

Subchronic Acephate Exposure Induces Immunotoxicity in White Leghorn Cockerels

Syamantak Mani Tripathi (✉ vjt93@yahoo.com)

Nanaji Deshmukh Veterinary Science University <https://orcid.org/0000-0002-5992-3980>

Prem Kumar Govindappa

Pennsylvania State University College of Medicine: Penn State College of Medicine

Megha Kadam Bedekar

CIFE: Central Institute of Fisheries Education

Yash Pal Sahni

Nanaji Deshmukh Veterinary Science University

B. C. Sarkhel

Nanaji Deshmukh Veterinary Science University

Rajesh Kumar Sharma

Nanaji Deshmukh Veterinary Science University

Laxmi Narayan Sankhala

Rajasthan University of Veterinary and Animal Sciences College of Veterinary and Animal Sciences: College of Veterinary and Animal Science Bikaner

Raghavendra Hallur Lakshmana Shetty

Universidade Estadual Paulista Julio de Mesquita Filho Faculdade de Medicina Campus de Botucatu

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Abstract

We investigated the subchronic immunotoxicity of the phosphoramidothioate organophosphorous insecticide, acephate in white leghorn cockerels (WLH). The cockerels were divided into five groups; C1 (plain control), C2 (vehicle control), T1, T2, and T3 which received acephate suspended groundnut oil for 60 days at doses of 21.3, 28.4 and 42.6 mgkg⁻¹ respectively. The live body weight gain, absolute and relative weights of the spleen, thymus, and bursa of Fabricius, hemoglobin (Hb), total erythrocyte counts (TEC), packed cell volume (PCV) and lymphocytes were significantly decreased. However, monocytes, eosinophils, heterophils, and basophils were significantly increased. Total protein, albumin and albumin to globin ratio, the antibody response to RD-F and delayed-type hypersensitivity response to DNCB dye or PHA-P, erythrocyte and brain Acetylcholinesterase activity was also significantly reduced in T2 and T3. At 40 and 60 days of acephate exposure, nitrate and nitric oxide production by RD-F and mitogen Con A stimulated peripheral blood and splenic lymphocytes, as well as lymphocyte proliferation in response to antigen RD-F and mitogen Con A stimulation, were significantly decreased in groups T2 and T3. Furthermore, dose-dependent increases in the frequency of micronuclei formation, varying intensity serum protein bands with different protein fractions (14.85KDa), and splenic DNA laddering (180 bp) were observed in groups T2 and T3. Histopathologically, the spleen and bursa showed morphological changes and mild lymphocyte depletion. In conclusion, low-level acephate exposure may affect acetylcholinesterase, lymphocytes, and immune responses in cockerels. As a result, it should be considered when assessing immunotoxicity and the risk to human and animal health.

Introduction

Unregulated uses of pesticides often associated with modern agriculture can threaten the viability of the ecosystem by reducing biodiversity (flora and fauna) and polluting natural resources, such as groundwater, which affect human health, communities and the environment [1, 2]. Approximately ninety per cent of insecticides are not reaching to the intended species but significantly contaminate the ecosystem. Since the introduction of several pesticide regulation acts, there has been greater understanding of the gain and losses of insecticides use [3, 4, 5]. The issue of pesticide toxicity and other associated chemicals to non-target species continues to be a major concern worldwide [6].

Acephate (O, S-dimethyl-acetylphosphoramidothioate (C₄H₁₀NO₃PS), a water-soluble phosphoramidothioate organophosphorous insecticide and extensively marketed worldwide as a foliar foam with an average estimated use of 4 to 5 million pounds of an essential element in agricultural practices (PubChem). This is a systemic insecticide with contact effects on the stomach. Due to its slow mammalian toxicity and cost, the use of acephate in control of household, agricultural, and forestry pests has increased significantly. Since acephate can be purchased in retail stores, a significant proportion of the general population runs a possible risk of exposure to Organophosphate [1, 7]. Although new inventions are being attempted to synthesize a novel insecticide with negligible harm to unintended species, still these present substantial immunosuppressive risk to health, which escape standard regulatory toxicology screening [6].

Acephate toxicities are associated with its metabolite methamidophos which serves as an acetylcholinesterase (AChE) inhibitor, neurotoxicant and breathing insufficiency agent [8]. Although many researchers documented the effects of insecticides on immune function of mammalian species [9], however, immunotoxicological

investigations on non-rodent models are scarce, particularly on species of economic importance such as a cockerel, and further research is required. Hence, we assessed acephate's immunotoxic potential in white leghorn cockerels following frequent, sustained application at environmentally appropriate rates of exposure. The proposed avian model for immunotoxicological evaluation of acephate, an organophosphorus insecticide of least toxicity, may play an important role for predicting the risk of environmental toxicant.

Materials And Methods

2.1 Chemicals

Acephate (Technical grade, 97.5 %, Meghmani Organics Ltd., Ahmedabad) was received as a gift from the Department of Veterinary Pharmacology and Toxicology, Veterinary College, Anand, AAU, Anand, Gujarat, India, PIN 388001. Serum total protein and albumin estimation kits were obtained from Aspen laboratories. DNA molecular weight marker was purchased from MBIFermentas GERMANY. Boric acid, chloroform, ethanol, glycerol, glycine, HCl, hydrogen peroxide (30 %), isopropanol, isoamyl alcohol, sodium carbonate, methanol were from Merck, Mumbai, INDIA. Ethidium bromide was procured from Hi Media, INDIA. Agarose, DMSO, Con-A, ATChI, PHA-P and other chemicals were purchased from Sigma chemicals, USA. All other reagents used were of analytical grade. Multi-well 24 well plate (35)3043, 96 well flat bottom plate with lid was purchased from Falcon, USA.

2.2 Experimental cockerels

The research was performed in day-old white male leghorn cockerels (WLH), procured from the poultry community Phoenix, Jabalpur (Madhya Pradesh). The cockerels were treated with the 12h of a dark-light period as suggested by Institute Animal Ethics Committee (IAEC) and were offered water ad libitum and regular ration obtained from the feed unit, poultry farm, College of Veterinary Science & Animal Husbandry, Adhartal (Jabalpur). On days 1 and 30, RD-F strain of the Newcastle Disease virus (Biological Products Division, IVRI, Izatnagar) was used to vaccinate experimental cockerels intranasally to check the serum antibody titer against NDV. On day 14, the cockerels were vaccinated against Infectious Bursal Disease using Gumboro Disease Vaccine (Live; intermediate plus strain type -VetriBiologicals, Venkateshwara Hatcheries).

2.3 Experimental design

One day-old cockerels were randomly distributed into husbandry control (C1) and vehicle control (C2), and three treatment (T1-1/40th of LD50, T2-1/30th of LD50, and T3-1/20th of LD50) groups with 20 cockerels in each. Reported LD50 (852 mg kg⁻¹) of acephate was used to calculate the different dose groups [10]. Doses of acephate 21.3 mg kg⁻¹ body weight (1/40 of LD50), 28.4 mg kg⁻¹ body weight (1/30 of LD50) and 42.6 mg kg⁻¹ body weight (1/20 of LD50) dissolved in peanut oil were given to the cockerels of group T1, T2 and T3, respectively, daily for 60 days from day 1. Cockerels from group C1 received regular feed and water only daily for 60 days from day 1, and acted as husbandry control. In group C2, the cockerels were given peanut oil along with usual feed and water daily for 60 days from day 1, and acted as vehicle control. The selection of dose(s) was based on previous studies [10] and the levels of acephate contamination / residue in drinking water or food supplies [11]. Acephate dissolved in peanut oil was injected directly into the crop through a catheter attached to

1 ml BD syringe. The cockerels were observed every day, and the body weights were reported at intervals of 7 days. At term, immune organs (Bursa of Fabricius, thymus, and spleen) were excised and weighed.

2.4 Body weight and relative lymphoid organ weights

The live body weight of birds was recorded prior to administration of acephate. Birds were observed daily and their live body weights were recorded at 7-day intervals for a period of 60 days in order to observe the manifestation of acephate. On day 61, after taking live body weight, ten cockerels of each experimental group were sacrificed and the spleen, thymus, and bursa of Fabricius were immediately removed and weighed separately for absolute organ weight. The relative body weights of the immune organs were determined by dividing the body weight of the organs. $O: BW = \text{Weight of the organ (g)} / \text{Body weight (g)} \times 10$

2.5 Blood collection

Blood sampling under strict asepsis was done from cardiac puncture, jugular vein and wing vein of the cockerels of 0-10 day old (pre-experimental for checking maternal antibody titer), 10-40 day old and 40 day old, respectively, until the termination of experiment for hemato-immunological studies. At 20day intervals peripheral blood lymphocytes and serum were collected from heparinized (with 20 IUml^{-1} heparin) and non-heparinized tubes, respectively. In immunotoxicity tests, peripheral blood lymphocytes were treated within an hour of collection, while serum was held at -80°C for further study.

