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Vitamin E pretreatment prevents the immunotoxicity of dithiocarbamate pesticide mancozeb in vitro: A comparative age-related assessment in mice and chick

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ABSTRACT

Pesticides used for crop protection cause life-threatening diseases affecting the immune system of non-target organisms including birds and mammals. Functionality of immune system is age-dependent; early- as well as old-life stages are more susceptible to toxic exposures because of less competent immune system. Vitamins are so far known to reduce toxic effect of several pesticides and/or xenobiotics. The present in vitro study elucidated immunotoxicity of fungicide mancozeb through comparable stages of immune system maturation in mice (1, 3, and 12 months) and chicks (4, 8, and 11 weeks). In vitro splenocytes viability on exposure to mancozeb was quantitatively assessed by MTT assay and qualitatively by acridine orange and ethidium bromide (AO/EB) double fluorescence staining. Mancozeb exposure dose dependently (250, 500, 1000, 2500, 5000 and 10,000 ng/ml) decreased the splenocytes viability. The in vitro preventive effect of Vitamin E has also been explored on toxicity induced by mancozeb. The increased susceptibility observed both in early and aged groups was due to less/decline competence of the immune system.

1. Introduction

Immune system plays a key role in the sustenance of organisms providing protection against infections, diseases and toxicity caused by several xenobiotics. There is age related functional changes in the immune system which attains a peak usually at young adult, followed by gradual decline with increase in age. Adaptive defenses are poorly developed in neonates and juveniles and become fully functional through growing age [1,2]. Mammals with short gestation periods e.g. rats, hamsters and mice have been reported to possess relatively immature immune system at birth compared to humans [3]. The birth in rodents is followed by a critical period of 30 days known as perinatal immunodeficient period in which a mature pattern of immune response has not been achieved. The activation of immune system in mice (lifespan 2–3 yrs) starts at about 1 month (immature immune response), attains a peak at approximately 3 months (peak immune response) which continues till 6 months, and followed by progressive decline in immune response till the life ends [2–4]. Further many differences exist on development as well as organization of the immune system between mammals and birds [5,6]. In chick whose lifespan (approx 6 months) is shorter than rodents, the activation of immune response starts comparatively late i.e. at 1 month (4 weeks), attains a peak at 2 months (8 weeks) and maintained till 9–10 weeks, followed by regression from 11 weeks [7]. Differential rates of development of the immune system in these two vertebrate classes are likely explained by the different costs and processes involved in the ontogeny of system [6]. Accordingly, the immune response may vary depending on the rate/time of development of immune system which creates a marked difference of susceptibility between these two classes.

Research on immunotoxicity is a challenging area and studies have revealed the susceptibility of immune system of vertebrates to xenobiotics including pesticides. The residues and metabolites of pesticides in the field crop are potentially hazardous to non-target organisms including birds and mammals. Pesticides of various groups are reported to affect several parameters of immune system of both the vertebrate classes. Increase in serum antibody production was seen in agricultural workers occupationally exposed to dithiocarbamate fungicide mancozeb (MCZ) [8]. Chronic administration of herbicide paraquat produces immunosuppression of T lymphocytes [9] and the insecticide triphenyltin hydroxide induced lymphopenia and lymphocyte depletion in thymus-dependent areas of spleen and lymph nodes in rats [10]. Immunotoxic effects of neonicotinoid (imidacloprid) [11], paraquat [12], and dinitrophenol (dinocap) [13] have been elucidated through histopathology of spleen and DTH response in mice. Combination of carbamate (carbofuran), organophosphate (malathion) and organochlorine (dieldrin) pesticides have shown immunosuppressive effect in mice by altering the functional activities of peritoneal

Abbreviations: MCZ, mancozeb; Vit E, Vitamin E; OD, optical density; AO/EB, acridine orange/ethidium bromide; ROS, reactive oxygen species.

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macrophages [14]. Studies on immunotoxicity of pesticides in birds are limited to few studies. While acephate [15] and carbendazim [16] decreased the humoral immune response in chicken, avermectin damaged the structure and physiology of spleen in pigeon [17]. Immunotoxic potential of pesticides also has been demonstrated in vitro. Dithiocarbamate MCZ was reported to cause lymphocyte toxicity in mice and human [18–20]. Lymphoproliferative response of mice thymocytes was suppressed on dinocap treatment [13]. Apoptotic effect was seen on mice splenocyte on exposure to triazine (atrazine) [21]. Organophosphate chlorpyrifos and organochlorine endosulfan are reported to cause dose dependent cytotoxicity in lymphocyte culture of poultry [22].

Exposures of pesticides at mild dose level (environmentally realistic exposure) during critical windows of development severely affect the functioning of the immune system. Pre-natal/lactational exposure of chlorpyrifos [23] and organochlorine chlordane [24] caused immunotoxicity in adulthood in mice. Prenatal atrazine exposure caused greater health complications as the mice aged [25]. Thus, studies have shown the vulnerability of the early life stage to pesticide exposure, and there are fewer studies available demonstrating the effect comparatively on various immunological stages.

