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COMPARATIVE ANALYSIS OF GENOTOXIC POTENTIAL OF THREE MOSQUITO REPELLENTS

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ABSTRACT

A comparative study of cytogenetic effects of three different mosquito repellents (coil, mat and liquid types) has been done. It is based on chromosome aberration (CAs) analyses, micronuclei (MN) and mitotic indices (MI) in pulmonary alveolar macrophages (PAMs) of rats following short term and long term exposure to mosquito coil smoke (MCS), mosquito mat vapour (MMV) and All Out (a liquid type mosquito repellent) vapour (AOV). Animals were exposed intermittently to different concentrations of smoke/vapour of mosquito repellents in a closed glass inhalation chamber. For the short term (1 day) the rats were exposed to static repellents collected for different periods (1, 5 or 10 min) for 15 min/h for 8 consecutive hours For the long term, the animals were subjected to 8h daily exposure (15min/h) for 1 week. They were killed 24 and 32 h after the final exposure, and pulmonary lavage fluid was collected and processed for CA and MN assays, respectively. All three repellents induced significant frequencies of chromosomal damage, compared to the respective controls, particularly at higher concentrations, indicating their genotoxic potential. The incidences of CAs, MN, damaged cells and MI induced by MCS, MMV and AOV, when compared, revealed the highest genotoxic ability of MCS. The highest effectiveness of MCS is assumed to be due to the combined effect of insecticide(s) and combustion products of other ingredients present in the coil.

RESUMEN

Se realizó una investigación sobre los efectos citogenéticos de tres diferentes repelentes de mosquitos (espiral, placa y líquido) con base en el análisis de aberraciones cromosómicas (CA), micronúcleos (MN) e índice mitótico (MI) de macrófagos alveolares de pulmón de ratas después de un periodo de exposición corto y uno largo al humo de espiral (MCS), al vapor de la placa (MMV) y al vapor de All Out (un repelente de mosquitos de tipo líquido) (AOV). Los animales se expusieron de manera intermitente a diferentes concentraciones de humo y vapor en una cámara de vidrio cerrada. Para el periodo corto (1 día) las ratas se expusieron a los repelentes colectados en diferentes periodos (1, 5 ó 10 min) por 15 min/h durante 8 horas consecutivas. Para el periodo largo los animales estuvieron sujetos a una exposición diaria de 8 horas (15 min/h) durante una semana. Los animales fueron sacrificados después de 24 y 32 h de la exposición final y se colectó y procesó el lavado de fluído pulmonar para los ensayos de CA y MN. Los tres repelentes indujeron frecuencias significativas de daño cromosómico al compararse con los controles respectivos, especialmente en las concentraciones elevadas, indicando su potencial genotóxico. Cuando se comparó la incidencia de CAs, MN, células dañadas y MI inducidos por MCS, MMV y AOV se observó una alta capacidad genotóxica de MCS. Se considera que la gran efectividad de MCS se debe a los efectos combinados del insecticida y de los productos de combustión de otros ingredientes presentes en la espiral.

INTRODUCTION

Presently mosquito repellents are being used widely to combat mosquito menace in tropical and subtropical countries. A variety of mosquito repellents are available on the market and they can be grouped mainly into three categories: coil, mat and liquid types (excluding the cream variety). They are either burnt or evaporated in closed or semiclosed rooms to keep the mosquitoes at bay. Mosquito repellents, while in use, therefore cause indoor air pollution to a great extent with vapour of insecticides and other ingredients, and users are exposed to it for hours, and night after night. The contaminated indoor air enters the lung

when inhaled and encounters the pulmonary alveolar macro-phages (PAMs) which remain in the alveolar space. Thus the PAMs constitute the first line of defence for the inhaled xenobiotic.

That smoke or vapour of mosquito repellents can induce cytogenetic damage has been demonstrated in PAMs of rats (Das *et al.* 1994, Sahu and Das 1995, Das and Sahu 1996). Smoke of coil type has been reported to induce chromosome damage in non-target bone marrow cells also (Moorthy and Murthy 1994).

In view of mosquito menace on one hand, and positive genotoxic effects of mosquito repellents on the other, it is very important to identify a genetically less toxic repellent. The present study deals with a comparative analysis of cytogenetic effects induced by three mosquito repellents of three different types: coil, mat and liquid. Our study is based on analyses of chromosome aberrations (CAs), micronuclei (MN) and mitotic indices (MI) in PAMs of rats.

