Sci. J. Fac. Sci. Minufiya Univ., 2011, Vol.XXV *ISSN: 1110-2195*

THE EFFECT OF BEE VENOM ON TUMOR BEARING MICE

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ABSTRACT

The present study was carried out to assess the effect of bee venom on Ehrlich's scites carcinoma (EAC) and its influence on antioxidant status in the liver. Female mice were divided into 5 groups; control, bee venom (0.3 mg/mouse subcutaneously s.c. for 7 days), Ehrlich ascites Carcinoma (EAC) ($1x10^6$ subcutaneously to induce solid tumor), Bee venom for 7 days. followed by (EAC) (protected group) and (EAC) followed by bee venom for 7 days (treatment group). A lipid peroxidation product was determined by measuring the malodialdehyde (MDA). Superoxide dismutase (SOD), catalase (CAT) and Glutathione-S-transferase (GST) activities; Glutathione (GSH) content and Immunohistochemistry were examined. In EAC-bearing mice, mean life span and antioxidants were significantly decreased, and lipid peroxidation was significantly increased. Nearly these changes were brought back to normal levels in protected and treatment groups. The findings are further confirmed by histopathological observations. Our data suggest that supplementation of bee venom is useful in reducing tumor in mice.

Keywords: Ehrlich's ascites carcinoma; Tumor; Bee venom; Antioxidant

INTRODUCTION

Cancer is a term for diseases in which abnormal cells grow and divide without control. This is usually the result of damage to a number of regulatory mechanisms within the cell (Cooper, 1992). Cancer chemotherapy using antioxidant formulations is an exciting pharmaceutical research involving the use

of either natural or synthetic components to delay, inhibit or reverse the development of cancer in normal or preneoplastic conditions.

The approach of testing venom as antitumor agents dates back to the beginning of the past century when Calmette et al. (1933) reported the antitumour activity of snake venom on adenocarcinoma cells. In addition to the snake venom, bee venom has been reported to have antiproliferative effect in vitro and reduction in tumor growth in vivo (Orsolic et al.2003).

Bee venom (BV) contains a variety of peptides including melittin, apamin, adolapin, and the Mast cell degranulating (MCD) peptide. It also contains enzymes (e.g., Phospholipase A2 (PLA_2)), biologically active amines (e.g., histamine and epinephrine) and nonpeptide components (including lipids, carbohydrates and free amino acids) (Lariviere and Melzack, 1996). BV has been used traditionally for the relief of pain and the treatment of chronic inflammatory diseases, such as rheumatoid arthritis and in oriental traditional medicine (Kwon et al., 2001). BV has also anti-cancer activity and has the capacity to kill cancer cells. The promise of this remedy exists with live honeybees, which make tumors disappear by killing cancer cells (Liu et al., 2002). The cytotoxic effect through the activation of PLA_2 by melittin is believed to be an important mechanism of anti-cancer activity of BV. The induction of apoptotic cell death through several cancer cell death mechanisms, including the activation of caspase and matrix metalloproteinases (MMP), is important for the melittin-induced anti-cancer effects (Moon et al., 2006).

Reactive oxygen species (ROS) are constantly produced during metabolic processes in all living species *(Sahu, 1991)*. Under normal physiological conditions, cellular ROS generation is counterbalanced by the action of antioxidant enzymes and other redox molecules. Oxidative stress is

potentially harmful to cells and reactive oxygen species (ROS) are implicated in the etiology and progression of many diseases including cancer *(Feillot-Coudray et al., 1999)*.

MATERIALS AND METHODS

Bee venom powder was purchased from Faculty of Medicine; Ain shams University, Cairo, Egypt. Bee venom was dissolved in distilled water (1gm/ml). Cell line of Ehrlich's ascites Carcinoma (EAC) obtained from National Cancer Institute, Cairo University, Egypt. In experimental protocols described, mice were injected subcutaneously (s.c.) of $1x10^6$ EAC cells into the right thigh of the lower limb of the mice to induce solid tumor.

