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Toxic Effects and Safety of Bee Venom Protein [Melittin] in Mice: Search for Natural Vaccine Adjuvants

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ABSTRACT

Melittin protein is the main component of Bee venom (BV) that contains twenty-six amino acids. It constitutes 40% to 50% of dry bee venom by weight and is used in traditional medicine to inhibit infection and reduce inflammation. This study aimed to determine the toxic effects of melittin in CD-1 Swiss albino mice. The study also aimed to determine the lowest safe melittin dose to be used as a potential vaccine adjuvant. One hundred and sixty mice were injected intra-dermally with increasing doses of melittin [9000, 4500, 2250, 900, 450, 250, 100, 50 and 30 µg/dose]. The mice were followed for toxicity with respect to body temperature, body movement, weight, function/ anatomical structure of vital organs [brain, liver, bone marrow, kidneys]. Injected mice with high doses [9000, 4500, 2250 µg/dose] died with hypothermia, ataxia and loss of weight. Thirty µg /dose of melittin was found to be the lowest safe dose with minimum side effects and no abnormalities in histological sections of vital organs. Result concludes, melittin is safe with minimum sides in low doses. 30 µg/dose of melittin can be taken forward to determine its effects on the immune system as a potential vaccine adjuvant.

1. Introduction

Bee venom (BV) is a natural toxin produced by the honey bee with various therapeutic and toxic effects that are closely linked. BV is an efficient and complex mixture of various peptides that include: Melittin, apamin, adolapamin, protease inhibitors, peptide 401 and mast cell degranulating peptide. Bee venom is used in traditional medicine to inhibit infection and reduce inflammation. BV stimulates the functions of immune system with release of cortisol which is a known natural anti-inflammatory agent. Whole BV is toxic when its therapeutic dose exceededs 200-500 times, while individual components show toxic effects with concentration 20-50 times greater than the therapeutic dose. Generally, BV components are preferable for specific medical applications [1-4]. The allergic response of BV proteins in a sting victim ranges from swelling, redness, pain, itching around the sting site and potentially life-threatening allergic effects, including anaphylactic shock [5-11]. Melittin, the major component of BV binds to secretary phospholipase A_2 ($sPLA_2$) and inhibits its enzymatic activity. $sPLA_2$ is abundantly released in severe inflammatory disorders and is found to cause tissue and organ degradation with loss of functions. Furthermore, melittin blocks the production of neutrophil superoxide [3, 12]. In addition to the peptides, BV contains non-peptide components such as amines that include histamine, dopamine and nor-adrenaline with small amounts of sugars, phospholipids, free amino acids and pheromones [13-16].

Melittin ($C_{131}H_{229}N_{39}O_{31}$) is the main component in honey bee venom, which accounts for 40-50% of the dried venom [11, 12]. This polypeptide is made up of twenty-six amino acids and its primary structure is Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro Ala Leu Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln [NH_2-GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$] [11, 17, 18]. The amino-terminal region (residues 1–20) is predominantly hydrophobic whereas the carboxy-terminal region (residues 21-26) is hydrophilic due to the presence of a stretch of positively charged amino acids. 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. Both the primary and secondary structures are similar to many antimicrobial peptides. Melittin has a molecular weight of 2.86 kD and high aqueous solubility [17, 19-22]. Melittin also appears to have toxic side effects as do some of the other individual compounds in BV. When whole venom is applied however, no side-effects have been shown, other than in allergic patients. In high doses, melittin may cause itching, inflammation and local pain; on the other hand, small doses of melittin produce broad anti-inflammatory effects [11, 19]. Numerous recent reports elucidated several anti-inflammatory mechanisms of melittin in different diseases [20-22].

