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# Phorate induced down regulation of chicken lymphocytes proliferation through oxidative stress due to *in vitro* exposure

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**ABSTRACT**
*Today chemical pesticides are playing vital role in controlling agricultural, industrial, home garden and public health pests globally though not without deleterious effects on human, animals and birds. Phorate is a broad spectrum, non-biocumulative organophosphorus insecticide and acaricide. It is extremely toxic to mammals and other non-target organisms. Present paper reports immunotoxic effects due to in vitro exposure of low level dose of phorate in chicken lymphocytes employing lymphocyte proliferation assay. Phorate induced oxidative stress was evaluated by nitric oxide estimation in the treated cells. There was significant decrease in B and T cell blastogenesis in chicken lymphocytes exposed to thousand times dilution of No Observable Effect Level (NOEL/$10^3$) dose of phorate. There was increase in oxidative stress on measuring nitric oxide level in phorate treated cells as compared to control cells. Thus in vitro exposure of phorate revealed its immunotoxic properties in chicken lymphocytes. It caused elevated oxidative stress even at low dose level. It is also proposed that such in vitro tests can be useful in preliminary evaluation of pesticide induced immunotoxicity and oxidative stress furthermore help in reducing the use of laboratory animals.*
***Key Words:*** *Phorate; immunotoxicity; oxidative stress; chicken lymphocytes.*

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## INTRODUCTION

Organophosphorus insecticides (OPIs) are some of the most useful and diverse classes of insecticides in use for almost five decades. OPIs have been used in agricultural and home formulations for many years and account for huge worldwide insecticide market [23]. Continuous exposure of pesticides even at low dose levels can exert adverse effects on immune system which is considered as a sensitive indicator [8-11]. OPIs are a group of insecticides derived from the phosphoric or phosphorothioic acid; its usage has been increased in the recent years [1]. Phorate (*IUPAC*: O,O-diethyl S-ethylthiomethyl phosphorodithioate; *Molecular formula*: $C_7H_{17}O_2PS_3$) is a broad spectrum, non-biocumulative OPI and acaricide, an indirect inhibitor of cholinesterase with good contact, stomach and fumigant action against target organisms. It is extremely toxic to mammals and other non-target organisms. Contamination of standing water and waterways must be avoided. Phorate is stable for at least two years at room temperature in media between pH 5 and 7.Phorate may be absorbed from the gastrointestinal tract, through the intact skin and by inhalation of spray mists or fine dust. Several metabolites of phorate inhibit the activity of both acetylcholinesterase and pseudocholinesterase [30].

The uncontrolled use of these insecticides in agriculture and public health operation has increased the scope of ecological imbalance and thus many non-target organisms have become victims [27]. It has been reported that OPIs are neurotoxic in nature by acting as inhibitors of neuronal cholinesterase activity [29] and serum cholinesterase [44]. Some studies reported that OPIs caused lipid peroxidation [28, 31, 13] in vertebrates.

The acute toxicity of many commonly used pesticides is well known and poisoning cases often documented. In contrast, much less is known about long-term impacts on different systems of the human

body including the nervous, hormone, reproductive and immune systems. The toxic effects and No Observable Effect Level (NOEL) doses of phorate are well documented [30]. Present communication evaluates immunotoxicity and oxidative stress in chicken lymphocytes due to *in vitro* exposure of low level dose of phorate employing lymphocytes proliferation assay and nitric oxide estimation. The pesticide dose selected for the study was NOEL/$10^3$ which is reported as suitable for the *in vitro* studies in avian lymphocyte cell culture system [24].

## MATERIALS AND METHODS

### Avian Lymphocytes

Spleens from healthy birds were collected from local slaughter house. Chicken lymphocytes were isolated from spleen collected aseptically under laminar air flow as per standard procedure [33]. Lymphocytes were separated through density gradient centrifugation as per the method described by Rose and Friedman [42].

### Cell Viability Assay

Percentage cell viability was determined by 0.1 per cent trypan blue dye exclusion test using haemocytometer [21] and final cell count was adjusted to $10^7$ cells/ml in RPMI-1640 medium and made into 1 ml aliquots in eppendorf tubes and cells were pelleted by centrifugation at 1,400 rpm for 10 min.

