IN VITRO ASSESSMENT OF APOPTOSIS IN AVIAN LYMPHOCYTES TREATED WITH FENVELERATE *VIS-À-VIS* IMMUNOSUPPRESSION IN POULTRY

Deena Khan, Lokesh K Singhal*, RK Banga* and RS Chauhan*

*College of Veterinary and Animal Sciences, GB Pant University of Agriculture and Technology, Pantnagar–263 145, India; *Centre of Advance Disease Research and Diagnosis, Indian Veterinary Research Institute, Izatnagar-243 122, India.*

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Introduction

Fenvelerate, a synthetic pyrethroid insecticide is used against a wide variety of insect pests in various agricultural, animal husbandry and public health operations. Indiscriminate and disproportionate use of pesticides has lead to their residues resulting in immunological disorders on man and animal. These days, a major concern of most of the researchers is to find alternative methods of animal experimentation. *In vitro* cellular models can be useful for immunopathological research and have predictive potential in the assessment of toxicity and consequently, safety evaluation. The main advantage of using *in vitro* cell culture system is that experimental conditions can be strictly controlled without causing any kind of pain to animals and ease for analysis of pathogenic mechanisms of action at cellular level. With the aim of replacing animal models with that of cell culture system in immunopathological studies, it was proposed to carry out the pesticide induced immunopathological studies in lymphocyte cell cultures.

Material and methods

Isolation of Lymphocytes: Spleen were collected from healthy adult birds under the sterilize conditions at the time of their slaughter. Spleen were minced in RPMI-1640 media and filtered through sterile sieve to remove the coarse material. The filtrate thus obtained was used as lymphocyte suspension for the study. The lymphocytes were washed twice with RPMI-1640 media supplemented with 10% FCS. The live cells were counted using trypan blue dye exclusion method and a final concentration of 10^7 cells per ml was prepared for further studies.

Pesticide Exposure: The cells were given treatment for 60 minute using $Nx10^{-2}$, $Nx10^{-3}$, $Nx10^{-4}$ (N=NOEL: No Observable Effect Level) dilutions of fenvelerate. NOEL dose of fenvelerate used was 20ppm.

Electron Microscopy: The electron microscopy was performed on treated and control avian lymphocytes at AIIMS, New Delhi according to the method described by Malorni *et al*. (1998) with slight modifications by Chauhan (2003).

Gel Electrophoresis of DNA: Apoptotic DNA was extracted from cells as described by Herrmann *et al*. (1994) method with some modifications was followed to extract apoptotic DNA from the cells. The extracted DNA was pelleted, dried and dissolved in Tris-EDTA buffer for analysis in 1.5% agarose gel electrophoresis.

Detection of apoptosis through immunoperoxidase technique: The apoptotic cells were detected by using apoptotic cell separation kit (Sigma) with slight modifications (Chauhan, 1998).

Result and discussion

1) Immunoperoxidase Staining: In the present experiment it was found that *in vitro* exposure of cells to the pesticide causes induction of apoptosis as evident by the demonstration of phosphatidylserine using annexin V-biotin conjugate and immunoperoxidase staining (Fig. 1). The number of mean percent apoptosis positive cells, after a treatment of the cells with $Nx10^{-2}$, $Nx10^{-3}$ and $Nx10^{-4}$ dilutions of fenvelerate for 60 minute of exposure, were 13.57±1.7, 11.86±1.5 and 5.607±1.5, respectively. However, in untreated control cells, phosphatidylserine could not be demonstrated using immunoperoxidase technique.

2) Electron microscopy: Both the transmission and scanning electron microscopy revealed the typical morphological characteristics of apoptosis in the pesticide treated cells. Scanning electron microscopic studies revealed the shrinking of the treated cells. Surface of the cells was smoothened due to loss of the microvillus structures. Blebbing was seen as the formation of pseudopodia and shedding of the apoptotic bodies. The apoptotic bodies were shed off which were seen phagocytized by the macrophages. Transmission electron microscopic studies conducted on the pesticide treated cells also showed the characteristic signs of apoptosis. It revealed that the cells were shrunken and became denser. Their chromatin became pyknotic and was packed into smooth masses applied against the nuclear membrane indicating the margination of chromatin. Karyorrhexis (breaking of nucleus) was observed in many cells. Cells started giving off the processes which were bud shaped and they were having tendency to break off from the cell and give rise to the apoptotic bodies (Fig. 2).

3) Electrophoresis of apoptotic DNA: Exposure of isolated avian lymphocytes to

NOEL/10^2, NOEL/10^3, NOEL/10^4 doses of fenvelerate for 60 min resulted in DNA fragmentation into multiples of approximately 200 bp of nucleotides which was detected by gel electrophoresis and compared with DNA markers. It was observed that fenvelerate causes fragmentation of DNA as the diffusion of smaller fragments leading to smearing and number of bands observed in treated group over control. The degree of fragmentation varied with pesticide's concentration. DNA isolated from untreated control cells did not reveal any fragmentation and bands on electrophoresis.

Most of the earlier work on fenvelerate induced immunotoxicity has been done *in vivo.* Significant suppression of macrophage function as indicated by reduction in number of Nitroblue Tetrazolium (NBT) dye reduction test positive cells and bactericidal phagocytosis in insecticides treated animals as compared to control was reported (Khurana *et al*., 1997). The total serum protein, globulin decreased significantly while A:G ratio increased in lambs treated with insecticide as compared to control (Khurana *et al*., 1997a). Khurana *et al*., (1999) reported that fenvelerate caused suppression of cell mediated immunity as indicated by reduction of delayed type hypersensitivity reaction in treated group of sheep. Fenvelerate has been found to suppress total leukocyte count, absolute count in lambs (Khurana *et al*., 2000) and birds (Singh *et al*., 2001). Fenvelerate contaminated feed is reported to decrease macrophage function in chicken thus leading to increased susceptibility for infectious diseases and recurrent infections poor response to antibiotics and vaccine failure (Singh *et al*., 2001a). B-lymphocyte (Singh *et al*., 2001b) and T-lymphocyte blastogenesis (Singhal *et al*., 2001) were found to be significantly reduced in fenvelerate treated birds as compared to controls.