2.6 Hematological studies

The day of the blood collection hematological tests were performed. The collected blood samples were diluted with 0.015 Toluidine blue and the total counts of leukocytes were assessed [12]. The differential leukocyte count was made in peripheral blood smears stained by Giemsa (Qualigens) [13].

2.7 Serum Protein profile

2.7.1 Serum chemistry

To determine the non-specific immune response in subchronicacephate-exposed cockerels serum total protein (gmdl^{-1}), serum albumin (gmdl^{-1}), and serum globulin (gmdl^{-1}) were estimated with standard protocol of Core System as-say kits using an autoanalyzer (Photometer BT-224, Biotechnica Instruments,Italy) (Bt 1975 Jul).

2.7.2 SDS Polyacrylamide-Gel Electrophoresis (SDS-PAGE) of serum protein

The changes in the serum protein profile were analysed by 12 % resolving gel prepared according to Laemmli (1970) containing 1.5 M Tris-HCl buffer (pH8.8), 10 % SDS, 10 % ammonium persulphate and TEMED along with a 5 % stacking gel. The stacking gel (5 %) was made using 1.5 M Tris-HCl (pH 6.8), 10 % SDS, 10 % ammonium persulphate and TEMED. The electrophoresis was accomplished at 150 V till dye came out of the gel using 1x Tris glycinebuffer. The molecular weights of the serum protein subunits were determined using pre-stained molecular weight standards run along with the samples. The volumes of samples were adjusted to a concentration of $50\mu\text{g}$ proteins per well. On completion of electrophoresis, the gels were stained for proteins with 0.1 % Coomassie brilliant blue according to conventional methods and destained in a solution of 50 % methanol

containing 10 % acetic acid. Prior to viewing, the gel was washed with distilled water until the background was clear. In reference to markers, the different protein fractions and molecular weights were estimated.

2.8 Acetylcholinesterase

2.8.1 Erythrocyte acetylcholinesterase

We assessed depression of Acetylcholinesterase enzyme (AChE) activity, reported for organophosphate toxicity in red blood cells and brain by measuring acetylthiocholine iodide (ATChI) according to standard protocols [14]. AChE activity was determined in blood samples (10 μ L) collected at 20 day intervals and reported as μ MATChI hydrolyzed $\text{min}^{-1} \text{mg}^{-1}$ protein.

2.8.2 Brain acetylcholinesterase

On day 61 all animals were sacrificed humanely by rapid decapitation. The brain was immediately removed from the head, and put in ice-cold saline. In order to measure brain acetylcholinesterase, dissection for discrete regions of the brain was performed on pre-chilled sterile Petri dishes. Great care has been taken to avoid tissue injury or inflammation of any portion of the brain when separating from the skull or dissecting the different areas [15]. The whole brains were suspended in a buffer (1 g of tissue plus 10 mL of sodium phosphate 0.1 M, pH 7.5) and homogenised in an ice bath using a homogenizer. The homogenate was then centrifuged (5,000g/30 min/5°C), discarding the soluble portion. The pellet was then re-suspended using the equal volume of 0.1 g percent of Triton X-100 in 0.1 M sodium phosphate, pH 7.5, solution, and re-homogenized. The supernatant resulting from the resolubilization of the pellet was collected after a second centrifugation (15,000g/90 min/5°C). Brain AChE activity was spectrophotometrically tested in 0.1 M sodium phosphate, Triton X-100 0.1 g percent pH 7.5 by modifying Ellman's method [14] as described by Silva Filho et al. [16].

2.9 Assessment of cell-mediated and humoral immunity

2.9.1 Delayed type hypersensitivity (DTH) reaction assay

In vivo, cell-mediated immune response was evaluated by assessing the DTH reaction to 2, 4-dinitro-1-chlorobenzene (DNCB) dye by following an earlier standard method with minor modifications [17]. Three cockerels from each group were arbitrarily selected and 2 \times 2 cm² aseptically defeathered skin areas on the left and right abdomen of each cockerel were marked with India ink at day 50. 100 μ L DNCB (10 mgml⁻¹) dye dissolved in acetone and olive oil in 4: 1 ratio was applied to the marked areas of the right side and 100 μ L acetone (vehicle control) applied to the marked areas of the left side of the cockerel abdomen. The birds were challenged by applying 100 μ L DNCB (2 mgml⁻¹) dye at the same areas of right side and 100 μ L acetone on the left side after 7 days of first application. The increase in dermal thickness (mm) was measured at 0, 6, 12, 24, 48 and 72 h post-application with a vernier caliper. Increase in skin thickness (mm) = (T_t-T₀) R - (T_t-T₀) L Where T_t= thickness at t, T₀=thickness at 0 h, R = right side, L = left side and t = time. DTH reaction in the form of increase in dermal thickness was also recorded in phytohemagglutinin-P (PHA-P). To this end, on day 57, three birds were randomly selected from each group. 100 μ L PHA-P (1mgml⁻¹) dissolved in PBS had been injected into the third interdigital space of the right leg and 100 μ L PBS (vehicle control) into the left leg. The increase in footweb thickness (mm) was measured at 0, 6, 12, 24, 48 and 72 h of post-application with a vernier

caliper and foot web index was calculated. Foot web index = $(T_t - T_0) R - (T_t - T_0) L$ Where T_t = thickness at t, T_0 = thickness at 0 h, R = right side, L = left side and t = time

2.9.2 Anti-NDV antibody assay

Cockerel humoral immunity was determined by assessing IgG antibody levels specific to Newcastle Disease Virus (NDV) in serum samples using ND AbSynbiotic ELISA kit (Hester Pharmaceuticals).

2.10 Lymphocyte mitogenesis assay

2.10.1 Separation of peripheral blood lymphocytes

The peripheral blood lymphocytes were isolated according to previously reported standard protocols [18]. Briefly, 5 ml of the heparinized fresh blood from each cockerel was layered over 3 ml of histopaque-1077 in 15-ml polypropylene centrifuge tube slowly through the wall and centrifuged at 1400 rpm for 40 minutes. Mononuclear blood lymphocytes circumscribed at the plasma and histopaque interface were collected in a separate centrifuge tube. The lymphocytes, after washing thrice with sterile PBS (pH 7.2), were suspended in 1–2 ml of red-free phenol RPMI-1640 medium supplemented with 10 % bovine foetal serum, 25 mM HEPES, 24 mM NaHCO₃, 2 mM l-glutamine, 100 IU penicillin ml⁻¹ and 100 g of streptomycin ml⁻¹.

2.10.2 Separation of lymphocytes from spleen

The spleen was excised gently after the cockerels were sacrificed at the end of experiment on day 61, and splenocytes were isolated by following previously reported standard protocols [19, 20]. The excised spleen was washed thrice in sterile ice-cold phosphate buffer saline (PBS). The spleen capsule was removed and the organ was sliced into sections with the forceps and the scissors in the PBS. With a 10 ml glass syringe, the fragments were gently pushed through the mesh into a Petri dish containing a chilled PBS. Cell suspension was collected in 15-ml centrifuge tubes and allowed to stand on the ice for 5 min to settle heavy tissue parts. The uppermost 12 ml of the cell suspension was collected and centrifuged for 10 min at 1400 rpm. Cell pellets were then re-suspended in PBS and layered over histopaque-1077 at a ratio of 1:1. The tubes were centrifuged for 40 minutes at 1400 rpm. Further processing of the collected splenic lymphocytes was done as stated earlier to differentiate peripheral blood lymphocytes.

2.10.3 Cell viability count

The viable splenocytes were counted by using 0.1 % trypan blue dye, and hemocytometer [21]. The percentages of T and B lymphocytes in the peripheral blood and experimental bird spleen were determined using Annexin-V FITC Apoptosis Kit (Sigma Aldrich). The samples were analyzed by flow cytometry (BD FACS Aria, CA)

2.10.4 Lymphoproliferative response

In vitro the mitogen stimulation assay was performed as defined in the literature with minor modifications to evaluate lymphocyte proliferation [22]. The active splenocytes reduced blue coloured 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) chemical dye to its purple colour insoluble formazan which was read at 492 nm by using 650 nm as a reference wavelength in ELISA reader (Multiskan EX, Thermo Lab Systems, Australia). The mitogen stimulation Index (SI) was determined as Stimulation Index (SI) =

$(OD_s - OD_{us}) / OD_{us}$ Where, OD_s and OD_{us} indicate optical densities of stimulated cells and unstimulated cells, respectively.

2.11 Assessment of mononuclear cell functional activity

2.11.1 Nitrite production by mononuclear cells

The functional activity of mononuclear cells stimulated by antigen Newcastle disease virus 'F' Strain (RD-F) or Lipopolysaccharides (LPS) was evaluated by assessing the concentration of nitrite produced by cultured cockerel splenocytes from control and treatment groups by following an established method [23]. The Nitrite level was assessed by measuring the absorbance at 550 nm within 30 minutes in the spectrophotometer microplate reader (Multiskan EX, Thermo Labsystems, Australia). The concentration of nitrite produced by splenocytes was determined by comparison to the nitrite standard reference curve drawn with known sodium nitrite concentrations within cell-free medium and exhibited as $\mu\text{mol nitrite} / 3 \times 10^6 \text{ cells}$. Nitrite (μM) = $(\Delta A_{550\text{nm}} - \text{intercept}) / \text{slope}$

2.11.2 Nitrate reduction by mononuclear cells (Phagocytic activity test)

The nitrobluetetrazolium (NBT) dye reduction test was performed to measure the phagocytic activity of splenic mononuclear cells following an established method [24]. The Nitrate level was assessed by measuring the absorbance at 710 nm immediately extraction of formazan using spectrophotometer (DU 640B, Beckman, USA). The concentration of nitrate reduction (formazan yield) by splenocytes was calculated as 1 nmol ml^{-1} formazan corresponds to an optical density 0.054 ± 0.003 in both untreated control and acephate treated splenocyte.

2.12 Cytokinesis block Micronucleus assay in Cockerel splenic lymphocytes

The "in vivo biomonitoring" micronucleus (MN) test was performed to determine the level of exposure and health risk, according to Krahn [25]. Briefly, cultured lymphocytes were taken and Colchicine (2×10^{-7} final) was added to each culture for the last 2 or 3 h of incubation. Metaphase cells were collected by "mitotic shake-off", sedimented by centrifugation, resuspended in 0.075 MKCl, and incubated at 37°C for 5 min. The cells were sedimented and resuspended (three times) in methanol:acetic acid fixative (3:1, v/v). Following a final centrifugation and resuspension in a small volume of fixative, the cells were dropped onto microscope slides. Slides were stained in 5 % Giemsa for 24 h, air dried, and mounted using permount. Air-dried and coded slides were analyzed using a light microscope. One thousand lymphocytes of each chick had been scored under oil immersion. Non-refractile particles, which in all respects resembled a nucleus, were considered to be micronucleus.

2.13 Internucleosomal DNA cleavage assay

In order to further evaluate the effect of acephate on apoptosis induction in splenic immune cells, an assay for internucleosomal DNA cleavage (a hall-mark of apoptosis) was carried out as previously reported [26]. Briefly, the fragmented DNA was extracted from splenocytes using a standard phenol:chloroform: isopropyl alcohol extraction (25:24:1), quantified by spectrophotometry, analyzed on a 0.8 % agarose gel, and photographed

(digitized) (GeneGenius Biolmaging System of SynGene, UK) and the electrophoretic pattern was utilized to determine total genomic DNA damage.

2.14 Histopathology

Bursa of Fabricius, spleen and thymus were fixed in 10 % formalin – saline solution from each cockerel. After processing, paraffin-embedded sections of each tissue were cut at a thickness of 4-6 μm and stained for histopathological examination using a microscope with hematoxylin and eosin (H and E) [27]. In splenic tissues, the degree of lymphoid depletion was assessed by the extent to which white pulp was reduced in size and distribution, and lymphoid follicles were measured using a microscope (200x fields) at 20 different fields per portion. The histomorphological lesions were blindly evaluated by a veterinary pathologist and these assigned to numerical scores 0 to 4, where a higher value corresponds to increasing severity.

Lymphoid necrosis score

0: No necrosis; 1+ : <5 % necrosis (mini); 2+ : 5–25 % necrosis (mild); 3+ : 25–50 % necrosis (moderate); 4+ : >50 % necrosis (severe).

Red pulp expansion score

0: No widening; 1+ : Minimal widening; 2+ : Mild widening; 3+ : Moderate widening; 4+ : Severe widening.

The number or size of lymphoid tissue follicles or their germ centers was scored between 1 and 4 in bursal tissues. 1: 0–10 % depletion of lymphoids; 2: 10–30 % depletion of lymphoids (mild variation of cortical lymphoid tissue); 3: 30–70 % depletion of lymphoids (moderate variation of cortical tissue and follicle medullary regions and increased stroma between follicles); 4: >70 % depletion of lymphoids (severe depletion of follicles with little to no cortex, increased stroma between follicles and decreased follicular size).

2.15 Statistical analysis

Data were analyzed one-way analysis of variance (ANOVA) using SPSS 17.0 software with Tukey's HSD post-hoc tests to determine whether there were any statistically significant differences among experimental groups. The results are given as mean \pm standard error mean ($\bar{X} \pm \text{SEM}$). The $p \leq 0.5$ was deemed significant differences.

Results

3.1 General assessment

An abrupt depression, decreased feed intake, dullness and ruffled feathers were shown in cockerels exposed to extremely toxic (T_3 - 42.6 mg kg^{-1}) doses of acephate over 60 days. While in medium (T_2 - 28.4 mg kg^{-1}) and low (T_1 - 21.3 mg kg^{-1}) toxic dose groups clinical signs were milder in appearance. The cockerels from control groups (C_1 and C_2) did not exhibit any abnormal behavior and symptoms of toxicity. Birds died in treatment groups with serious nervous symptoms and clinical signs of toxicity before death. In the eighth week of experiment, mortality reached six, four and two in group T_3 (21. Mg kg^{-1}), group T_2 (28.4 mg kg^{-1}) and group T_1 (42.6 mg kg^{-1}) respectively. The remaining birds of all treatment groups revealed characteristic signs of organophosphate toxicity.

3.2 Body weight and lymphoid organ weights

Supplementary FIGURE 1 presents the stress induced toxicological impact of oral administration of acephate over 60 days on the live body weight of cockerels. Acephate exposure altered the feed intake in the cockerels of an extremely toxic dose group (data not shown) that led to significant decrease in live body weight of cockerels weighed at 7-day intervals in dose dependent manner compared to controls. TABLE 1 illustrates the effects of acephate exposure in cockerels upto 60 days on absolute organ weights and relative weights of cockerels. Absolute and the relative weights of immune organs *viz* spleen, bursa of Fabricius, and thymus, were significantly decreased dose dependently in medium and extremely toxic dose groups (T2 and T3) in comparison to controls except for the low dose treatment group (T1).

3.3 Hematology

Table 2 and 3 illustrate the impact of subchronic exposure to acephate on hematological indices, peripheral blood lymphocytes, total leukocyte counts and differential leukocyte counts (DLC) in WLH cockerel from treatment and control groups. Blood testing of cockerels exposed to acephate *per os* for 20 days did not show any significant alterations in Hb, TEC, and PCV whereas these were significantly reduced in cockerels from treatment groups (T2 and T3) on days 40 and 60 (TABLE 2). TLC (FIGURE 2) and lymphocyte concentrations were also significantly reduced dose dependently on days 20, 40, and 60. On the contrary, there was a dose dependent rise in the number of heterophils, monocytes, eosinophils, and basophils in the cockerels from treatment groups in comparison to controls (TABLE 3).

3.4 Serum chemistry

3.4.1 Total protein

Acephate exposure for 20, 40, and 60 days caused the significant reduction in the serum total protein level in the cockerels from medium (T2) and extremely toxic (T3) treatment groups compared with controls (FIGURE 3A).

3.4.2 Serum albumin

Acephate exposure for 20, 40, and 60 days caused the significant reduction in the serum albumin level in the cockerels from medium (T2) and extremely toxic (T3) treatment groups compared with controls (FIGURE 3B).

3.4.3 Serum globulin

Acephate exposure in cockerels with medium toxic (T2) and extremely toxic (T3) dose(s) caused significant decrease in serum globulin level at day 20 while it was non significantly diminished in all treatment groups at day 40 and significantly decreased in T2 and T3 at day 60 compared with controls (FIGURE 3C).

3.4.4 Serum Albumin: Globulin ratio

Serum albumin showed a significant decline in albumin: globulin ratio at medium and extreme toxic dose levels compared with controls (FIGURE 3D).

3.4.5 Changes in serum protein profile

Electrophoretic analyses of chicken serum in the treatment groups reflected the appearance of varying intensity protein bands compared to controls. Ninemolecular entities ranging from 16.42-175 kDa molecular weight were detected in chicken serum at a low toxic dose (21.3 mgkg^{-1}) of acephate and control groups. However, after exposure to 28.4 mg kg^{-1} and 42.6 mgkg^{-1} acephate, chicken serum proteins were separated into 9 and 10 MW bands ranging from 15.61-173.95 to 14.65-171.96 KDa, respectively. New protein expression was also stimulated as evidenced by the presence of new bands after treatment with a higher toxic dose of acephate. Two common protein bands were shared between dose groups of 21.3 mgkg^{-1} , 28.4 mgkg^{-1} and 42.6 mgkg^{-1} of acephate and controls with MW 18.25 KDa and between 28.4 mgkg^{-1} and 42.6 mgkg^{-1} of acephate with 14.85 KDa. One different fraction of protein (16.48 KDa) was observed in the serum protein of Acephate exposed cockerels at an extremely toxic dose (42.6 mgkg^{-1}) of up to 60 days (FIGURE 4). This was confirmed by the reduction of total proteins as shown in the Figure 3A. The expression of all fractions had become too weak to be detected in all test groups.