Animals

Forty female mice (obtained from National Research Center, EL- Doky, Cairo, Egypt) were used. All mice were between 8 and 12 weeks old weighing about 20-25 g and maintained at the animal house of Genetic Engineering and Biotechnology Institute, Minufiya University. Mice were housed at 23 ± 2°C and in daily dark/light cycle. They were maintained under standard condition and fed standard chow and water ad libitum. The mice divided randomly into 5 groups (8 in each).

Group I- Normal control group consisted of healthy female mice.

Group Π- Animals of this group were injected subcutaneously into the left footpad with 0.3 mg of bee venom / mouse for seven days.

Group Ш- Animals of this group were implanted with EAC to form a solid tumor.

Group IV-Animals of this group were protected by injecting bee venom subcutaneously *(s.c.)* in a dose of 0.3 mg / mouse for seven days and after 24h mice were injected with EAC.

Group V- Animals of this group were injected with EAC and after 2weeks mice were injected subcutaneously *(s.c.)* with bee venom in a dose of 0.3 mg / mouse for seven days.

Total experimental period was 3 weaks. Tumor and liver tissues samples were taken for analysis.

Preparation of liver homogenate

Liver tissue was homogenized in ice-cold homogenate buffer (0.34 M sucrose and 1 mM potassium dihydrogen phosphate, pH 7.0) for analysis.

Assessment of oxidative capacity

The MDA content of homogenates was determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances (TBARS) (Uchiyama and Mihara, 1978). Results are expressed as nmol/g wet tissue.

SOD enzyme activity determination was based on the ability of SOD to inhibit the phenazine methosulphate-mediated (PMS) reduction of nitroblue tertrazolium (NBT) dye to formazan as previously described (Nishikimi et al., 1972).The product was evaluated spectrophotometrically at 560 nm. Results are expressed as U/g wet tissue .

CAT enzyme activity was determined according to the method of (Bergmeyer, 1974). The principle of the assay is based on the determination of

the H2O2 decomposition rate at 240 nm. Results were expressed as μmol/sec/g wet tissue.

GSH was determined by the method of Beutler et al. (1963). This method utilized metaphosphoric acid for protein precipitation and the water soluble 5, 5`-dithiobis (2-nitrobenzoic acid), DTNB, for color development. Results are expressed as μmol/min/g wet tissue.

GST activity was assayed spectrophotometrically using 1-chloro-2-4-dinitrobenzene (CDNB) and glutathione as described by (Habig et al., 1974). Results are expressed as nmol/g wet tissue.

Immunohistochemistry procedures

Immunohistochemistry was performed in tumor tissue section for the detection of B-cell lymphoma 2 (Bcl-2) expression. The selected paraffin blocks for immunohistochemical staining are sectioned at 4 um and stained with two monoclonal antibodies, using the avidin-biotin peroxidase method.

Histological procedures:

Liver and tumor tissues of two animals from each group were fixed in 10% neutral buffered formalin, 4μm paraffin sections were dehydrated in graded alcohol, embedded in paraffin section, and stained with hematoxylin and eosin (H&E), and viewed under microscope (Mallory, 1938).

Statistical analysis:

The results were expressed as mean ±standard deviation (SD) for eight animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) . Post hoc testing was performed for inter-group

comparisons using the Tukey test; Differences were considered significant at $P<$ 0.001.

RESULTS

Effect of bee venom on lipid peroxides production, SOD, CAT, GST activities and GSH content.

