Original Article

A Cytogenetic Study of the Effect of Bee Venom on the Genetic Material in the Laboratory Mice

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Abstract

Bee venom (BV) therapy was used to treat various diseases. It is a tiny essential peptide with a powerful hemolytic feature composed of a known series of amino acids. Since melittin is a non-specific cy¦tolytic pe¦ptide attacking lip¦id m¦embranes which contribute to toxicity, it is suspected that it could have important therapeutic benefits. This study aims t¦o use a multi-biomarker approach to assess the cytogenotoxic ef¦fects o¦f melittin i¦n periph¦eral blo¦od lym¦phocytes of mice and th¦e mole¦cular mechan¦isms invol¦ved. Control and treatment groups are divided into one hundred and fifty mice. Bee venom has been combined with four separates dose of melittin in the peritoneal (500, 350, 300 and 250) µg/ml. The results of the experiment showed that significant difference in the first group which injected with melittin intra peritoneal 0.2 ml and the second group in mitotic index (MI), where the differences were respectively 8.00 ± 0.56 for the first group and the second group 6.90 ± 0.31. In the case of micro nuclei examination, we also showed significant differences in the third group, where the difference was 14.7 ± 0.29. In the case of micronuclei examination, also there were significant differences in the third group, where the difference was 14.7 ± 0.29.

Keywords: Bee venom, Cytogenetic, Micro nuclei, Mitotic index.

Introduction

Africanized honey bee-AHB (Apis mellifera L.) produces the bee venom (BV), a complex mixture of proteins, lipids, amino acids, carbohydrates and peptides such as melittin and apamine. Many studies have studied bee venom and melittin's biological and pharmacological functions, indicating that they have radio-protective, anti-inflammatory, antibacterial, antiviral and anti-cancer activities (1). Melittin (C131H229N39O31) is the main component of HBV (2), and it accounts for about 50% of venom's dry weight (3). The cationic peptide is made up of 26 amino acid residues, and has five positive bond-free charges (4). The amphipathic properties of Melittin help the peptide bind with the phospholipid membranes and turn them into a water-soluble compound (5). Of red blood cells too, Melittin has elevated hemolytic activity (6). Several studies have shown that Melittin prevents apoptosis, necrosis and lysis induction from the proliferation of various cancer cells (7).

Melittin molecular structure, determined from crystals grown in aqueous solutions is a bent α-helical rod. The bending is due to the presence of proline, a feature common to anti-microbial and toxin peptides, and has a molecular weight of 2.86 kD and high aqueous solubility. Melittin also appears to have toxic side effects as some of the other individual compounds in BV (8). There are numerous biological activities in this peptide, including high anti-inflammatory activity (9). BV toxin has also been used to treat some immune-related diseases, notably rheumatoid arthritis, in aqua acupuncture therapy.

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Using traditional oriental medicine, satisfactory results are obtained (10). Many studies examined the biological and pharmacological activities of bee venom toxin. Anti-inflammatory and anti-rheumatoid arthritis effects (11), pain-relief effect (10), anti-cancer cell proliferation (12, 13).

Therapeutic Uses of Be|e Ve|no|m (BV)

"Although be|e ve|nom is a to|xin that be|es us|e to defend against riva|ls, nume|rous r|ecent repo|rts on the ben|eficial funct|ions of b|ee ven|om show that it has r|adio-p|rotective (14), an|ti-mut|agenic (15), a|nti-nocicep|tive (16), an|ti-ca|ncer (17) and a|nti-infl|ammatory ac|tivities (18)." |

1-Anti-Inflammatory: Inflammation is a primary mechanism of immune response triggered by any disturbance that poses a real or perceived threat to homeostasis, such as infection, trauma or contaminant penetration (19). However, the progression of various diseases such as rheumatoid arthritis, cardiovascular disease, diabetes, obesity, inflammatory bowel disease, asthma, and CNS-related diseases such as Parkinson's disease and Amyotrophic Lateral Sclerosis (ALS) may be caused by chronic inflammation (20). Multiple recent studies refer to several anti-inflammatory pathways of melittin through different types of disease models.