3.5 Assessment of acetylcholinesterase enzyme inhibitor interaction

3.5.1 Erythrocyte acetylcholinesterase

In vivo effects of acephate on erythrocyte acetylcholinesterase activity are presented in TABLE 4. Acephate exposure in white leghorn cockerels caused a significant decrease in erythrocyte acetylcholinesterase activity in a dose-dependent manner as compared to control groups. The maximal inhibition on the acetylcholinesterase was observed 20 days onwards which was in a dose-dependent manner.

3.5.2 Brain acetylcholinesterase

In vivo effects of acephate on brain acetylcholinesterase activity are shown in TABLE 4. Acephate exposure in white leghorn cockerels caused significant decrease in acetylcholinesterase activity as compared to control groups.

3.6 Assessment of cell mediated and humoral immune response

3.6.1 Delayed type hypersensitivity (DTH)

Cell mediated immunity was assessed through measuring DTH reaction using DNCB dye in response to the acephate exposure at varying toxic doses to cockerels over 60 days. The mean rise of dermal thickness (mm) was seen in all treatment groups at 6, 12, 24, 48, and 72 h after pre challenge and 7- days' later post-challenge sensitization with DNCB is shown in FIGURES 5A–5B. The rise in dermal thickness after sensitization with DNCB dye was significantly decreased in medium (T2) and extremely (T3) toxic dose groups at 12, 24, 48, and 72 h in dose dependent manner compared with the controls. However, a non-significant increase in dermal thickness was also recorded in a low toxic dose (T1) treatment group at 12 and 24 h (FIGURE 5A). However, the rise in dermal thickness of cockerels following post-challenge sensitization after 7-days of pre-challenge sensitization was significantly reduced at 12, 24, 48 and 72 h in medium (T2) and extremely (T3) toxic dose groups comparison with control groups, and this impact was also evident as a non-significant increase in dermal thickness in cockerels exposed to low toxic dose (FIGURE 5B). DTH response was also evaluated, followed by interdigital PHA-P injection and measurement of footweb index (mm) at 6, 12, 24, 48 and 72 h post-injection. Acephate decreased the PHA-P post-injection footweb index at 12, 24, 48, and 72 h (FIGURE 5C).

3.6.2 Assessment of humoral immune response

The immunological reaction in response to the antigen challenge is the best way to evaluate the immune system. That approach has therefore been applied in this analysis. Humoral immunity in response to RD-F antigen in cockerels exposed to acephate over 60 days was recorded by assessing the level of antiNDV antibody titer using ELISA and shown in FIGURE 6. On the 20th day of acephate exposure anti NDV ELISA values were non-significantly higher in cockerels from low toxic dose group (T1) and significantly lower in medium toxic (T2) and extremely toxic (T3) dose groups. While on days 40 and 60 after the start of exposure to acephate, these values were significantly lower in all treatment groups in a dose dependent manner compared with controls and this decreasing effect on antibody production persisted till the end of study (FIGURE 6).

3.7 Assessment of lymphoproliferative response

3.7.1 Cell viability

The viability of splenocytes was found to be around 70 per cent at the treated concentrations. Viable cells appear translucent, and dead cells stained blue due to increased permeability of the cells.

3.7.2 Splenic and peripheral blood lymphocyte proliferation

The effects of acephate exposure over 60 days on immune responses of cockerels determined by assessing in vitro proliferation of splenic or peripheral blood lymphocytes stimulated with antigen RD-F or mitogen Con A. Results of lymphocytes' stimulation index are shown in FIGURE 7A, 7B, and 7C. The toxic effect of acephate on peripheral blood lymphoproliferation after stimulation with antigen or mitogen was evidently significant from day 40 onwards to the end of study (FIGURE 7A and 7B). On Day 20, lymphoproliferation was significantly increased in cockerels from the low toxic dose (T1) group, while it was significantly decreased in cockerels exposed to medium and highly toxic acephate levels compared to cockerels from the control group. Significant reductions in lymphoproliferation were observed in cockerels from medium (T2) and extremely (T3) toxic dose groups compared to cockerels from control groups from day 40 to the end of the study. However, the lymphocyte proliferation of splenocytes stimulated with antigen and mitogen was significantly reduced in all treatment groups dose dependently, and the effect was more marked in the birds subjected to extremely toxic dose (T3) (FIGURE 7C).

3.8 Functional activity evaluation of mononuclear cells

3.8.1 Assessment of non-specific immune response (nitrite production)

The concentrations of nitrite were determined to assess the production of nitric oxide (NO) by peripheral blood and mononuclear splenocytes. Acephate induced development of nitrite by peripheral blood lymphocytes stimulated with antigen LPS and RD-F or mononuclear splenocytes of cockerels stimulated with mitogen Con-A is shown in FIGURES 8A, 8B, and 8C respectively. Acephate exposure over 20 days resulted in significant reduction in the production of nitrite by peripheral blood lymphocytes stimulated with LPS and RD-F in the birds from medium (T2) and extremely (T3) toxic dose groups as compared to controls. However, the production of nitrite by peripheral blood lymphocytes was significantly reduced in birds of all toxic dose groups (stimulated with LPS) and dose-dependent manner (stimulated with RD-F) compared to controls at day 40, and at day 60,

significantly decreased in dose-dependent manner (stimulated with LPS) and in birds of all toxic dose groups (stimulated with RD-F) compared to controls (FIGURES 88A and 8B). The similar dose dependent significant reductions in nitrite production by mononuclear splenocytes stimulated with mitogen Con-A were also recorded (FIGURE 8C).

3.8.2 Assessment of Macrophage Function test (nitrate production)

The synthesis of nitrate (formazan) in 1 mL supernatant of harvested splenocytes was used to assess phagocytic activity of splenic mononuclear cells. FIGURE 9 shows nitrate production by splenic mononuclear cells (stimulated with ConA) in birds exposed to Acephate. The results indicate that treatment with Acephate for 60 days reduced nitrate production by ConA stimulated splenic mononuclear cells in a dose-dependent manner, with a non-significant increase in nitrate production by low toxic dose (21.3 mg/kg) exposed birds at 24 h, and that this significant change persisted until the end of the study (FIGURE 9).

3.9 Cytokinesis block micronucleus (CBMN-Cyt) assay

Acephate increased the development of micronucleus, nuclear buds and nucleoplasmic bridges in a concentration-dependent manner in the cytokinesis block micronucleus cytome (CBMN) test when compared to the control. At higher concentrations, however, these increases were evident. The internucleosomal assay revealed that acephate significantly increased DNA damage. In addition to the induction of micronuclei (TABLE 5 and FIGURE 10A-D), the acephate also caused other nuclear anomalies in the treated sequence, such as vacuolated nuclei and binucleated cells with unequal nuclear content. In certain cases, chromatin disintegration and karyorrhexis were also observed.

3.10 Molecular detection of apoptosis by DNA ladder assay

The results showed the formation of DNA ladders in all splenic cells treated with acephate that indicate apoptotic cell death (FIGURE 11). The splenocytes of cockerel exposed to acephate triggered intense laddering and increased smearing, which suggests spontaneous cleavage of DNA and necrotic cell death. Splenic cells from control cockerels have a vivid top lane band with intact DNA and less evident apoptotic laddering.

3.11 Histopathology

Spleen and bursa of Fabricius histopathology exams showed changes primarily in group T3 (42.6 mg kg⁻¹ body weight). Severe depletion of lymphocytes, necrotic foci and red pulp expansion was observed in the spleen (FIGURE 12A-D), whereas there was no histopathological lesion in the thymus (data not shown). Bursa of Fabricius showed lymphoid depletion and decreased follicular size with interfollicular fibrosis (FIGURE 12E-H). These histopathological severities were in a dose-dependent manner and the scorings are depicted in TABLE 6.