Presently an attempt has been made to elucidate the immunotoxic potential of MCZ comparatively at different immunological ages of mice. Keeping in view the differential immunological maturity, a parallel study was also carried out in chick to compare the susceptibility with that of mice. The assessment was made through in vitro exposure of pesticide MCZ to splenocytes of various immunological ages. The immunotoxicity of MCZ was shown to be due to oxidative stress effect [26]. The oxidative stress can contribute towards normal process of aging of any organisms causing gradual accumulation of damage to macromolecules by ROS [27]. Thus, the oxidative stress may have its impact on immune system as well. Environmental enrichment including diet in critical stages of immune system development is reported to have positive impact on health. Although age related changes in immune system are not reversible but supplementation of some micronutrients and vitamins are effective in maintaining an efficient immune functioning [28]. Antioxidative vitamins are the most important free radical scavengers in extracellular fluids that protect biomembranes from peroxidative damage [29–31]. In vitro administration of Vitamin E (Vit E) prevents the toxic effect of different chemicals and pesticides including dithiocarbamates [19,30]. We hypothesized that Vit E might exert preventive effect against MCZ induced toxicity. Therefore, the preventive effect of Vit E pretreatment against MCZ induced splenocyte toxicity was assessed.

2. Material and methods

2.1. Animals

2.1.1. Mice

SWISS albino mice, 4–6 weeks old, were purchased from Central Drug Research Institute, Lucknow, India. They were housed in woodchip bedded polypropylene cages with free access to food and water with a 12/12 light–darkness cycles and at ambient temperature of 20–25 °C. Two female and one male mouse were kept together in one cage for breeding to get the mice of required age.

2.1.2. Chicks

One day old male chicks (Cobb 400) were purchased from commercial hatchery (Khushboo poultry, Allahabad, India.) and acclimatized in laboratory condition (25 °C temperature and 60–80% humidity) in a thermostatically-controlled aviary for one week. Half of them were vaccinated by Ranikhet and IBD vaccines (Indovax Pvt. Lmt. Siswala, Hisar, Haryana, India) on 2nd day and 14th day of birth respectively for prevention of diseases and better health management. Chicks were kept in this environment till the desired age is obtained. There was ad libitum supply of poultry feed and water.

The experimental protocols were approved by the Institutional Animal Ethical Committee of the University according to the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

2.2. Experimental design

The studies were conducted at three stages of immunological maturity in mice (male and female): 1, 3 and 12 months as well as chicks (vaccinated and nonvaccinated): 4, 8 and 11 weeks. The three corresponding stages indicated initiation (1 month, 4 weeks), mature pattern of immunofunctioning (3 months, 8 weeks) and regression (12 months, 11 weeks) in mice and chick respectively.

Mancozeb (Indosal M-45, Saral crop science, India) was initially dissolved in DMSO and appropriate final doses were adjusted in complete RPMI 1640 medium (Solvent concentration < 1%). Six different doses of MCZ (250, 500, 1000, 2500, 5000 and 10,000 ng/ml) were used in the study, decided on the basis of reports indicating toxicity of the pesticide on splenocytes and other cell types [18,19]. Vit E (Evion 400 tocopherol acetate IP, Merck Lmt. Goa, India) was prepared in 90% ethanol and added to the medium to make final concentration of 50 μg/ml. The dose selected for Vit E was effective in reducing toxicity induced by dithiocarbamate pesticide, Zineb [30].

2.3. Splenocyte toxicity assay

2.3.1. Isolation of splenocyte

Animals (chick and mice) were anesthetized by using chloroform and spleens were immediately dissected out. Dissected spleens were immediately transferred to chilled Phosphate buffer saline (PBS, pH 7.4) followed by mincing and homogenization. The suspension was mixed with equal volume of NH_4Cl, incubated at 0 °C for 15 min and then centrifuged (at 2000 rpm) for 15 min at 4 °C. After centrifugation the cells were resuspended in PBS (pH 7.4) and placed in a sterile petridish for 1 h at 37 °C to remove the macrophages by adherence. The unadhered cells were decanted and centrifuged (at 2000 rpm) for 15 min at 4 °C. The precipitate was washed twice in incomplete media (RPMI 1640 + Antibiotics). Cell viability (~85%) was checked by trypan blue exclusion test (0.4% trypan blue was mixed with equal amount of cell suspension and incubated for 2–5 min). Counting was done in 1 × 1 mm sq. of Neubauer chamber of Hemocytometer.

2.3.2. Seeding and culture

The cells were resuspended (1.5 to 2×10^7 cells/ml) in a culture medium RPMI 1640. For cytotoxicity assay, 1×10^6 cells were cultured in triplicate in 200 μl of complete media RPMI 1640 supplemented with penicillin (100 UI/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. Cultures were grown in 96-well flat-bottomed microtiter plates and maintained at 37 °C with supply of 5% CO_2 in a humidified atmosphere in CO_2 incubator (Midi 40 Thermo Scientific, USA) for 48 h. Fourteen different groups were maintained. Group I was without any exposure treated as control, Group II was pretreated with Vitamin E, Group III to Group VIII were exposed to different concentrations of MCZ (250, 500, 1000, 2500, 5000 and 10,000 ng/ml) and Group IX to XIV were Vit E pretreated splenocytes exposed to different concentrations of MCZ (250, 500, 1000, 2500, 5000 and 10,000 ng/ml). Treatment with MCZ was done after 24 h of pretreatment with Vit E.

2.4. Analysis of culture

The splenocyte culture was analyzed after 48 h as follows for quantitative and qualitative estimation of viability: