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Original Article

"EVALUATION OF CYTOTOXICITY, OXIDATIVE STRESS, NUCLEAR CHANGES AND PRO-INFLAMMATORY CYTOKINES INDUCED BY MONOCROTOPHOS IN HUMAN KERATINOCYTE CELLS *IN VITRO*"

NATHIYA SOUNDARARAJAN, DURGA MOHAN, RAJASEKAR ABBU, THIYAGARAJAN DEVASENA*

Centre for Nanoscience and Technology, AC Tech Campus, Anna University, Chennai-600025, Tamil Nadu, India.
Email: tdevasenabio@gmail.com

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ABSTRACT

Objective: This study was explored to identify the toxicological behaviour of monocrotophos against human keratinocyte cells (HaCaT cells) **Methods:** In this study HaCaT cell line was used to identify the inflammatory effect of monocrotophos on cell viability, nitric oxide secretion (NO), lactate dehydrogenase release (LDH), malondialdehyde release (MDA), nuclear changes, reactive oxygen species generation and cytokine expression

Results: From the *in vitro* cell viability study, it appears that the monocrotophos was toxic to HaCaT cells; IC_{50} value was 408.2 µg/mL during 24 h of incubation period. Significant increase in NO, LDH, MDA, nuclear changes, pro-inflammatory cytokine and ROS generation was observed compared with the control. $1/5^{th}$ IC_{50} value treatment of HaCaT cells with monocrotophos resulted in 9.97, 8.78 and 9.45 times increase in TNF-α, IL-6 and IL-8 expression higher than the control.

Conclusion: This study gives perceptiveness about the toxicity of monocrotophos and provides wide acquaintance to restrict the use of monocrotophos.

Keywords: Cytokine, Pesticide, Cell viability, HaCaT cells, Toxicity.

INTRODUCTION

Pesticides became hazardous because of carcinogenic, reproductive, oncogenic, teratogenic and mutagenic. It causes toxic effects on the skin, lung, mucous membrane, immune system, liver and blood [1-3]. Monocrotophos is used in large quantities in India, especially in cotton growing areas. Several reports are available proving that organophosphates, including monocrotophos, are the major agent of self-poisoning, with increased fatality rates. The prolonged use of monocrotophos in protection of plants may lead to the significant effect on dermal exposure and causes severe impact on genotoxicity, cardiotoxicity and cholinesterase activities [4-6].

In vivo bioassays are costly and time-consuming; hence there is a need for rapid and short-term *in vitro* bioassays to screen the toxicity of pesticides using biochemical techniques and to identify the cellular and molecular mechanisms involved in cytotoxicity [7]. Pesticides can be toxic by ingestion, dermal exposure, inhalation, or ocular exposure. Dermal exposure of pesticide causes inflammation in skin cells. Hence human keratinocyte cell line was used to identify toxic effect of monocrotophos on skin cell line. Exposure of pesticides is the second major component of risk assessment. Exposure profile can be described as

(i) Time period of exposure and sources

(ii) Impact of variability

(iii) Transport of pesticides and pathways of exposure

Pesticides absorbed on the outer surface of the skin enter into inner surface or into the body, finally enters into the blood stream causes severe health problems and its major action is away from the site of entry. Feldmann and Maibach [8] studied the penetration of 12 radio labelled pesticides and the skin absorbed herbicides including monocrotophos in male volunteers, by topical application maximum of 22% of monocrotophos and it causes damage to the skin cells. It is essential to identify the cytotoxic effect of monocrotophos on cell proliferation under *in vitro* conditions in human HaCaT cell line. This study gives astuteness about the toxicity of pesticides and delivers extensive knowledge regarding the environmentally relevant concentration causes toxic effects on man.

MATERIALS AND METHODS

Chemicals

Monocrotophos pure form was obtained from Sigma Aldrich. HaCaT cell line was purchased from National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in DMEM (Dulbecco's Minimal Eagle's Medium), supplemented with 10% Fetal Bovine Serum (FBS), at 37°C in humidified atmosphere with 5% CO_2. Nitric oxide (NO) level determination kit obtained from Biovision Inc. Lactate dehydrogenase (LDH) and Malondialdehyde (MDA) level determination kit obtained from Sigma-Aldrich, India. Also reagents used were of analytical grade and obtained from Hi-Media India Ltd.

Cytotoxicity studies

Cell viability assay: Cell viability assay was performed following the method described by Carmichael et al [9] and percentage of cell viability was determined by spectrophotometer determination of accumulated formazan derivative in treated cells at 570 nm in comparison with the untreated ones. For the MTT assay, the cells were grown in 25 cm × 25 cm × 25 cm tissue culture flasks containing DMEM medium. When a cell density in a culture flask reached 70-80% confluence, they were trypsinized and seeded in 96 well plates at varying cell number according to the size and shape of the HaCaT cells were seeded in the density of 3000 cells per well in 100 µL and incubated for 24 h at CO_2 incubator. Test items were prepared as 1 mg/mL stocks by adding directly in to the DMEM medium. The working stock of 2X (2000, 600. 200, 60 and 20 µg) concentration to the cell in 100 µL volumes and the final concentration range was: 1000, 300, 100, 30 and 10 µg/mL. The plates were further incubated for 24 h in the CO_2 incubator. MTT solution was composed of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide at 5 mg/mL in phosphate buffered saline (1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4), from this solution 50 µL was pipette out into each well to achieve 1 mg/mL as final concentration. The plate was further incubated for 2.30 h in the incubator and the medium was carefully decanted. The formazan crystals were air dried in a dark place and dissolved in 100 µL dimethyl sulphoxide (DMSO) and the plates were mildly shaked at room temperature and the OD was measured using Synergy H4 microplate reader at 570 nm.

Measurement of NO and LDH

For the measurement of NO and LDH 1/5th (P1) and 1/10th IC_{50} values (P2) of monocrotophos was taken. HaCaT cells were seeded into 6-well plates at a density of 2.0×10^5per mL in 2.5 mL culture medium and allowed to proliferate attach and cover around 80% of the plate surface area before the treatment with monocrotophos for 12 and 24 h. After 12 and 24 h of exposure, the culture supernatant was collected to determine the levels of NO and LDH.

Measurement of intracellular MDA

For the measurement of MDA level, HaCaT cells were treated with 1/5th (P1) and 1/10th IC_{50} values (P2) of monocrotophos. HaCaT cells were seeded into 6-well plates at a density of 2.0×10^5per mL in 2.5 mL culture medium. Cells allowed proliferating, dose and time dependent experiments were performed. After 12 and 24 h treatments of HaCaT cells with monocrotophos, cells were washed using phosphate buffered saline (PBS), trypsinization was done and followed by immediate disruption by freeze-thaw process about 3 times and the cell lysates were analysed for intracellular MDA levels.

Apoptosis measurements and 4'-6-diamidino-2-phenylindole (DAPI) staining

To determine the level of apoptosis in pesticide treated HaCaT cells, DAPI staining was performed as described by Sandra et al [10]. Briefly, the cells were seeded onto glass slides and treated for 12 and 24 h in the concentration 1/5th IC_{50} of pesticide (P1). Untreated and treated cells were rinsed with phosphate buffered saline (PBS), fixed with ice-cold 10% trichloroacetic acid, and further washed with cold 70, 80, 90% absolute ethanol. The cells were permeabilized with Triton-X (10% v/v) and stained with 1 µg/mL DAPI for 3 min. To reduce the background, the stained cells were washed with PBS, cover-slipped with 90% glycerol and observed under a fluorescence microscope (Labomed- Carl zeiss Lens with blue filter Olympus India).

Measurement of reactive oxygen species (ROS)

The ROS production was examined by staining with 2',7'-dichlorofluorescin diacetate (DCFDA). It enters into the cell where it reacts with ROS to produce the fluorescent compound dichlorofluorescein (DCF). HaCaT cells were seeded into 6 well plates at a density of 2.0×10^5per mL in 2.5 mL culture medium and allowed to proliferate. Dose and time dependent experiments were performed. HaCaT cells were treated with 1/5th IC_{50} concentration of pesticide (P1), DMEM was replaced by PBS with glucose (5.5 mM) and the cells were treated with 1 µM CM-H2DCFDA for 30 min at 37°C in darkness. The excess probe was washed out; in glucose-enriched PBS the cells were suspended and transferred to a fluoro slide. The cells were monitored at different time intervals. The fluorescence was recorded at 495 nm excitation and 530 nm emission (excitation/emission slit = 10 nm/5 nm) by a luminescence fluorescent microscope. The treated cells were observed in 10x resolution.

Expression of cytokines

HaCaT cells were incubated in tissue culture flask containing DMEM, FBS, streptomycin and penicillin (100 U/l) in a humidified atmosphere containing 5% CO_2 at 37°C for confluence. After discarding the media, 1 mL of Trypsin (0.25%) was added and kept in the incubator for 5 min. To detach the cells the flask was gently tapped and then the flask was completely rinsed by adding 3 mL of DMEM-FBS media and the contents were centrifuged at 1000 rpm for 10 min. After discarding the supernatant, the cell pellet was dissolved in DMEM-FBS media. The viability of the trypsinised (Trypsin-EDTA) HaCaT cells was checked after staining with 0.4% trypan blue staining solution (1:4 diluted in phosphate buffered saline) and the cells were counted using neubeuer chamber and then diluted based on the requirement.

HaCaT cells (10^6 cells/mL) were seeded and incubated with monocrotophos at 1/5th IC_{50} concentration (P1) (dissolved in 5% DMSO in serum free DMEM medium). The setup was incubated for 12 and 24 h at 37°C in a humidified atmosphere containing 5% CO_2. Unstimulated cells were served as negative control. After 24 h, the quantitative measurement of pro-inflammatory cytokines (IL-8, TNF-α and IL-6) was done in the supernatant of cultured cells using commercial ELISA assay kits (ebiosciences, USA), according to the manufacturer's recommendations. The samples and standards were all run in duplicates, and the data were then averaged.

RESULTS

Cell viability (MTT) assay

Monocrotophos is listed as a Prior Informed Consent chemical, and it is still used extensively in developing countries. HaCaT cells were treated with 1000, 300, 100, 30 and 10 µg of monocrotophos for 24 h (fig. 1**)**. Growth inhibition was observed at the lower concentration of pesticide and the IC_{50} value of monocrotophos was found to be 408.2 µg/mL (Table 1). Cytotoxicity of monocrotophos was evaluated by cell viability and changes in biochemical factors such as NO and LDH present in cell culture supernatants at 12 and 24 h incubation. Lower concentrations 1/5(P1) and 1/10th (P2) IC_{50} values were chosen to identify the NO, LDH and oxidative stress in cell culture supernatant at 12 and 24 h incubation. Monocrotophos causes toxic effects on skin cells if it is exposed through dermal route to humans, where exposure of 10 µg of monocrotophos causes toxicity in HaCaT cells.

Table 1: Percentage growth of HaCaT cell line against monocrotophos

	Percentage growth					Growth inhibition in µg		
	1000µg	300µg	100µg	30µg	10µg	IC_{50}	TGI	LC_{50}
Monocrotophos	35	55	70	89	94	408.2	1000.0	1000.0

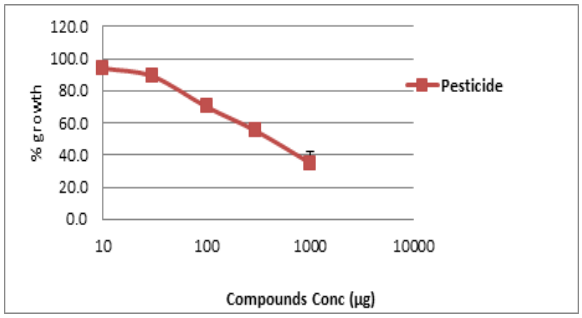

Fig. 1: Cell viability of monocrotophos exposed to HaCaT cells after 24 h exposure was determined. Data are expressed as mean ± SD of three repeated experiments

Measurement of NO and LDH

Monocrotophos incubation in HaCaT cells resulted in significant release in NO in P1 and P2 treatments compared with control in a dose dependent manner. At 12 h of incubation P1 and P2 treatments resulted in 1.34 and 2.63 times higher secretion of NO when compared with the control.

At 24 h of P1 and P2 treatments showed 1.78 and 2.92 times increase in NO secretion higher than the control (fig. 2). The effect of monocrotophos on LDH release in supernatant fluids resulted in the significant increase compared with the control.

P1 and P2 treatments resulted in 1.14 and 1.30 times increase in LDH release at 12 h incubation higher than the control. At 24 h incubation 1.22 and 1.38 times increase in LDH release was observed, which was higher compared to control (fig. 3).

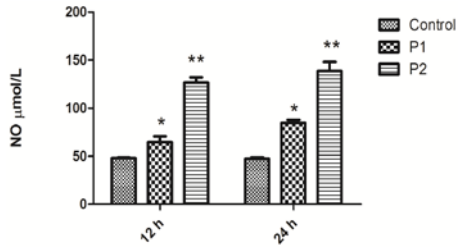

Fig. 2: NO levels in HaCaT cells exposed to monocrotophos. Data expressed as mean ± SD. *Denotes a significant difference from the control

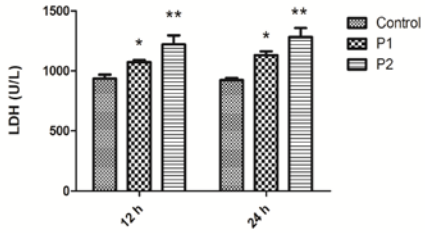

Fig. 3: LDH levels in HaCaT cells exposed to monocrotophos. Data expressed as mean ± SD. *Denotes a significant difference from the control

Measurement of intracellular enzyme (MDA)

The effect of monocrotophos on oxidative stress in HaCaT cells was examined by investigating MDA intracellular enzymes. In the present study dose dependent toxic effect was observed in all the parameters. To ameliorate the toxicity of pesticides MDA involves in molecular mechanisms. Due to cell membrane damage MDA is released in the medium. At 12 h incubation P1 and P2 treatment resulted in 1.57 and 1.69 times increase in MDA level higher than the control. At 24 h incubation P1 and P2 treatment resulted in 1.76 and 1.95 times increase in MDA level compared with the control (fig. 4).

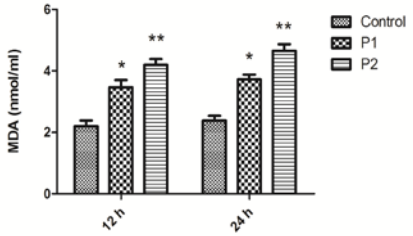

Fig. 4: Oxidative stress induced by monocrotophos in HaCaT cells after 12 and 24 h exposure. Data expressed as mean ± SD. *Denotes a significant difference from the control

Apoptosis measurement

Monocrotophos induced morphological changes in HaCaT cells were determined using DAPI staining. In the present investigation loss of nuclear construction, formation of apoptotic bodies, chromatin condensation was observed in monocrotophos treated cells. These changes were not observed in control cells.

Nuclear changes in arbitrary units were determined for 12 and 24 h incubation. 1/5th IC_{50} value (P1) was used to determine apoptosis, it was observed that 40 and 41% increase in nuclear changes higher than the control at 12 and 24 h incubation. Significant nuclear changes were not observed at time-dependent manner (fig. 5).