Discussions

The continuous usage of pesticides pollutes the environment and cause a detrimental effect on the health system, Half-life of acephate dissipation in aerobic and anaerobic soils is less than 3 and 6 days, respectively, and degrades to immobile compounds in 20 days [28]. The toxic doses of acephate were selected based on the basis of residues and predicted concentrations reaching non-target species through feed and water after one to

two consecutive half-lives. In this context, we hypothesized that avian species, due to having an important immune organ bursa of Fabricius, may act as an indicator for detecting environmental contaminants present in low concentrations. Often it has been observed that environmental toxicants present at low concentrations affect immune organs, which leads to immunosuppression. Here, we chose cockerel as the most appropriate and widely used experimental model to investigate the immunotoxic effects of acephate. We recorded a substantial decrease in cockerel live body weights as well as the weights of immune organs such as the thymus, spleen, and Fabricius bursa, and these findings were corroborated by other studies in broiler chickens [29]. The weights of the spleen, thymus, and bursa of Fabricius were found to be associated with the dosage and length of acephate exposure, as well as the relative immune status of the animals. Our results indicate a dose-dependent relationship between reduced organ weights and humoral and cell-mediated immunity in cockerels from treatment groups relative to controls after 8 weeks of exposure. Blood provides a significant profile for studying the toxicological effect on animal tissues. In current exploration, acephate administration negatively affects Hb, TEC, PCV, and TLC, these changes correlated with dose and duration of exposure [30]. This shows the target action of acephate on erythropoiesis, which eventually results in hemolysis and anemic conditions of cockerel. White blood cells (WBCs), also called leukocytes, are part of the body's immune system, which helps to fight infection and other diseases. In cockerels, there are five major kinds of leukocytes (heterophiles, eosinophils, basophils, lymphocytes, and monocytes), each responding differently to protect against foreign organisms. We have analyzed the reduced level of TLC (leukopenia) in the acephate exposure cockerels, and this shows hematological dysfunctions and immune deficiency. Our previous study has shown a similar finding (leukopoiesis suppression) after short term exposure (28 days) of acephate [10] and also this results in agreement with findings of imidacloprid exposure in male WLH cockerels [31]. On the contrary, we observed significantly increased heterophile, monocytes, eosinophil and basophil percentage in blood smear of acephate treatment groups. The most common and important cause of heterophilia is a systemic infection and these findings are corroborated with histopathological changes of kidney *viz.*, intertubular haemorrhages, tubular degeneration, tubular dilatation and fatty alterations (data not shown). Although the exact mechanism for this effect is not yet well-known but earlier investigators have reported that serine hydroxylases associated with cell membranes of lymphocytes and monocytes altered in such a way that may induce structural/functional changes in immune cells including lymphoproliferation in response to antigens [32]. The albumin globulin ratio and total serum proteins can provide useful information about the function of the liver and lymphocytes. Our findings show that after acephate intoxication, total protein and the albumin/globulin ratio both drop significantly. The amounts of globulin, on the other hand, were insignificant. As a result of its stressogenic effects, acephate can impair protein metabolism and liver functions, affecting cockerel weight gain (Supplementary FIGURE 1), and these are typical insecticide side effects. As compared to controls, the homogenates of Acephate exposed and non-exposed chicken serum reflected the presence of varying intensity protein bands in a dose dependent manner. Following 60 days of exposure to various concentrations of Acephate in developing WLH cockerels, the electrophoretic pattern of chicken serum protein revealed clear differences in band numbers and molecular weights. These findings support previous research that showed the appearance of a new protein band with a molecular weight of 74 KDa after 15 days of cyanophos insecticide treatment of Albino rats [33]; pyriproxyfen, Bioneem, Garlic Gard, Nat-1, and deltamethrin caused an increase in the amount of bands in separated soluble proteins of *Spodoptera littoralis* eggs [34]; Glyphosate and CdCl₂ displayed marked differences in serum enzyme activity and polyacrylamide gel electrophoretic patterns of serum proteins in an in vitro sample, but not paraquat [35]. The appearance of a new protein band, similar to the

cyanophos pesticide in human serum protein, could be due to the Acephate's affinity for either high molecular weight lipoprotein or binding to transferrin or albumin [36]. Our findings indicate a strong link between inhibition of the activity of the brain and erythrocyte AChE and the dose of acephate. These findings are in agreement with other reports [37]. Also, this result is new and there are limited reports on AChE inhibition activity after subchronic exposure of acephate. Reduction of cholinesterase is common in all organophosphate pesticides in both the blood and tissues. Cholinesterase inhibition causes the accumulation of acetylcholine in synapses, resulting in nervous system (neurobehavioral activity) and gastrointestinal tract (hypophagic activity) dysfunctions [38][39]. The DTH and foot web index assay in response to DNCB dye and PHA-P, respectively, is of great usage for assessing the overall competency of cell-mediated immunity within the host and is an inflammatory response mediated by T-cells. Subchronic acephate exposure suppressed the foot web index at 12, 24, 48 and 72 h post-injection PHA-P. The findings indicate that exposure to acephate at moderate and high toxic dose rate induced dysfunction of another T-cell subpopulation (T effector cells) and toxic to cell-mediated immune response. These cells produce various cytokines following interaction with a particular antigen that plays a crucial role in DTH. In the DTH reaction, the primary lymphocyte response appeared to be responsible for the accretion of mononuclear cell infiltrates, interaction and increased vascular permeability that occurs in the vicinity of a stimulus [6]. This finding gets further support as we reported a reduced number of total leukocyte counts and absolute counts of lymphocytes in groups treated with acephate. We recorded significantly increased levels of antibodies against ND vaccine (RD-F), which were contained in serum obtained from low-dose groups of cockerels. However on days 40 and 60 of the experiment, these were significantly lower in subsistence treatment groups. The decline in vaccine response suggests that subchronic acephate exposure has impaired the production of B-cell antibodies and memory response [40]. This finding led to an obvious immunostimulation and subsequent immunosuppression at minimal and higher acephate concentrations. The exact mechanism of this immunosuppression has not been defined. The subchronic acephate exposures resulted in a significant reduction in peripheral blood lymphocyte and mononuclear splenic lymphocyte proliferation in response to antigen LPS and RD-F and mitogen Con A. The effect was noticeable after 20 days of exposure in all treatment groups and persisted until the end of the study except for a significant increase in the low toxic dose treatment group at day 20 [41]. A significant decrease in B-lymphocyte proliferation suggests the decreased capacity of B-lymphocytes to form clones and turn them into plasma cells. Plasma cells are responsible for the secretion of antibodies, suggesting that B-lymphocytes have become less receptive to antigen, leading to suppression of humoral response. The impaired humoral response could increase the risk for vaccine failure and susceptibility to infection. The decrease in cockerel lymphocytes proliferation in response to antigen LPS and RD-F and mitogenic Con-A may be indirectly correlated with the decrease in proinflammatory cytokines, lymphocyte enumeration, and differentiation. However, the mechanisms involved in the above-mentioned effects are not well understood. All the treatments in this study significantly suppressed nitrite production by peripheral blood and splenic mononuclear lymphocytes stimulated with antigen (RD-F) and LPS and mitogen Con A. Supporting our results, it has been reported that rats exposed to a low level of acephate inhibited iNOS production which was also similar to iNOS production by splenic mononuclear splenocytes stimulated with LPS [42]. It has been indicated that a suppressive effect on NO production could take place at different molecular levels [43]. Phagocytic cells are considered to be an important component of the defense mechanisms, since they act against any foreign invasion not only to kill and remove them from the body, but they also act as antigens presenting cells and actively participate in the specific immunity. In this study, all treatments significantly suppressed the generation of nitrate by splenic mononuclear cells stimulated by the mitogen Con-A, except for a

non-significant increase in cockerels in the low-toxic dose group at 12 h of stimulation. Similar to the present observations on the effect of acephate on lymphocyte activity, significant depression in the functional activity of macrophages as assessed by the nitrobluetetrazolium reduction test has been reported in broiler chickens fed a diet carbaryl at 20 ppm [44]. Contrary to the present findings in chickens, the NBT test showed a decrease in the number of active phagocytic cells in spleen at 0.1 µg/egg and 0.05 µg/egg doses of combination insecticide treatment, while 0.01 µg/egg did not show any changes [45]. Decrease in lymphocyte nitrate production (NBT) indicates the reduced functional status of phagocytic cells. Impaired phagocytosis may also be due to the adversely modulated immunogenic potency of lymphocytes. Reduction in the number of active phagocytic cells in Acephate treated birds may also lead to a decrease in natural resistance or innate immunity to infections. The micronucleus test is one of the most reliable cytogenetic bioassays to detect mutagenic environmental agents [46]. Micronuclei indicate acentric chromosome fragments or whole chromosomes that have not been incorporated into the main nucleus at cell division. Significant induction of MN in all three doses was observed in the current study in cultured splenocytes. The significant increase in acephate-induced MN in cultured splenocytes demonstrates its genotoxicity in the chick. Similarly, increases in acephate-induced small and large micronucleus in cultured splenocytes have been observed in chicks [47]. Any agent capable of inducing micronucleus is considered to be a clastogen or aneugen [48]. Significant increases in small and large acephate-induced micronucleus in splenocytes indicate the clastogenic and aneugenic potential of the chick. DNA ladder assays were performed to determine apoptotic or necrotic type of specific cell death which resulted in cleaving of cockerel splenic DNA into 180 bp as a ladder pattern from treatment groups in comparison to the control groups. The presence of the DNA ladder in splenocytes confirmed the acephate-induced apoptotic death. DNA cleavage has been suggested as one of the first irreversible and characteristic phenomena that trigger apoptosis in fully grown immune cells [10]. The degree of DNA smearing is indicative of necrotic cellular events, including spontaneously splitted DNA. In the course of time the intensity of smearing increased. We carried out histopathological evaluations of lymphoid organs, spleen, thymus, and bursa of Fabricius to assess acephate induced immunotoxic effect. The results showed that necrotic cells were increased in the splenic corpuscles dose dependently, which indicates that excessive necrosis in the spleen would eventually compromise the B and T cell based immune response. Severe lymphoid depletion of follicles with reduced follicular size and increased stromal follicle means variations in cortical lymphoid and development of interfollicular fibrosis. Similarly, lymphocyte depletions in focal areas of spleen and bursa of Fabricius have been observed in broiler chickens [49]. Acephate's immunotoxic effect is consistent with our previous research on other organophosphate pesticides. It has been shown that acephate inhibits both humoral and cellular immunity among chickens [10]. There is little information about the way acephate pesticides act on the immune system. We suggest that inhibition of AChE can be the hierarchical event that contributes to the production of immunotoxicity in cockerels exposed to acephate (Supplementary FIGURE 2). Immunosuppression in cockerels may result from direct action of acetylcholine on the immune system, and/or may be due to the toxic chemical stress related cholinergic poisoning. This is unlikely to require a direct cholinergic process, since the introduction of multiple cholinergic agonists will not inhibit immune reactions in vitro. Carbamates and organophosphate have been reported to work through serine esterase inhibition to deflect the generation and manifestation of an immune response [42].

