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Research Article

Assessment of oxidative stress induced immunotoxic effect mediated by *in vitro* exposure to methyl parathion in lymphocytes isolated from chicken spleen

Sonu Ambwani, Tanuj Kumar Ambwani, Ramswaroop Singh Chauhan

Abstract

Immunotoxicology examines the adverse effects of xenobiotics- including pesticides, heavy metals from emissions, drugs and other substances- on the body's defense system of humans and animals. The term pesticide encompasses a diverse array of substances, including insecticides, fungicides, herbicides, rodenticides, etc. Pesticides are widely used around the globe; however, there has been an increase in reports highlighting their harmful effects. The broad-spectrum activity of methyl parathion allows elimination a vast range of pests, making it a popular choice for pest control in agriculture. However, its use is increasingly regulated due to its high toxicity to humans, non-target organisms and the environment. Methyl parathion, a widely used organophosphate pesticide, is reported to pose significant health risks through its neurotoxic, immunotoxic and oxidative stress-inducing effects. Methyl parathion primarily exerts toxicity by inhibiting acetylcholinesterase, leading to neural overstimulation. This study investigates the *in vitro* immunotoxic effects of methyl parathion induced due to increased oxidant stress (OS) in mitogen-stimulated avian lymphocytes. Utilizing lymphocyte proliferation and nitric oxide (NO) estimation assays, the findings showed a marked reduction in B and T-cell proliferation following exposure to a thousand-fold dilution of the No Observable Effect Level (NOEL/10^3) dose of methyl parathion. Additionally, oxidative stress, as indicated by NO levels, was significantly elevated in methyl parathion treated cells as compared to control cells. Thus, our results indicate that *in vitro* exposure to methyl parathion caused considerable immunotoxic effects in the exposed lymphocytes that could be linked with enhanced OS.

Keywords chicken lymphocytes, methyl parathion, nitric oxide estimation, oxidative stress

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Introduction

In veterinary medicine, immunotoxicology primarily addresses the impact of prevalent environmental toxicants, such as pesticides. Consequently, veterinary immunotoxicology is often referred to as ecoimmunotoxicology, reflecting its focus on the intersection of ecological toxicants and immune system health. Pesticides, although widely used in agro-sector for eliminating pests for saving crops from their attack, are a

cause for concern because of their detrimental effects on the environment, animal and human health. Organophosphorus insecticides (OPIs) have been the most effective as well as versatile class of pesticides. They have been widely used in both agricultural and household formulations and represent a significant portion of the global insecticide market [1]. Continuous exposure to pesticides, even at low doses, causes oxidative stress (OS) and adversely affects the immune system, which is considered a sensitive indicator of toxicological effects [2-5]. Recently, there has been a growing emphasis on *in vitro* studies examining the immunotoxicity and OS induced by pesticide exposure [4, 6-8]. Low-level exposure to OPIs is known to cause a variety of bio-chemical alterations that could be responsible for unfavorable health impacts on human beings and laboratory animals [9]. Belonging to the OPIs group of pesticides, methyl parathion (O, O-dimethyl O-(4-nitrophenyl) phosphorothioate) is reported to exhibit high mammalian toxicity (Figure 1). Methyl parathion can be absorbed through contact with skin or through fumes in the air [10]. It functions as a broad-spectrum insecticide that works through contact and ingestion, with some fumigant properties. It effectively targets most insects, however, due to its high toxicity; it is not recommended for household use [11]. Besides neurotoxicity, methyl parathion is reported to impact the reproductive and immune system. OS is a key mechanism underlying pesticides' toxic effects, which occurs due to an imbalance between generations of reactive oxygen species (ROS) and cellular antioxidant machinery [12-15].