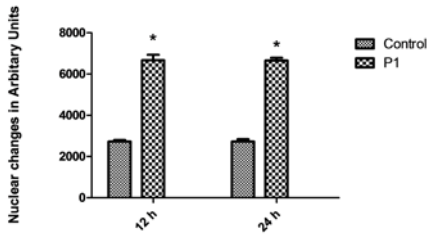

Fig. 5: Nuclear changes induced by monocrotophos in HaCaT cells

Measurement of ROS

ROS generation was calculated for 12 and 24 h time period. The percentage ROS generation was increased in 24 h time period compared to 12 h time period. 1/5th IC_{50} value (P1) treatment of monocrotophos in HaCaT cells resulted in 20.1 and 26.7% increase in ROS generation at 12 and 24 h higher than the control (fig. 6A). The possible reason for our findings is due to generation of free radicals such as superoxide can be converted into reactive oxygen species such as hydroxyl radical, hydrogen peroxide and peroxynitrite to facilitate a variety of oxidative reactions. The induction of apoptosis and ROS generation is shown in fig. 6B.

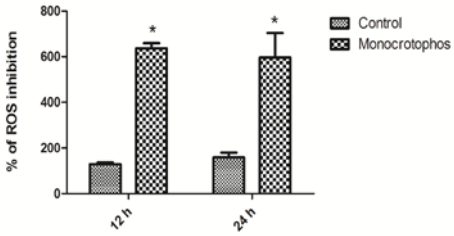

Fig. 6A: Percentage of ROS inhibition in HaCaT cells exposed to monocrotophos. Data expressed as mean ± SD. *Denotes a significant difference from the control

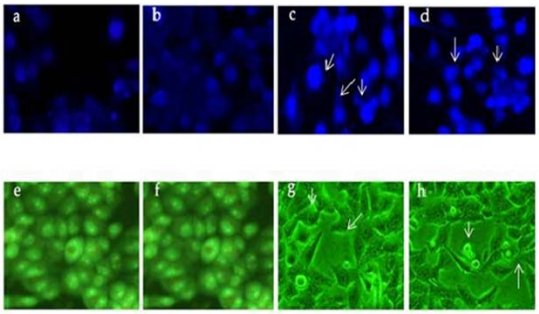

Fig. 6B: Induction of apoptosis and ROS in HaCaT cells.

a. Control, HaCaT cells stained with DAPI, incubated for 12 h.

b. Control, HaCaT cells stained with DAPI, incubated for 24 h.

c. Induction of apoptosis in HaCaT cells exposed to monocrotophos with the incubation period of about 12 h.

d. Induction of apoptosis in HaCaT cells exposed to monocrotophos with the incubation period of about 24 h.

e. Control, ROS generation in HaCaT Cells studied using chloromethyl derivative of H2DCFDA, incubation period of about 12 h.

f. Control, 24 h.

g. ROS generation in HaCaT cells exposed to moncrotophos with incubation period of about 12 h.

h. ROS generation in HaCaT cells exposed to Moncrotophos with incubation period of about 24 h.

Expression of Pro-inflammatory cytokines

Monocrotophos induced pro-inflammatory cytokines in HaCaT cells at 24 h incubation. The expression of TNF-α was high compared to other pro-inflammatory cytokines. By detecting the expression of TNF-α cytokine, the direct information about the pro-inflammatory response towards the pesticide was identified in HaCaT cells. 1/5th IC_{50} value (P1) treatment of HaCaT cells resulted in 9.97 times increases in TNF-α expression, 8.78 times increase in IL-6 expression and 9.45 times increases in IL-8 expression higher than the control. Comparative study between the expression of TNF-α, IL-6 and IL-8 cytokines in HaCaT cells showed that, TNF-α induced in higher amount in monocrotophos treated HaCaT cells (fig. 7). There is a significant increase in the expression of TNF-α, IL-6 and IL-8 compared with the control.

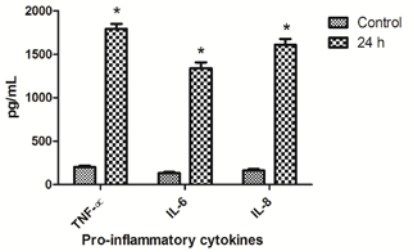

Fig. 7: Expression of pro-inflammatory cytokines. Data expressed as mean ± SD. *Denotes a significant difference from the control

DISCUSSION

In recent years monocrotophos pesticide toxicity have been extensively investigated, but there are few reports on the cytotoxicity of organophosphate pesticides on skin cell lines. In the previous study, cytotoxicity of methyl parathion was measured in FG-9307 cells using the neutral red uptake assay, cell protein assay and tetrazolium assay [7]. The stimulatory effect of environmentally relevant concentrations of pesticides such as chlorpyrifos and resmethrin in inducing tumors was analyzed on breast cancer cell lines, this gives information about the exposure of pesticides results in inducing different tumors [11].

In the present study, the control group showed no significant changes in cell viability or the increase in LDH activity and NO generation. It was observed that in cytotoxicity evaluation compared to 1/5th IC_{50} value treatment (P1), 1/10th IC_{50} value (P2) resulted in increased cytotoxicity. LDH is a cytosolic enzyme released in the culture supernatant upon cell death due to damage of cell membrane [12]. Thus in the present study significant increase in NO and LDH release was observed compared to control in both P1 and P2 treated groups. Use of pesticides in an uncontrolled manner leads to ecological imbalance and it affects the public health [13]. MDA involves in the molecular mechanism to scavenge the free radicals generated by pesticides [14]. Monocrotophos treated HaCaT cells resulted in increased MDA levels compared to control, it is because HaCaT cells generate MDA to overcome the oxidative damage induced by pesticide.

Apoptotic pathways such as mitochondrial dependent and independent pathways are triggered in normal cell line due to ROS generation [15]. In the present study, monocrotophos induced changes in ROS generation in the course of apoptosis induction in HaCaT cells were determined. DAPI binds to the minor groove of A-T rich sequences of double-stranded DNA; the amount of DAPI bound to cell depends upon the degree of chromatin condensation. Reactive oxygen species involved in the toxicity of various pesticides. ROS serves as common mediators of programmed cell death in response to pathological conditions and toxicants like pesticides [16]. Due to exposure of toxicants, ROS (ions or very small molecules) increase dramatically results in damage to cell structures called as oxidative stress. In the present investigation compared to control the significant increase in ROS level was observed in the monocrotophos treated groups. Pro-inflammatory cytokines promotes systemic inflammation, increased expression of cytokines are due to exposure of pathogenic substances. In this study, we used quantitative ELISA to compare the profile of cytokine expression such as IL-6, TNF-α and IL-8 in the monocrotophos treated HaCaT cells. IL-6 uses gp130 known as IL-6 signal transducer in their signalling complexes, it not only involved in promoting inflammation but also act as anti-inflammatory myokine. This phenomenon was reported in earlier studies, IL-6 inhibits the lipopolysaccharide induced TNF-α in cultured human monocytes [17-18]. In the present investigation induction of IL-6 cytokine expression is less compared to TNF-α and IL-8. TNF-α involved in signalling events within the cells, it is an inflammatory cytokine causes necrosis or apoptosis [19]. TNF-α act as complex network of cytokines, it is induced due to a variety of stimuli, including lipopolysaccharide, cytokines, oxygen free-radical mechanisms, etc [20]. Monocrotophos involved in the induction of IL-8 due to increased oxidative stress.

CONCLUSION

The use of monocrotophos is banned in several developed countries but still it's being used in many developing countries. The pesticide at environmental concentrations induced toxic effects on different life forms. In the present investigation monocrotophos induced cytotoxicity, generation of NO, LDH, MDA levels were briefly analyzed. Monocrotophos induced apoptosis in HaCaT cells and the reactive oxygen species generations at the time of programmed cell death in HaCaT cells were ephemerally studied.

CONFLICT OF INTERESTS

Declared None.

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