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Evaluation of Sex Sensitivity in Local Lymph Node Assay Using Acephate and α- Hexylcinnamaldehyde

Dalal V [a]., Chhimwal R [a]., Verma R[a] and Suresh B [b].
[a]*Jai Research Foundation, Gujarat, India*
[b]*Department of Zoology, Faculty of Science, The M.S. University of Baroda, Vadodara, Gujarat, India*

Abstract: *The current OECD Test Guideline for the conduct of the Local Lymph Node Assay (LLNA) recommends the use of only female mice for the assessment of skin sensitization potential for a given chemical. The NIH publication N° 99-4494 recommends that only female CBA mice be used, as they reportedly develop a stronger contact dermatitis response when compared to males however if male to be used, systematic studies evaluating potential sex differences should be conducted. Moreover, males were reported to display a larger variation in response due to a greater tendency to fight and to be involved in 'social ranking' behavior when group housed. However, there are several advantages to consider with the inclusion of male mice in LLNA testing including a more refined and responsible use of animals. Therefore, to begin to systematically assess the appropriateness of using male mice in the LLNA a comparative guideline study was conducted with individual housing of mice using a non-sensitizer and a sensitizer viz., Acephate 97 DF and α-hexylcinnamaldehyde (HCA) respectively. We have conducted an in-house study using a vehicle (dimethyl formamide) that is recommended by OECD 429 for the testing of mixtures in the LLNA for assessing skin sensitisation. We conducted the test according to the methods described by OECD 429 (Skin Sensitization: Local Lymph Node Assay). One common pesticide formulation, Acephate 97 DF and one known skin sensitizer - HCA, were each applied to the dorsum of both ears (25 µL per ear) of groups of 5 female CBA/J mice (male and female). Acephate 97 DF was applied at 3 separate concentrations and one concentration for HCA for 3 consecutive days (days 0, 1 and 2). A further group was given the vehicle (dimethyl formamide), alone. On day 5, all mice were injected intravenously (tail vein) with approximately 20 (±1) µCi of tritiated methyl thymidine. Five hours post-administration the uptake of 3H-thymidine into the auricular (local) lymph nodes draining the site of chemical application was measured in order to assess the proliferative response of the lymph node. The DPM values were measured individually for each mouse. Stimulation Index (SI Value) was calculated and are given below:*

	(conc) SI	(conc) SI	(conc) SI	Historical rage of Female mice (in JRF)
HCA (Male)	(25%) **4.31**	-	-	**3.89 to 12.25**
HCA (Female)	(25%) **5.44**	-	-	
Acephate 97 DF (Male)	(10%) **1.16**	(25%) **1.58**	(50%) **1.59**	-
Acephate 97 DF (Female	(10%) **1.39**	(25%) **1.55**	(50%) **1.56**	-

Values shown in bold have a SI three or more times greater than the control (Control SI = 1) and are considered to have the potential to cause skin sensitisation.
The results obtained in the present study for HCA are comparable with the Historical Control Data of Laboratory (Jai Research Foundation).

I. Objective

The mouse local lymph node assay (LLNA) is an accepted test for evaluating the dermal sensitising potential of chemicals. The method is described in the OECD guideline 429: Skin Sensitization: Local Lymph Node Assay; however, limited guidance is given on its use for the testing of formulations and mixtures. At JRF, to expand our LLNA capabilities to include the testing of formulations and mixtures, we conducted an in-house validation study using male and female mice. We conducted a test to check the reliability, sensitivity and reproducibility of the method using HCA as positive control and made a comparison with the results of positive control data obtained from our laboratories.