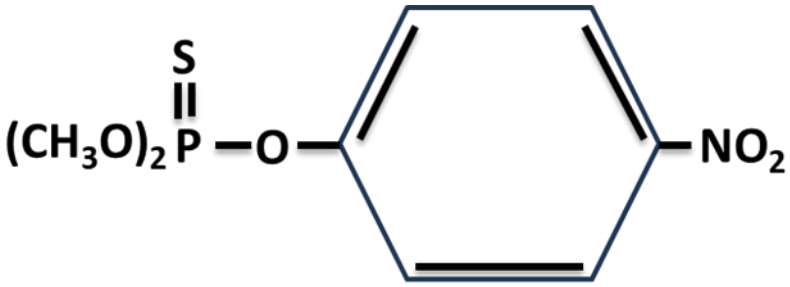

Figure 1. Structural formula of Methyl parathion

Methyl parathion exposure increases ROS through metabolic activation and mitochondrial dysfunction, overwhelming antioxidant defenses [16-18]. The resultant ROS causes damage/ alterations in macromolecules like proteins, lipids and even mutations in DNA, leading to cellular dysfunction and even death. Chronic exposure can lead to immunosuppression, increased infection susceptibility, exacerbation of autoimmune diseases and onset of chronic situations such as cardiovascular diseases. Regulatory measures, including exposure limits and protective guidelines, are essential to mitigate these risks [19]. Furthermore, promoting antioxidant-rich diets and safer pest control practices can enhance protection against methyl parathion-induced toxicity [14, 20-22]. This study assesses immunotoxic and OS-inducing effects in lymphocytes isolated from healthy chicken spleen due to methyl parathion *in vitro* exposure by conducting lymphocytes proliferation assay' (LPA) and 'nitric oxide estimation (NO) assay'. The dose of methyl parathion used for conducting these assays was NOEL/10^3 in the chicken lymphocyte culture system [12].

Methodology

Avian lymphocytes

Healthy chickens from a local slaughterhouse were used for isolating the lymphocytes from the spleen under strict aseptic conditions inside a laminar air flow employing regular protocol. The density gradient centrifugation was used for the splenocyte separation using LSM (Himedia, India) as described earlier by Ambwani et al., [12, 23].

Assessment of viability of separated lymphocytes

The trypan blue dye method was used to assess the percentage viability of isolated lymphocytes in the hemocytometer [24]. The cell count was made to 10^7 cells/ ml in animal cell culture media (RPMI-1640, Himedia, India). One ml aliquots were prepared in 1.5 ml micro-centrifuge tubes and a cell pellet was obtained for giving methyl parathion treatment [12].

In vitro exposure of methyl parathion

Commercially available preparation of methyl parathion from the market was obtained and a dose of NOEL/10^3 (3.0 mg/ kg body weight) was used for giving treatment to isolated lymphocytes (2 hrs, 37°C). The control untreated and methyl parathion-treated lymphocytes were pelleted and washed two times and finally suspended in 1 ml of 10 percent fetal bovine serum (Sigma, USA) supplemented animal cell culture media (RPMI-1640) as described earlier [12].

Lymphocyte proliferation assay (LPA)

LPA or lymphocyte blastogenesis assay was conducted following the procedure of Ambwani et al., [12]. Two mitogens *viz*., lipopolysaccharide (LPS) and Concanavalin-A (ConA) (Sigma, USA) were used in the experiment at concentrations of 5 µg/ml, each, in RPMI-1640 medium for proliferation of B and T lymphocytes, respectively.

Nitric oxide estimation Assay (NO assay)

NO assay using Griess reagent (Sigma) was performed by 96 well microplate assay procedure to assess the NO content in the macrophages culture media in control untreated and methyl parathion treated cells [23, 25]. NO production was measured using different dilutions of $NaNO_2$ for the standard curve preparation and assessment of NO in the test samples.

Statistical analysis

To find out the significant difference between untreated-control cells and methyl parathion treated cells, analysis of variance (ANOVA) and student's t-test were employed. The values are shown as mean optical density±standard error (mean OD±SE) [26].

Results and Discussion

Lymphocyte proliferation assay (LPA)

After cell counting, 10^7 cells/ ml were kept finally in 1.5 ml micro-centrifuge tubes. These cells were used for giving methyl parathion exposure as described earlier. The NOEL/10^3 dose of methyl parathion used for giving *in vitro* exposure to LPS-stimulated cells led to the significant reduction of about 87% in B lymphocyte proliferation in comparison to the untreated control cells (Table 1, Figure 2). Similarly, ConA-stimulated cells caused a marked reduction of about 68% in T lymphocyte proliferation in methyl parathion-treated cells in comparison to untreated- control cells (Table-1; Figure 3).

Table 1. *In vitro* effects of methyl parathion on B and T cell blastogenesis in avian lymphocytes

S.N.	Treatments	Mean Δ O.D. ± S.E.**	Percentage change	Mean Δ O.D. ± S.E.**	Percentage change
1	Control	0.265 ± 0.016	–	0.294 ± 0.008	–
2	Methyl parathion	0.034 ± 0.004	-87.23	0.093 ± 0.006	-68.25
CD at 1% = 0.048		CD at 5% = 0.034	CD at 1% = 0.037		CD at 5% = 0.027

*** Significant at p< 0.01*

Nitric oxide estimation Assay (NO assay)

OS was detected by measuring the NO content in methyl parathion treated cells in comparison to untreated control cells. Marked enhancement was detected in the NO production in methyl parathion treated cells as compared to control-untreated cells (Table 2, Figure 4).