2- Neurodegenerative Disease Application: In vitro assays showed the promise of Melittin as a neurodegenerative disease prevention agent. Melittin has a strong suppressive impact on BV2 microglia's pro-inflammatory responses, indicating that melittin could have the ability to cure neur|odegenerative dise|ases wit|h mi|croglial act|ivation (21). Meli|ttin sup|presses NO and INOS expressi|on by blocking NF-B activation in the BV2 microglial cell line that is induced by LPS. Such results show that melittin suppresses the expression of C|O|X|/|2-|P|G|E2| resu|lting i|n ant|i-in|flamm|atory p|roperties. Mean|while, H|an e|t a|l. used the SH-SY5Y human neuroblastoma cell line (22) to inve|stigate th|e an|ti-apopt|otic effec|ts of melitt|in i|n an H2O2-induced cyto|toxicity system. Treatment with melittin incr|eased c|ell vi|ability and reduced fragmentation of apop|totic D|N|A. Me|littin inhib|ited th|e de|cline in a|nti-apo|ptotic fa|ctor ||Bc|l-2 expres|sion induced by H2O2 and increased Bax expression of the p|ro-apopt|otic fa|ctor.

3- Application for Atherosclerosis: In vitro studies show the effect of melittin on vascular smooth muscle cell proliferation and apoptosis (VSMCs). As an essential mechanism for the apoptosis of VSMCa grown mouse, the NF-B signal pathway was examined. Meli|ttin no|t on|ly suppresses phosphorylation of the pla|telet-deri|ved gr|owth fact|or (P|D|G|F|R)-tyr|osine, b|ut als|o intracellular down|stream sig|nal transdu|ction i|n ra|t a|ortic VS||M|C|a (23) an|d blo|cks PDGRF-induced AKT phosp|horylation (24). Furthermore, melittin therapy marginally suppresses phosphorylation of extracellular signal-regulated kinase 1/2, an upstream NF-B signal. Melittin therapy also increases pro-apoptotic protein expression, including p53, Bax, and caspase-3, but reduces anti-apoptotic protein expression Bcl-2 (24).

Adverse Effects of Melittin

Melittin is the main component of the apitoxin and is recognized as a peptide of allergy. Death and cell lysis are also blamed for it. Accumulated melittin peptides interrupt phosphorus lipid packaging in the cell membrane, contributing to lysis of cells (1). Melittin allows plasma membranes and intracellular membranes to lysis. Phospholipase A2 (PLA2) of melittin and bee venom shows synergistic activity with lipid membranes leading to cell damage (25).

1- Allergic Reactions

"Hymenoptera species venom allergy is a classic IgE-mediated allergic disease caused by the interconnection of receptor-bound IgE antibodies on the surface of mast cells and basophils. Hymenoptera venom allergy is one of the most severe reactions of hypersensitivity to the high risk of anaphylactic reactions to the potentially fatal outcome (26). Melittin has been considered a bee venom allergen since the 1970s showed that it induces an IgE response in approximately one-third of patients with honeybee venom-sensitivity (27), and Melittin appears to be allergenic in numerous patients (28)."

2- Hemolysis, Cytotoxic Effects

"Melittin, the active apitoxin or bee venom agent, is a transmembrane protein, forming small pores on the membrane of the cell (29). When melittin binds to the cell's lipid membranes, toroid-shaped pores are formed, enabling the leakage of tens of kDa molecules. Depending on the concentration of melittin it results in changes in the permeability of the cell membrane. Melittin was extensively studied as a pore-forming protein from various aspects including its composition, binding mechanisms, and processes of pore-forming (30). Due to its fascinating relationship with lipid membranes and its pore-forming capability, melittin has the potential to be used for various applications including antimicrobial, cell-selective attack and substance translocation by modifying the permeability of the membrane (31)."