Conclusions

In conclusion, subchronic exposure to acephate at concentrations relevant to the environment significantly altered the immunological functions tested in cockerels and could pose a significant risk of pathogen infection. Our experimental findings, in summary, endorse acephate's immunotoxic nature. Cellular immunosuppression can be measured almost precisely only by several parameters. Therefore, before concluding, it is necessary to analyze multiple parameters, as set out in this report.

Declarations

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Authors' contributions: All the authors made significant contributions to the work presented in this manuscript. The experiments were supervised by YPS, MKB, RKS, and BCS. The experiment under this project was conceptualized by SMT, MKB, and YPS. The entire experiment under this project was carried out by SMT. The findings were arranged, analyzed and interpreted by SMT, PKG, MKB, BCS, and RHL. The manuscript draft was written by SMT and PKG. The manuscript was read by RHL, LNS and PKG and provided critical assessment and conceptual insights.

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals

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References

1. Anu Kalia, Gosal SK (2011) Effect of pesticide application on soil microorganisms. *Arch Agron Soil Sci* 57(6):569–596
2. Travis W, Reynolds SR, Waddington CL, Anderson A, Chew Z, True, Alison Cullen. Environmental impacts and constraints associated with the production of major food crops in sub-Saharan Africa and south Asia. *Food Security*, 7(4):795–8222015
3. TA Miller (1975) Federal and state pesticide regulations and legislation. *Ann Rev Entomol* 20:119–131
4. Culleen LE. Pesticide registration in the united states: overview and new directions. *Quality Assurance (San Diego, Calif.)*, 3(3):291–299, 1994
5. Mcclintock JTHOMAS, Kough JOHNL, Sjoblad RD (1994) Regulatory oversight of biochemical pesticides by the us environmental protection agency: health effects considerations. *Regul Toxicol Pharmacol* 19(1):115–124
6. Volodymyr I, Lushchak TM, Matviishyn VV, Husak (2018) Janet MStorey, and Kenneth B Storey. Pesticide toxicity: a mechanistic approach. *EXCLI J* 17:1101
7. James R, Roberts, Catherine J, Karr et al (2012) Pesticide exposure in children. *Pediatrics* 130(6):e1765–e1788
8. Mirjana B, Colovic DZ, Krstic TD, Lazarevic-Pasti (2013) Aleksandra M Bondzic, and Vesna M Vasic. Acetylcholinesterase inhibitors: pharmacology and toxicology. *Current neuropharmacology* 11(3):315–335
9. Maryam Hedayatirad A, Mirvaghefi MA, Nematollahi (2020) Mohammad Navid Forsatkar, and Culum Brown. Trans generational disrupting impacts of atrazine in zebrafish: Beneficial effects of dietary spirulina. *Comparative Biochemistry and Physiology Part C. Toxicology & Pharmacology* 230:108685
10. Syamantak M, Tripathi AM, Thaker CG (2012) Joshi, and Laxmi Narayan Sankhala. Acephate immunotoxicity in white leghorn cockerel chicks upon experimental exposure. *Environmental toxicology pharmacology* 34(2):192–199
11. Subir K, Nag, Mukesh K, Raikwar (2008) Organochlorine pesticide residues in bovine milk. *Bull Environ Contam Toxicol* 80(1):5–9
12. NC Jain (1986) Clinical interpretation of changes in leukocyte numbers and morphology. *Schalm's Veterinary Hematology*, Lea and Febiger, Philadelphia
13. Beutler ERNEST, Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U (2001) Anemia due to other nutritional deficiencies. In: *Williams Hematology*, 6th edn. McGraw Hill, New York, pp 472–473
14. Ellman GL, Courtney KD. Andres V, RM feather stone. 1961, a new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7a88-95
15. Glowinski J (1966) The disposition of [3H] norepinephrine, [3H] dopamine and [3H] dopa in various regions of the brain. *J Neurochem* 13:655–669
16. Moacelio V, Silva Filho MM, Oliveira J, ao B, Salles, Vera LF, CunhaBastos (2004) Vicente PF Cassano, and Jayme Cunha Bastos. Methyl-paraoxon comparative inhibition kinetics for acetylcholinesterases from brain of neotropical fishes. *Toxicology letters* 153(2):247–254
17. Huynh V, Chubb RC (1987) The induction of delayed type hyper sensitivity to dinitrochlorobenzene in the chicken. *Avian Pathol* 16(3):383–393

18. Eid E, Haddad, Magdi M, Mashaly (1992) Augmentation of natural cell-mediated cytotoxic activity by supernatant from in vitro mitogen-stimulated, in vivo hormone-treated lymphocytes in immature male (K strain) chickens. *Immunol Investig* 21(4):365–375
19. Sharma JM, Lee LF (1983) Effect of infectious bursal disease on natural killer cell activity and mitogenic response of chicken lymphoid cells: role of adherent cells in cellular immune suppression. *Infect Immun* 42(2):747–754
20. Athena HJ, Ding, Lam KM (1986) Enhancement by interferon of chicken splenocyte natural killer cell activity against marek's disease tumor cells. *Vet Immunol Immunopathol* 11(1):65–72
21. SP SINGH and GK CHOUDHARY (2016) Immune response following prolonged pesticide exposure in cockerels. *Journal of Veterinary Pharmacology Toxicology/June 15(1/77):80*
22. Denise I, Bounous RP, Campagnoli, Brown J. Comparison of MTT colorimetric assay and tritiated thymidine uptake for lymphocyte proliferation assays using chicken splenocytes. *Avian Diseases*, pages 1022–1027, 1992
23. Lepoivre M, Flaman JM, Henry Y (1992) Early loss of the tyrosyl radical in ribonucleotide reductase of adenocarcinoma cells producing nitric oxide. *J Biol Chem* 267(32):22994–23000
24. KUNIHICO OKAMURA, YUKINORI YONEMOTO, HISASHI HI-GASHIIWAI, and AKIRA YAJIMA. Quantitative NBT reduction test with or without stimulation of peripheral polymorphonuclear cells from pregnant women. *The Tohoku journal of experimental medicine*, 151(1):89–95,1987
25. David F, Krahn and KRAHN DF. Chinese hamster cell mutagenesis: a comparison of the cho and v79 systems. 1983
26. Xuesong Liu P, Li P, Widlak H, Zou Xu, Luo, William T, Gar-rard, Wang X. The 40-kda subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. *Proceedings of the National Academy of Sciences*, 95(15):8461–8466, 1998
27. LG Luna. Armed forces institute of pathology (us), armed forces institute of pathology (us) (1968) manual of histologic staining methods of the armed forces institute of pathology. New York, Blakiston Division. pp xii,258, 1968
28. Elizabeth Downing. Environmental fate of acephate.PDF. *Environmental Monitoring and Pest Management*. Department of Pesticide Regulation. Sacramento, CA. 11pp, 2000
29. Umesh K, Garg AK, Pal GJ, Jha, Sanjay B, Jadhao (2004) Haemato-biochemical and immuno-pathophysiological effects of chronic toxicity with synthetic pyrethroid, organophosphate and chlorinated pesticides in broiler chicks. *Int Immunopharmacol* 4(13):1709–1722
30. Khan MZ, Zishan AA, Hrar, Javed MT, Hussain I (2015) Impact of chlorpyrifos on health biomarkers of broiler chicks. *Pestic Biochem Physiol* 122:50–58
31. Tarun Balani S, Agrawal, Thaker AM (2011) Hematological and bio-chemical changes due to short-term oral administration of imidacloprid. *Toxicology International* 18(1):2
32. Asim Shahzad A, Khan MZ, Khan F, Mahmood ST, Gul, Kashif Saleemi M (2015) Immuno-pathologic effects of oral administration of chlorpyrifos in broiler chicks. *J Immunotoxicol* 12(1):16–23
33. Afify AMR, Hossam S, El-Beltagi et al (2011) Effect of the insecticide cyanophos on liver function in adult male rats. *Fresen Environ Bull* 20(4a):1084–1088