II. Materials & Methods

Test Materials and Vehicle

Test Material	Purity	Source
a-Hexylcinnamaldehyde (HCA)	85%	Sigma-Aldrich Chemie GmbH

Acephate 97 DF	97%	United Phosphorous Limited

Test Animals

CBA/J male and female mice (9 to 10 weeks old) comprising 5 females per groups (source: Animal Breeding Facility, JRF) were used for study. Mice were housed individually. Feed (Teklad Certified Global High Fiber Rat/Mice feed manufactured by Harlan, USA, was provided ad libitum.) and water (UV sterilised water (Kent Reverse Osmosis water filtration system) was provided ad libitum.) was provided ad libitum. Environmental conditions were maintained as per international requirements.

Experimental Design

Dimethyl formamide (DMF) was used as the vehicle. This vehicle was recommended by the OECD 429. One known non skin sensitizer pesticide formulation, Acephate 97 DF and one known skin sensitizer - Hexylcinnamaldehyde (HCA) were selected for the test. The dose levels were based on preliminary assay for Acephate 97 DF.

The study design followed that recommended by OECD 429[1] and the following dose regimen was selected for the main study.

Treatment Procedure

Animals were treated topically for three consecutive days (days 0, 1 and 2) on the dorsal surface of both ears (25 µL/ear) using a calibrated micropipette. No treatment was applied on days 3 and 4. On day 5, all mice were injected intravenously (tail vein) with 250 µL of sterile phosphate buffered saline (PBS) containing approximately 20 (±1) µCi of tritiated methyl thymidine (Board of Radiation and Isotope Technology, DAE, India). Five hours post-injection of 3H-methyl thymidine, the animals were euthanized and the draining auricular (local) lymph node from both ears of each animal was excised and collected into PBS.

A single cell suspension of lymph node cells (LNC) was prepared for each mouse by gentle mechanical disaggregation through 200-210 µm-mesh stainless steel gauze with the plunger of the syringe. The gauze was washed with PBS into the base of a petri dish and the single cell suspension was transferred into a centrifuge tube and made up to 10 mL with PBS and centrifuged at approximately 190 to 200 g for 10 minutes at 4 (±2) °C. This procedure was performed twice. After the final wash each supernatant was removed leaving just a small volume of supernatant and then re-suspended with 3 mL of 5% trichloroacetic acid (TCA) and kept for precipitation for 18 at 4 (±2) °C. Thereafter each precipitate was recovered by centrifugation, the supernatant was removed and 1 mL of 5% TCA.was added. Each precipitate was transferred to a scintillation vial containing (Hionic flour) scintillation fluid.

The uptake of 3H-thymidine into the auricular (local) lymph nodes draining the site of chemical application was measured using a β –scintillation counter to assess the lymph node proliferative response in disintegrations per minutes (dpm).

Parameters as such body weight and ear thickness measurement also considered for evaluation.

Evaluation of Results

The test item was not regarded as a skin sensitiser ifas the SI for a dose group is ≤ 3 together with consideration of a dose-response relationship.

EC_3[2] value (theoretical concentration resulting in a SI value of 3) was calculated using the equation given below.

$$EC_3 = c + [(3 - d)/(b - d)] \times (a - c)$$

Where a = the lowest concentration giving stimulation index > 3; b = the actual stimulation index caused by a; c = the highest concentration failing to produce a stimulation index of 3; and d = the actual stimulation index caused by c.

Categorization of contact allergens on the basis of relative skin sensitization potency, is conducted using EC3

[1] OECD N° 429, "Skin Sensitisation: Local Lymph Node Assay". The Organisation for Economic Co-operation and Development (OECD) Guideline for Testing of Chemicals, adopted by the Council on July 22, 2010.

[2] Basketter, D.A., Lea, L.J., Dickens, A., Briggs, D., Pate, I., Dearman, R.J., and Kimber, I. (1999): A comparison of statistical approaches to the deviation of EC_3 values from local lymph node assay dose responses. *J. Appl. Toxicol.* **19,** 261-266.

values derived from the LLNA[3].

Any test material that produces a SI < 3 in the LLNA is considered negative for contact sensitization potential and therefore, an EC3 is not determined.