It has been shown that melittin inhibits TNF-α secretion and expression of IL-1 β and IL-6 in TNF-α-treated hepatic stellate cells (HSCs). In addition, melittin attenuates inflammation and fibrosis by inhibiting the NF-κB signaling pathway in thioacetamide-induced liver fibrosis [18]. Melittin is known for its high lytic activity on human erythrocyte cells, directly binds on erythrocytes and releases hemoglobin with an initial phase of increased permeability of ions [23-25].

This study aimed to determine the toxic effects of melittin on CD-1 Swiss albino mice and to determine the lowest safe dose that can be studied further for melittin potential vaccine adjuvant effects.

2. Experimental Methods

2.1 Ethical Considerations

The National regulations for experimentation on animals and the European manual of Ethics committee for the use of laboratory animals were followed for the care of the study mice.

2.2 Study Animals

One hundred and sixty CD-1 Swiss albino mice were selected to determine the toxicity and safety of Bee Venom [melittin].

2.3 Melittin Preparation

2.3.1 Melittin Purification

One gram of raw Bee Venom (China International Express (EMS) Company, Hong Kong, China) was dissolved in 5 mL of double distilled H_2O in sterile 50 mL Falcon tube in a water path at 56 °C for two hours. The solution was centrifuged at 40,000 rpm for 40 minutes. The supernatant was filtered using 0.3 and 0.2 µm filters and divided to 1 mL aliquots in sterile 1.5 mL Eppendorf tubes and stored at -20 °C until use.

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2.3.2 Melittin Precipitation by Acetone

Cooled acetone (-20 °C) to the purified melittin in a ratio of 6:1 by volume in glass test tubes. The solution was then vortexed and incubated for 2 hours in -20 °C freezer and then the solution was centrifuged at 13,000-15,000 rpm for 15 minutes. The supernatant was disposed properly and carefully so as not to dislodge the protein pellet.

The pellet was washed three times in 100 µL of cold 90% acetone and centrifuged at 13,000-15,000 rpm for 5 minutes. The pellet was dried at room temperature (overnight) and later dissolved in 1 mL normal saline.

The concentration of melittin in solution was ermined spectrophotometrically [280 nm] using (Nanophotometer® P_{300}, IMPLEN GmbH, Munich, Germany).

2.4 Study Animals Sourcing, Care and Feeding

The CD-1 (Swiss albino mice) donated by the Department of Clinical Pathology and Immunology, University of Khartoum [Animal House] were housed in hanging plastic cages (using a wooden dust-free litter as bedding material) under controlled light conditions (12 hour light/ 12 hour dark regime) with relative humidity of (50 ±5%) and temperature of (17 ±2 °C). A well balanced diet was prepared from carbohydrates (flour), vegetables (carrots) and proteins (eggs and meat) were offered to the mice. The mice were fed daily and supplied with water using water bottles.

2.4.1 Animals Follow Up and Monitoring

During the experiment the followings variables were monitored for the following variables:

- General condition [well, ill].
- Locomotion [active, immobile aggressive].
- Weight was measured by an electronic balance [increased, decreased and stable].
- Temperature was measured by a digital Themometer-7013300, Fugison Company SLR-Romania [increased, decreased, stable].
- Injection site [color "Normal, redness", size "Normal, swollen", injury].

2.5 Safety and Toxicity of Melittin

One hundred and sixty CD-1 Swiss albino mice (age 20-22 weeks and weight 25-33 grams) were divided into sixteen groups (10 mice/ group). Mice received one to three doses [two in footpads and one in ear pinna] on days 0 "screening", 7 and 14. The body temperature, body weight, movement and breathing were monitored. Group F1 and F2 received 450 µg/dose of melittin in 10 µL normal saline; Group G1 and G2, 225 µg/dose melittin in 10 µL normal saline; Group I1 and I2, 100 µg/dose melittin in 10 µL normal saline; Group J1 an J2, 50 µg/dose melittin in 10 µL normal saline; Group K1 and K2, 30 µg/dose melittin in 10 µL normal saline; Group A, as control group received 10 µL normal saline injection. Blood samples were collected by the Intra-cardiac Puncture in EDTA blood samples (0.5 mL -1.0 mL) to determine hematological parameters using (Sysmex KX-21N™ Automated Hematology Analyzer, Sysmex, Norderstedt, Germany). Heparinized blood samples (1.5 mL) for the measurement of biochemical parameters [Serum totals protein/serum albumin/globulin, creatinine, urea, ALT/AST]. Fluoride/oxalate tubes were used for plasma collection for glucose level determination (Biosystem BTS_{310}, Biosystem, Barcelona, Spain) [26-32].