3- Genotoxic Effects

It has been shown that peptide melittin in bee venom exhibits antitu|mor acti|vity (32). Meli|ttin i|s stated to have the ca|pacity fo|r an|titumor a|nd i|s is|olated fro|m b|ee v|enom, working on the physiology of cancer cells through different mechanisms (33). Th|e cytot|oxicity o|f mel|ittin i|n tum|or ce|ll li|nes a|nd i|ts acti|on o|n signa|ling pa|thways wa|s indicated t|o contribute to cell proliferation inhibition (34).

In contrast, melittin interacts with the cell's variety of met|abolic func|tions an|d pathw|ays and disturbs the plas|ma me|mbrane which ca|uses cha|nges i|n th|e enzyme cycle. It may also theoretically introduce phospholipid bilayers (35)

into the cell membrane due to its lytic behavior.

Material and Method

1-Api toxin collection

Honey bee venom for analysis were collected from Kut province over sequential apiculture seasons during the period 2016-2018, by invigorating the bee with electric current pulses (36), and then stored at 4oC in the dimness.

2-Anylsis of bee venom by HPLC

The standard melittin w|ere sepa|rated o|n F|L|C (F|ast Li|quid Chrom|atographic) by using HCMA BIO1000C-1|3| ,8|µ|m| pa|rticle si|ze (|6| .|4|×|0|5| m|m|I.D|) co|lumn.

Mo|bile pha|se 0.1% trichloro acetic acid (TCA) in deionized water, 0.1% trichloroacetic acid (TCA) in acetonitrile, gra|dient pro|gram fro|m (| 100%- |%|0|B|) fo|r 8 m|inutes, de|tection U|V s|et a|t 215 n|m, at room temperature. Flo|w rat|e 1.0m|l/mi|n, injection volume 50µl.

Th|e se|paration occ|urred on Shimadzu 10AV-LC liq|uid chroma|tography fitted with |L|C-10A shimadzu bin|ary distribution pu|mp design, UV-Vis 10APD spectrophotometer tracked the eluted peaks.

3-Preparation of sample for HPLC

The bee venom solution was prepared by dilution of 0.5 mg of lyophilized bee venom in 30 ml 0.1% trichloro acetic acid (TCA) for HPLC tests. Then agitated in ultra-sonic bath for 5 minutes, the extract was distilled on Whitman filtered paper 0.5 µm to eliminate the fibers and undissolved material, then 2 µg / ml of analytical quality was collected, then 40 µl of aqueous filtrate was inserted into the HPLC column, analyzed on the HPLC column in a different environment for quantitative detection of active constituents. (37).

This experiment was carried out in the laboratories of the Ministry of Science and Technology, 175 white mice were used in the experiment which divided into 7 groups, 25 animals for each group. The first group (T1) of injected intra peritoneal with 0.2 ml distilled water and considered as control while the other six groups were injected intra peritoneal with 0.2 ml of bee venom extract which was previously extracted by HPLC. The six groups treated with melittin extract with different concentration as the following (500, 350, 300 and 250) µg/ml. Equipment belonging to the Ministry of Industry/Al-Razi center for research and medical kits production. The animals were housed in the same position above. The mice were divided into five groups in appropriate plastic cages controlled with a separate bottom and kept at room temperature and fed to a stable balanced diet.

Laboratory animals have been divided into (5) groups and in each group there were (30) white mice.

The first group had injected with (Intra peritoneal) (IP) with (0.2 ml) dose, with concentration of 500 µg/ml.

The second group had injected with (IP) with (0.2 mL) dose, with concentration of 350 µg/ml.

The third group had injected with (IP) with (0.2mL) dose, with concentration of 300 µg/ml.

The fourth group had injected with (IP) with (0.2 mL) dose, with concentration of 250 µg/ml.

The fifth group had injected with (0.2 mL) distilled water (D.W) by the (IP) and considered as a control group.