### Pesticide Treatment

Commercial preparation of phorate was purchased from local market and it's thousand times diluted NOEL (6.0 mg/kg body weight) dose in RPMI-1640 medium (Hi-Media, India) was used for the *in vitro* exposure of avian lymphocytes for 2 h at 37°C. After incubation cells were washed twice and finally suspended in 1 ml of RPMI-1640 medium supplemented with 10 per cent FCS (Sigma, USA).

### Lymphocyte Proliferation Assay (LPA)

Lymphocyte proliferation assay or B and T cell blastogenesis assay was carried out as per the method described by Rai-el-Balhaa *et al*. [38] with minor modifications [25]. Concanavalin-A (ConA) (Sigma, USA) was used as T cell mitogen, whereas lipopolysaccharide (LPS) as B cell mitogen at a concentrations of 5 µg/ml, each, in RPMI-1640 medium.

### Oxidative Stress Assay

Nitric oxide (NO) production by macrophages in the medium was measured by microplate assay method [46]. The standard curve to calculate the NO production was prepared using different dilutions of $NaNO_2$.

### STATISTICAL ANALYSIS

Analysis of variance and Student's *t*-test were used to estimate significant difference between control and treated cells. The values were expressed as mean delta optical density ± standard error (mean Δ OD± SE). Student's *t*-test was employed for comparing the means ODs [45].

## RESULTS

### Cell Viability Assay

After cell counting, the final cell count was adjusted to $10^7$ cells/ml in RPMI-1640 medium and made into 1 ml aliquots in eppendorf tubes. These cells were pelleted and used for giving pesticide exposure as described earlier.

### LPA

The *in vitro* exposure of avian lymphocytes to NOEL/$10^3$ dose of phorate showed significant decrease in B cell blastogenesis in the presence of B cell mitogen LPS in comparison to the control (Table 1 and Figure 1). In case of T cell proliferation studies, phorate treated avian lymphocytes showed sharp decrease in T cell blastogenesis in the presence of mitogen ConA (Table 2 and Figure 2). Overall, there was significant decrease in B and T cell blastogenesis, both.

### Oxidative Stress Assay

Oxidative stress was detected by NO estimation. In case of dimethoate treated cells, more NO production was detected as compared with control untreated cells (Table 3 and Figure 3).

## DISCUSSION AND CONCLUSIONS

Exposure to low level OPIs is known to produce a variety of biochemical changes, some of which may be responsible for the adverse biological effects reported in humans and experimental animals [36]. Recently much emphasis is being given on *in vitro* system-based studies on pesticide induced immunotoxicity, oxidative stress and apoptosis [14, 15, 16, 26, 34, 4, 6, 9-11]. The present study was planned to examine *in vitro* immunotoxic effects of phorate and its possible mechanism employing chicken lymphocytes cell culture system.

In the present study, there was a decrease in B and T cell blastogenesis in phorate treated cells as compared with control cells. This was in good agreement with several other studies conducted on OPIs [35, 47]. Studies have revealed dimethoate, an OPI to be toxic to the immune system [32, 2, 3, 4]. *In vitro* studies on malathion exposure showed that high cholinergic doses and prolonged exposure to low doses (subchronic) suppressed humoral immune response [19, 20, 22]. *In vitro* exposure of malathion (250 µg/ml) in murine splenocytes induced partial cytotoxicity. Also, purified malathion as low as 25 µg/ml suppressed cell mediated immunity by inhibiting cytotoxic T lymphocytes response to alloantigens [40, 41]. Institoris *et al.* [32] determined immunotoxicity of repeated small doses of dimethoate and methyl parathion administered to rats over three generations. Effect of both the substances on immunological variables were detectable at the 1/75 $LD_{50}$ dose level, but different parameters were affected in the three consecutive generations. Cytogenicity of methyl parathion in human peripheral lymphocytes showed an increase in SCEs due to pesticide exposure [43, 34].