Conclusion

From the present study it can be concluded that avian lymphocytes even if exposed to very minute amount of fenvelerate for shorter duration, demonstrate typical changes of apoptosis. Apoptosis leads to the decreased number of functional cells which eventually results in reduced capacity to mount immunity through cell mediated or humoral immune response. The present study also advocates that the cell culture system can be used as a promising replacement for animal experimentation.

References:

1. *Chauhan, R.S. 1998. Laboratory Manual of Immunopathology. G.B.Pant University of Agriculture & Technology. Pantnagar. 96pp.*
2. *Chauhan, R.S. 2003. Veterinary Laboratory Diagnosis. 1st Edition, IBDC, Lucknow. pp. 293-297.*
3. *Herrmann, M.; Lorenz, M.H.; Grunke,M; Woith, W. and Kalden, J.R. 1994. A rapid and simple method for isolation of apoptotic DNA fragments. Nucleic Acid Research, 22: 5506-5507.*

4. *Khurana, R. and Chauhan, R.S. 2000. Immunopatholgical effects of fen on cell mediated Immune response in sheep. J Immumnol Immunopathol, 2: 56-59.*
5. *Khurana, R., Mahipal, S.K. and Chauhan, R.S. 1997. Effect of Insecticides on peritoneal macrophages in sheep. Indian J Vet. Res. 6:32-36.*
6. *Khurana, R., Mahipal, S.K. and Chauhan, R.S. 1997a. Insecticide induced biochemical alterations in sheep. Indian J Vet. Res.8:31-38.*
7. *Khurana, R., Mahipal, S.K. and Chauhan, R.S. 1999Effect of pesticide on Delayed type Hypersensitivity Reaction in sheep. Ind J Anim. Sci 69:880-881.*
8. *Malorni, W.; Fais, S. and Fiorentini. 1998. Morphological aspects of apoptosis. Apoptosis: Laboratory Manual of Experimental Methods (Eds. Andrea Cossarizza and Diana Boraschi). Purdue University, West Hafayette, U.S.A.*
9. *Singh, B.P., Singhal, L.K. and Chauhan, R.S. 2001. Down Regulation of macrophage functions in chicken due to fenvelerate contaminated feed. In: Livestock Community and Environment 10th Int. Conf. AITVM Copenhagen Denmark p-64.*
10. *Singh, B.P., Singhal, L.K. and Chauhan, R.S. 2001a. Fenvelerate induced cell–mediated immunological alterations in chickens. J Immunol Immunopathol, 3: 59-62.*
11. *Singh, B.P., Singhal, L.K. and Chauhan, R.S. 2001b. Effect of fenvelerate induced stress on humoral immunity in chickenc Int Symp Anim Welfare Consd Livestock Has, Syst, Oct. 23-25, Poland.*
12. *Singhal, L.K., Singh, B.P. and Chauhan, R.S.2001. Fenvelerate induced stress and its effect on cell mediated immunity in chickens. Proceeding. Int Symp. End Tech. Sec. CIGR Anim. Welf. Consider. Livestock Hasing Syst. Oct. 23-25, 2001 Poland.*

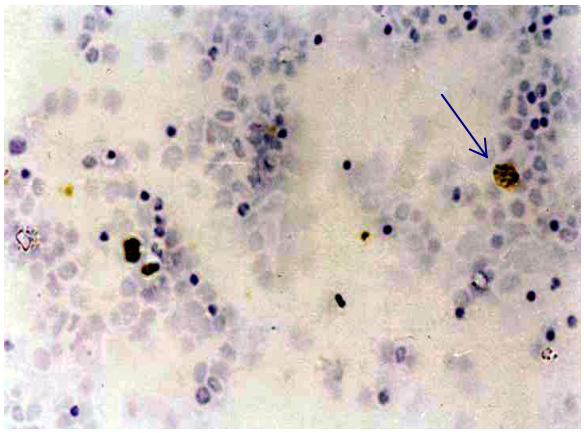

Fig. 1. Photomicrograph of apoptotic avian lymphocytes stained with immunoperoxidase technique- Apoptotic cell showing blebbing (x 400)

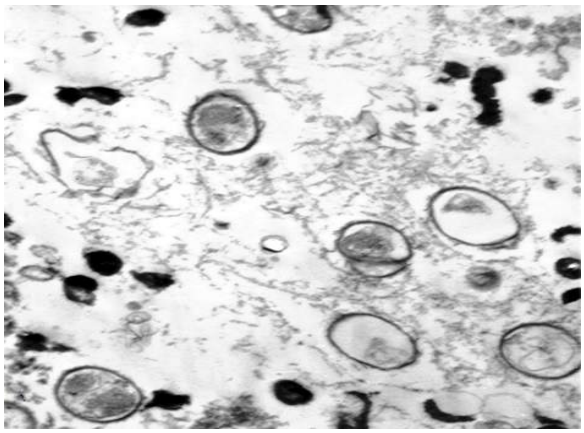

Fig. 2. Transmission electron microphotograph showing formation of apoptotic bodies (x11000)