2.6 Histopathological Analysis

After the mice were bled, its hearts, brains, cerebella, lungs, livers, spleens and kidneys were removed and stored in 10% buffered neutral Formaldehyde for further sections preparation and H and E staining.

2.7 Statistical Analysis

Statistical analyses were performed using Epidemiological Information (Epi Info) software version 7. The mean, standard deviation (SD) and P-values were calculated for weight, temperature, biochemical and hematological parameters. P value less than 0.05 was considered significant.

3. Results and Discussion

One hundred and sixty CD-1 Swiss albino mice with mean ages and weights of 21±1 weeks and 29 ± 4 grams respectively, with a Male: Female ratios of 2:1 were studied.

3.1 Movement and Behavior Patterns in Melittin-Toxicity Study Mice

Mice injected with melittin (Group B to Group J2) became unwell and ataxic with sluggish movement within one hour of injection. Later the mice became immobile with decrease in respiratory rates. The injection site (footpad) became red and swollen. There was significant differences in movement patterns between Group A (Control Group) and Groups B to J2. There were no differences in movement patterns between Group K and Control Group (A). The mice perished after two to six hours of injection in Groups B to F2, while 50% (25/50) of mice in Groups G1 to I2 died after one day compared to 25% (10/40) in Group J. All injected mice in Group K became normal in movement patterns after three hours of injection (Table 1).

Table 1 Experimental melittin administration schedule

Mice Group *(n=10)*	Amount of Melittin Injected	No of Doses	Route/Site	Percentage of dead mice	Remarks
A	000 µg	-	-	-	Control Mice
B	9000 µg	One	SQ/ Footpad	100%	-
C	4500 µg	One	SQ/ Footpad	100%	-
D	2250 µg	One	SQ/ Footpad	100%	-
E	900 µg		SQ/Footpad; ear pinna	100%	Three doses in days 0,7 & 14.
F1	450 µg	One	SQ/ Footpad	100%	-
F2	450 µg	Three	SQ/Footpad; ear pinna	100%	Three doses in days 0,7 & 14.
G1	225 µg	One	SQ/ Footpad	50%	-
G2	225 µg	Three	SQ/Footpad; ear pinna	50%	Three doses in days 0,7 & 14.
H	180 µg	Three	SQ/Footpad; ear pinna	50%	Three doses in days 0,7 & 14.
I1	100 µg	One	SQ/ Footpad	50%	-
I2	100 µg	Three	SQ/Footpad; ear pinna	50%	Three doses in days 0,7 & 14.
J1	50 µg	One	SQ/Footpad	25%	-
J2	50 µg	Three	SQ/Footpad; ear pinna	25%	Three doses in days 0,7 & 14.
K1	30µg	One	SQ/ Footpad	Zero%	-
K2	30 µg	Three	SQ/Footpad; ear pinna	Zero%	Three doses in days 0,7 & 14.

***SQ** = Subcutaneous. **Safe dose will be taken for vaccination*

3.2 Temperature Variation in Melittin Toxicity Groups

The mean mice body temperature decreased significantly in Groups F1 and F2 (450 µg/dose); Groups G1 and G2 (225 µg/dose); Groups I1 and I2 (100 µg/dose); Groups J1 and J2 (50 µg/dose) compared to the control group (p= 0.000001- 0.0000) (Table 2).