Present study showed enhanced oxidative stress through NO estimation in phorate treated cells. Banerjee *et al.* [15, 16] clearly established that pesticides exposure leads to oxidative stress. There is a clearly established relationship between ROS/free radicals and apoptosis. As ROS/free radical intermediates mediate many immune cell functions and apoptosis has been established in immune cell populations [2, 39, 4,10], it is likely these two events could arise simultaneously during certain chemical exposures. In summary, there is sufficient evidence for a relationship between ROS generation, immune cell regulation and apoptotic process [12]. Peña-Llopis [37] discussed role of antioxidants as potentially safe antidotes for OP poisoning.

Analysing the outcome of the present studies, it can be concluded that *in vitro* exposure of low level dose of phorate caused immunotoxic effects and significantly enhanced oxidative stress in the treated cells. The *in vitro* system was found to be a versatile and scientifically convenient system for such immunotoxicity studies and can prove helpful in preliminary screening of low-level pesticide exposure and reduce experimental animal testing.

**Table 1 *In vitro* effects of phorate on B cell blastogenesis in avian lymphocytes**

| S. No. | Treatments | Mean Δ O.D. ± S.E.** | Percentage change |
|---|---|---|---|
| 1. | Control | 0.329 ± 0.017 | – |
| 2. | Phorate | 0.105 ± 0.090 | -68.09 |
| CD at 1% = 0.074 | | CD at 5% = 0.053 | |

** Significant at p< 0.01

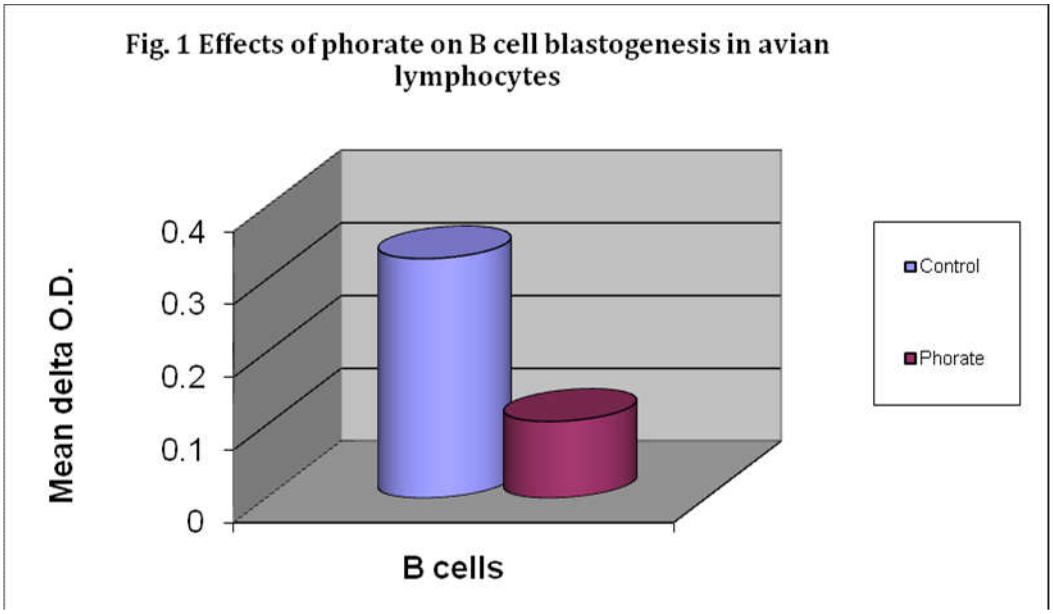


**Table 2 *In vitro* effects of phorate on T cell blastogenesis in avian lymphocytes**

| S. No. | Treatments | Mean Δ O.D. ± S.E.** | Percentage change |
|---|---|---|---|
| 1. | Control | 0.368 ± 0.017 | – |
| 2. | Phorate | 0.066 ± 0.006 | -82.07 |
| CD at 1% = 0.040 | | CD at 5% = 0.028 | |

