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In-vitro assessment of carbaryl-induced immunotoxicity in chicken lymphocytes

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ABSTRACT

In present study, an insecticide, carbaryl was evaluated for *in-vitro* assessment of immunotoxicity in chicken lymphocytes. The splenic lymphocytes were collected and treated with NOEL/10^2, NOEL/10^3, NOEL/10^4, NOEL/10^5, NOEL/10^6 and NOEL/10^7 dose of carbaryl for 30, 60 and 90 min and a group of cells were kept control. The Concanvalin A (Con-A) and lipopolysaccharide (LPS) stimulated proliferation of T and B lymphocytes were assessed using MTT dye method. The proliferation of T- and B-lymphocytes responses to Con-A and LPS were markedly reduced in treated group in comparison to control indicating the down regulation of not only the number of the variable lymphocytes but also their blastogenic activity required to mount immune response in chicken. It can be concluded from the present study that the carbaryl, even in very low doses can reduce the proliferation of chicken lymphocytes leading to state of immunosuppression.

Key words: Carbaryl, Chicken lymphocytes, Immunotoxicity

Carbaryl ((1-Naphthol N-methyl carbamate; CAS registry number is 63–25–2 (Carbaryl Health Advisory, Draft Report 1987)) controls over hundreds of species of insects on fruits, cotton, forest, lawns, nuts, trees, crops, livestock and poultry. It is also used as topical molluscicide and an acaricide (Nollan and Robertson 1988). The use of agrochemicals is gaining importance in toxicity evaluation, though very low level dietary intake through food residues may decrease the disease resistance and cause breakdown to vaccination (Rodgers 1996). Due to wide application humans, animals and birds may be exposed to carbaryl residues through food, water and other routes. It can be absorbed through the skin (Graines 1969). Singh *et al*. (2007) reported *in-vivo* immunosuppressive effect of carbaryl. Considering the immunosuppressive effects of the carbaryl, it was planned to study its *in-vitro* immunotoxicity in chicken lymphocytes.

MATERIALS AND METHODS

Cell culture: White leghorn broiler chickens were kept under standard husbandry conditions at Poultry Research Centre of the University. Chicken lymphocytes were separated from the spleen of birds and collected under aseptic conditions. Small pieces of spleen were cut and suspended in media for separation of lymphocytes. The cells were counted in filtrate using trypan blue (0.5%) dye exclusion test. Finally, the lymphocytes were adjusted to 10^7cells/ml in media.

Media: RPMI-1640 supplemented with 20 mM N-[2–hydroxy-ethyl]piperazine-N2 -[2-ethanesulfonic acid] (HEPES) buffer and 5% fetal calf serum was used to culture the avian lymphocytes and for preparation of various dilutions of cabaryl. One vial of RPMI-1640 with hepes buffer was dissolved in 1 litre triple glass distilled water filtered through 0.22 µ membrane filter and stored in 100 ml aliquots at 4°C for further use. Streptomycin (100 µg) and penicillin (1000 IU) were added to check the bacterial contamination and nystatin (2000 IU) was added to check the contamination of fungus in 100 ml of culture medium.

Carbaryl: Non observable effect level (NOEL) dose of this insecticide is 20 ppm. In this study, different dilutions of carbaryl, i.e. NOEL/10^2, NOEL/10^3, NOEL/10^4, NOEL/10^5, NOEL/10^6 and NOEL/10^7 were used. *In vitro* cultures of chicken lymphocytes were treated with each dilution for different intervals, i.e. 0, 30, 60 and 90 min followed by washing and final suspension in media.

Lymphocyte proliferation assay: Lymphocyte proliferation assay was carried out to evaluate the induction of *in-vitro* lymphocyte proliferation by mitogens as per Rai-

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el-Balhaa *et al*. (1987) with slight modifications (Chauhan 1998) using RPMI-1640 as test medium and lipopolysaccharide (LPS) and concanvalin (Con-A) as a mitogen for T and B-lymphocytes. To assess lymphoproliferative response, 1.0×10^7 viable cells per well were cultured in 96-well micro titre plates. Triplicate wells of culture were prepared. Cells were incubated with concentration of con-A (10µg/ml) and LPS from *E.coli* (O26:B6; 10µg/ml) in 5% carbon dioxide (CO_2) for 68 h at 40°C. Equal volume of media was added to control wells.

