Production Of Polyclonal Antibodies In Milk For Protection Against Deltamethrin Poisoning

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Citation

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Abstract

Background: As most infectious agents are developing resistance to antibiotics; immunotherapy may provide a convenient treatment alternative. Unlike antibiotics, immunotherapy could be used to treat poisoning and being used as cancer therapy. Deltamethrin; a common insecticide; was chosen in this study as the poisoning agent to assess the effectiveness and practicality of using neutralizing antibodies in the milk of sheep. Methods: Two Merino ewes were immunized against deltamethrin. Antibody levels in the serum and milk of both ewes were measured to confirm antibody production. Then, milk was orally fed to 3 groups of adult Balb/c mice after overdosing with deltamethrin. Results: Continuous visual monitoring of the poisoned mice showed that the mean time to recovery with milk enriched with polyclonal antibodies (pAb) was shorter than that for control group treated with sheep's milk without pAb ($p < 0.05$). Conclusions: Polyclonal antibodies derived from sheep's milk may be a promising therapeutic tool against toxicity.

INTRODUCTION

The primary way of fighting infection is by use of antibiotics, while cancers are treated by chemotherapy. Treating poisoning episodes is mainly by way of an antidote (if available) and supportive therapy, such as anticonvulsants. Nevertheless, these methods for treating poisoning may lead to the accumulation of harmful residues in the liver and kidneys. Immunotherapy using polyclonal antibodies may provide a convenient alternative. It is a practical method since it can be produced naturally in domestic animals. Being a protein that is naturally occurring, it does not accumulate as harmful residues in the body. Immunotherapy is friendly to the environment and it is biodegradable, chemical free solution.

This study used antibodies in the milk of sheep as a treatment against deltamethrin poisoning. Deltamethrin is one of the widely used insecticides because of its low level of toxicity to human [11]. More or less, deltamethrin is probably ingested with daily diet [23]. It is a Type II Pyrethroid developed by Elliot et al (1974) [15]. Deltamethrin is widely used in agriculture, in pest control in the home and to control disease vectors [1, 5, 20]. Nevertheless, deltamethrin has been shown to cause negative effects on the environment including humans and animals, mainly fish [19]. Systemic poisoning can occur due to misuse or inadequate user protection [21]. There have been reported death cases due to deltamethrin poisoning [22]. There were 325 cases of acute deltamethrin toxicity (158 occupational and 167 accidental) reported in Chinese medical literature during the period 1983-1988 [10, 16]. Occupational exposures primarily resulted from mishandling during agricultural application and accidental ingestion of commercial deltamethrin-containing insecticides [11, 16]. In addition, there have been reports on exposure to dangerously high levels of deltamethrin in operators applying insecticides on a routine basis [9, 16].

Deltamethrin causes prolonged sodium influx along neuronal axons leading to persistent nerve depolarization and blockage of their conduction [15, 17, 24]. It also blocks the inhibitory pathway of the central nervous system through binding and altering gamma-aminobutyric acid-receptor-mediated chloride channels [15, 17]. In humans, large exposure to deltamethrin results in pronounced salivation, course whole body tremors resembling that caused by dichlorodiphenyltrichloroethane (DDT) poisoning, choreoathetosis, vomiting, diarrhea and seizures. Death may occur if untreated [5, 11]. Direct skin contact causes burning, numbness and tingling [3].

There is no specific effective treatment against deltamethrin toxicity [14, 15, 18]. Current approaches to manage deltamethrin poisoning are supportive and required for bronchospasm. The treatment of anaphylaxis includes the administration of antihistamines and benzodiazepines is also prescribed [7, 15]. As a result, the development of an effective therapy that neutralizes deltamethrin overdose is necessary.

The first objective of this study was to conduct preliminary experiments to evaluate the effectiveness of sheep immunization with a deltamethrin hapten conjugated to bovine serum albumin (BSA) as a mean to produce polyclonal antibodies (pAb) against deltamethrin. It is known that the deltamethrin hapten alone is unable to provoke the immune system in sheep [12, 13]. However, conjugation with a carrier molecule such as BSA and emulsifying the conjugate with an adjuvant such as complete Freund's adjuvant (CFA) will make this substance immunogenic [2].