In Groups G1, G2 and I1, I2 the majority (92%, 37/40) of dead mice developed hypothermia (30 ± 0.1 °C) an hour after injection that persisted at seven hours. After 24 hours, the mice body temperature was back to the normal (38.8 ± 0.5 °C). Groups K1, K2 and A (Control group) had baseline temperature levels all through the experiment (Table 2).

In Groups J1 and J2 the majority (80%, 16/20) of dead mice developed hypothermia (31 ± 0.2 °C) an hour after injection that continued low at seven hours. After 2 days the body temperature was back to the normal levels (38.7 ± 0.5 °C) (Table 2).

In Groups K1 and K2 no mouse died and the body temperature stayed at the normal baseline level (38.9 ± 0.4 °C) similar to the control group (Group A) where the temperature stayed at baseline levels (Table 2).

Table 2 Temperature variation following melittin injection

Mice Groups (10 mice/group)	Temperature (°C)								
	Weight(g)	00 min	30 min	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
A (000 µg)	27.2±1.7	38.2±0.5	37.8±0.2	37.9±0.3	37.6±0.3	37.9±0.7	37.8±0.4	37.8±0.3	37.8±0.4	38.0±0.5
F1 (450 µg)	27.1±0.7	38.2±0.3	37.4±0.4	*36.8±0.5	*36.0±0.6	*35.7±0.5	*35.2±0.9	*34.4±1.3	*33.1±0.1	*32.5±0.2
F2 (450 µg)	27.5±1.0	37.8±0.4	37.4±0.4	*36.0±0.5	*36.3±0.8	*34.9±0.8	*34.6±0.7	*34.0±0.9	*32.0±0.1	*32.3±0.2
G1 (225 µg)	27.7±0.9	38.8±0.5	37.9±0.5	37.5±0.4	*36.6±0.4	*35.5±0.5	*34.4±0.5	*33.9±0.2	*32.9±0.1	*32.2±0.2
G2 (225 µg)	27.9±0.7	38.6±0.4	37.5±0.6	*36.9±0.6	*36.2±0.9	*35.5±0.6	*34.1±0.5	*33.9±0.2	*32.9±0.1	*32.2±0.2
I1 (100 µg)	27.1±0.9	38.2±0.3	37.5±0.6	*36.5±0.6	*36.1±1.0	*35.5±0.7	*34.8±0.5	*34.1±0.2	*33.6±0.4	*32.7±0.5
I2 (100 µg)	27.1±0.9	37.7±0.5	37.7±0.5	*35.4±0.6	*35.5±0.8	*35.4±0.7	*34.0±0.6	*33.5±0.4	*32.6±0.5	*32.0±0.2
J1 (50 µg)	26.2±0.3	38.5±0.5	37.4±0.5	*36.5±0.5	*35.5±0.6	*34.7±0.8	*34.1±0.6	*33.5±0.7	*33.1±0.5	*33.8±0.9
J2 (50 µg)	27.0±0.5	38.9±0.4	37.0±0.6	*35.8±0.6	*34.9±0.7	*34.4±0.7	*33.1±0.6	*33.6±0.8	*34.0±0.7	*34.5±0.7
K1 (30 µg)	25.1±0.5	38.8±0.4	37.3±0.8	37.0±0.5	36.7±0.6	36.7±1.1	36.9±0.9	37.2±0.7	37.4±0.4	37.6±0.4
K2 (30 µg)	26.6±0.4	39.0±0.5	38.0±0.4	37.2±0.6	36.5±0.5	36.4±0.7	37.0±0.7	37.5±0.7	38.0±0.4	38.6±0.3