Table 2. *In vitro* effects of methyl parathion on NO concentration (µM/ml) in mononuclear cells

S. No.	Treatments	Mean Conc. ± S.D.**
	Control	48.18 ± 0.974
	Methyl parathion	80.48 ± 0.955
CD at 1% = 4.223		CD at 5% = 3.013

** Significant at p< 0.01

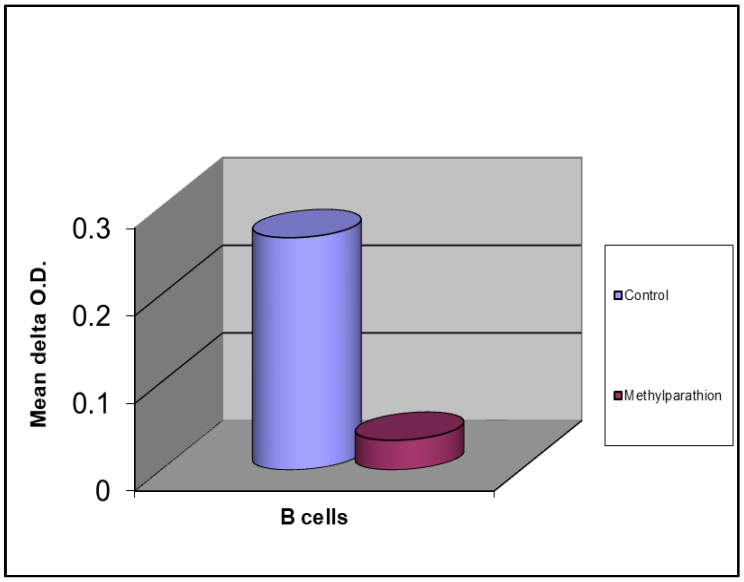

Figure 2. Effects of methyl parathion on B cell blastogenesis in avian lymphocytes

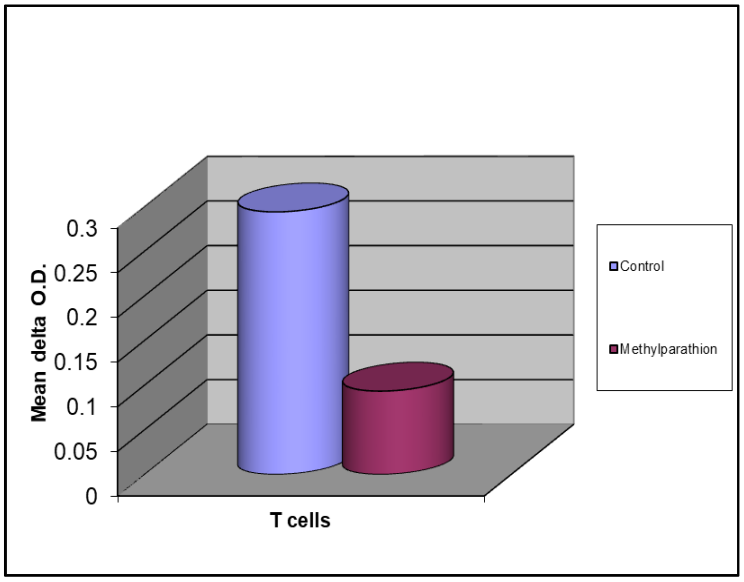

Figure 3. Effects of methyl parathion on T cell blastogenesis in avian lymphocytes

Methyl parathion has been widely used on various crops, including cotton, rice, wheat, and fruit trees. Its broad-spectrum activity makes it effective against a variety of insect pests such as aphids, boll weevils, and leafhoppers. The typical application methods include aerial spraying, ground spraying and seed treatment. Methyl parathion is a highly toxic organophosphate insecticide extensively used in the farming sector to eliminate pests [27]. Its primary function is to inhibit the enzyme acetylcholinesterase, leading to the accumulation of acetylcholine in the nervous system of insects, which leads to paralysis [3]. Despite its effectiveness, the application of methyl parathion has raised significant health and environmental concerns, leading to strict regulatory controls in many countries. In many countries, its use is either restricted or banned. Exposure to methyl parathion may be there through air inhalation, or oral or skin absorption. Acute contact can lead to symptoms such as headaches, dizziness, nausea, respiratory distress, and, in severe cases, convulsions and death.