The second objective was to use milk enriched with anti-deltamethrin pAb to treat deltamethrin overdose. To assess the presence of these antibodies in milk, enzyme-linked immunosorbent assay (ELISA) was used. The milk containing pAb was used in a challenge study involving a Balb/c mouse model for deltamethrin overdose. The effectiveness of the antibodies in neutralizing the toxin was evaluated.

METHODS

PREPARATION OF DELTAMETHRIN HAPTEN-BSA CONJUGATE

The hapten of deltamethrin: cyano (4-phenoxyphenyl) methyl 3-(2,2-dibromovinyl)-2,2- dimethylcycloprpoane carboxylate (Sigma-Aldrich, USA) was conjugated to BSA (Sigma-Aldrich, USA) according to the method described by Lee et al. (2002). Specifically, 0.05mmol hapten was dissolved in 4ml of ethanol and treated with 0.6ml of 1.0N hydrochloric acid. The resulting solution was stirred in an ice bath while 0.4ml of 0.2M sodium nitrite was added. Subsequently, 0.4ml of dimethylformammide (DMF) was added drop-wise to the solution to ensure thorough mixing.

In a separate step, 45mg of BSA was dissolved in 5.0ml mixture of 0.2M borate buffer (76.2g ml^{-1} borax and 12.37g L^{-1} at pH 8.8) and 1.2ml DMF. The activated hapten solution was added drop-wise to the stirred BSA solution. The resulting mixture was stirred in an ice bath for 45 minutes and then dialyzed against phosphate buffered saline (PBS) for 72 hours at 4°C. The purified conjugates were suspended in sterile distilled water and stored in aliquots at -20°C. The concentration of hapten-BSA was 2.8µg ml^{-1} after measurement by UV-absorbance at 280nm. The product of conjugation contained large amounts of precipitate. The precipitate was included in the vaccination mixture to elicit an immune response in lactating sheep.

ANIMALS

Two Merino adult non-pregnant lactating ewes of approximately 2 years old (Animal Care Unit, Australia) were used for the immunization experiment. They were numbered and housed in a communal area with their lambs at Large Animal Facility at University of Western Australia. Lambs were kept with their mothers for the welfare of animals and to maintain milk flow and production. They were provided with feed and water ad libitum and were maintained on a 12 hour light cycle.

Thirty nine adult female Balb/c mice, with approximate 30g body weight were used for the study (Animal Resources Centre, Australia). They were separated into five cages for the poisoning experiment. The mice were housed at Preclinical Animal Facility at University of Western Australia. They had free access to feed and water over the course of these experiments and were maintained on a 12 hour light cycle. All experimental procedures were performed after an initial one week adaptation period, using methods that were approved by Animal Ethics Committee at University of Western Australia (AEC permit number RA/3/100/811).

VACCINE FORMULATION

The inoculation formula used for sheep was 0.5ml of deltamethrin hapten-BSA conjugates per ml PBS (pH 7.0-8.0) emulsified in 0.5ml CFA. The booster formula was 0.5ml of deltamethrin hapten-BSA conjugates per ml PBS (pH 7.0-8.0) emulsified in 0.5ml incomplete Freund's adjuvant (IFA).

IMMUNIZATION OF SHEEP

The two ewes (No. 1 and 2) received an intramuscular injection in the left hind quadriceps with the inoculation formula at Day zero. Then, they received two boosters on Day 15 and 29 of the immunization period.

SAMPLING

Milk samples (50ml each) were taken on Day 0, 8, 22 and 36. The samples were collected by manual milking. After

collection, the samples were centrifuged at 10,000 for 10 minutes and stored in aliquots at -20°C. Samples from Day 0 were collected before the inoculation and used as the control for later milk samples.