SD=*Standard Deviation* ****P value****= Significant difference*

Table 3 Biochemical changes following melittin injection

Mice Groups (10 mice/ group)	Chemical Parameters							
	AST	ALT	Globulin	Albumin	T.Protein	Creatinine	Urea	Glucose
	(Unit/L)	(Unit/L)	(g/dL)	(g/dL)	(g/dL)	(mg/dL)	(mg/dL)	(mg/dL)
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
A (000 µg)	104.2±21.5	48.6 ±16.1	3.7±0.6	2.5±0.1	6.2±0.7	0.5±0.1	42.2±11.1	119.0±22.3
F1 (450 µg)	*842.0±272.8	*235.8±60.3	2.8±0.5	2.7±0.3	5.5±0.6	0.2±0.1	36.2±2.9	133.2±16.6
F2 (450 µg)	*986.0±152.9	*244.2±45.1	3.0±0.5	2.9±0.4	6.0±0.9	0.3±0.1	39.8±3.0	125.0±15.2
G1 (225 µg)	*1396.0±1303.9	*285.0±88.2	4.6±0.9	3.7±1.1	8.3±1.9	0.2±0.2	52.8±15.9	131.8±16.8
G2 (225 µg)	*1534.0±1300.0	*301.0±80.1	4.3±1.1	3.9±0.9	10.0±1.4	0.2±0.1	57.9±13.1	149.2±11.7
I 1 (100 µg)	*747.0±278.4	*207.0 ±48.2	4.5±1.7	2.4±0.5	7.0±1.8	0.2±0.1	40.5± 8.1	N
I 2 (100 µg)	*842.2±270.7	*231.2 ±50.0	4.0±2.0	2.5±0.2	8.3±1.3	0.3±0.1	35.1± 10.0	133.3±16.3
J 1 (50 µg)	*682.4±187.8	*110.4 ±36.3	2.2±1.3	3.5±0.6	5.8±1.9	0.3±0.1	40.6± 9.3	N
J 2 (50 µg)	*621.1±199.0	*98.9 ± 29.6	2.7±1.2	3.9±0.5	6.8±0.9	0.3±0.1	50.1± 6.0	144.8±15.8
K1 (30 µg)	169.0±30.3	62.5 ± 10.6	0.6±0.3	3.8±1.3	5.4±0.9	0.4±0.1	47.2±10.1	151.0±16.0
K2 (30 µg)	138.8±27.1	53.0 ± 13.9	1.8±0.5	3.1±0.6	5.9±0.7	0.5±0.1	43.5±10.6	139.0±18.2

AST= *Aspartate aminotransferase* ***ALT=*** *Alanine aminotransferase* ***T.Protein=*** *Total protein* ****P value=*** *Significant difference* ***N****= Not done*

Table 4 Hematological changes following melittin injection

Mice Groups (10 mice/ group)	Hematological Parameters					
	HGB	HCT	WBCs	NEUT	LYM	PLT
	g/dL	%	/µL	/µL	/µL	/µL
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
A (000 µg)	13.3±0.7	42.4±2.9	6.0 ± 2.7	1.7±1.2	4.0±2.0	588.4±556.0
F1 (450 µg)	15.1±1.1	45.7±4.6	12.2±9.6	3.1±2.9	9.0±10.4	*1267.8±193.9
F2 (450 µg)	14.1±1.0	46.4±4.2	11.8±9.9	5.1±1.8	8.8±10.5	*1303.1±199.0
G1 (225 µg)	15.7±2.4	47.1±6.2	7.6 ±2.7	3.7±3.9	3.7± 4.0	*674.5±425.5
G2 (225 µg)	15.3±2.5	50.0±6.9	8.9 ±1.9	3.6±3.8	5.1± 3.9	*693.2±444.2
I 1 (100 µg)	14.9±1.1	44.7±3.6	10.2±4.2	2.2±1.9	7.0±7.1	*891.3 ±415.7
I 2 (100 µg)	14.4±1.4	46.3±2.8	11.9±3.8	1.8±1.6	10.0±6.4	*902.1 ±389.2
J 1 (50 µg)	14.2±0.8	42.6±2.4	7.6 ±2.5	3.5±3.1	3.9±4.0	*1024.2±777.6
J 2 (50 µg)	15.5±0.5	43.3±2.3	8.0 ±2.3	4.2±2.6	6.0±2.7	*999.9 ±790.0
K1 (30 µg)	15.1±0.2	43.8±2.9	6.8±3.1	1.4±1.0	5.4±2.4	*1263.8±97.8
K2 (30 µg)	14.1±0.3	43.0±2.9	6.2±3.2	1.6±1.1	4.9±2.1	*893.9±111.2