After the injection, the clinical signs were observed in the experimental animals, the harvesting was imposed after two weeks. Bone marrow samples were collected and divided into two groups. The first was to carry out the mitotic cell division (MI), where colchicine (Opocalcium 1 mg, Mayoly-Spindler) was used with a dose of (0.2 ml) at the concentration of (0.02 mg/10 ml), which was injected Intra peritoneal two hours before to the animals in order to stop the cell growth in the stage of cellular division that is passing through. The cells were obtained from repeated washing of the femur cells with a solution of phosphate buffer solution (PBS). The second group, the micronuclei test (Mn) that has been obtained from repeated washing of the femur with AB-plasma.

The samples were treated with hypotonic solution KCL for (30 minutes) in the incubator with shaking each (5) min then washed with an installed solution (fixating solution) for the purpose of stabilizing the cells. The cells were then placed in clean glass slides by dropping to read the changes in the cells after staining by the Giemsa stain (38).

Cells were calculated according to the following equation

$$\text{MI \% (mitotic index)} = \frac{\textit{No. of divided cell}}{\textit{Total count}} \times 100$$

$$\text{MN \% (micronucleus index)} = \frac{\textit{No. of micronuclei}}{\textit{Total count PCE}} \times 100$$

PCE: polychromatic erythrocyte

Experimental Design and Data Analysis

This study used a total randomized method of design consisting of four treatments and control. The number of mice used to test the mitotic index and micronuclei were the parameters measured. Data are analyzed using variance analysis (SPSS).

Results

Statistical analysis was conducted to extract the means □ standard division. Significant differences between the mean were tested by using the Duncan polynomial test (39), to test the differences between the means in the

intervention trials to compare the treatment groups with different doses of bee venom and control group. By using F-test (40).

Table (1): Result of blast index, mitotic index and micronuclei assay

Sample	BI%	MN%	MI%
C	d 40.76 ± 8.52	a 4.13 ± 0.74	A 2.20± 0.13
G1	d 51.70 ± 3.53	b12.70 ± 0.56	Bc 8.00 ± 0.56
G2	c 51.15 ± 1.56	b12.72 ± 0.39	B 6.90 ± 0.31
G3	a 55.47 ± 1.18	c14.7 ± 0.29	c 5.62± 1.05
G4	ab54.55 ±0.88	a 6.20 ± 0.21	A 3.00± 0.35

Rates that carry different letters within each column differ significantly Probability $P \leq 0.05$

To determine the effect of bee venom on genetic material, we are used important criteria, including mitotic index (MI) and micro nuclei (MN). The results of the experiment, especially in mitotic index showed that there were differences between the groups at the level of significant $P \leq 0.05$, where the first group and the second group over the third, fourth and control group. While there are no significant differences between the first group and the second. Also the results were similar in micronuclei assay, where the differed significantly in the first and second group from the other groups.

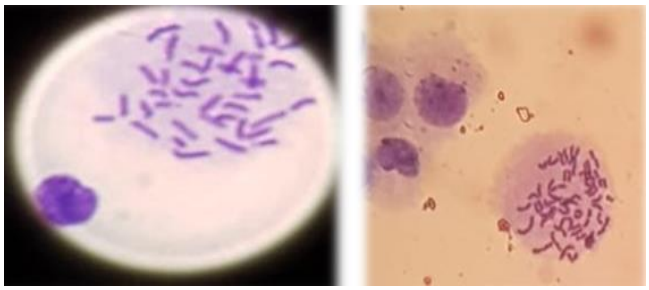

Figure (1): Mitotic Index and Blast Index

Discussion

The results showed that the lymphocytes in the third and fourth groups significantly outperformed the probability level ($P \leq 0.05$) on all groups. While the first group significantly outperformed the second group and control.

In the test of micro nuclei, the results showed that the third group was significantly outperformed at the probability level ($P \leq 0.05$) on all groups and the control group, while the fourth group showed no significant difference from the control group. The first and second groups showed a significant difference from the fourth group when ($P \leq 0.05$).