After 68.0 h, 50µl of MTT dye [3-(4, 5–dimethyl-thiazaol-2-yl)-2, 5-diphenyl tetrazolium bromide] was added in each well and then incubated again for 4 h. MTT solution was dissolved in RPMI-1640 medium @ 4 mg/ml. It was passed through 0.45µ filter to remove any amount of insoluble residue. After 4.0 h, the liquid content of the wells were discarded by careful inverting and blotting the plates. Acid isopropanal (0.04N HCl, 100µl) was added in each well and mixed thoroughly by pipetting to solibulize the farmazan crystals to produce a solution of suitable for measurement of absorbance. The results were reported as optical density (OD) measured at wavelength of 570 nm.

RESULTS AND DISCUSSION

The mean delta OD of *in vitro* effects of different doses of carbaryl exposure for different time interval on T-cell blastogenesis along with normal chicken lymphocytes are presented in Table 1 and Fig. 1. Mean delta OD for control group (no exposure to pesticide) for 30, 60 and 90 min decreased in the lymphoid cells exposed to NOEL$\times10^{-2}$ dose of carbaryl for 30, 60 and 90 min, respectively. There was significant decrease in lymphocytic proliferation up to NOEL$\times10^{-5}$ dilution in comparison to control untreated cells. The percentage decrease in proliferation of T-cells was highest at NOEL$\times10^{-2}$ dilution for 90 min. (82.40%) and lowest at 10^{-5} for 60 min (14.545%).

The proliferation assay of lymphocytes to Con-A and LPS mitogens was suggested as a measurement of lymphocyte proliferation capacity (Toivanen and Taivanen 1983, Lee 1977). Mitogens stimulate the proliferation of lymphocytes independent to their antigen specificity. This proliferation response is considered to reflect clonal expansion of that follows antigen sensitization *in-vivo*. Concanavalin (Con-A) and lipopolysaccharide (LPS) stimulate T and B-lymphocytes respectively. Lymphocyte proliferation assay using MTT dye is rapid calorimetric assay and has number advantages over the conventional methods. This test is based on capacity of mitochondrial enzymes succinate dehydrogenase to transfer the tetrazolium salt of MTT into formazon, a blue color product which can be measured spectrophotometrically (Mossman 1983, Bounous *et al*. 1992). The lymphocytes exposed to carbaryl showed marked decrease in delta-OD after stimulation with mitogen Con-A and LPS indicating lowered T and B-lymphocyte blastogenesis. The significant depression in proliferation of T-lymphocytes including T-helper cells indicated lowered mounting of both cells mediated and humoral immune response. *In-vitro* carbaryl impaired the immune function of splenocytes (Rodgers 1996) and inhibited proliferation of IL-2 dependent T-cells (Baveri *et al.* 1989).

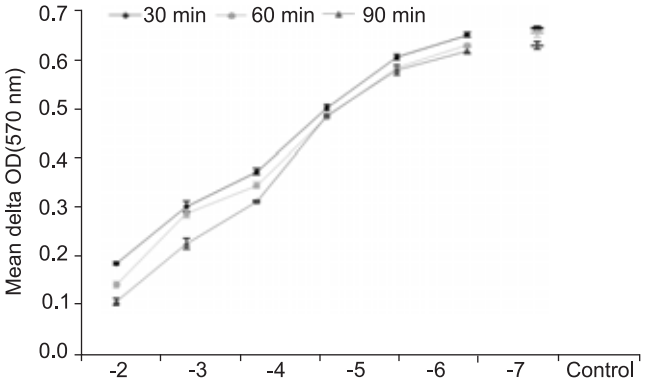

Fig. 1. *In vitro* effects of carbaryl on T-cell blastogenesis

Table 1. *In vitro* effects of carbaryl on T-cell blastogenesis of chicken splenic lymphocytes (mean delta OD±SE)

Min	Control	Nx10^{-2}	Nx10^{-3}	Nx10^{-4}	Nx10^{-5}	Nx10^{-6}	Nx10^{-7}
30	0.642±0.004	0.182±0.003*	0.294±0.010*	0.362±0.006*	0.486±0.006*	0.585±0.005	0.629±0.004
60	0.632±0.007	0.140±0.006*	0.279±0.007*	0.335±0.006*	0.467±0.004*	0.563±0.003	0.608±0.003
90	0.608±0.007	0.107±0.007*	0.221±0.010*	0.304±0.002*	0.471±0.002*	0.510±0.010	0.598±0.007

*Significant difference (P≤ 0.05) between rows (control and treatment).