HGB *= Hemoglobin* ***HCT*** *= Hematocrit* ***WBCs*** *= White Blood Cells* ***NEUT*** *= Neutrophils* ***LYM*** *= Lymphocyte* ***PLT****= Platelets* ****P value****= Significant difference*

3.3 Biochemical and Hematological Parameters Derangement in Melittin Toxicity Groups

There were no statistically significant differences in total proteins, globulins and albumin levels in all melittin toxicity groups (F to K2) compared to the control group (p=0.5 to 1.0) (Table 3). Creatinine, urea and glucose levels were not significantly different between melittin-injected groups (F to K2) and the control group (p=0.15 to 0.9). There were significant differences in ALT and AST in melittin groups (F to J2) compared to the control group (Group A) (p=0.0002-0.04 and 0.0000002-0.00007 respectively). Groups K1 and K2 showed an increase of AST and ALT enzymes (p= 0.7 and 0.2 respectively). This was attributed to mild hemolysis that is not detected by the naked eye (Table 3).

Hematological parameters were measured after four weeks following melittin injection from surviving mice. There were no significant differences in the hemoglobin and hematocrit (HCT) between melittin-injected groups (F to K2) and the control group (Group A) (p=0.5 to 1.0) (Table 4). There were no significant difference in total white cells, neutrophil and total lymphocytes between melittin injected groups (F to K2) and the control group (p=0.57 to 0.93). The increase in platelet counts was significant in melittin injected groups (Group F1 to K2) compared to Group A (control group) (p=0.0000 to 0.004) (Table 4).

3.4 Weight Monitoring During the Three Doses Vaccination of the Study Groups

The weights of mice injected with three doses were not significantly different in melittin injected groups compared to the control group during the follow up period (p= >0.05).

3.5 Histopathological Appearances of Vital Organs in Melittin Injected Mice Groups

Histological sections from brain, cerebellum, heart, lungs, liver, spleen and kidneys of the different melittin groups and control group showed no edema, hemorrhages, loss of vascularity, cell integrity. No cell death (necrosis or apoptosis) was observed.

This study determined the toxic effects and a safe dose of the BV protein, melittin as a preliminary step to test its adjuvant effects for anti-leishmania vaccines. The study reported that high doses of melittin can cause death in mice; death was preceded by hypothermia, ataxia and elevated liver [ALT/AST] enzymes [11, 22]. Despite the deaths and elevated liver enzymes, the histological sections of the dead mice brains and livers showed intact neurons/hepatocytes and vascularity with lack of edema/hemorrhages. No cell death (necrosis or apoptosis) were reported. This is in agreement with previous reports [33]. The increase in the liver enzymes with higher doses of melittin was reported previously and attributed to hepatotoxic effects [11, 18]. This is discordant with our findings, but could be explained by submicroscopic changes that were not

detected in the H & E sections of vital organs. Alternatively, the increase in liver enzymes could probably be due to melittin-indcued hemolysis due to melittin high lytic activity on human erythrocyte cells as was shown previously [23, 24]. Melittin in high doses may cause itchiness, inflammation and local pain; but in small doses it produces minimum side effects with broad anti-inflammatory effects. This was confirmed by our findings where a low dose of melittin was reported to be safe with minimum side effects [5, 19].

4. Conclusion

Bee Venom protein, melittin is safe in low doses [≤30 µg/dose]. Low doses of melittin can be tested further to determine its adjuvant effects on anti-leishmania vaccines.

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