ANTIBODY MEASUREMENTS

Indirect Enzyme-Linked Immunosorbent Assay (ELISA) was used to measure the levels of anti-deltamethrin antibodies in the milk samples collected. A 96-well microtiter plate (Bio-Rad, NSW) was coated overnight at 4°C with 100µl/well of 20µg of deltamethrin hapten-BSA conjugate per ml of 0.1M carbonate coating buffer (0.03g Na_2CO_3 and 6.0g $NaHCO_3$ in 1,000ml distilled water at pH 9.6). The plate was washed twice with washing solution (0.05% Tween 20 in 10% phosphate buffered saline). The remaining protein-binding sites in the coated wells were blocked by adding 200µl carbonate blocking buffer (5.3g Na_2CO_3, 4.2g $NaHCO_3$ and bovine serum albumin in 1000ml distilled water at pH 9.6) then incubated overnight at 4°C prior to the assay. Next, the wells were washed twice to remove the blocking agent.

Serial dilutions of sheep's immunoglobulin (BioCore, NSW) were prepared in the blocking buffer at the following concentrations: 0.1, 0.2, 0.3, 0.4 and 0.5µg/ml. Samples at dilutions 1:10, 1:100 and 1:1000 were added to wells and incubated 2 hours at room temperature. After incubation, the plates were washed and anti-sheep-peroxidase antibodies produced in donkey (Sigma-Aldrich, USA) were added. These antibodies were diluted to 1:5000 in blocking buffer to decrease non-specific binding. The plate was incubated for 1 hour at room temperature. To develop colour, 3,3',5,5'-tetramethylbenzidine (Sigma, USA) was added to wells and incubated for 30 minutes in a dark place at room temperature. Finally, the reaction was stopped by adding 100µl stop solution (3.0M HCl) to each well. The absorbance readings were measured at 450nm using Plate Reader 200/2.0 (Bio-Rad, NSW). The concentration of anti-deltamethrin polyclonal antibodies was calculated by extrapolating the absorbance values using a constructed standard curve.

NEUTRALIZATION STUDIES USING A MOUSE MODEL

Thirty nine adult female Balb/c mice were randomly separated into five groups (two study, one control and two placebo). Each mouse had a unique number assigned. The two study and control groups were given a dose of deltamethrin LD_{50} (20mg of deltamethrin per kg of body weight) [6, 7]. Group 1 received 0.2ml of deltamethrin LD_{50} followed by 0.2ml of sheep's milk with pAb. Group 2 received 0.1ml of deltamethrin LD_{50} followed by 0.2ml of sheep's milk with pAb. Group 3 received 0.1ml of deltamethrin LD_{50} followed by 0.2ml of sheep's milk without pAb as a control for the study. Further details are on Table 1. Special precautions were adopted during handling chemicals, especially, deltamethrin. Handling was according to precautions and conditions specified on Material Safety Data Sheet (Sigma-Aldrich, USA).

Figure 1

Table 1 The design of testing milk immunotherapy as a treatment of deltamethrin overdose. The number, type and description of the five groups of Balb/c mice used for all experiments

Group No.	Group type	Brief Description	Full description
1	Study	0.2ml deltamethrin/+pAb [a]	Eight mice were orally poisoned with 0.2ml of 20mg deltamethrin per kg body weight followed by oral neutralization with 0.2ml of sheep's milk WITH pAb
2	Study	0.1ml deltamethrin/+pAb	Eight mice were orally poisoned with 0.1ml of the deltamethrin dose followed by oral neutralization with 0.2ml of sheep's milk WITH pAb
3	Control	0.1ml deltamethrin/-pAb [b]	Eight mice were orally poisoned with 0.1ml of the deltamethrin dose followed by 0.2ml of sheep's milk WITHOUT pAb
4	Placebo	0.1ml saline/+pAb	Seven mice were orally given 0.1ml sterile normal saline followed by 0.2ml milk WITH pAb
5	Placebo	0.1ml saline/-pAb	Eight mice were orally given 0.1ml sterile normal saline followed by 0.2ml of sheep's milk WITHOUT pAb