Results showed a significant increase in lipid peroxidation level ($P<0.001$) in the liver of Ehrlich's ascites (EAC) group as compared with protected group (BV/EAC), treated group (EAC/BV) and also normal control group. On the other hand, MDA was decreased significantly ($P<0.001$) in liver of BV group as compared with that of (BV/EAC) group, (EAC/BV) group and also normal control group (Table1). SOD,CAT,GST activities and GSH content significantly decreased ($P<0.001$) in the liver of (EAC) group as compared with (BV/EAC) group, (EAC/BV) group and also normal control group. On the other hand, catalase activity was increased significantly ($P<0.001$) in liver of BV group as compared with that of (BV/EAC) group, (EAC/BV) group and also normal control group (Table 2). The tumor implant has decreased the activities of enzymes like SOD, CAT, GST, and GSH levels in the liver. Nearly treatment with BV was found to restore the above levels to normal.

Immunohistochemical of bcl-2

Figures (1,2 and3) depict the effect of bee venom on bcl-2 expression in tumor cells of EAC-bearing mice and are compared with the bcl-2 expression in untreated tumor cells of EAC-bearing mice. Untreated tumor cells show very focal expression of bcl-2 (fig.1). Fig (2) shown bcl-2 positivity in tumor cells of bee venom protected group. On the other hand, fig.(3) elevated complete negativity focal bcl-2 expression in tumor cells of bee venom treated group.

Histological findings

Both bee venom treated and untreated tumor were examined histologically. The tumor from EAC-bearing mice appeared to contain highly malignant tumor (fig.4). In contrast, bee venom protected tumors were found to contain necrosis cells with an adjacent viable sheets of cells (fig.5). In addition, fig.(6) showed extensive necrosis with few viable tumor cells in bee venom treated tumor.

Table 1: Level of MDA as the end product of lipid peroxidation in the liver of different animal groups.

Parameters	Control	BV	EAC	BV/EAC	EAC/BV
MDA(nmol/g wet tissue)	$239.63\pm2.13^{a,b}$	231.75 ± 3.95^{c}	419.12 ± 5.11^{c}	$248.12\pm 2.03^{a,b}$	$246.5\pm4.81^{a,b}$

Values are expressed as mean ± SD of eight mice.

(^{a}): *P < 0.001 vs. EAC group.*

(^{b}): *P < 0.001 vs.BV group.*

(^{c}): *P < 0.001 vs. control group.*

Table 2: Levels of SOD, CAT, GST activities and GSH content in the liver of the different animal groups.

Parameters	Control	BV	EAC	BV/EAC	EAC/BV
SOD(U/g wet tissue)	138.12± 2.8[a,b]	194.12±6.4[c]	99.04±1.7[c]	162.4±3.9[a,b]	176.4± 1.06[a,b]
CAT(µmol/sec/g wet tissue)	8.97± 0.4[a,b]	11.66±0.9[c]	4.37±0.26[c]	8.2± 0.52[a,b]	8.85± 0.21[a,b]
GST(µmol/min/g wet tissue)	0.57± 0.05[a,b]	0.68± 0.02[c]	0.28± 0.04[c]	0.6± 0.03[a,b]	0.46±0.03[a,b]
GSH(µmol/min/g wet tissue)	5.81± 0.2[a,b]	7.22± 0.83[c]	3.17± 0.24[c]	5.67± 0.27[a,b]	4.24± 0.18[a,b]

Values are expressed as mean ± SD of eight mice.

([a]): *P < 0.001 vs. EAC group.*

([b]): *P < 0.001 vs. BV group.*

([c]): *P < 0.001 vs. control group.*

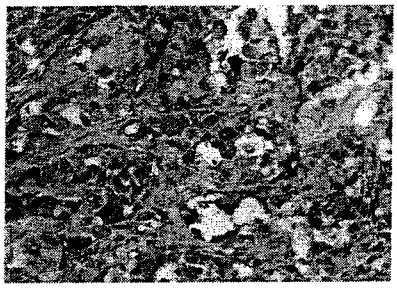

Fig.1.Immunohistochemistry observation of untreated tumor shows very focal expression of bcl-2 in tumor cells. (Immunohistochemistry) x400.