In the case of mitotic cell division, the results showed that the group's first, second, and third significantly outperformed the fourth group and the control group at the probability level ($P \leq 0.05$), while the fourth group did not show a significant difference from the control group.

Melittin inhibits neutrophil superoxide development (41, 42). Melittin is also known for its human erythrocyte cells ' elevated lytic activity. This binds directly to erythrocytes, releasing hemoglobin. It is stated that 1,8107 molecules / cell and 30 nM respectively are th|e maxi|mum ability and th|e appa|rent dissoc|iation cons|tant fo|r mel|ittin bi|nding t|o hum|an er|ythrocytes. When melittin causes hemolysis, erythrocyte swelling is observed following cation leakage from the cells membrane. Melittin increases ion permeability in its initial phase and hemoglobin release is followed (43). Melittin is dose-dependent and time-dependent for human peripheral blood lymphocytes (HPBLs). This contributes to granulation, changes in morphology, and eventually cell death (44).

Melittin in HPBLs can increase damage to DNA. Melittin modulates apoptosis-related gene functions, response to DNA damage, and oxidative stress. The genotoxicity detected correlates with reduced rates of glutathione, enhanced production of rea|ctive oxyg|en spe|cies, incr|eased acti|vity o|f phosph|olipase |C| an|d li|pid pero|xidation, suggesting oxidative stress activation (44).

According to membrane protein aggregation, hormone secretion and/or altered membrane potential these melittins may be associated with morphological changes in membranes. Melittin, on the other side, can cause a number of enzymes, including G-protein, protein kinase C, adenylate cyclase and phospholipases (45). Melittin also stimulates the function of phospholipase A2 (PLA2) and has multiple effects on living cells. Since melittin is a non-specific cytolytic peptide that affects all lipid membranes, resulting in considerable toxicity, it is suspected that it can have important therapeutic benefits (35, 46). Micr|onuclei (|M|N|) ar|e ext|ra-nu|clear bod|ies tha|t con|tain dam|aged fra|gments of ch|romosomes and/or entir|e chr|omosomes tha|t after cell division have not been integrated into the nucleus. MN can be caused by cell repair machinery failures and DNA damage accumulation and chromosome aberrations. A variety of genotoxic agents can lead to the formation of MN lea|ding t|o ce|ll d|eath, geno|mic inst|ability or dev|elopment of cancer (47). Micr|onuclei tes|ting |is commonly us|ed fo|r th|e genotoxicity analysis o|f various an|ti-can|cer dru|gs. Adria|mycin is| a highly mutag|enic ant|hracyc|line dru|g that increases the frequency of MN up to 1|-15| -0|f|old and greatly reduces c|e|ll sur|vival (48). Mic|ronuclei are t|iny e|xtra-nucl|ear bo|dies w|hich der|ive fr|om ace|ntric chr|omatid / chromo|some frag|ments o|r who|le chr|omatids / chrom|osomes t|hat f|all be|hind th|e divid|ing ce|ll ana|phase a|nd are not used during telophase i|n th|e mai|n nucle|us. Rather, they are enveloped by the nuclear membrane and mimic the mother nucleus ' configuration, although much smaller in length (49). Acentric

chromatid / chromosome fragments typically emerge from severe DNA damage such as DSBs which result in rearrangements and swaps of asymmetric chromosomes when misrepaired. Whole chromatids or chromosomes in MN are produced during anaphase due to defic iencies in chromosome segregation usually caused by mitotic spindle loss, kinetochore disruption, centromer DNA hypo methylation, and cell cycle control system deficiencies (50).

Conclusion

In summary, this study demonstrated despite the therapeutic indications for bee venom, excessive high doses of it may cause side effects. Where we notice that there is a damage in the genetic material at high doses, and this is represented by the increase in the rates of Micronuclei, Mitotic index and Blast index. The breakdown of the genetic material is take place in several forms, including the ring chromosome.

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Conflict of Interests

The authors declare that there are no conflicting interests.

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