Evaluation of the in vitro immunotoxic effect of bromadiolone rodenticide in poultry

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In the present study, rodenticide bromadiolone was evaluated for in vitro immunotoxicity in poultry. The lymphocytes were separated from spleen and treated with NOEL/10^2, NOEL/10^3, NOEL/10^4, NOEL/10^5, NOEL/10^6 and NOEL/10^7 (No observable effect level) dose of rodenticide for 30, 60 and 90 min and a group of cells were kept untreated (control). The cells were collected for detection of T and B-lymphocyte proliferation. Concanavalin-A (Con-A) and lipopolysaccharide (LPS) stimulated blastogenesis of T and B-lymphocytes was assessed by MTT dye method. The proliferation of both lymphocytes was 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 an immune response in birds. It can be concluded from the present study that the bromadiolone rodenticide, even in very low doses can reduce proliferation of poultry lymphocytes leading to a state of immunosuppression.

Keywords: Bromadiolone, Immunotoxicity, Poultry

Pesticides are used all over the world in different branches of agriculture, industry, healthcare and animal husbandry. Their wide application and inadequate information about the means of their use and their toxic effects pose a real hazard to the health of both animals and humans. To control the harmful rodents (mice, rats, field mice etc.), it requires the precise and adequate use of chemical means for restriction of their population containing active substances with various compositions and the anticoagulant effect is the most frequent of such means. Anticoagulant rodenticides are probably the most commonly used of such means around the world. It has been estimated that approximately 95% of all rodenticides used are anticoagulant. Anticoagulant rodenticides are classified into two principal groups: derivatives of coumarin and indanedione. Bromadiolone is a hydroxyl-coumarin with the full chemical name: 4-hydroxy-3-coumarinyl-3-phenyl-4-bromo-4-biphenyl-1-propanol-1. The preparations from the coumarin group are more extensively used and provoke severe injury to vascular permeability, resulting in massive haemorrhages and the rapid death of rodents.

The indirect effect on non-target animal species consequent on inadvertent environmental pollution has become the major concern of the scientists. The immune system is considered to be a more sensitive indicator for toxicity assay especially for environmental pollutants which may have residual effects in the ecosystem. The continuous use of pesticides even in normal recommended doses may cause deleterious effects on the physiological functions that may range from lowered immunity of animals to reduced production performance. The use of agrochemicals is gaining significance in toxicity evaluation, as very low-level dietary intake through food residues may decrease the disease resistance and cause breakdown to vaccination. Considering the immunosuppressive effects of the bromadiolone, it was planned to evaluate the in vitro effect on T and B-cells in poultry lymphocytes.

Materials and Methods

Cell Culture

White leghorn broiler chickens were kept under standard husbandry conditions at Poultry Research Centre, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India. 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 infiltrate using trypan blue (0.5%) dye exclusion test. Finally, the lymphocytes were adjusted to 10^7 cells/mL in the media.

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Media

RPMI-1640 supplemented with 20 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES) buffer and 5% fetal calf serum (Sigma) was used to culture the avian lymphocytes and for preparation of various dilutions of bromadiolone. One vial of RPMI-1640 with HEPES buffer was dissolved in 1.0 L triple glass distilled water filtered through 0.22 µm 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.

Rodenticide

Nonobservable effect level (NOEL) dose is the dose at which we do not observe any clinical change in the individuals after exposure but, long term exposure at NOEL dose leads to immunosuppression. The NOEL dose of the bromadiolone is 3.0 ppm for in vivo conditions. The poultry lymphocytes treated with NOEL and NOEL/10 dose under in vitro condition did not show any growth so lower dilutions of bromadiolone i.e., “No Observable Effect Level” (NOEL)/10², NOEL/10³, NOEL/10⁴, NOEL/10⁵, NOEL/10⁶, and NOEL/10⁷ were used. The vehicle used to dissolve bromadiolone was RPMI-1640 media. In-vitro cultures of poultry 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. The cells of the control group were treated with RPMI-1640 media only.