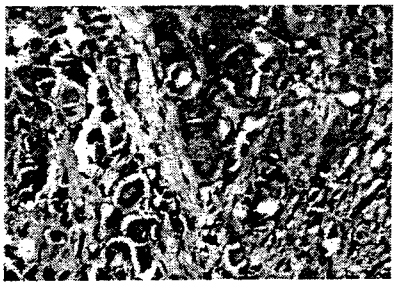

Fig.2.Immunohistochemistry observation shows bcl-2 positivity in tumor cell was protected with bee venom. (Immunohistochemistry) x400.

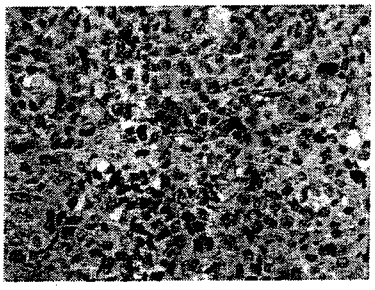

Fig.3. Immunohistochemistry observation shows complete negativity focal bcl-2 expression in tumor cells in cases treated with bee venom. (Immunohistochemistry) x400.

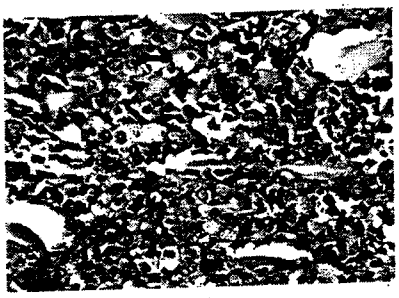

Fig.4.Histopathological observation of tumor cells shows highly malignant tumor induced by Ehrlich's ascites (EAC) (H&E, 200x).

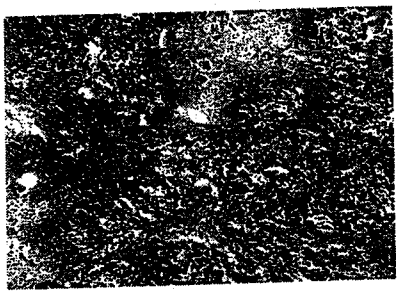

Fig.5.Histopathological observation of tumor cells Shows necrosis with an adjacent viable sheets of tumor cells in mice protected with bee venom (BV) (H&E, 100x).

Fig.6.Histopathological observation of tumor cells Shows extensive necrosis with few viable tumor cells in mice treated with bee venom (BV) (H&E, 100x).

Discussion

The present study was carried out to investigate the protective and treatment activity of bee venom against solid tumor induced by Ehrlich's ascites carcinoma (EAC) in female mice.

The potential antioxidant therapy includes natural free radicals scavenging enzymes and agent which are capable of augmenting the activity of these enzymes including SOD and CAT. Cancer cells have highly elevated protective mechanism to prevent lipid peroxidation (Gauchez, 1995). SOD is the key enzyme in scavenging the superoxide radicals; CAT is also another key enzyme in the scavenging, which helps in cleaning the H_2O_2 formed during incomplete oxidation. As a whole, these antioxidant enzymes play an important role in the body defense mechanism against the harmful effects of the reactive oxygen species (ROS) and free radicals in biological systems (Halliwell and Gutteridge, 1999). GSH is a naturally occurring antioxidant important in the antioxidant defense of the body. It has been reported that determination of total GSH can serve as a key to know the amount of antioxidant reserve (Soni et al., 2008). Therefore, the observed elevated levels of lipid peroxidation in liver of tumor-bearing animals could attribute to observed decrease in GSH levels and GST activities. GST is biotransformation enzymes involved in the detoxification of xenobiotics, carcinogens, free radicals, and peroxides by conjugating these toxic substances with GSH, ultimately protecting the cells and organs from oxidative stress (Devasena et al., 2002).

