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Immunological Effects of Honey Bee Venom in Mice with Intracerebral Candidiasis

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Bee Venom (BV) is a natural substance, reported to booster the immune system. This study aimed at evaluating the effects of bee venom in stimulation of the immune system in mice infected with intracerebral Candidiasis probing for its possible use in treatment of this ailment. *In vivo* study included three groups of mice; bee venom-treated *Candida* infected group, untreated *Candida*-infected group and control group. Mice in the first two groups received intracerebral (i.c.) inoculation of *C. albicans*, while the control mice were inoculated i.c. with sterile saline. Mice of first group, the BV-treated group, received thereafter SC injection of BV for 4 days. On 5th day, mice of all groups were sacrificed and *Candida* infection was evaluated by culture of homogenized brain tissue on Sabouraud dextrose agar. Stimulation of the immune system was assessed by determining TNF- α and IFN- γ mRNA expression in splenocytes by real-time PCR (RT-PCR). In the *in vitro* study, direct effect of BV as antifungal agent and its effect on phagocytic function of neutrophils were evaluated. The results revealed that BV-treated mice had significantly low fungal load in the brain, with significantly high TNF- α but insignificantly high IFN- γ mRNA expression in splenocytes. BV had no direct antifungal effect, but it enhanced phagocytic activity of neutrophils. It was concluded that treatment with BV could result in significant therapeutic effect in intracerebral Candidiasis and thereby could be effective in resistant life-threatening infections.

Key words: Bee venom (BV), Candidiasis, innate immunity, cytokines, meningitis, TNF- α

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INTRODUCTION

Endemic and opportunistic fungal infections constitute an important clinical problem. The morbidity and mortality rates caused by normally harmless fungal species, e.g., *Candida*, *Aspergillus*, *Fusarium* and *Trichosporum*, are relatively high. Fungal invasion in human is associated with a wide variety of diseases, ranging from benign colonization, superficial skin infections, allergy, to life-threatening systemic mycosis (Cramer and Blaser, 2002; Murciano *et al.*, 2006).

Garber (2001) stated that systemic Candidiasis is, Sometimes, associated with high morbidity and mortality rates, even life-threatening conditions, because diagnosis is difficult and current antifungal therapies often fail. One form of invasive Candidiasis is candidemia, that was reported as the fourth most common bloodstream infection among hospitalized patients in the United States. *Candida meningitis* has been seen in low birthweight neonates with septicemia, as well as in patients with hematological malignancies, complicated neurosurgery and in patients with ventricular shunts or after lumbar puncture (Lunel *et al.*, 2004).

Netea *et al.* (1999) stated that treating Candidiasis solely with medication might not give the desired results and that *Candida albicans* could possibly develop resistance to the drugs used for treating it. They added that additional therapies directed toward the augmentation of host defense mechanisms would be a rational approach.

Bee Venom (BV) is a quite complicated chemically compound, that contains several biochemical or pharmacologically active substances. There are at least eight protein fractions of Honeybee Venom, of which phospholipase A, mellitin and apamin are the three major ones. BV is believed to have potential use in the medical field, as it was found to stimulate natural immunity through activation of the pituitary and adrenal glands and to stimulate the body to produce natural cortisone. Furthermore, BV was shown to be effective in the remission of tumors of many different types of malignant diseases. It was, also, found to have an anti-inflammatory and anti-infectious agents. phospholipase A₂-activating protein (PALP), that shares antigenic and biochemical similarities with mellitin, was shown to stimulate human neutrophil aggregation and release of lysosomal enzymes, as well as release of superoxide ions and eicosanoids (Bomalaski *et al.*, 1989).

In addition, BV was shown to induce the synthesis of IL-1 and TNF- α in human monocytes (Bomalaski *et al.*, 1995) and to induce, as well as, Th1 lineage development from CD4 (+) T cells by increasing the expression of a

Th1-specific cytokine (Nam *et al.*, 2005). Th1 clones have been found earlier to synthesize mRNA for IL-2, IFN- γ and TNF- α (Cherwinski *et al.*, 1987).

Considering the aforementioned information, together with the observation that polymorphnuclear cells and macrophages exhibit potent innate anticandidal activity and that specific T cells participate in the regulation and expression of acquired immunity to *Candida albicans* (Blasi *et al.*, 1993), it seemed possible that BV could be a potential therapeutic drug against *Candida* infection. Therefore, this study was planned to assess the immunological effects of BV in cases of intracerebral Candidiasis, a life-threatening infection, since the antifungal efficiency of BV in such condition has not been reported, aiming at introducing a possibly helpful means in therapy of i.c. Candidiasis.