EC_3 value	Category
< 0.1%	extreme sensitizer
0.1 - < 1%	strong sensitizer
1 - < 10%	moderate sensitizer
10 - 100%	weak sensitizer

Body weight and radioactive disintegrations per minute (dpm) were subjected to Bartlett's test to meet the homogeneity of variance before conducting Analysis of Variance (ANOVA) and Dunnett's t-test to assess statistical significance from the control.

III. Results

Body Weight

The mean body weight of treatment group animals was comparable to that of the control group.

Local Irritation Response and Ear Thickness Percent Change

No erythema was observed in any treated mice at 10%, 25% and 50% (w/v) of Acephate 97 DF on day 0 to day 5. Very slight erythema (barely perceptible) was observed in dose group of 25% HCA (on days 1 to 4) in all mice (5/5 male mice and 5/5 female mice).

Ear thickness measurements showed much individual variation but revealed maximum increases on day 2 at 25% concentrationof HCA; around 18%. Acephate 97 DF treated group shows 8 to 12% increases on day 2 at 10%, 25% and 50% (w/v) concentrations.

Summary of Ear Thickness Percent Change

Male

Group N°	Dose Concentration (%)	N° of Mice Used	Mean Ear Thickness (Percent Change)			
			Left Ear Thickness (% Change)		Right Ear Thickness (% Change)	
			On Day 2	On Day 5	On Day 2	On Day 5
G1	Control (DMF)	5	4.008 ± 0.681	2.915 ± 0.443	3.970 ± 0.671	3.036 ± 0.579
G2	10% Acephate 97 DF (w/v) in DMF	5	7.920 ± 1.350	5.226 ± 0.791	8.271 ± 1.323	4.932 ± 0.675
G3	25% Acephate 97 DF (w/v) in DMF	5	10.833 ± 0.399	7.918 ± 0.542	10.984 ± 0.307	7.687 ± 0.633
G4	50% Acephate 97 DF (w/v) in DMF	5	14.823 ± 0.435	11.782 ± 1.158	14.673 ± 0.533	11.023 ± 1.741
G5	25% HCA (w/v) in DMF	5	17.768 ± 0.810	14.689 ± 0.848	18.016 ± 0.746	14.421 ± 0.869

Note: Values are in mean ± standard deviation.

[3] Kimber I., Basketter, D. A., Butler M., Gamer A., Garrigue J.L., Gerberick, G.F., Newsome, C., Steiling, W., Vohr, H.W. (2003). Classification of Contact Allergens According to Potency: Proposal. *Fd. Chem. Toxicol.* **41**, 1799-1809.