Lymphocyte proliferation assay

Lymphocyte proliferation assay was carried out to evaluate the induction of in vitro lymphocyte proliferation by mitogens with slight modifications using RPMI-1640 as test medium and lipopolysaccharide (LPS) and concanavalin-A (Con-A) as a mitogen for T and B-lymphocytes. To assess lymphoproliferative response, 1.0 × 10⁷ viable cells per well were cultured in 96-well microtiter plates (Corning M/S Sigma, USA). Triplicate wells of culture were prepared. Cells were incubated with concentration of Con-A (Sigma, 10 µg/mL) and LPS from E. coli (O26:B6; Sigma, 10 µg/mL) in 5% carbon dioxide (CO₂) for 68 h at 40°C. An equal volume of media was added to triplicate control wells.

Results

The mean delta OD of in vitro effects of different doses of bromadiolone exposure for different time interval on T-cell blastogenesis along with normal poultry lymphocytes is presented in Table 1 and Fig. 1A. Mean delta OD for the control group

<table>
<thead>
<tr>
<th>Min</th>
<th>Control</th>
<th>N × 10⁻²</th>
<th>N × 10⁻³</th>
<th>N × 10⁻⁴</th>
<th>N × 10⁻⁵</th>
<th>N × 10⁻⁶</th>
<th>N × 10⁻⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.590±0.011</td>
<td>0.101±0.010*</td>
<td>0.250±0.010*</td>
<td>0.340±0.004*</td>
<td>0.390±0.005*</td>
<td>0.440±0.005*</td>
<td>0.520±0.002</td>
</tr>
<tr>
<td>60</td>
<td>0.550±0.010</td>
<td>0.130±0.003*</td>
<td>0.260±0.005*</td>
<td>0.341±0.006*</td>
<td>0.410±0.002*</td>
<td>0.470±0.010*</td>
<td>0.560±0.002</td>
</tr>
<tr>
<td>90</td>
<td>0.520±0.010</td>
<td>0.140±0.003*</td>
<td>0.280±0.010*</td>
<td>0.370±0.006*</td>
<td>0.419±0.003*</td>
<td>0.498±0.012</td>
<td>0.592±0.006</td>
</tr>
</tbody>
</table>

*Significant difference (P≤0.05) between rows (control and treatment)
(no exposure to rodenticide) for 30, 60 and 90 min decreased in the lymphocytic cells exposed to a NOEL × 10^2 dose of rodenticide for 30, 60 and 90 min, respectively. There was a significant decrease in lymphocytic proliferation up to NOEL × 10^-6 dilution for 60 min exposure in comparison to control untreated cells. The percentage decrease in proliferation of T-cells was highest at NOEL × 10^-2 dilution for 30 min (82.88%) and lowest at 10^-6 for 60 min (14.545%).

In B-lymphocyte proliferation, the mean deltas OD of \textit{in vitro} effects of different doses of bromadiolone exposure for different time intervals are presented in Table 2 and Fig. 1B. There was a 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 (79.104%) and lowest at 10^-5 for 60 min (25.484%).

### Discussion

The mean delta OD of \textit{in vitro} effects of bromadiolone at different dilutions and exposure time on T-cell blastogenesis was a significant decrease in cells up to NOEL × 10^-6 dilution for 60 min exposure in comparison to control group. The proliferation assay of lymphocytes to Con-A and LPS mitogens have been suggested as a measurement of lymphocyte proliferation capacity. Mitogens stimulate the proliferation of lymphocytes independent to their antigen specificity. This proliferation response is considered to reflect the clonal expansion of that follows antigen sensitization \textit{in vivo}. Concanavalin-A (Con-A) and Lipopolysaccharide (LPS) stimulate T and B lymphocytes respectively. Lymphocyte proliferation assay using MTT dye is rapid calorimetric assay and has a number of advantages over the conventional methods. This test is based on capacity of a mitochondrial enzymes succinate dehydrogenase to transfer the tetrazolium salt of MTT into formazan, a blue color product which can be measured spectrophotometrically. The lymphocytes exposed to bromadiolone rodenticide showed marked decrease in delta OD after stimulation with mitogen Con-A and LPS which indicated lowered T and B-lymphocyte proliferation. The significant depression in the proliferation of T-lymphocytes including T-helper cells indicated lowered mounting of both cell-mediated and humoral immune response.