Table 2. *In vitro* effects of carbaryl on B-cell blastogenesis of chicken splenic lymphocytes (mean delta OD±SE)

Min	Control	Nx10^{-2}	Nx10^{-3}	Nx10^{-4}	Nx10^{-5}	Nx10^{-6}	Nx10^{-7}
30	0.695±0.002	0.175±0.006*	0.287±0.004*	0.362±0.002*	0.474±0.005*	0.572±0.006	0.673±0.010
60	0.672±0.002	0.154±0.008*	0.270±0.007*	0.349±0.006*	0.452±0.002*	0.541±0.010	0.664±0.005
90	0.659±0.003	0.126±0.008*	0.218±0.006*	0.331±0.010*	0.431±0.002*	0.561±0.003	0.665±0.006

*Significant difference (P≤ 0.05) between rows (control and treatment)

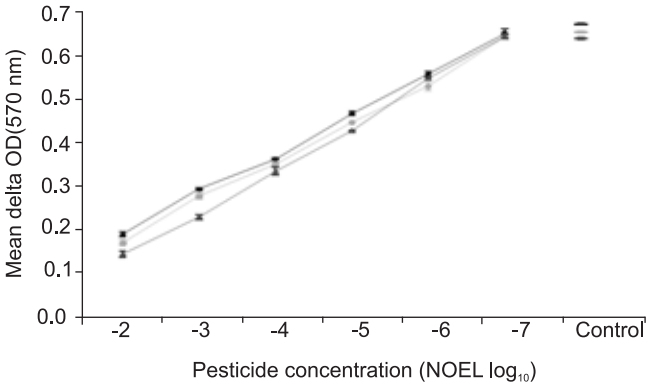

Fig. 2. *In vitro* effects of carbaryl on B-cell blastogenesis

In B-lymphocyte proliferation, the mean delta OD of *in-vitro* effects of different doses of carbaryl exposure for different time intervals are presented in Table 2 and Fig. 2. There was significant decrease in lymphocytic proliferation up to NOEL×10^{-5} dilution in comparison to control untreated cells. The percentage decrease in proliferation of B-lymphocytes was highest at NOEL×10^{-2} dilution for 90 min (80.88%) and lowest at 10^{-5} for 30 min (31.798%).

There was significant reduction in blastogenic activity of cabaryl exposed B-lymphocytes, indicating lowered capacity of B-lymphocytes to form clones and convert into plasma cells. Plasma cells are responsible for synthesis of immunoglobulins and thus it was indicated that B-lymphocytes were less responsive to antigen. Carbaryl inhibited humoral response in chicken at NOEL dose (Singh *et al*. 2007), mice (Wiltrout *et al*. 1978, Ladies *et al.* 1994) and lowered resistance in quails (Fournier *et al.* 1988). Carbofuron suppresses both humoral (Khurana *et al.* 1998) and cellular immunity (Street and Sharma 1975). Another carbamate pesticide, previcur was studied for its immunotoxicological properties in mouse and suppressed both humeral and cell mediated immune response (Elasabbagh and El-Tawil 2001). Carbendazim also down-regulates the humoral immunity in chicken (Singhal *et al*. 2003). Chronic exposure to aldicarb or ethyl carbamate lowered humoral immune response in mice (Olson *et al*. 1987, Hajouiv *et al*. 1992, Cha *et al*. 2000). Decrease in functional cells responsible for reduced capacity to mount immunity through cell mediated or humoral immune mechanism thereby leads to immunosuppression.

On the basis of above findings, it can be concluded from the present study that carbaryl even at every minute concentrations and short exposure time reduces the proliferation in chicken lymphocytes leading to suppression of both cell mediated and humoral immune response. As a result the immune competence of birds is altered leading to increased susceptibility to various infections and increased veterinary costs resulting in loss in poultry industry.

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REFERENCES

Bavari S, Duszynski C and Casale G P. 1989. Effects of carbaryl and its metabolites alpha-naphthol, alpha-naphthyl-betaglucornide and alpha naphthyl sulfate on cell cycle traverse by CTLL-cells, *Toxicologist* **9**: 203.