MATERIALS AND METHODS

Healthy adult inbred rats (Charles Foster strain) of both sexes, age 8-12 weeks, with body weights of 80-100 g, were employed. Standard laboratory rat chow and tap water were available to them *ad libitum* except during the period of exposure.

Two sets of animals were employed: one for analysis of CAs and the other for MN. For MI, no separate treatment or preparation was done, slides for MN assay were examined for a second time. Similarly, the incidence of damaged cells was determined from CA-preparation. In each set, four animals (2 males and 2 females) were used for each point of each repellent, and for control.

Repellents tested

'Tortoise' brand of coil type, 'Good Knight' of mat type and 'All Out' (AO) of liquid type were chosen for testing because of their wide use. They were bought from the local market.

Inhalation chamber and exposure schedule

Details of the inhalation chamber and exposure schedule have been described by us elsewhere (Das *et al.* 1994). In brief, rats were exposed to smoke or vapour (henceforth referred to as repellent) in a closed glass chamber (67.5 L) being fitted with a top door. 1. The repellent was collected in the chamber by keeping a smouldering mosquito coil (MC), or mosquito mat (MM) or AO with its electrical device inside for a particular period (1, 5 or 10 min). 2. The MC, MM or AO with its device was then removed quickly from the chamber and animals were released into it, and kept there for 15 min. 3. The animals were then taken out and the repellent was removed from the chamber. Steps 1-3 were repeated for each exposure, and every time the repellent was collected afresh.

For each repellent two modes of exposure, short term (1-day) and long term (7-days), were followed. For the former, rats were exposed for 15 min/h, 8h (consecutive)/day and for the latter for 15 min/h, 8h/day and 7 days/week. The animals were thus exposed intermittently to static repellent. The repellents were collected in the chamber for different periods (1, 5 or 10 min) just to obtain differential 'concentration' for concentration-response analysis done for short term exposure; for long term exposure they were collected for 10 min only.

Exposure schedule for CA and MN assays remained the same and both assays were conducted for short term as well as long term exposure.

Control

The age and sex matched rats maintained side by side in identical condition as the experimental animals but without exposure to the repellent were used as control. Separate controls were kept for CA and MN assays.

Collection and processing of pulmonary lavage

The animals were killed by CO_2 asphyxiation followed by cervical dislocation 24 and 32 h after the final exposure for CA and MN studies respectively. For the CA assay, but not for MN, rats were treated ip with colchicine (4mg/kg) 3h before killing. Pul-monary lavage was collected and slides were prepared and stained for CA and MN studies according to the schedules described by us earlier (Das *et al.* 1994, Sahu and Das 1995). In brief, about 10 ml of pulmonary lavage fluid was collected in 0.56% aqueous KCl solution by repeated infusion of the salt solution into the lungs through exposed trachea and withdrawal of the same. The lavage was then processed following conventional acetic acid-methanol-flame-drying schedule for metaphase chromosome analysis. The slides were stained next day in 10% Giemsa diluted with buffer solution (pH 6.8). To evaluate the occurrence of CAs, 50 well spread intact metaphases were examined from each animal. Cell containing CAs, irrespective of number and type, was referred to as a damaged cell, and its frequency was also considered.

For MN preparation, the lavage fluid without incubation was centrifuged, the supernatant was decanted off and a concentrated suspension of pulmonary cells was prepared in the left over drop of KCl solution for smear preparation. The smears were fixed in absolute methanol for 10 min and stained next day in Giemsa diluted (1:10) with buffer (pH 6.8) for 15 min. The slides were rinsed in deionized water, air-dried and mounted with DPX. 500 PAMs were examined from each animal. Data for MI (% of dividing cells) were scored from MN preparation.

Statistical analysis

Analysis of variance (F test) and Student's 't' test were performed to know if the treatment values differed from each other and from the control ones significantly. Correlation coefficient (r) test was conducted to find out if the data were concentration-dependent.