MATERIALS AND METHODS

This study was carried out in the Faculty of Medicine, Ain Shams University, Cairo, Egypt, in the period April to August, 2008. The study consisted of *in vivo* as well as *in vitro* approaches.

In vivo study: This was carried out on male mice, weighing 25-30 g, divided into three groups:

- Bee venom(BV)-treated mice, received BV after being inoculated intracerebrally (i.c.) with *Candida albicans* (n = 5). Mice were treated with bee venom dissolved in saline, in a dose of 20 $\mu\text{g kg}^{-1}$, given s.c for 4 days (Lipps, 2002)
- *Candida*-infected mice, inoculated i.c. with *Candida albicans* and received no further treatment (n = 7)
- Control group, consisted of healthy male mice, of the same weight and age as above 2 groups, inoculated i.c. with sterile saline (n = 7)

Yeast *C. albicans* was isolated from clinical specimens and maintained by daily passage on Sabouraud dextrose agar (Oxoid, England). Yeast cells were harvested from agar plates, washed twice in saline by low speed centrifugation and diluted in saline prior to i.c. inoculation (Blasi *et al.*, 1993), the inoculum size being = 10^5 yeast cell per mouse.

Inoculation site and technique: The i.c. inoculation of mice was performed as described by Chiavolini *et al.* (2004). Mice were inoculated with *C. albicans* through a soft point located along the skull midline, 3.5 mm rostral to the bregma (the intersection of the coronal and sagittal sutures of the skull). The stereotaxic coordinates of such

inoculation point are 0 mm (x plane), 3.5 mm rostral (y plane) and 2 mm ventral (z plane) from the bregma.

Bee Venom (BV, Apitoxin), purchased from the Egyptian Organization for Biological Products and Vaccines (VACSERA), El-Agouza.

Experimental procedure: On the 5th day following i.c. inoculation, mice of all groups were sacrificed, the brains and spleens dissected out. Spleens were kept in PBS for quantitation of cytokine (TNF α and IFN- γ) mRNA expression by real-time (RT)-PCR to evaluate the stimulation of Cell-Mediated Immunity (CMI). The brains were removed aseptically to be homogenized for quantitation of *C. albicans* in infected brains.

Quantitation of *C. albicans* in infected brain homogenates: The brains of individual mice, removed aseptically, were placed in tissue homogenizer with 3 mL of sterile distilled water (Blasi *et al.*, 1993). Brain homogenates were cultured on Sabouraud dextrose agar to evaluate *Candida* infection in the brains of both the BV-treated and the untreated *Candida*-infected groups. Plates were incubated at 37°C for 24-48 h and the yeast cell count was evaluated by using calibrated loop.

Quantitation of cytokine (TNF α and IFN- γ) mRNA expression by real-time (RT)-PCR: Levels of both cytokines expression were assessed by real-time reverse transcription-PCR. Measurement of IFN- γ and TNF- α mRNA expression were performed in splenocytes. Spleens were obtained from each mice under complete aseptic conditions. Each spleen was put in PBS. Spleens were homogenized by gentle grinding between the frosted ends of autoclaved glass slides. Total RNA was extracted immediately using Magna Pure Compact RNA Isolation Kit (Cat No.04802993001) supplied by Roche-Germany. The yield of total RNA obtained was determined spectrophotometrically. Then, cDNA was synthesized immediately using Prime RT Premix (2X) supplied by Genet Bio Korea (Cat No. R-2000). The kit contains Prime MMLV RTase, reaction buffer, dNTPs mixture, RNase inhibitor and protein stabilizer. Two μ g of the total RNA was mixed with 0.5 μ g of the oligo dt primer in RNase free (DEPC-treated) water to reach a total volume of 10 μ L, incubated at 70°C for 5 min and then chilled on ice then 10 μ L of Prime RT Premix was added to have a total reaction volume of 20 μ L and mixed by pipetting gently up and down. The mixture is incubated for 60 min at 37°C. The reaction was stopped by heating at 70°C for 10 min then chilling on ice.

The set of primers used for amplification of IFN- γ and TNF- α were as follows: IFN α forward primer AAT GCA

GGT CAT TCA GAT G and reverse primer AAC TGA CTT GAA TGT CCA A (accession NM 000619.2) and TNF- α forward primer AGACCCCCCTCGGAATCG and reverse primer CCGGATCATGCT TTCAGTGC (accession NT 113891.1) As internal control GAPDH primers were used with forward primer ACCACAGTCCATGCCATCAC, and reverse primer : TCCACCACCCTGTTGCTGTA (accession NM 002046.3).

A final reaction volume of 20 μ L was prepared using Light Cycler-DNA Master SYBR Green I kit (Cat No. 2 015 099) - Roche diagnostics, Germany. Each mix is formed of 1 μ L of each primer (0.5 μ M), 2 μ L Light Cycler DNA Master SYBR Green I (1x), 2.4 μ L MgCl₂ stock solution (4 mM), 11.6 μ L H₂O sterile PCR grade and 2 μ L of cDNA template (30 ng μ L⁻¹). PCR reactions were done in Light Cycler (Roche, Germany). Light Cycler system has been designed to reduce the time taken to achieve results from PCR reactions and to enable the user to monitor the amplification of the PCR product simultaneously, in real time and on line. The PCR occurs in specially designed borosilicate glass capillaries which can hold up to 20 μ L of sample. The combination of using air for rapid thermal cycling and the high surface to volume ratio of the capillaries allows a single PCR cycle to be completed in less than 30 sec.

The temperatures for PCR cycles was denaturation 95°C for 2 sec, annealing at 60°C for GAPDH, 56°C for TNF- α and 58°C for IFN- γ for 10 sec and elongation 72°C for 15 sec then analysis by melting curve to discriminate between primer dimer and specific product.

In vitro study: The direct antifungal effect of BV on *Candida* cells was assessed *in vitro* by using:

- Agar diffusion method, carried out according to the method described by Miles and Amyes (1996) and Cvetni \acute{a} and Knezevi \acute{a} (2004)

The selected strain of *C. albicans* was cultured overnight in broth and its concentration was adjusted to 0.5 Mc Farland scale (1 \times 10⁸ cfu mL⁻¹). A swab was immersed in the broth, squeezed against the wall of the tube and spread on the surface of Sabouraud dextrose agar media.

In the center of the agar plate 25 μ L of bee venom (5 μ g mL⁻¹ %) solutions was poured inside a sterile plastic cylinders (8 \times 6 \times 10 mm in diameter). Antimicrobial effect was assessed by measuring inhibition zone around the cylinder.

- Serial dilution method to determine the Minimum Inhibitory Concentration (MIC) of BV, was done

according to the method described by Miles and Amyes (1996)

In vitro effect of honey bee venom on phagocytic activity of neutrophils: Blood samples were taken from 15 mice, 7 samples were incubated with bee venom and 8 samples were used as controls. Fresh blood taken from mice was immediately placed on a clean dry glass slide and BV dissolved in saline, was added to the blood to obtain a final concentration of 5 µg BV mL⁻¹ blood. The polymorphnuclear leucocytes (PMNL) were obtained following lysis of the erythrocytes. One hundred microliters of PMNLs suspensions was mixed with 100 mL of preopsonized *C. albicans*, before Hank's Balanced Salt Solution (HBSS) containing 0.5% Bovine Serum Albumin (BSA) was added to a final volume of 1 mL. This provided an initial fungus to PMNL ratio of 10:1. The mixtures were rotated at 37°C for 15 min. Then, the PMNL suspension was spread and stained by Leishman stain. Phagocytosis was measured cytomorphologically by determining the *C. albicans* phagocytic index according to Ballaret *et al.* (1987) using the following equation:

$$\text{Phagocytic index} = \frac{\text{Total No. of } C. \text{ albicans cells} \times 100 \text{ PMNL}}{100}$$

In other words, the average number of ingested yeast cells (cytic number) per 100 neutrophils (PMNL) was enumerated and expressed as the phagocytic index.

Statistical analysis (Armitage and Berry, 1987): Data are expressed as Mean±SEM. Statistical significance for unpaired data was determined using a one-way Analysis of Variance (ANOVA) with post-hoc test, significance calculated by LSD (least significant difference) multiple range-test to find inter-group significance. A probability of p<0.05 was considered statistically significant. Statistical significance for data of fungal load in the brain as well as phagocytic function of neutrophils was determined using non parametric Mann-Whitney test. The level of significance was accepted as p<0.05.

RESULTS

The Fungal load (yeast cell count) expressed in colony forming units (cfu) collected from brain homogenates was significantly higher in the untreated *Candida*-infected mice as compared to BV-treated mice, indicating significant therapeutic effect of bee venom (Table 1).