In our study, solid tumor induced by EAC caused a significant increase ($P<0.001$) in the level of lipid peroxidation. While levels of antioxidative statue have been significantly disminished. Several studies have demonstrated that lipid peroxidation is significantly decreased in tumor cells and tissues compared with that of corresponding normal cells (Gauchez, 1995). Treatment with bee venom

for 7 days prior to EAC injection resulted in a reduction in the level of lipid peroxidation and improved antioxidant enzymes as well as non-enzymatic markers concentration in liver as shown in table (1and2). Level of lipid peroxidation measured as MDA is decreased significantly *(P<0.001)* in liver homogenate of mice maintained on bee venom as shown in table (1). While, levels of SOD and CAT showed significantly increased values *(P<0.001)* in liver homogenate of mice protected with bee venom as compared to those of tumor control group. Also, hepatic tissues indicated remarkable increases *(P<0.001)* in GSH and GST levels in mice protected with bee venom.

Several studies have demonstrated that tumor-bearing animals can experience a systemic change of antioxidant enzymes in organs distant from the tumor (Sardar et al., 1993). A study by Zatmoski and Nathan (1991) suggested that tumor cells produce substantial amount of hydrogen peroxide (H2O2), which may be released into circulation for being transported to the liver for detoxification. Furthermore, growing tumors sequester essential antioxidants from host tissues and meet their demand (Bhuvaneswari et al., 2004). The superoxide radical has been shown to directly inhibit the activities of enzyme catalase.

Likewise, it appears that treatment with bee venom for 7 days post EAC lead to a remarkable reverse of oxidative damage. This is shown by a significant reduction in level of lipid peroxidation and enhancement in level of GSH as compared to group III. Similarly, treatment with bee venom resulted in significant amelioration in the activities of enzymes CAT, SOD and GST.

Our results are in line with this report of (Balasubashini et al., 2006) who revealed that treatment with fish venom(FV) effectively reduce the oxidative stress in EAC-bearing mice and thereby restored the activities of enzymic antioxidants. Similar results were reported by Ramanaiah and Venkaiah (1992) that, scorpion venom has the SOD activity whose action is inhibited by specific

antivenom. Apart from this, in the past few years several peptides have been reported to exert different mechanisms of action in free radical mediated oxidative sequences by radical scavenging and metal ion chelation (Niranjan et al., 2004). In addition , Our finding are in agreement with those of (Balamurugan et al., 2010) who reported that treatment with different doses of sea nettle nematocyst venom(SNV) peptide was observed to decrease the tumor burden, hence decrease the Oxidative stress, which in turn results in the restored activities of GST and GSH levels. Also administration of SNV peptide at different doses increased the SOD and CAT levels in a dose –dependent manner, which may indicate the antioxidant and free radical scavenging property of SNV peptide. Previous study revealed the presence of SOD and GST in venom gland of the honey bee which have peroxidase activity in insects and thus may be involved in the reduction of hydrogen peroxide (Peiren et al., 2008).

In general, cell death has long been considered to occur via two distinct processes: apoptosis and necrosis. Previous studies have considered autophagy as an additional mechanism via which cells may undergo programmed cell death (Adams, 2003). Apoptosis is characterized by condensation of cell cytoplasm and chromatin, cell shrinkage, nuclear breakdown, DNA fragmentation, and cell fragmentation into apoptotic bodies (Woodle et al., 1997). On the other hand, necrosis is characterized by the swelling of the cells, formation of microvessicles, and leakage of the cytoplasm (Pang and Geddes, 1997). Our histochemical analysis of the untreated tumor cells revealed very expression in bcl-2 as shown in figures (1). These cells have suffered apoptotic cell death .On the other hand, the tumor cells that were treated with bee venom for 7days revealed complete negativity of bcl-2 expression as shown in figures(3). These findings demonstrated that bee venom induces apoptotic cell death.

Since bee venom contain cocktails of many different types of proteins with a variety of pharmacological activities, and they are also known to cause damage selectively to different cell types, it is likely therefore that some component(s) of venom may cause cell death via apoptosis, whereas different component(s) may cause cell death via necrosis.

