell wall proteins of *Candida albicans*. M: Marker, CWP: Cell Wall Protein.

Macrophage culture and stimulation

Male BALB/c mice Six- to eight-week-old were used throughout the experiments. Macrophages were harvested by lavage of the peritoneal cavity with 10 ml of RPMI 1640 (Sigma Chemical Co). The cells were centrifuged at 200×g and washed, then adjusted to 1.5×10^6 cells/ml in RPMI medium supplemented with 11 mM sodium bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 5% fetal bovine serum. The 3×10^5 cell suspensions were plated (200 µl/well) onto 96-well flat-bottomed plates (Nunc) and incubated for 4 hr to achieve adherence in a humidified atmosphere with 5% CO_2 at 37 °C. The nonadherent cells were removed by washing the wells three times with PBS (pH=7.2). Adherent cells containing macrophages incubated for 48 hr in RPMI medium. To stimulate the NO production by macrophages, different amounts of killed *C. albicans* (100, 200 and 300 killed organisms/well), some doses of cell wall fraction (1, 10, 50 and 100 µg/ml) and cytoplasmic proteins (1, 10, 50 and 200 mg/ml) of *C. albicans* were added to the cultures as triplicated wells. Wells containing macrophages with RPMI medium alone and also wells stimulated with 50 IU/ml IFN-γ, considered as negative and positive controls, respectively (14, 15).

Measurement of nitrite concentration

NO released into the supernatants of cultured macrophages. NO is unstable and rapidly converts to nitrite and nitrate. Accordingly, we estimated the level of NO synthesis by macrophages via measuring the amount of nitrite accumulating in the cultures, using the method of Stuehr & Nathan (16). Briefly, nitrite concentration was determined with the standard Griess reaction, by adding 50 µl of test solution (supernatants of macrophage culture) to 96-well flat-bottomed plates containing 50 µl of Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl) ethylenediamine dihydrochloride/2.5% H3PO4] (Merck). The samples were assessed in triplicate. After 15 min at room temperature, the absorbance of each well was measured in a

Multiskan MS microplate reader at 540 nm and the nitrite concentration was determined from a standard curve of sodium nitrite (15). Mean of nitrite concentration (µM) was expressed.

MTT assay

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] reduction assay is widely used to evaluate cell viability. MTT reduction is interpreted to be indicative of cellular metabolic activity (17). MTT (Merck) was dissolved in PBS (5 mg/ml), filtered and stored at –20 °C until use. MTT solution was added to each well at one tenth of its volume. Briefly, 20 µl of MTT (5 mg/ml in PBS) was added to wells and the plates were incubated for 4 hr. Then, the supernatants were gently removed and 100 µl of acidic isopropanol (0.04 M HCl in isopropanol) was added in order to dissolve the formazan crystals generated with MTT reduction by the living cells (18). The plates were incubated for 20 min at 37 °C and the absorbance was read at 540 nm in a microplate Labsystem Multiskan MS reader (21). The result of the test was expressed as a Stimulation Index (SI), which is OD_{540} of the test samples/OD_{540} of negative control (21).

Statistical analysis

Data are presented as mean±SD. Results of NO assay and 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 cell wall proteins of C. albicans albicans on NO production and MTT reduction

After extraction of whole cell wall proteins, concentration of cell wall protein fractions was determined as 2 mg/ml by the Bradford method. Doses of 1, 10, 50 and 100 µg /ml of cell wall proteins were added to each well of microplate of macrophage cultures. NO amount in the collected supernatants was measured. As shown in Figure 4, suppressive effect of cell wall proteins on NO release was significant (P=0.010) at the dose of 100 µg /ml compared to the negative control (macrophages). In the case of MTT test, cell viability of macrophages in 50 µg/ml (P=0.003) and 100 µg/ml (P=0.002) of cell wall proteins was significantly lower than the negative control.

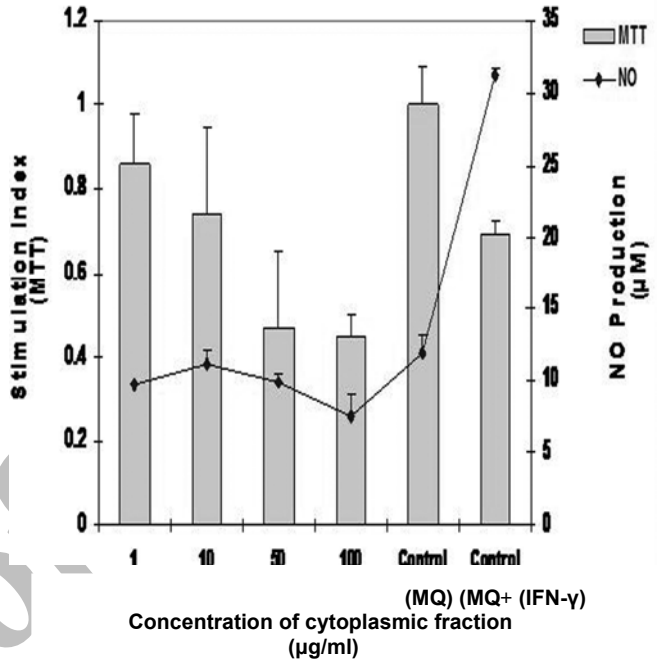

Figure 4. The values (mean±SD) of MTT reduction and NO production by mouse peritoneal macrophages and macrophages treated with various concentrations of cell wall fraction or 50 IU/ml IFN-γ. The number of macrophages was 3×10^5/well. NO production by macrophages was inhibited at the dose of 100 µg/ml (P=0.01). Also, MTT reduction ability of macrophages was significantly diminished at the concentration of 50 (P=0.003) and 100 µg/ml (P=0.002).

The effect of cytoplasmic proteins of C. albicans on NO production and MTT reduction

Cytoplasmic proteins of *C. albicans* were extracted and its effect on NO release and macrophage viability was evaluated. Concentration of cytoplasmic protein was determined as 1 mg/ml by the Bradford method. Doses of 1, 10, 50 and 200 µg/ml of this fraction were added to each well of microplate of macrophage cultures. Supernatants were collected and NO assay was performed. As shown in Figure 5, increase of NO amount was significant (P=0.003) at the dose of 1 mg/ml compared to the control group (macrophage cells without cytoplasmic

protein, as a negative control). For the evaluation of macrophage viability, MTT assay was done. The results in Figure 5 demonstrate that the differences between Stimulation Index (SI) of groups stimulated with cytoplasmic fraction and the negative control (macrophages) group were not statistically significant (P>0.05).

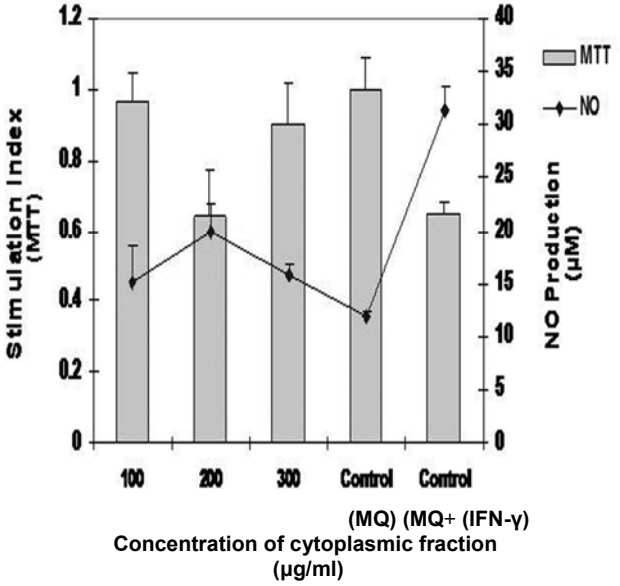

Figure 5. The values (mean±SD) of MTT reduction and NO production by mouse peritoneal macrophages and macrophages treated with various concentrations of cell wall fraction or 50 IU/ml IFN-γ. The number of macrophages was 3×10^5/well. NO production by macrophages was enhanced at the concentration of 1 mg/ml (P=0.003), but the MTT reduction ability of macrophages was not statistically different between groups (P>0.05).

The effect of killed C. albicans on NO production and MTT reduction

In order to examine the effect of killed *C. albicans* on NO production and macrophage viability, *C. albicans* cells were killed by heating at 56 °C for 1 hr. Then 100, 200 and 300 killed organisms were added to each well. Our findings expressed in Figure 6, indicate that SI, thus the macrophage viability in the group stimulated with 200 killed *C. albicans* /well was significantly (P=0.006) lower than the negative control (macrophages). NO amount was higher than the other groups treated with 100 and 300 killed *C. albicans* (P<0.05) and the negative group (P=0.008).

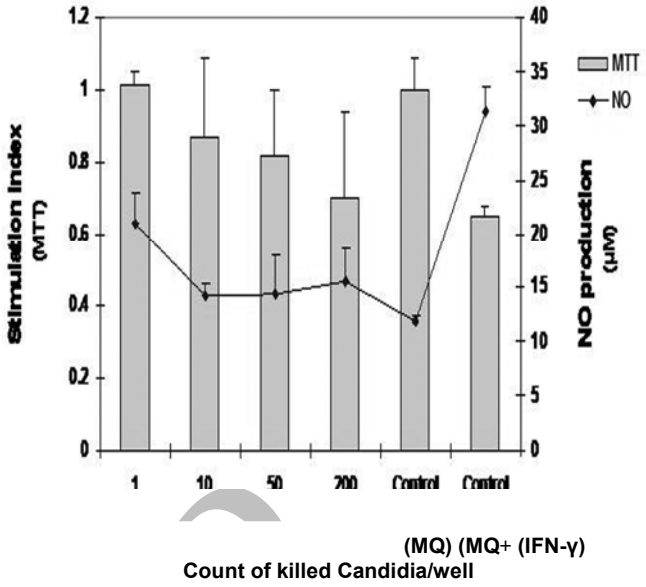

Figure 6. The values (mean±SD) of MTT reduction and NO production by mouse peritoneal macrophages and macrophages treated with various amount of killed *C. albicans* or 50 IU/ml IFN-γ. The number of macrophages was 3×10^5/well. MTT reduction ability of macrophages was diminished at 200 killed *C. albicans*/well (P=0.006), while at the amount of 100 and 300 killed *C. albicans*/well, macrophages ability to MTT reduction did not alter. The amount of 200 killed *C. albicans*/well, induced an augmentation of NO production (P=0.006)

Discussion

The major immune defense against fungi is CMI response (6). Macrophages are important for the defense against experimental disseminated candidiasis. Nitric oxide generated by the inducible isoform of NO-synthase (iNOS or NOS_2) is thought to contribute to candidacidal effector functions by activated macrophages. It is shown that *C. albicans* effectively inhibited the production of NO via suppression of total NOS_2 protein and enzyme activity (19). Nitric oxide reacts with oxygen to form nitrogen dioxide radical ($NO_2^{\bullet}$) and also reacts with superoxide to generate peroxynitrite anion ($ONOO^-$), a more potent suppressant of mitochondrial respiration than nitric oxide (20). *C. albicans* evades the host defense systems and multiplies in host tissue. Several investigators have demonstrated alterations by *C. albicans* of the fungicidal activity of phagocytic cells. For example, live *C. albicans* suppressed the release of H_2O_2 by neutrophils,

superoxide anion production and release of azurophilic and specific granule components by activated neutrophils (21). Inhibition of NO production is another mechanism that allows *C. albicans* to resist macrophage fungicidal activity (22).

The cell wall protein component of *C. albicans* includes both mannoprotein and nonmannoproteins, is composed of some 40 or more moieties. *C. albicans* cell wall proteins have been shown to adhere to host tissues and ligands. Surface mannoproteins are strong immunogenes that trigger and modulate the host immune response during candidiasis (23).