** Significant at p< 0.01

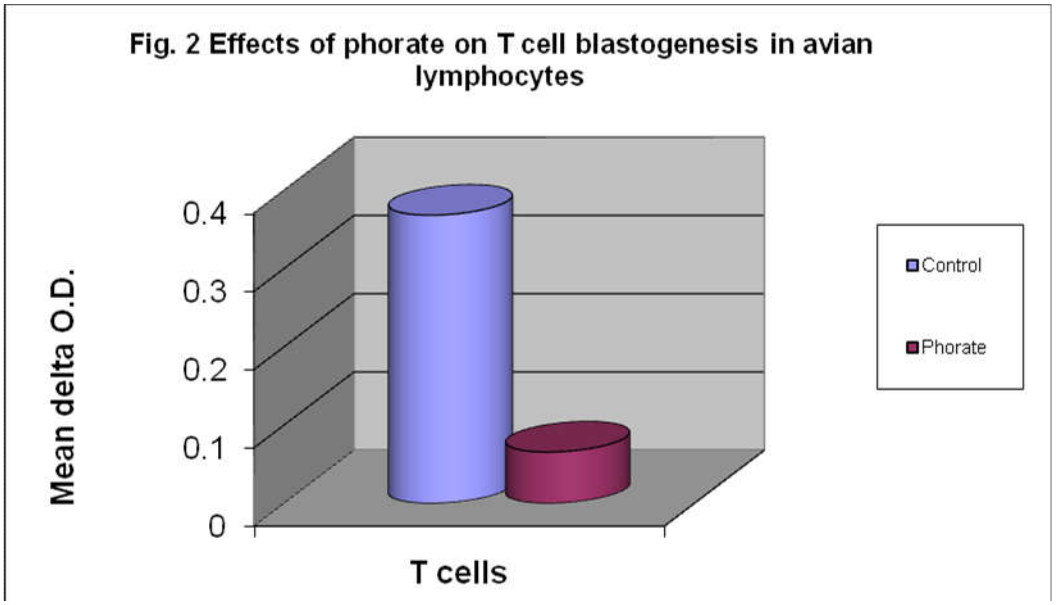
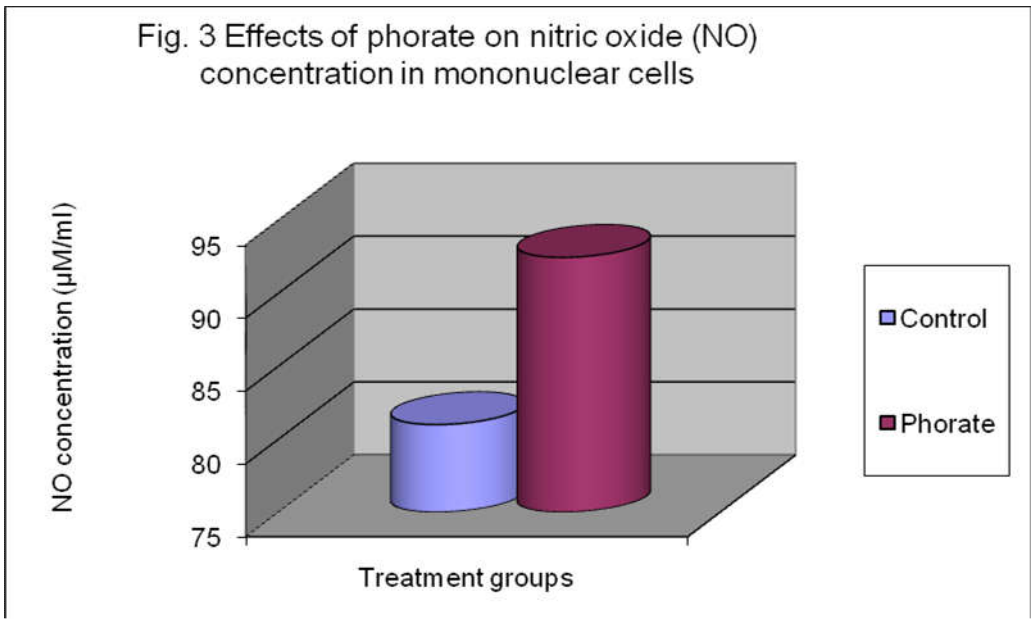



**Table 3*In vitro* effects of phorate on NO concentration (µM/ml) in mononuclear cells**

| S. No. | Treatments | Mean Conc. ± S.E.** | Percentage change |
|---|---|---|---|
| 1. | Control | 81.00 ± 1.034 | – |
| 2. | Phorate | 92.46 ± 5.167 | +14.15 |
| CD at 1% = 9.797 | | CD at 5% = 6.991 | |

** Significant at p< 0.01



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