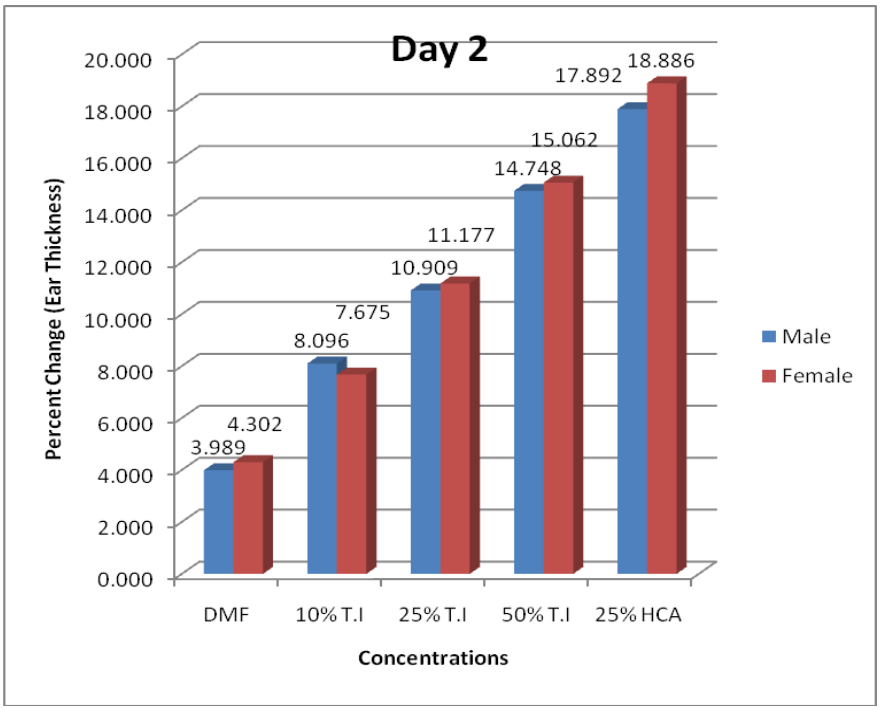
Female

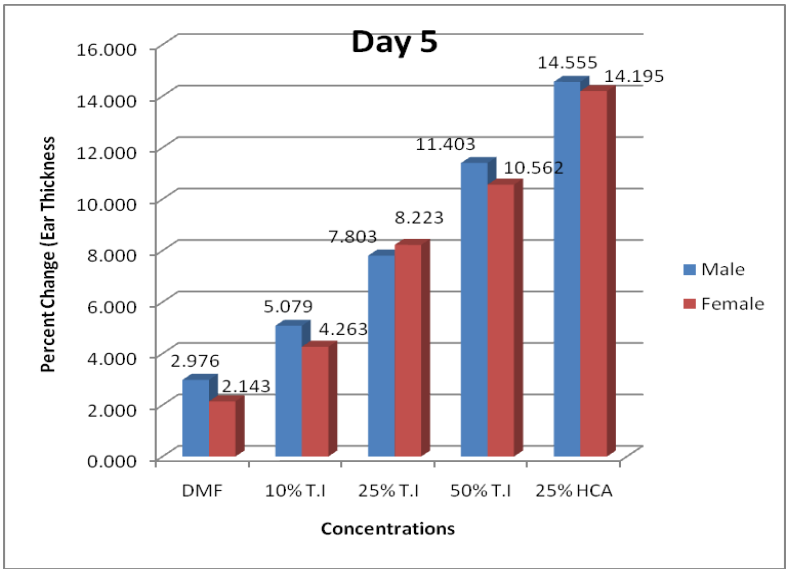
Group N°	Dose Concentration (%)	N° of Mice Used	Mean Ear Thickness (Percent Change)			
			Left Ear Thickness (% Change)		Right Ear Thickness (% Change)	
			On Day 2	On Day 5	On Day 2	On Day 5
G6	Control (DMF)	5	4.145 ± 0.281	2.262 ± 0.491	4.459 ± 0.561	2.024 ± 0.377
G7	10% Acephate 97 DF (w/v) in DMF	5	7.384 ± 0.561	4.224 ± 0.723	7.965 ± 1.436	4.302 ± 0.222
G8	25% Acephate 97 DF (w/v) in DMF	5	11.020 ± 0.939	8.316 ± 0.438	11.333 ± 2.270	8.129 ± 0.656
G9	50% Acephate 97 DF (w/v) in DMF	5	15.017 ± 0.982	10.799 ± 0.651	15.106 ± 1.521	10.324 ± 0.464
G10	25% HCA (w/v) in DMF	5	18.911 ± 0.795	14.248 ± 0.452	18.861 ± 0.976	14.141 ± 0.476

Note: Values are in mean ± standard deviation.

Mean of Left and Right Ear Thickness (Percent Change)

Dose Concentration (%)	N° of Mice Used	Mean Ear Thickness (Percent Change)			
		Male		Female	
		On Day 2	On Day 5	On Day 2	On Day 5
Control (DMF)	5	3.989	2.976	4.302	2.143
10% Acephate 97 DF (w/v) in DMF	5	8.096	5.079	7.675	4.263
25% Acephate 97 DF (w/v) in DMF	5	10.909	7.803	15.062	10.562
50% Acephate 97 DF (w/v) in DMF	5	14.748	11.403	18.886	14.195
25% HCA (w/v) in DMF	5	17.892	14.555	4.302	2.143