Our result is in line with this report of (Gao et al., 2007) who revealed that some venom has also been shown to induce both apoptosis and necrosis. In addition, (Hu et al., 2006) who also reported that BV inhibits proliferation and induces apoptosis of human hepatoma cells. Furthermore, (Ip et al., 2008a) investigated the molecular mechanisms of apoptosis induced by BV in human breast cancer cells.

Another results are in agreement with those of (Jang et al., 2003) who reported that BV treatment resulted in an increase in the expression of Bax, a pro-apoptotic protein, and a decrease in the expression of Bcl-2, a protein that heterodimerizes with Bax, suppressing cell death. Also, (Moon et al., 2006) shown that melittin kills tumor cells by apoptosis through several cancer cell death mechanisms. Similar results were reported by (Moon et al., 2008) who suggest that melittin-induced apoptosis contributes to the decreased proliferation of U937 cells. This apoptotic response was associated with the upregulation of Bax and caspase-3 activation and downregulation of Bcl-2 (inhibitor of apoptosis).

Our results are supported by the histopatholoigical study. The observation of the tumors that were treated and protected with bee venom showed distinct areas of necrosis as shown in figure (5and6).These suggested that necrosis may be the primary cause of bee venom induced cell death. Our findings are in consistence with histopathological results of (Mohamed et al., 2009) who reported that

tumors that were treated in vivo with snake venom, all tumors showed the presence of mainly necrotic cells.

In conclusion, protected and treatment with bee venom include the inhibition of lipid peroxidation, increasing the content of GSH, elevating the expression of antioxidant enzymes, and induces apoptotic cell death.

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Sci. J. Fac. Sci. Minufiya Univ., 2011, Vol.XXV *ISSN: 1110-2195*

تأثير سم النحل علي الفئران البيضاء الحاملة للأورام السرطانية

طارق سالم، أيمن المغاوري، علاء سعدالدين، أسماء جابر، ولاء خالد، محمد عثمان

قسم البيولوجيا الجزيئية و قسم المعلوماتية الحيوية معهد الهندسة الوراثية و التكنولوجيا الحيوية و كلية الطب جامعة المنوفية

- يحتوى سم نحل العسل على مواد عديدة نشطه مثل الميليتين والأبامين والببتيدات المسببه لإنحلال الخلايا الحلمية والدوبامين وإنزيم الفوسفولايبيزأ والهيستامين والدهون والكربوهيدرات والأحماض الأمينية. وقد أستخدم سم نحل العسل لتخفيف الألم وعلاج الأمراض الإلتهابية المزمنة كما أن له القدرة على قتل الخلايا السرطانية.

لهذا تم تصميم هذه الدراسة للكشف عن دور سم نحل العسل للوقاية والعلاج من تأثيرالاورام السرطانيه فى الفئران البيضاء. وقد اشتملت الدراسة علي ٤٠ فأراً ابيض تتراوح أوزانهم من ٢٠ الي ٢٥ جرام تم تقسيمهم الي خمس مجموعات: المجموعة (١): تتعايش الفئران بهذه المجموعة تحت الظروف العادية من حيث التغذية والشراب المجموعة (٢): تم حقن الفئران بسم النحل بتركيز ٠,٣ (مللى جرام) لكل فأر لمدة ٧ يوم .المجموعة (٣): تم حقن الفئران بالإستسقاء المسبب للسرطان المجموعة (٤): تم حقن الفئران بسم النحل لمدة ٧ أيام ثم الإستسقاء .المجموعة (٥): تم حقن الفئران بالإستسقاء ثم بسم النحل لمدة ٧ أيام. وقد تم إجراء العوامل الكيميائية الحيوية والعوامل المناعية والعوامل الهيستولوجية. وتتلخص النتائج التي تم الحصول عليها أن العلاج أو الحمايه بسم نحل العسل يستعيد مستويات انزيمات الكبد أشبه بالطبيعيه ويخفض مستوي تأكسد الدهون وأيضا يؤدى إلى الموت المبرمج للخلايا السرطانيه.