[a] +pAb: Milk with anti-deltamethrin polyclonal antibodies
[b] -pAb: Milk without anti-deltamethrin polyclonal antibodies

Assessment of toxicity was based on continuous visual observation[7, 10, 22, 24]of the mice through all experiments. The mice were observed for an early toxicity sign displayed after the oral dose of deltamethrin LD_{50}. The early sign was somnolence which is a general indicator of depressed activity. The control group was designed to investigate whether the therapeutic effect was due to pAb or due to other milk components. To examine whether milk with or without pAb had any negative effect on the mice; two placebo groups were given saline orally instead of deltamethrin followed by milk with pAb for Group 4 and without pAb for Group 5.

Normal behavior of mice included general grooming, normal appetite, normal standing position, rounded abdominal lateral sides, inquisitive activity and widely open shiny eyes. The early symptoms of deltamethrin poisoning were somnolence (general depressed behavior), flat posture and eye squinting, while the later symptoms were twitching, tremors and convulsions (at a very advanced stage). Each

mouse was given milk once she displayed somnolence. This was to avoid the severe pain of convulsions and to give the suggested therapy enough time to act.

QUALITY CONTROL

The design of all experiments was based on previous studies of acute toxicity due to deltamethrin[7, 10, 22, 24]. The test subjects became distinctly depressed and generally, there was less social activity among the animals. The mice retreat to a corner of their pen and show signs of distress. In addition, somnolence was the target stage of toxicity; an early sign of deltamethrin poisoning. The experiments were conducted by a veterinarian, Dr Shreen Nusair. The experiments were monitored by Dr Veronica Anderson, the resident veterinarian at the University of Western Australia and a member of the Animal Ethics Committee at UWA. The two independent observations made were filmed for validation.

STATISTICAL ANALYSIS

The time each mouse succumbed to poisoning was recorded as well as the recovery time after receiving the treatment regime. The statistical package SPSS (SPSS Inc, USA) was used to analyze the data and to compare the study groups with each other and with the control. Mean ± Standard Deviation (M ± SD) were calculated. For all of the statistical comparisons, the value $p < 0.05$ was considered significant. All statistical analysis was conducted at the Statistics Clinic at University of Western Australia.

RESULTS

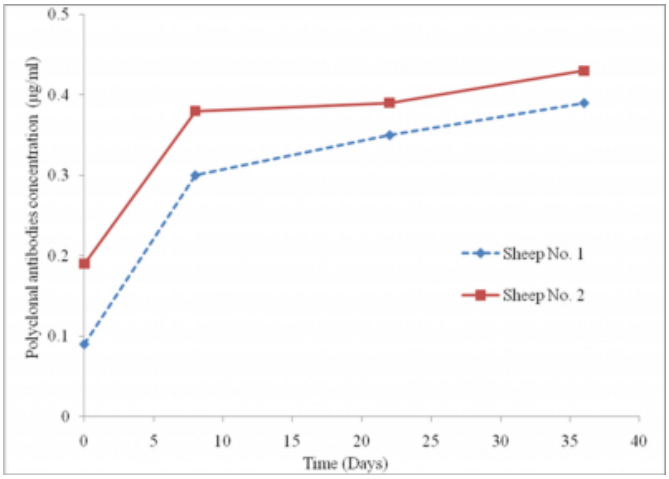
SPECIFIC ANTIBODY MEASUREMENTS

The physiochemical characters of the milk were yellowish white in color with pH of 6.8 and a freezing point of -0.56°C. The composition of the milk was not measured in this specific study. The average fat content in the milk of Merino sheep is 6.1% (w/v); the average protein content is 6.5% (w/v) and the lactose content is 4.09% (w/v) [4].