DPM and SI Value

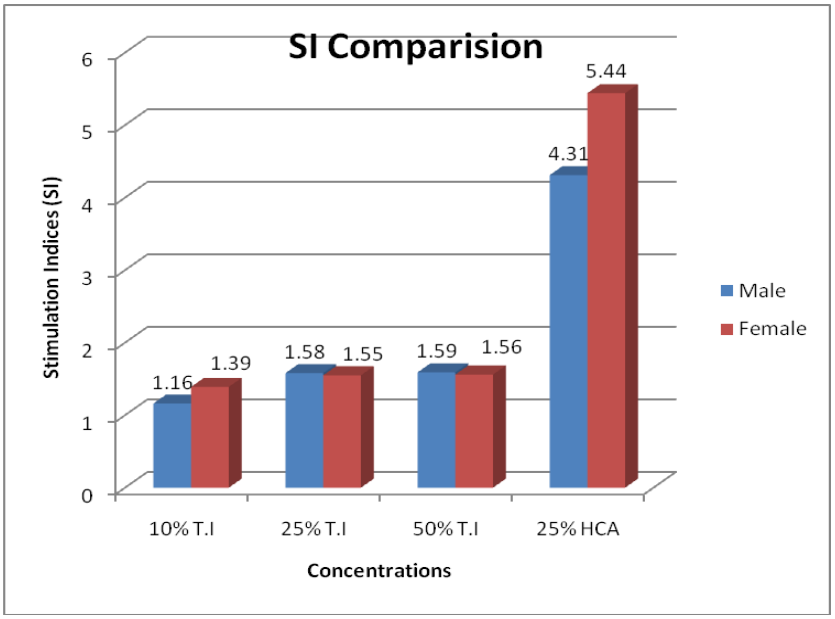
DPM value observed at all the treatment groups of Acephate 97 DF statistically insignificant and in dose dependent manner for male as well as female mice. DPM value of HCA treated groups statistically significant in male as well as female mice.

Male Mice:

Group N°	Dose Concentration (%)	N° of Mice Used	Group Mean DPM	Stimulation Index (SI)
G1	Control (DMF)	5	376.30 ± 88.64	1.00
G2	10% Acephate 97 DF (w/v) in DMF	5	435.20 ± 121.86	1.16
G3	25% Acephate 97 DF (w/v) in DMF	5	593.60 ± 183.83	1.58
G4	50% Acephate 97 DF (w/v) in DMF	5	599.90 ± 251.29	1.59
G5	25% HCA (w/v) in DMF	5	1623.20 ± 499.89*	4.31

Female Mice:

Group N°	Dose Concentration (%)	N° of Mice Used	Group Mean DPM	Stimulation Index (SI)
G1	DMF	5	493.10 ± 160.28	1.00
G2	10% Acephate 97 DF	5	683.38 ± 267.31	1.39
G3	25% Acephate 97 DF	5	762.60 ± 380.01	1.55
G4	50% Acephate 97 DF	5	770.70 ± 321.69	1.56
G5	25% HCA	5	2680.90 ± 390.50*	5.44



IV. Interpretation Of Results

Based on the SI value, HCA is categorized as weak sensitizers. SI for the three Acephate 97 DF treatment groups were 1.16, 1.58 and 1.59 for male and 1.39, 1.55 and 1.56 for females, respectively and SI for the HCA treated group were 4.31 for male and 5.44 for females. The SI of 4.31 for male and 5.44 for females obtained for the concurrent positive control, HCA, showed greater than a three-fold increase over the control value indicating a clear positive response for this known weak sensitiser that confirmed the reliability of this test procedure.

V. Conclusion

The results revealed no significant difference between samples means or variability between the sexes and the SI values observed for both male and female mice of positive control were within the range of historical control data for female mice. These data provide initial support for the use of male mice in the LLNA and will be followed by further experimentation.

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