Using quantitative RT-PCR to investigate the effects of BV on the expression of cytokine mRNA in

Table 1: Fungal load in brain of mice infected with *C. albicans*

Mice	cfu (10 ⁶) recovered from brains	No. of observation
Bee venom-treated	0.0026±0.001	5
<i>Candida</i> -infected	59±21.01*	7

*Significance calculated by Mann-Whitney test, *: p<0.05

Table 2: Quantitative IFN-γ and TNF-α mRNA expression in splenocytes

Expression	Bee venom-treated	<i>Candida</i> -infected	Control
IFN γ (copy mL ⁻¹)	1.21±0.75 (5)	0.63±0.56 (7)	0.67±0.35 (7)
TNF-α (copy mL ⁻¹)	13.36±5.95ab (5)	0.35±0.28 (7)	2.33±0.96 (7)

Value in parenthesis is the number of observations. a: Significance calculated by Least Significant Difference (LSD) at <0.05 from control group, b: Significance calculated by LSD at <0.05 between bee venom-treated and *Candida*-infected groups

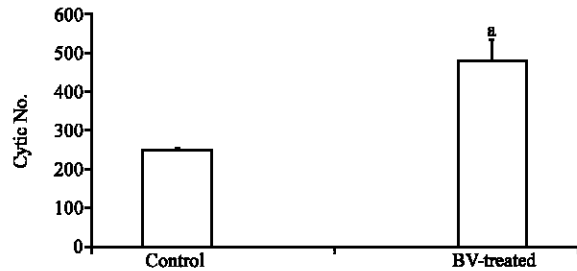


Fig. 1: Effect of honey bee venom on phagocytic activity of neutrophils: a: Significance calculated by Mann-Whitney test: p<0.001

splenocytes, a significant increase in expression of TNF-α mRNA was observed in the BV-treated *Candida*-infected mice as compared to both the untreated *Candida*-infected mice as well as the controls. On the other hand, the mRNA IFN-γ expression showed insignificant increase in the BV-treated group as compared to the untreated *Candida*-infected and control groups (Table 2).

Further, the present results concerning the direct antifungal effect of BV on *Candida* cells revealed no direct antifungal activity of the venom, in either the agar diffusion or the serial dilution methods.

With regard to the effect of *in vitro* BV challenge on phagocytosis of *Candida* cells by neutrophils. BV was found to increase significantly the phagocytic activity of neutrophils against *Candida* cells, indicated by phagocytic index with p-value<0.001 (Fig. 1).

DISCUSSION

The incidence of systemic fungal infection, specially by *C. albicans*, is increasing. There is a growing body of literature on the resistance to the current antifungal therapies (Murciano *et al.*, 2006). Researches suggest that besides the efforts to develop more effective and safer antifungal agents, a new therapeutic approach to augment

the antifungal capacity of the host's immune system should be investigated (Káposzta *et al.*, 1998). It was, thus, intriguing to probe the role of BV, known to be capable of stimulating the immune system, thereby boosting the host's defence against this fungal infection.

As evident from the present results, a much lower fungal load of *C. albicans* was recovered from the brains of BV-treated mice compared to the infected non-treated group. Such effect seems to entirely rely on enhancement of the host's immune response, as the BV did not exert any direct antifungal effect on *Candida* cells when tested *in vitro*. Furthermore, TNF- α and IFN- γ mRNA expression in the spleen from BV-treated mice was higher than in the non-treated group, the increase being more marked and statistically significant for TNF- α mRNA expression only.

Bomalaski *et al.* (1989) had stated that resistance to candidal infection requires the coordinated action of innate and adaptive immune defenses and that neutrophils can clear the pathogen via phagocytosis. In accordance with this view is the significant increase in the phagocytic activity of neutrophils in presence of BV, seen in the present study. This increased neutrophil phagocytic activity towards *C. albicans* might have possibly helped in lowering the fungal load of the brains taken from mice treated with BV. In addition, the BV peptide melittin was found to express functional and antigenic similarities to Phospholipase A₂-activating protein (PLAP), which was shown to induce neutrophil aggregation, secretion of lysosomal enzymes and release of superoxide ions and eicosanoids (Bomalaski *et al.*, 1989).