Chronic exposure has been associated with long-term health effects, including neurological disorders and potential carcinogenicity. Methyl parathion is highly toxic to birds, beneficial insects, fishes, and other aquatic organisms [28]. It can be bioaccumulated and can enter in the food chain, posing risks to wildlife and potentially affecting biodiversity. Methyl parathion exerts its toxic effects primarily due to its inhibitory effect on acetylcholinesterase (AChE), an enzyme crucial for the breakdown of acetylcholine in the nervous system. Inhibition of AChE leads to the accumulation of acetylcholine at synaptic junctions, causing prolonged neural stimulation and resulting in a range of symptoms from mild (headaches, dizziness) to severe (convulsions, respiratory failure) [29]. Undeğer and Başaran [30] displayed genotoxicity in the human peripheral lymphocytes due to *in vitro* exposure to dimethoate and methyl parathion. Institoris et al., [31] displayed immunotoxic effects in rats over three generations due to exposure to repeated small doses of dimethoate and methyl parathion. There were enhanced SCEs due to methyl parathion exposure in human peripheral lymphocytes [6, 32]. Several studies displayed immunomodulating effects due to OPIs exposure [12-13, 33-34].

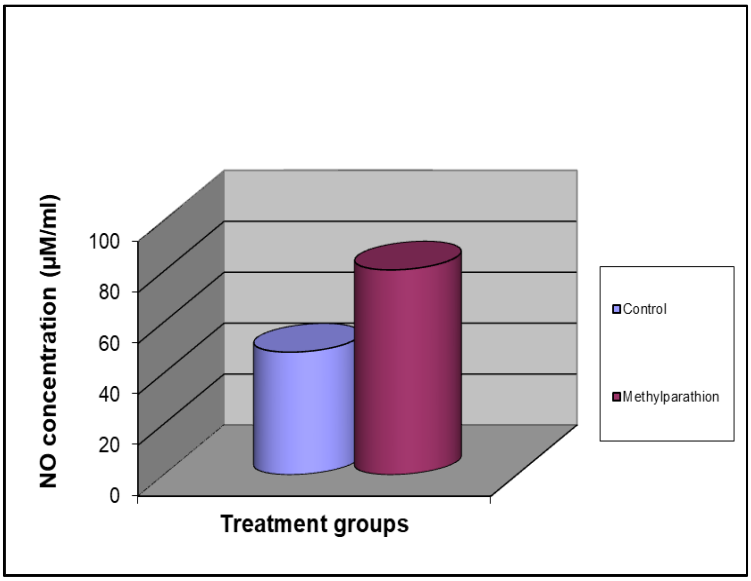

Figure 4. Effects of methyl parathion on nitric oxide (NO) concentration in mononuclear cells

OS is caused by to continuous generation of ROS and the inability of cellular antioxidant machinery to combat these ROS. This can cause macromolecular damage, interference with the cellular processes, and even cell death. Excessive ROS is generated due to methyl parathion administration as observed in several *in vitro*/ *in vivo* experimental model systems. The body has

evolved a complex system of antioxidant defenses to neutralize ROS and mitigate their harmful effects. Studies have demonstrated that methyl parathion can decrease the activity of SOD, catalase, and glutathione peroxidase [35]. Furthermore, it can increase lipid peroxidation [9, 36], which is the oxidative degradation of lipids that can compromise cell membrane integrity, leading to increased permeability and cell death. Methyl parathion exposure can cause DNA damage [37], which can cause apoptosis, mutations and even contribute to carcinogenesis. Sivapiriya et al., [38] reported dimethoate-induced altered antioxidative status in the liver and kidney in experimental rats. It could be inferred that there is a clear correlation between OS, immunity status, and apoptosis [4, 5, 12-14].

In conclusion, methyl parathion, while effective as a pesticide, poses significant risks to health through its immunotoxic and oxidative stress-inducing effects. The disruption of immune function and the imbalance in oxidative homeostasis can lead to increased susceptibility to infections, chronic diseases, and other health complications. Understanding these mechanisms underscores the importance of stringent regulation, protective measures, and ongoing research to develop safer alternatives to organophosphate pesticides.

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