Mannan and oligosaccharide fragments of mannan are potent inhibitors of cell-mediated immunity and appear to reproduce the immune deficit of patients with the mucocutaneous form of candidiasis (24). To elucidate possible supressive mechanisms of immune supression by *C. albicans*, we examined the effect of cell wall proteins on macrophage activity. According to our results, with increasing concentration of cell wall fraction, viability of macrophages was diminished. The most decrease of cell viability was occurred in 100 µg/ml of cell wall fraction accompanied by decrease in NO production. Thus, we demonstrated that cell wall of C. *albicans* have a cytotoxic effect on macrophages. Diminished production of NO may be due to the cytolytic effect of the culture supernatant on the cells, because the number of viable macrophages, estimated by MTT method, was reduced 48 hr after initiation of cultures.

According to the results of Chinen, *et al* inhibition of NO release can mediate mainly by soluble molecules derived from *C. albicans* (22). These molecules can be glycoproteins such as mannoproteins which may release from cell wall of *C. albicans*.

Also, it has been reported that β1, 2 oligomannoside, a fragment of cell wall proteins has an inhibitory effect on NO release (25). Accordingly, suppression of NO production by murine peritoneal macrophages due to the whole cell wall fraction may be related to β1, 2 oligomannosides of cell wall proteins. As the enhanced formation of NO by the macrophage greatly contributes to tissue injury in human inflammatory disorders such as arthritis (26), cell wall components of *C. albicans* could be consider as a modulator of NO production.

Cytoplasmic fraction of *C. albicans* contain several different antigenic proteins such as enollase, metalopeptidase, HSPs,… with pathogenic, immunogenic and frequently immunomodulatory effects (27). These properties must be considered for further investigation in the future.

Concentration of 1 mg/ml of cytoplasmic fraction significantly enhanced NO synthesis compared with the control groups (P=0.003). We suggest that this fraction could be considered as a stimulator for inflammatory function of macrophages in further studies. These cytoplasmic proteins may improve inflammatory response in immunocompromised hosts and could be used for immunotherapy in fungal infections.

It is considerable that Chinen, *et al* findings showed live *C. albicans* has inhibitory effect on NO production while killed *C. albicans* has not such suppression effect (22). In the present research, albeit heat- killed *C. albicans* diminished macrophages viability as shown in MTT test, simultaneously caused enhancement in NO level and suggested a stimulatory property for killed *C. albicans* on the functions of macrophages.

Further studies must be planned to define the exact effect of these fractions or different ingredients of these fractions on macrophages activity and other inflammatory responses.

Acknowledgment

We wish to thank Tarbiat Modares University, Tehran, Iran for the financial support. The authors declare that they have no conflict of interests.

References

1. Romani L. Innate and adaptive immunity in *C. albicans* infections and saprophytism. J Leukoc Biol 2000; 68:175-179.
2. Crameri R, Blaser K. Allergy and immunity to fungal infections and colonization. Eur Respir J 2002;19:151-157.