Further, more recently Stuhlmeier (2007) stated that venom of apis mellifera (bee venom) and melittin mediate immune-modulating effects and large quantities of oxygen radicals are produced in a dose-dependent manner in leukocytes exposed to BV. The changes in leukocytes activity in presence of BV could possibly contribute to the role of BV in promoting the host's defense mechanisms against this infection. Further, the present study revealed a marked and significant increase in TNF- α mRNA expression: TNF- α is known to play an important role in immune and inflammatory responses. It was previously demonstrated that the pro-inflammatory cytokine TNF- α , secreted by monocytes and activated macrophages, is critical in protecting the host against disseminated Candidiasis (Netea *et al.*, 1999). The researchers added that endogenous TNF- α contributes to host resistance to disseminated Candidiasis and its absence in mice renders the animals more susceptible through impaired recruitment of neutrophils and impaired phagocytosis of *C. albicans*.

Similarly, Brieland *et al.* (2001) reported that TNF- α plays a key role in host defense against systemic Candidiasis caused by *C. albicans*, as the absence of endogenous TNF- α activity was associated with enhanced tissue burden in infection models.

Earlier, Blasi *et al.* (1994) reported that IL-1 secretion, as a sequel of *Candida* infection, is mediated by endogenous TNF- α . The researchers stated that IL-1 has a protective role in systemic and localized *Candida* infections.

Bomalaski *et al.* (1995) had reported that the BV peptide melittin induces the synthesis of TNF- α and that this cytokine has anticandida effect.

Besides the role of neutrophils in clearing the pathogen via phagocytosis and the macrophage activation that leads to the release of several key mediators, such as pro-inflammatory cytokines, it is accepted that antifungal CD4 + T helper 1-mediated responses play a central role in anti-*C. albicans* defenses. These responses are believed to provide control of fungal activity through the production of gamma interferon (IFN- γ), which is required for optimal activation of phagocytes and for helping in the generation of a protective antibody response. Moreover, helper T cells play a central role in regulating immune responses to the fungus, *C. albicans*, by providing critical cytokine-mediated activation and deactivation signals to fungicidal effector cells. In experimental models of candidiasis, protection correlates with the generation of CD4 + Th1 cells producing IL-2 and IFN- γ and requires the concerted action of several cytokines, including IL-12. Susceptibility to infection correlates with the generation of CD4 + Th2 cells producing IL-4 and IL-10 that have been implicated in inhibiting the development of protective Th1 cells and in opposing the IFN- γ -mediated activation of fungicidal macrophages (Murciano *et al.*, 2006)

Earlier, *in vivo* and *in vitro* studies indicated IFN- γ as a major activating factor for fungicidal phagocytes. IFN- γ was reported to exert complex effects on the regulation of immunity against *C. albicans* infection, affecting both innate and acquired Th1-dependent immune responses (Cenci *et al.*, 1998).

In the present study, the encountered results showed that treatment with BV results in only slight insignificant increase in IFN- γ . These findings which agree with other studies do not rule out the therapeutic effect of Bee venom. As previous study (Brieland *et al.*, 2001) showed that murine resistance to primary *C. albicans* infections was dependent on TNF- α and independent of IFN- γ . The controversy between different literatures may be accounted to different reasons; firstly the time of assessment of cytokine mRNA. Previous report

(Brieland *et al.*, 2001) studied the temporal induction of cytokine mRNA for interferon- γ in kidneys of infected mice, it reported a delay of induction for 2-3 days after infection. Similarly, it could be a delay in induction of cytokine mRNA for interferon- γ after Bee venom administration, since our study protocol was only four days.

An alternative explanation could be that both absolute and relative amounts of secreted cytokines depended on the PLAP concentration. At low PLAP concentrations, it was found that little or not IFN- γ was produced, whereas at higher PLAP concentrations significant amounts of IFN- γ were obtained (Carballido *et al.*, 1992). As in the current study the dose of Bee venom used was a physiological one, this could contributed to the insignificant increase of IFN- γ .

CONCLUSIONS

The findings of the present study provide further evidence that BV boosts the body's immune system, manifested by significant decrease in the fungal load in the brain, significant increase in TNF- α mRNA expression, together with slight increase in IFN- γ expression in mice treated with BV compared to the untreated mice. Also, BV enhanced phagocytic activity of neutrophils *in vitro*. It had no direct antifungal effect, indicating that the lower fungal load in brains of BV-treated mice is the sequel of enhanced activity of the immune system induced by BV.

Based on the present and former data, it is possible to recommend bee venom as an alternative medicine, providing a strong and effective therapeutic tool in resistant and life threatening infectious conditions, helping conventional medical therapy by augmenting the antimicrobial capacity of the host's immune system.

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