RESULTS

The CAs as well as MN induced by three types of mosquito repellents tested by us did not differ qualitatively. CAs

TABLE I. INCIDENCE OF CHROMOSOME ABERRATIONS IN PAMs OF RATS EXPOSED TO DIFFERENTE MOSQUITO REPELLENTS FOR ONE DAY

Collection of smoke/ vapour (min)	mosquito repellent	metapahses scored/animals	«Break type» aberrations					Damaged cells mean % ± SE
			Chr. Br.	Frag.	Exch.	Ring	Total (mean % ± SE)	
Control	---	600/12*	6	3	---	1	1.83 ± 0.90	1.50 ± 0.43
1	MCS	200/4	3	2	---	---	2.50 ± 0.43	2.50 ± 0.43
	MMV	200/4	3	---	---	---	1.50 ± 0.82	1.50 ± 0.82
	AOV	200/4	2	1	---	---	1.50 ± 0.43	1.50 ± 0.43
5	MCS	200/4	5	4	1	---	5.50 ± 0.82b	4.00 ± 0.70a
	MMV	200/4	3	3	1	---	4.00 ± 0.70a	3.50 ± 0.43a
	AOV	200/4	2	1	1	---	2.50 ± 0.83	2.00 ± 0.70
10	MCS	200/4	12	4	---	1	9.00 ± 1.11b	8.50 ± 1.47b
	MMV	200/4	8	7	2	---	9.50 ± 2.04b	7.00 ± 1.12b
	AOV	200/4	11	---	1	---	6.50 ± 0.43b	5.50 ± 0.86a

* Contol data for three repellents were pooled for convenience in analysis
a, b: significantly higher than the control value (t test) at a = $p < 0.05$; b = $p < 0.01$

encountered were mainly of chromatid type and comprised of chromatid breaks, acentric fragments of untraceable origin (unpaired and paired), ring chromosomes (resulted from sister chromatid union) and exchanges. Among the aberration types the first two constituted the major bulk. As the aberration types mentioned above resulted from breaks they were considered under 'break' type aberration for convenience in quantitative analysis. Since each of the exchanges and rings needed 2 breaks for its formation they were considered as two breaks. For MN analysis only a few PAMs were recorded to contain two MN of different size, otherwise, one MN per affected PAM was of general occurrence.

The data for CAs induced in PAMs by the three repellents (**Table I**) exhibited a significant correlation with the concentrations (for MCS $r = +0.999$, $p < 0.001$; for MMV $r = +0.982$, $p < 0.01$; for AOV $r = +0.929$, $p < 0.05$; **Fig. 1a**). The values for the lowest concentration for the 3 repellents remained close to each other and also close to the control value; marked differences in their effect were noted at higher concentrations. A two-way analysis of variance clearly demonstrates significant differences among the values obtained at different concentrations for three repellents (F between conc.= 59.64, $p < 0.01$; F between variety = 7.20, $p < 0.05$; **Fig. 1a**). Mosquito coil smoke (MCS) showed the maximum effect, while the minimum effect was found for 'All Out' vapour (AOV). A similar trend was revealed when damaged cells were taken into consideration (F between conc. = 64.41, $p < 0.001$; F between variety = 8.95, $p < 0.05$; **Fig. 1b**).

Data on MN-PAMs (**Table II**) showed concentration dependent increase of the effect for all the repellents, and revealed the highest incidence for MCS and the lowest for AOV at any particular concentration (**Fig. 1c**). The data for the three repellents differed from each other significantly at the highest concentration level (F = 12.33, $p < 0.01$).

The mitotic indices for the three repellents remained close to each other at any particular concentration level (**Fig. 1d**). The MI for any repellent obtained at the highest concentration level was significantly higher than that for the two lower concentrations as well as that for the control.

TABLE II. INCIDENCE OF MN-PAMs AND MITOTIC INDICES IN RATS EXPOSED TO DIFFERENT MOSQUITO REPELLENTS FOR ONE DAY

Collection of smoke/ vapour (min)	Mosquito repellent	No. of PAMs scored/ animals	PAMs with MN (mean % ± SE)	Mitotic Index (Mean ± SE)
Control	---	6000/12*	0.16 ± 0.06	0.80 ± 0.09
1	MCS	2000/4	0.25 ± 0.04	0.98 ± 0.12
	MMV	2000/4	0.20 ± 0.07	1.10 ± 0.14
	AOV	2000/4	0.15 ± 0.08	1.15 ± 0.15
5	MCS	2000/4	0.50 ± 0.09a	1.20 ± 0.19
	MMV	2000/4	0.35 ± 0.08	1.00 ± 0.13
	AOV	2000/4	0.25 ± 0.04	1.18 ± 0.09
10	MCS	2000/4	1.10 ± 0.15c	2.13 ± 0.16c
	MMV	2000/4	0.65 ± 0.04b	1.70 ± 0.13b
	AOV	2000/4	0.35 ± 0.04a	1.75 ± 0.13c

*Control data for three repellents were pooled for convenience in analysis
a, b, c: significantly higher than the control value (t- test) at a = $p < 0.05$; b = $p < 0.01$; c = $p < 0.001$

Data on CAs for 7-day exposure revealed the same trend as that exhibited by the 1-day data, MCS showing the highest effect, while AOV the lowest (**Table III, Fig. 2a**). The data for AOV were, however, significantly higher than the control values. Further, the data for CA as well as damaged cells for three repellents were significantly different from one another (for breaks, $F = 4.76$, $p < 0.05$; for damaged cells $F = 6.06$, $p < 0.05$) (**Fig. 2a, b**). For MN-PAMs the highest incidence was also recorded for MCS and the lowest for AOV (**Table IV, Fig. 2c**).

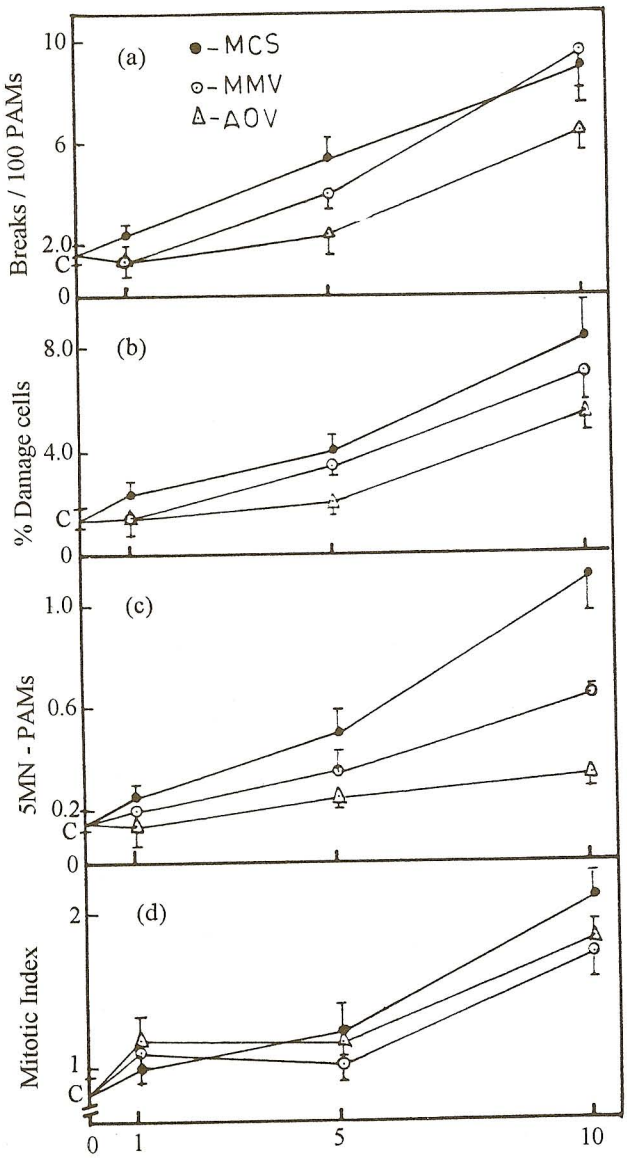

Fig. 1. Comparative analysis of the incidences of (a) chromosome aberrations, (b) damaged cells, (c) micronuclei and (d) mitotic indeces in PAMs of rats exposed to MCS, MMV and AOV for one day (15 min/h, 8h/day)

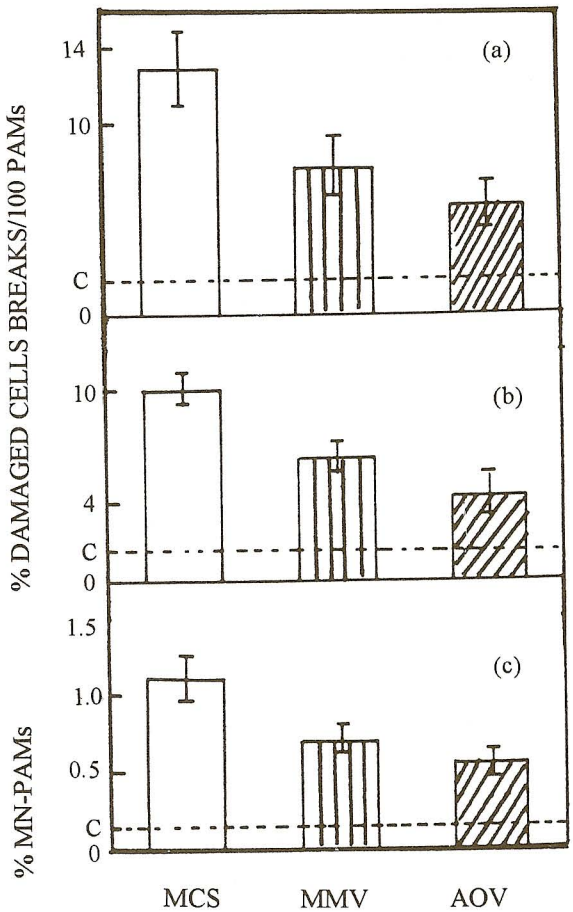

Fig. 2. Histogram analysis of frequencies of (a) chromosome aberrations, (b) damaged cells and (c) micronuclei in PAMs of rats exposed to MCS, MMV and AOV for one week (15 min/h, 8h/day and 7 days/ week

DISCUSSION

The baseline incidence of CAs in PAMs noted in the present study is low and close to the values obtained earlier by others in the same species (Rithidech *et al.* 1989, 1990). Significantly elevated incidences of CAs, damaged cells and MN at least at higher concentrations clearly indicate positive genotoxic capacity of the mosquito repellents tested by us. A similar trend in concentration-response data as well as for short-term and long-term exposures data was seen for all the experimental conditions. Data obtained from identically conducted experiments for three repellents unequivocally demonstrate the highest genotoxic effectiveness for MCS and the lowest for AOV.

In the present study, the repellent collection period inside the inhalation chamber was expressed as repellent «concentration». As evaporation or smouldering takes place almost uniformly at a constant temperature it is expected that the amount of repellent in a closed chamber will be nearly proportional to the period of evaporation or smouldering. Although this is a crude method to express the concentration, the correlation between

TABLE III. INCIDENCE OF CHROMOSOME ABERRATIONS IN PAMs OF RATS EXPOSED TO DIFFERENTE MOSQUITO REPELLENTS FOR ONE WEEK

Collection of smoke/ vapour (min)	mosquito repellent	metapahses scored/animals	«Break type» aberrations					Damaged cells mean % ± SE
			Chr. Br.	Frag.	Exch.	Ring	Total (mean % ± SE)	
Control	---	600/12*	6	3	---	1	1.83 ± 0.90	1.50 ± 0.43
10	MCS	200/4	17	1	2	2	13.00 ± 2.06b	10.00 ± 0.70c
	MMV	200/4	11	4	---	---	7.50 ± 1.89a	6.50 ± 0.82b
	AOV	200/4	6	3	---	1	5.50 ± 1.29a	4.50 ± 1.29

*Control data for three repellents were pooled for convenience in analysis
a, b, c: significantly higher than the control value (t- test) at a = $p < 0.05$; b = $p < 0.01$; c = $p < 0.001$

the vapour/smoke amount and the chromosome damage is clearly evidenced.

In normal conditions, alveolar macrophages rarely divide. But toxic exposure not only induces them to divide but causes their influx into the bronchoalveolar air space also (Evans *et al.* 1973, White and Garg 1981). Marked increase in MI particularly at higher concentrations for all three repellents as well as occurrence of mitosis in both resident and influxed macrophages, clearly indicate a toxic nature of the repellents .

Based on the present study it is not possible to identify the genotoxic agent present in the repellents. Pyrethroid insecticides are the commonly used active principle in mosquito repellents. As mentioned on the packets, the mat contains 4% allethrin, a synthetic pyrethroid, and AO contains 3.6% allethrin, a natural pyrethroid, but nothing is known about the MC's ingredients and their proportion. A number of synthetic pyrethroids have earlier been reported to be clastogenic and/or mutagenic in mammalian cells *in vivo*, plant and *Drosophila* systems (Amer and Aboul Ela 1985, Batiste-Alentom *et al.* 1986, Chouhan *et al.* 1986, Bhunya and Pati 1988, Pati and Bhunya 1989), though shown to be nonmutagenic in *Salmonella*/microsome assay (Pluijmen *et al.* 1984). There are many agents which show lack of effect in *Salmonella* assay but demonstrate a positive effect in *in vivo* mammalian system (Das and Roy 1990, Roy and Das 1990). As no mosquito repellent (except the cream variety) is covered under the Indian Drug (Control) Act, sometimes dishonest manufacturers use more toxic and cheaper insecticides like chlorinated hydrocarbons, organophosphorous compounds and carbamate insecticides, just to elevate mosquito knock down effect and to reduce the market price. Insecticides belonging to chlorinated hydrocarbon, organophosphorous and carbamate groups are known to be highly genotoxic (Wild 1975, Behera and Bhunya 1989, Bhunya and Jena 1992). It is quite possible that any of these insecticides present in the mosquito repellents is the causative agent for the chromosomal damage. But the role of other ingredients and combustion products cannot be ruled out.

TABLE IV. INCIDENCE OF MN-PAMs IN RATS EXPOSED TO DIFFERENT MOSQUITO REPELLENTS FOR ONE WEEK

Collection of smoke/ vapour (min)	Mosquito repellent	No. of PAMs scored/ animals	PAMs with MN (mean % ± SE)
Control	---	6000/12*	0.16 ± 0.06
10	MCS	2000/4	1.12 ± 0.17c
	MMV	2000/4	0.70 ± 0.08b
	AOV	2000/4	0.55 ± 0.08b

*Control data for three repellents were pooled for convenience in analysis
b, c: significantly higher than the control value (t- test) at b = $p < 0.01$; c = $p < 0.001$

From mass spectrophotometric and gas chromatographic analyses, the presence of 60 organic compounds and 8 metals has been detected in a mosquito coil of Japanese brand and in its smoke (Liu *et al.* 1987, 1989). These include, besides allethrin, toluene, xylene and phenols in high quantities. The major bulk of all MCS including the one tested here is constituted by saw-dust and/or coconut shell powder. Phenols, xylene and toluene are the common products of wood combustion. Burning of wood also releases a little amount of polynuclear aromatic hydrocarbons (PAH). Smoke condensates from varieties of wood have been reported to induce mutation in *Salmonella* in a dose-dependent manner (Lofroth 1978, Alfheim *et al.* 1984, Asita *et al.* 1991, McCrillis *et al.* 1992). That toluene, xylene and phenols are genotoxic is also on record (Dean 1985, Mavournin *et al.* 1990). The highest incidence of chromosomal damage noted for MCS may be explained by the combined action of insecticide(s) and combustion products of wood and other ingredients.

Quality and quantity of other ingredients for 'Good Knight' are not mentioned on the packet. Here a small mat or biscuit like structure is prepared with the ingredients on a piece of paper board; it does not contain saw dust. This may be one of the factors that cuases less cytogenetic effect of MMV.

AO contains, in addition to allethrin (3.6%), dibutyl hydroxy toluene (0.31%), perfume (0.15%) and deodorized kerosine (95.94%). The effect of kerosine, besides allethrin and toluene, cannot be ignored, particularly in view of a recent report on induction of mutation in *Salmonella* Ames test by the organic compounds emitted from kerosine space heater (Mumford *et al.* 1992). However, emission of PAH from oil-burning is much less than that of wood-burning (Cooper 1980). The lower effectiveness of AOV with regard to chromosome damage compared to that of MCS and MMV, may be due to the lower concentration of allethrin and/or other combustion products like toluene and PAH.

In response to injury or inflammation caused on the alveolar surface due to toxic exposure, macrophages and other phagocytes

are known to produce reactive oxygen species (superoxide radicals, hydrogen peroxide, hydroxyl radical, singlet oxygen) (Tate and Repine 1984, Doelman *et al.* 1990). Acute smoking has also been shown to increase super oxide production by unstimulated alveolar macrophages (Richter *et al.* 1986). However, it is not known if the causative factor(s) (the insecticides, other ingredients and/or their combustion products) for cytogenetic damage act(s) directly or indirectly through production of reactive oxygen species.

Compared to other two repellents, AO is, thus, less harmful. In view of having potential hazardous effect on users' health, other brands of mosquito repellents also warrant testing.

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