34. Zidan ZH, Abdel-Megeed MI, Watson WM, Sobeiha AK (1987) Ovicidal activity of certain mineral oils, organic insecticides and their mixtures against the cotton leaf worm, *spodoptera littoralis*. *Boisd: Lepidoptera:NoctuidaeApplied Entomology Zoology* 22(3):241–247
35. El-Demerdash FM, Yousef MI, Elagamy EI (2001) Influence of paraquat, glyphosate, and cadmium on the activity of some serum enzymes and pro-teín electrophoretic behavior (in vitro). *Journal of Environmental Science Health Part B* 36(1):29–42
36. Larsen GL, Davison KL, Bakke JE, Bass NM. Isolation of pesticide-binding protein from rat blood. 1994
37. EL-Nahhal Yasser and Raed Lubbad (2018) Acute and single repeated dose effects of low concentrations of chlorpyrifos, diuron, and their combination on chicken. *Environ Sci Pollut Res* 25(11):10837–10847
38. Kevin B, Temeyer, Alexander P, Tuckow (2016) Tick salivary cholinesterase:A probable immunomodulator of host–parasite interactions. *J Med Entomol* 53(3):500–504
39. Jinxin Wang J, Matias ER, Gilbert (2019) Tetsuya Tachibana, and Mark A Cline. Hypothalamic mechanisms associated with corticotropin-releasing factor-induced anorexia in chicks. *Neuropeptides* 74:95–102
40. Lee G-H, Choi K-C. Adverse effects of pesticides on the functions of immune system. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, page 108789, 2020
41. Manoj Aggarwal SB, Naraharisetti S, Dandapat GH, Degen, Malik JK (2008) Perturbations in immune responses induced by concurrent subchronic exposure to arsenic and endosulfan. *Toxicology* 251(1–3):51–60
42. Singh AK, Jiang Y (2003) Lipopolysaccharide (LPS) induced activation of the immune system in control rats and rats chronically exposed to a low level of the organothiophosphate insecticide, acephate. *Toxicol Ind Health* 19(2–6):93–108
43. Iris Estrada, Emma S, Calderon-Aranda et al (2002) DDT inhibits the functional activation of murine macrophages and decreases resistance to infection by *mycobacterium microti*. *Toxicology* 174(3):201–210
44. Singh BP, Singhal L, Chauhan RS. Immunotoxicity of carbaryl in chicken. 2007
45. Afrin S, Sohrab MH, Ahmed MS et al (2019) Terpenoids and steroids from the stem bark of *sesbania grandiflora* and biological studies of the plant extracts. *Pharm Pharmacol Int J* 7(6):307–313
46. Sylwester Sommer I, Buraczewska, Kruszewski M (2020) Micronucleus assay: The state of art, and future directions. *Int J Mol Sci* 21(4):1534
47. Mandil R, Prakash A, Rahal A, Singh SP, Sharma D (2020) Rahul Kumar, and Satish Kumar Garg. In vitro and in vivo effects of flubendiamide and copper on cytogenotoxicity, oxidative stress and spleen histology of rats and its modulation by resveratrol, catechin, curcumin and α -tocopherol. *BMC Pharmacology Toxicology* 21:1–17
48. Wilson A, Grabowski P, Elloway J, Ling S, Stott J, Doherty A (2021) Transforming early pharmaceutical assessment of genotoxicity: applying statistical learning to a high throughput, multi end point in vitro micronucleus assay. *Scientific reports* 11(1):1–12
49. Sodhi S, Sharma A, Brar RS (2006) A protective effect of vitamin e and selenium in ameliorating the immunotoxicity of malathion in chicks. *Vet Res Commun* 30(8):935–942

Tables

TABLE 1: Trends of absolute and relative weights of immune organs in white leghorn cockerels upon daily exposure to acephate dissolved in peanut oil over 60 days

Groups	Organ Weight (g)			Organ: Bodyweight (g/100 g body weight)		
	Thymus	Spleen	Bursa of Fabricius	Thymus: Bodyweight	Spleen: Bodyweight	Bursa of Fabricius: Bodyweight
C1	2.19±0.02	9.54±0.17	3.57±0.08	0.24±0.00	1.08±0.04	0.33±0.03
C2	2.16±0.03	9.67±0.17	3.69±0.05	0.25±0.01	1.07±0.04	0.34±0.03
T1	2.25±0.02	9.76±0.15	3.49±0.09	0.25±0.004	1.02±0.02	0.34±0.03
T2	0.70±0.04***	7.33±0.15***	2.17±0.07***	0.12±0.00***	0.97±0.03*	0.24±0.02**
T3	0.29±0.03***	6.25±0.14***	1.01±0.03***	0.05±0.00***	0.93±0.03**	0.16±0.02***

Results (mean ± SEM, n = 10) of absolute organ weight (g) and relative weight in the same column bearing (*) decreased significantly ($p < 0.05$), (**) strongly significantly ($p \leq 0.01$) and (***) profoundly significantly ($p \leq 0.001$) in Duncan's multiple comparison post hoc test.

Note: C1: Control; C2: Vehicle control; T1: LD50/40; T2: LD50/30; T3: LD50/20

TABLE 2: Trends of hematological indices in white leghorn cockerels upon daily exposure to acephate dissolved in peanut oil over 60 days

Parameters	Days	Groups				
		C1	C2	T1	T2	T3
Hb	0	9.53±0.17	9.98 ±0.14	9.90 ±0.13	9.82±0.14	9.88±0.13
	20	10.53±0.17	10.60±0.07	10.77±0.12	10.33±0.15	10.27±0.13
	40	11.37±0.23	11.27±0.19	11.47±0.15	10.70±0.12**	10.53±0.13***
	60	12.10±0.24	12.17±0.20	12.23±0.20	11.33±0.13**	8.90±0.21***
TEC	0	1.68±0.01	1.70±0.01	1.68±0.01	1.68±0.02	1.68±0.02
	20	2.85±0.02	2.88±0.02	2.87±0.02	2.89±0.02	2.88±0.02
	40	3.20±0.07	3.21±0.08	3.23±0.09	3.02±0.03	2.91±0.05**
	60	3.35±0.04	3.40±0.04	3.43±0.05	3.29±0.03	3.22±0.05*
PCV	0	26.67±1.20	26.83±1.28	26.67±1.20	26.83±1.17	26.17±1.64
	20	30.67±1.15	30.17±1.08	31.33±1.20	29.50±1.12	28.00±1.07
	40	34.67±0.92	34.80±1.10	33.77±1.06	31.93±1.05	30.74±1.08*
	60	36.08±0.69	36.04±0.93	35.65±0.89	34.95±0.71	32.35±0.68**

Results (mean ± SEM, n = 10) of Haemoglobin (g %), Total erythrocyte count (1×10^6 cells/ m^3) Total leukocyte count (1×10^3 / μ l) and Packed cell volume (PCV) (%) in the same row bearing (*) diminished significantly ($p < 0.05$), (**) strongly significantly ($p \leq 0.01$) and (***) profoundly significantly ($p \leq 0.001$) in Duncan's multiple comparison post hoc test.

Note: C1: Control; C2: Vehicle control; T1: LD50/40; T2: LD50/30; T3: LD50/20

TABLE 3: Trends of heterophils, lymphocytes, monocytes, eosinophils and basophils counts in white leghorn cockerels upon daily exposure to acephate dissolved in peanut oil over 60 days

Parameters	Days	Groups				
		C1	C2	T1	T2	T3
Heterophils (%)	0	39.17±0.87	39.33±0.84	39.67±0.92	39.33±0.76	39.00±0.82
	20	34.83±0.60	34.00±0.58	33.83±0.79	35.67±0.42	36.33±0.84
	40	33.17±0.60	33.00±0.58	31.83±0.70	36.00±0.37***	39.33±0.62***
	60	32.50±0.76	32.67±0.72	36.33±0.76***	41.67±0.56***	44.50±0.76***
Lymphocytes(%)	0	25.83±0.70	25.50±0.62	25.17±0.79	25.67±0.62	25.33±0.72
	20	58.83±0.70	58.50±0.76	60.67±0.88	56.00±0.73	54.17±0.60**
	40	62.00±0.58	62.67±0.72	64.50±0.76	56.67±0.76***	49.17±0.60***
	60	66.83±0.79	66.67±0.49	64.33±0.33*	52.33±0.43***	45.83±0.60***
Monocytes (%)	0	2.14±0.26	2.13±0.30	2.00±0.22	2.00±0.33	2.11±0.20
	20	2.29±0.36	2.25±0.25	2.57±0.30	2.75±0.37	2.78±0.22
	40	2.29±0.29	2.38±0.18	2.71±0.29	3.25±0.25**	4.11±0.26***
	60	2.43±0.20	2.63±0.18	3.29±0.18*	3.88±0.23***	4.67±0.33***
Eosinophils (%)	0	0.86±0.14	0.89±0.11	0.88±0.13	0.89±0.11	0.90±0.10
	20	1.29±0.18	1.33±0.17	1.25±0.16	1.56±0.18	2.10±0.10***
	40	2.14±0.14	2.11±0.11	2.00±0.19	2.67±0.24	3.30±0.21***
	60	2.14±0.14	2.22±0.15	2.75±0.16*	3.22±0.22***	3.70±0.15***
Basophils (%)	0	Rare	Rare	Rare	Rare	Rare
	20	Rare	Rare	Rare	0.25±0.16	0.38±0.18
	40	Rare	Rare	0.13±0.13	0.50±.19	0.75±0.25**
	60	Rare	Rare	0.25±0.18	0.63±0.16*	1.13±0.15**

Results (mean ± SEM, n = 10) of heterophils (%), lymphocytes (%), monocytes (%), eosinophils (%) and basophils (%) in the same row bearing (*) diminished significantly ($p < 0.05$), (**) strongly significantly ($p \leq 0.01$) and (***) profoundly significantly ($p \leq 0.001$) in Duncan's multiple comparison post hoc test. Rare: Scanty observation.