Bounous OI, Campernoh RP and Brown J. 1992. Comparision of MTT colorimetric assay and tritated thymidine uptake by lymphocyte proliferation assay using chicken splenocyte. *Avian Diseases* **36**: 1022.

Carbaryl Health Advisory: Draft Report 1987. (U.S. Environmental protection Agency (USEPA), Office of Drinking Water), August 1987.

Cha S W, Lee M H, Kim K H, Kim E J, Han S S and Leong T C. 2000. Role of corticosterone in ethyl carbamate induced suppression of antibody response to sheep red blood cells in female BALB/C mice, *Toxicologist* **54**: 161.

Chauhan R S. 1998. *Laboratory Manual of Immunopathology.* G.B. Pant University of Agriculture & Technology. Pantnagar. Pp96.

Elsabbagh H S and EI-Tadwil O S. 2001. Immunotoxicity of cupravit and previcur fungicides in mice. *Pharmacological Research* **43**: 71.

Fournier M, Chevalier G, Nadeau D, Trotter B and Krzystynial K. 1988. Virus pesticide interactions with murine cellular immunity after sublethal exposure to dieldrin and aminocarb. *Journal of Toxicology and Environmental Health.* **1**: 103.

Graines T B. 1969. Acute toxicity of pesticide. *Toxicology and Applied Pharmacology* **14**: 515.

Hajouiv O, Flipo D, Mansou S, Fournier M and Krzystyniak K. 1992. Immunotoxicity of subchronic versus chronic exposure to aldicarb in mice, *International Journal of Immuno-pharmacology* **14**: 1203.

Khurana R, Mahipal S K and Chauhan R S. 1997. Humoral immune response in carbafuron fed sheep. *Indian Journal of Toxicology* **4**: 78.

Ladies G S, Smith C, Heaps K and Loveless S E. 1994. Evaluation of the humoral immune response of CD rats following a 2 week exposure to the pesticide carbaryl by the oral, dermal and inhalation routes. *Journal of Toxicology and Environmental Health* **42**: 143.

Lee L F. 1977. Chicken lymphocyte stimulation by mitogen: a microassay with whole blood culture. *Avian Diseases* **22**: 296.

Mosmann T. 1983. Rapid calorimetric assay for cellular growth and survival, application to proliferation and cytotoxicity assay. *Journal of Immunological Methods* **65**: 55.

Nollan M P and Robertson E L. 1988. External parasite control. *Veterinary Pharmacology and Therapeutics.* 6th edn. Pp. 969. (Eds) Booth N H and Mc Donald L E. Iowa state university Press, Ames.

Olson O, Erickson B and Hins dale R. 1987. Aldicarb immunomodulation in mice, An inverse dose-response to parts per billion levels in drinking water. *Archives of Environmental Contamination and Toxicology* **16**: 322.

Rai-el-Balhaa G, Pellerin J L, Bodin G and Muller M. 1987.

Importance of the hour sampling in the lymphoblastic transformation assay of sheep peripheral blood lymphocytes. *Veterinary Immunology and Immunopathol*ogy **16**: 67–76.

Rodgers K. 1996. Immunotoxicity of pesticides. *Experimental Immunotoxicology* pp. 245. (Eds) Smialowicz R J and Holsapple M P. CRC Press, New York.

Singh B P, Singhal L K and Chauhan R S. 2007. Immunotoxicity of carbaryl in chicken. *Indian Journal of Experimental Biology* **45**: 890–95.

Singhal L K, Bagga S, Kumar R and Chauhan R S. 2003. Down regulation of humoral immunity in chicken due to carbendazim, *Toxicology in-vitro* **17**: 687.

Steel J C and Sharma R P. 1975. Alteration in induced celluliar and humoral responses by pesticides and chemicals of environmental concern: quantitative studies of immuno-supression by DDT, arochlor 1254, carbaryl, carbofuron and methyl parathion. *Toxicology and Applied Pharmacology* **32**: 587.

Toivanen P and Toivanen A. 1983. Selective activation of chicken T-lymphocyte by concanavalin-A. *Journal of Immunology* **111**: 1602.

Wiltrout R W, Ercegovich C D and Ceglowski W S. 1978. Humoral immunity in mice following oral administration of selected pesticides. *Bulletin of Environmental Contamination and Toxicology* **20**: 423.