The relative amount of anti-deltamethrin pAb in milk was measured following the primary (Day 0) and the two booster immunizations (Day 15 and 29). The milk samples collected at Day zero (before immunization) were used as controls (i.e. no pAb) for later samples (i.e. with pAb). Figure 2 shows that following primary immunization there was a 2.3-fold increase in anti-deltamethrin pAb in the milk of sheep No. 2 (0.43 µg/ml relative to 0.19 µg/ml) at 1:1000 dilution and a 4.3-fold increase in the milk of sheep No. 1 (0.39 µg/ml relative to 0.09 µg/ml) at the same dilution.

Figure 2

Figure 1 The concentration of anti-deltamethrin polyclonal antibodies (pAb) in the milk of sheep No. 1 and 2 measured by ELISA at a 1:1000 dilution during the immunization experiment. The measurements were taken at Day 0, 8, 22 and 36



NEUTRALIZATION STUDIES USING A MOUSE MODEL

The mice were visually monitored on continuous basis through all the experiments. The time for two events were recorded (Table 2), time elapsed before the mice responded to deltamethrin toxicity and time elapsed before the mice responded to immunotherapy (milk with pAb) and milk therapy (milk without pAb). The first elapsed time was recorded for each normal mouse when it displayed somnolence (general depressed activity). The second elapsed time was recorded for each poisoned mouse when it started to show recovery symptoms and return to normal behavior. An exception was in the control (Group 3); the mice displayed advanced symptoms after milk therapy without pAb. These symptoms were twitching and tremors. These animals failed to show normal behavior despite treatment with standard milk samples (i.e. without specific anti-deltamethrin antibodies).

The average time for Group 1 to respond to delatmethrin LD_{50} dose and to display toxicity symptoms was 6.06 minutes, while that for Group 2 was 14.68 minutes. Group 2 and control took almost double the average time needed by Group 1 to respond to the oral dose of deltamethrin and to show somnolence. Group 1 took the shortest time for a group to develope toxicity symptoms in this study. Group 1 was compared to Group 2 to show the dose effect of deltamethrin toxicity and its role in the effeciency of the suggested immunotherapy.

Figure 3

Table 2 The time elapsed to respond to toxicity and the time elapsed to respond to immunotherapy was recorded for every mouse in Group 1 and 2. Then, results were compared to those of animals that received milk alone in Group 3.

Group	Study						Control		
	Group 1			Group 2			Group 3		
Mouse Number	Response to TOXICITY (min)	Response to IMMUNO THERAPY (min)	Total Elapsed Time (min)	Response to TOXICITY (min)	Response to IMMUNO THERAPY (min)	Total Elapsed Time (min)	Response to TOXICITY (min)	Response to MILK THERAPY (min)	Total Elapsed Time (min)
1	5.17	40.05	45.22	14.11	10.00	24.11	13.30	>64.20	77.50
2	4.20	41.07	45.27	13.35	17.30	31.05	18.20	>102.14	120.34
3	3.36	45.54	49.20	16.01	15.20	31.21	15.11	>96.42	111.53
4	9.50	60.08	69.58	14.53	18.54	33.07	18.09	>98.32	116.41
5	10.00	80.33	90.33	13.44	22.03	35.47	18.36	>94.60	112.96
6	4.05	70.50	74.55	15.55	24.16	40.11	14.02	>96.42	110.44
7	6.04	84.00	90.04	17.00	24.06	41.06	15.55	>102.14	118.09
8	6.12	67.55	74.07	13.44	25.12	38.56	13.33	>94.28	108.01
Mean	6.06	61.14	67.20	15.08	19.55	34.23	15.75	>93.57	>109.31
± SD	± 2.48	± 17.37	± 18.74	± 1.37	± 5.28	± 5.64	± 2.19	±>12.25	±>13.50
Toxic dose received	0.2ml of 20mg of deltamethrin per kg body weight			0.1ml of 20mg of deltamethrin per kg body weight			0.1ml of 20mg of deltamethrin per kg body weight		
Antibody treatment	YES			YES			NO		

Table 2 shows that the average time for Group 1 to respond to immunotherapy was 61.14 minutes, while that for Group 2 was 19.55 minutes; almost one third of Group 1. The control (Group 3) had the longest average time to respond to milk therapy (if at all); it was more than 93.57 minutes. The control mice displayed advanced symptoms of toxicity, twitching and tremors.

Table 3 shows the total response time during all experiments for the study, control and placebo groups. The average response time was the sum of the average time to develop toxicity symptoms and return to normal behavior. The average total elapsed time for Group 1 was 67.20 minutes. This was almost double that for Group 2 which was 34.23 minutes. The longest time was for the control group (>109.31 minutes). This group failed to show normal behavior when the experiment was terminated. The animals developed twitching and tremors instead. To test if the displayed symptoms were due to deltamethrin or milk itself, Group 4 and 5 were given saline as a placebo. Then, Group 4 was given milk with pAb and Group 5 was given milk without pAb. Only Group 4 displayed signs of discomfort due to milk ingestion with pAb. Data for Group 5 was not shown. The signs were flat back, inward sucktion of their abdomenal lateral side walls and seized inquzitive behavior for less than five minutes (Table 3).

For further illustration, Figure 2 illustrates the responce time to toxicity and the total elapsed time of the experiments for the study groups compared to control. In general, the Mean ± Standard Deviation (M ± SD) for the three groups was remarkably diffrent when compared to each other and the p-value was significant ($p < 0.05$) by a two way ANOVA.

Figure 4

Figure 2 The response time to toxicity and the total elapsed time for Group 1, 2 and Control (Group 3).

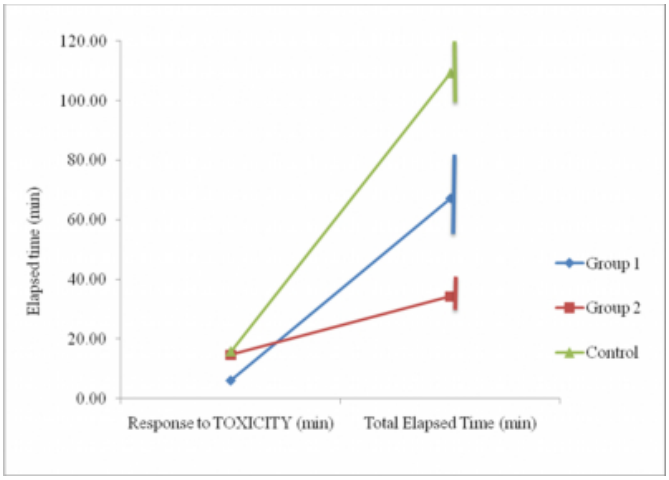


Figure 5

Table 3 The average time to develop symptoms compared to the average time to return to normal behavior. The average response time for the study, control and placebo groups following poisoning by deltamethrin then treatment by milk dose with or without antibodies.

Group	Study		Control	Placebo
	Group 1 (n=8)	Group 2 (n=8)	Group 3 (n=8)	Group 4 (n=7)
Toxic dose	0.2ml toxic dose	0.1ml toxic dose	0.1ml toxic dose	Saline
Milk type	WITH antibodies	WITH antibodies	WITHOUT antibodies	WITH antibodies
Average response time (min)	6.06 min Development of somnolence 67.20 min Return to normal behavior	14.67 min Development of somnolence 34.23 min Return to normal behavior	15.75 min Development of symptoms * >109.31 min Fail to show normal behavior	< 5.00 min Return to normal behavior

* Symptoms: somnolence, twitching and tremor

DISCUSSION

There was a marked increase in the level of pAb in the milk collected at Day 8, 22 and 36 compared to control milk samples (Figure 1). The level of pAb was slightly higher in the milk of sheep No. 2 than that of sheep No. 1. This might be due to individual variability, especially, in the immune response to certain antigens. In addition, sheep No. 1 was

producing more milk compared to sheep No. 2, who was weaning her lamb. This could have concentrated the antibodies in the milk of sheep No.2.