3. Fukazawa Y, Cassone A, Bistoni F, Howard DH, Kagaya K, Murphy J W, *et al*. Mechanisms of cell mediated immunity in fungal infections: a review. J Med Vet Mycol 1994; 32:123.
4. Shoham S, Levitz SM. Immune response to fungal infections. Br J Haematol 2005; 129:569–582.
5. Badauy CM, Barbachan JJ, Rados PV, Sant Ana Filho M, Chies JAB. Relationship between Candida infection and immune cellular response in inflammatory hyperplasia. Oral Microbiol Immunol 2005; 20:89-92.
6. Bistoni F, Cenci E, Mencacci A, Schaffolla E, Mpsci P, Puccetti P, *et al*. Mucosal and systemic T helper cells function after intragastric colonization of adult mice with Candida albicans. J Infect Dis 1993; 168:1449–1457.
7. Santoni G, Boccanera M, Adriani D, Lucciarini R. Immune cell-mediated protection against vaginal Candidiasis: evidence for a major role of vaginal $CD4^+$ T cells and possible participation of other local lymphocyte effectors. Infect Immun 2002; 70:4791–4797.
8. Fidel Jr PL. Candida-host interactions in HIV disease:relationships in oropharyngeal candidiasis Adv Dent Rec. 2006; 19:80-84.
9. Fang FC, Vazquez-Torres A. Nitric oxide production by human macrophages: there's NO doubt about it. Am J Physiol Lung Cell Mol Physiol 2002; 282:L941- L943.
10. Gonzalez A, Gregori W, Velez D, Restrepo A, Cano LE. Nitric oxide participation in the fungicidal mechanism of gamma interferon-activated murine macrophages against paracoccidioides brasiliensis conidia. Infect Immun 2000; 68:2546–2552.
11. Kawakami K, Zhang T, Qureshi MH, Saito A. *Cryptococcus neoformans* inhibit nitric oxide production by murine peritoneal macrophages stimulated with interferon-g and lipopolysaccharide. Cell Immunol 1997; 180:47–54.
12. Elorza M, Murgui A. Dimorphism in *Candida albicans*; contribution of mannoprotein to the architecture of yeast and mycelial cell wall .J Gen Microbiol 1985;131:2207-2216.
13. Casanova M, Chaffin WL. Cell wall glycoproteins of *Candida albicans* as released by different methods. J Gen Microbiol 1991; 137:1045–1051.
14. Ribeiro-Dias F, Russo M, Nascimento FRF, Barbuto JAM, Timenetsky J, Jancar S. Thioglycollate-elicited murinemacrophages are cytotoxic to mycoplasma arginini-infected YAC-1 tumor cells. Braz J Med Biol Res 1998; 31:1425-1428.
15. Scuro LS, Simioni PU, Grabriel DL, Saviani EE, Modolo LV, Tamashiro WM, *et al*. Suppression of nitric oxide production in mouse macrophages by soybean flavonoids accumulated in response to nitroprusside and fungal elicitation. BMC Biochem 2004; 21; 5:5.
16. Stuehr DJ, Nathan CF. Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J Exp Med 1989; 169:1543–1555.
17. Sladowski D, Steer SJ, Clothier RH, Balls M. An improved MTT assay. J Immunol Methods.1993; 157: 203-7.
18. Ribeiro-Dias F, Russo M, Nascimento FRF, Barbuto JAM, Timenetsky J, Jancar S. Thioglyollate-elicited moraine macrophages are cytotocxic to mycoplasma arginini – infected YAC-1 tumor cells. Braz J Med Biol Rec. 1998; 31:1425-1428.
19. Schroppel K, Kryk M, Herrman M, Leberer E, Rollinghoff M ,Bogdan Ch. Suppression of type 2 NO-synthase activity in macrophages by *Candida albicans*. Int J Med Microbiol 2001; 290: 659-668.
20. Pacher P, Joseph S, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007; 87:315- 424.
21. Smail EH, Kolotila MP, Ruggeri R, Diamond RD. Natural inhibitor from *Candida albicans* blocks release of azurophil and specific granule contents by chemotactic peptide-stimulated human neutrophils. Infect Immun 1989; 57:689–92.
22. Chinen T, Qureshi MH, Koguchi Y, Kawakami K. *Candida albicans* suppresses nitric oxide (NO) production by interferon-gamma (IFN-g) and lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages. Clin Exp Immunol 1999; 115:491–497.
23. Lajean Chaffin W, Lopez-Ribot JL, Casanova M, Gozalbo D, Martínez JP. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol Mol Biol Rev 1998; 62:130-180.
24. Nelson RD, Shibata N, Podzorski RP, Herron MJ. Candida mannan: chemistry, suppression of cell-mediated immunity, and possible mechanisms of action. Clin Microbiol Rev 1991; 4:1-19.
25. Jouault T, Fradin C, Trinel P, Poulain D. Candida albicans-derived β-1,2-linked mannooligosaccharides induce desensitization of macrophages. Infect Immun 2000; 68:965-968.
26. Cartney-Francis NM, Allen JB, Mizel DE, Albina JE, Xie QW, Nathan CF, *et al.* Suppression of arthritis by an inhibitor of nitric oxide synthase. J Exp Med 1993; 178:749-754.
27. Montagnoli C, Sandini S, Bacci A, Romani L. Immunogenicity and protective effect of recombinant enolase of *Candida albicans* in a murine model of systemic candidiasis. Med Mycol 2004:42; 319-324.