T lymphocytes are the precursor of cell mediated immune response. Previous investigations demonstrated that subchronic epicutaneous application of bromadiolone anticoagulant to rat skin resulted in lymphopenia and reduced proliferative response of splenocytes to lectin stimulation. These studies corroborated \textit{in vitro} data on warfarin immunotoxicity as judged by inhibition of human T lymphocyte proliferation by warfarin and by its additive effect on other immunosuppressive substances in T-cell proliferation. Depression of cell-mediated immunity in mice on exposure to chlordane (chlorinated hydrocarbon pesticide) has been found. The reduction in proliferation observed in response to stimulation with Con-A and PHA in lymphocytes of the spleen due to the effect of insecticides on T-helper subpopulations in spleen. Insecticide, carbaryl lowers the cell-mediated immune response by decrease in the lymphocyte proliferation response to the mitogen in chicken at NOEL dose. This carbaryl insecticide even at very minute concentrations and short exposure time under \textit{in vitro} conditions reduces the proliferation of chickenT-lymphocytes. Lindane and quinalphos insecticides decrease the proliferation of T-lymphocytes under \textit{in vitro} conditions in chicken lymphocytes. Carbofuran has been found to suppress the generation of T-cell mediated cytolytic response in murine splenocytes.

The mean delta OD of \textit{in vitro} effects of different doses of bromadiolone rodenticide exposure for different time intervals on B-cell blastogenesis was significantly reduced. The proliferative activity of bromadiolone rodenticide exposed B-lymphocytes indicated the lowered capacity of B-lymphocytes to form clones and convert into plasma cells. Plasma
cells are responsible for the synthesis of immunoglobulins and thus it was indicated that B-lymphocytes were less responsive to antigen. B-lymphocytes are the precursors of humoral immunity. The significant decrease in the number of plaque forming cells in cypermethrin treated group and the overall diameter of plaques was also reduced\textsuperscript{16}. In inbred mice, organophosphate induced immunosuppression. Subsequently, the number of Ig M plaque forming cells was reduced by 65% in mice given parathion 2 days after immunization with sheep red blood cells and it was further observed that the reduction of haemagglutination titre coincided with reduction of the number of splenic plaque forming cells\textsuperscript{17}. Reduction in the number of hemolytic plaque means a decrease in the number of Ig M producing cells including B cells. The antibody producing B cells have well-developed rough endoplasmic reticulum where the immunoglobulins are synthesized. DDT induces disorganization of endoplasmic reticulum and reduction in the number of zymogen granules of exocrine pancreas and vesiculation of rough endoplasmic reticulum of hepatocytes in chickens\textsuperscript{18}. The antibody titre of fenvalerate intoxicated strain-19, tissue culture rinderpest vaccine and alum precipitated \textit{P. asteurella} multocida vaccine was highly reduced.

Carbaryl has been shown to inhibit humoral response at NOEL dose in chicken, mice and lowered resistance in quails\textsuperscript{19}. This carbaryl even at very minute concentrations and short exposure time under \textit{in vitro} conditions reduce the proliferation of chicken B-lymphocytes\textsuperscript{14}. Lindane and quinalphos insecticides also downregulates the humoral immunity in chicken and decrease the proliferation of B-lymphocytes under \textit{in vitro} conditions\textsuperscript{15}. The decrease in functional cells responsible for reduced capacity to mount immunity through cell-mediated or humoral immune mechanism thereby leads to immunosuppression. From the present study, it can be concluded that bromadiolone rodenticide reduces proliferation in poultry lymphocytes even at very minute concentration and short exposure time leading to suppression of both cell-mediated and humoral immune response. Decreased proliferation of T and B-lymphocytes results altered immune competence of birds leading to increased susceptibility to various infections and increased medicine costs resulting in a loss in poultry rearing. The lymphocytic proliferation in poultry can also be used as a marker of pesticide exposure in animals and birds.

\section*{Acknowledgment}

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\section*{References}