The results shown on Table 2 provide experimental evidence for the potential therapeutic effect of anti-deltamethrin pAb in a mouse model of deltamethrin overdose. The therapeutic effect of pAb was dose-dependent. Comparing Group 1 which was given double the volume of the deltamethrin toxic dose given to Group 2; there was a significant difference in the time elapsed to show recovery after administration of milk enriched with pAb between the two groups. The effect of anti-deltamethrin pAb in overcoming the toxic effect of deltamethrin for Group 1 was overwhelmed by doubling the volume of the deltamethrin dose for Group 2. Thus, Group 2 returned to normal behavior in almost one third the time needed for Group 1. This can be explained by the level of pAb given to Group 1 was more than that of deltamethrin antigens leading to more effective neutralization. When Group 1 was compared to control (Group 3), the results indicated that milk without anti-deltamethrin pAb failed to overcome the effect of the deltamethrin overdose. This resulted in that the control animals displaying advanced symptoms of toxicity (twitching and tremors).

Continuous visual observations revealed obvious overdose symptoms which were somnolence (general depressed activity), eye squinting, flat posture, twitching and tremors. Twitching and tremors were absent in Group 1and 2 during all the experiments (Table 3). They were only displayed in the control (Group 3) even after oral administration of milk without pAb. This indicated that milk alone failed to overcome the deltamethrin overdose. The control group had the longest average time to show response after the milk dose. This was due to absence of anti-deltamethrin pAb. As a result, these mice failed to return to normal behavior. Milk enriched with pAb had a slight effect on mice of Group 4. This group displayed flat abdominal walls and seized inquisitive behavior for less than 5 minutes. The time of anti-deltamethrin pAb action depended on the ratio of these pAb to the toxic dose. This was well-represented by Group 2 which significantly recovered faster than Group 1 after receiving half the volume of the deltamethrin dose.

Mice of Group 5 were not included in the results as they displayed no symptoms. They were normal during all the experiments. This indicated the relative safety of oral administration of sheep's milk to mice. All these results were further illustrated on Figure 2.

A remarkable point was that the toxic dose of deltamethrin given to mice was LD_{50}. This dose was supposed to cause death to half of the mice number in this study. On the contrary, there was no death among the animals, even when milk without antibodies was given to control animals. This could be due to the dilution effect of milk volume on the administered toxic dose. Milk could also have a demulcent effect due to its lipid molecules that could have adsorbed to some of the deltamethrin molecules which are known to be lipophilic [15]. Furthermore, the milk collected before immunization containing pAb against deltamethrin at low levels could not be excluded. This could have happened due to potential previous exposure of the selected sheep to this insecticide or other structurally related insecticides as residues in their rations. However, this is unlikely. If present, the levels will be lower than the threshold of the ELISA test to quantify deltamethrin.

The data presented in this study show that milk enriched with anti deltamethrin pAb may be an effective, practical and non-invasive therapeutic tool against deltamethrin toxicity. However, these antibodies will not be a 'Magic Wand'. The subject may be overcome by any approach based on increasing deltamethrin toxicity such as exposure to larger doses; or by additive effect of other insecticides such as cybermethrin. It has been reported that increasing the dose of most substances was able to overcome the effects of their specific antibodies [8]. Moreover, the proposed therapeutic milk was administered at an early stage of deltamethrin toxicity; when animals displayed somnolence. This suggests that if the toxicity case was at later stages; milk immunotherapy might not be effective.

The finding that anti-deltamethrin pAb in sheep's milk inhibited symptoms of deltamethrin overdose is important. It leads this line of research in a direction of greater potential clinical relevance using a non-invasive practical therapeutic method. This method; if not completely effective to treat deltamethrin toxicity; could be a supportive therapy with a reduction effect on deltamethrin overdose. Further research will be required to develop an effective immunotherapy for deltamethrin overdose in humans. In fact, this may not only be effective against deltamethrin alone, but also against other structure-similar toxic substances (e.g. cybermethrin).

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