Note: C1: Control; C2: Vehicle control; T1: LD50/40; T2: LD50/30; T3: LD50/20

TABLE 4: Trends of erythrocyte acetylcholinesterase activity ($\mu\text{Mol ATChI hydrolyzed/min /mg protein}$) and brain acetylcholinesterase activity ($\mu\text{Mol ATChI hydrolyzed/min /mg protein}$) in white leghorn cockerels upon daily exposure to acephate dissolved in peanut oil over 60 days

Groups	Acetylcholinesterase				
	Erythrocyte (nMolATChI hydrolyzed/min/mg protein)				Brain (μ MolATChI hydrolyzed/min/mg protein)
	0 day	20 day	40 day	60 day	60 day
C1	15.16 \pm 0.145	16.25 \pm 0.063	17.57 \pm 0.032	18.86 \pm 0.037	22.4 \pm 0.008
C2	15.54 \pm 0.103	16.62 \pm 0.078	18.24 \pm 0.020	19.08 \pm 0.028	22.6 \pm 0.010
T1	16.22 \pm 0.072	14.36 \pm 0.096	12.12 \pm 0.028***	9.46 \pm 0.029***	19.4 \pm 0.007*
T2	15.01 \pm 0.135	12.12 \pm 0.095**	10.25 \pm 0.027***	8.07 \pm 0.031***	7.5 \pm 0.005***
T3	14.69 \pm 0.101	6.73 \pm 0.089***	5.20 \pm 0.022***	4.17 \pm 0.029***	4.7 \pm 0.003***

Results (mean \pm SEM, $n = 10$) of erythrocyte acetylcholinesterase activity in the same column bearing (**) decreased marked significantly ($p \leq 0.01$) and (***) highly significantly ($p \leq 0.001$) and of brain acetylcholinesterase activity in the same column bearing (*) decreased significantly ($p < 0.05$) and (***) profoundly significantly ($p < 0.001$) in Duncan's multiple comparison post hoc test.

Note: C1: Control; C2: Vehicle control; T1: LD50/40; T2: LD50/30; T3: LD50/20

TABLE 5: Trends of frequency micronuclei formation in cultured splenic lymphocytes of white leghorn cockerels upon daily exposure to acephate dissolved in peanut oil over 60 days.

Groups	Frequency of micronuclei formation in cultured lymphocytes
C1	2.90 \pm 0.46
C2	3.10 \pm 0.23
T1	3.90 \pm 0.31
T2	6.40 \pm 0.43***
T3	7.10 \pm 0.53***

Results (mean \pm SEM, $n = 10$) of frequency of micronuclei formation in the same column bearing (*) decreased significantly ($p \leq 0.01$) and (***) highly significantly ($p \leq 0.001$) in Duncan's multiple comparison post hoc test.

Note: C1: Control; C2: Vehicle control; T1: 1/40th LD50; T2: 1/30th LD50; T3: 1/20th LD50

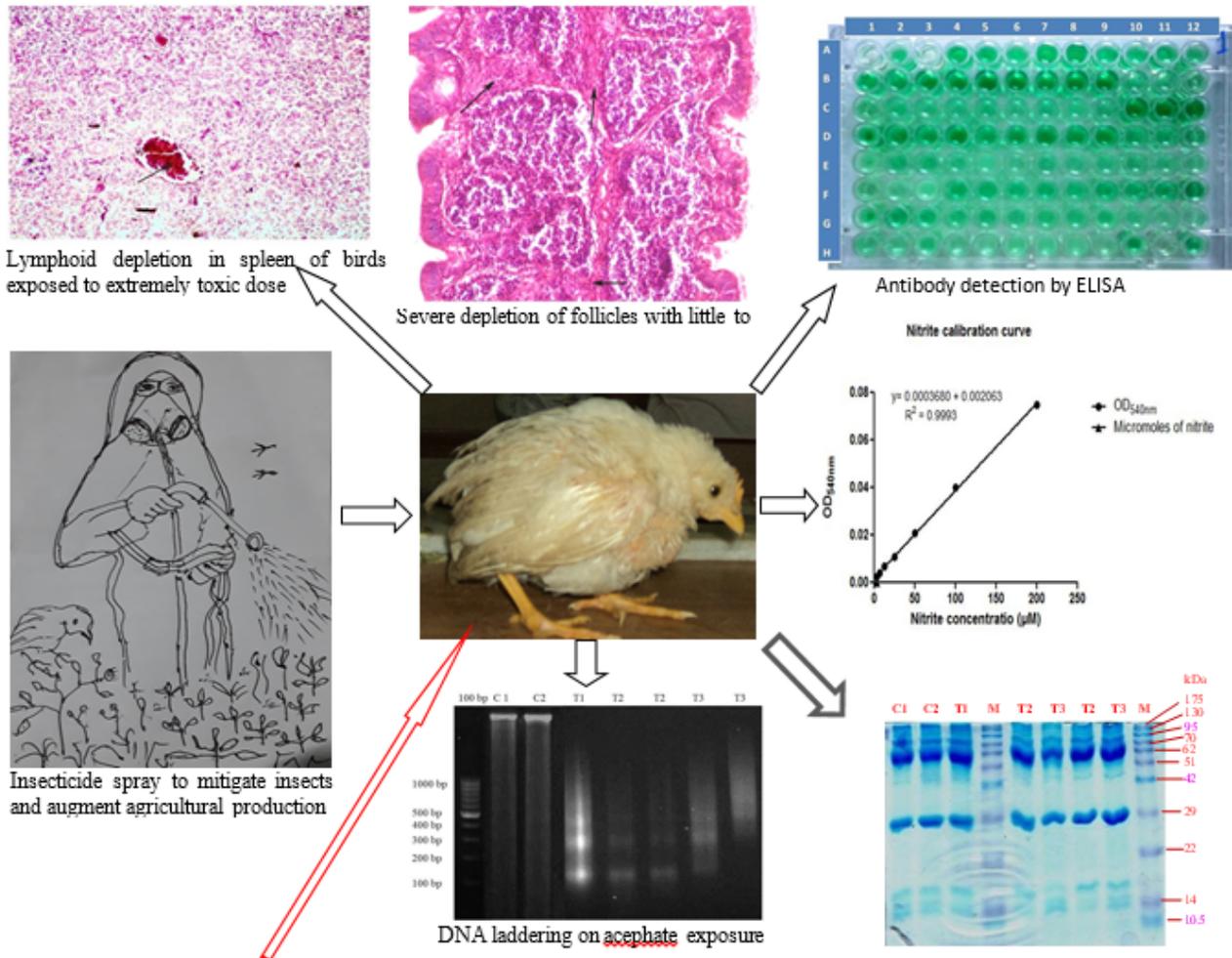
TABLE 6: Trends of pathological score for changes in spleen and Bursa of Fabricius architecture in white leghorn cockerels upon daily exposure to acephate dissolved in peanut oil over 60 days

Groups	Histopathological scores	
	Spleen	Bursa of Fabricius
C1	0.20±0.133	0.10±0.100
C2	0.30±0.153	0.20±0.133
T1	0.80±0.250*	0.50±0.167*
T2	2.80±0.200***	3.10±0.100***
T3	3.60±0.163***	3.70±0.153***

Results (mean ± SEM, n = 10) of spleen and Bursa of Fabricius architecture in the same column bearing (*) decreased significantly ($p \leq 0.01$) and (***) highly significantly ($p \leq 0.001$) in Duncan's multiple comparison post hoc test.

Note: C1: Control; C2: Vehicle control; T1: LD50/40; T2: LD50/30; T3: LD50/20

Figures



CURRENT STUDY: White leghorn chicks sub-chronically exposed to acephate for immunotoxicological study

Figure 1

Reduced Nitrite and nitrate production by peripheral blood lymphocytes stimulated antigen RD-F and LPS and splenic mononuclear cells stimulated with mitogen ConA and reduced humoral immunity in all treatment groups on days 40 and 60 after the start of exposure to acephate compared with the controls.

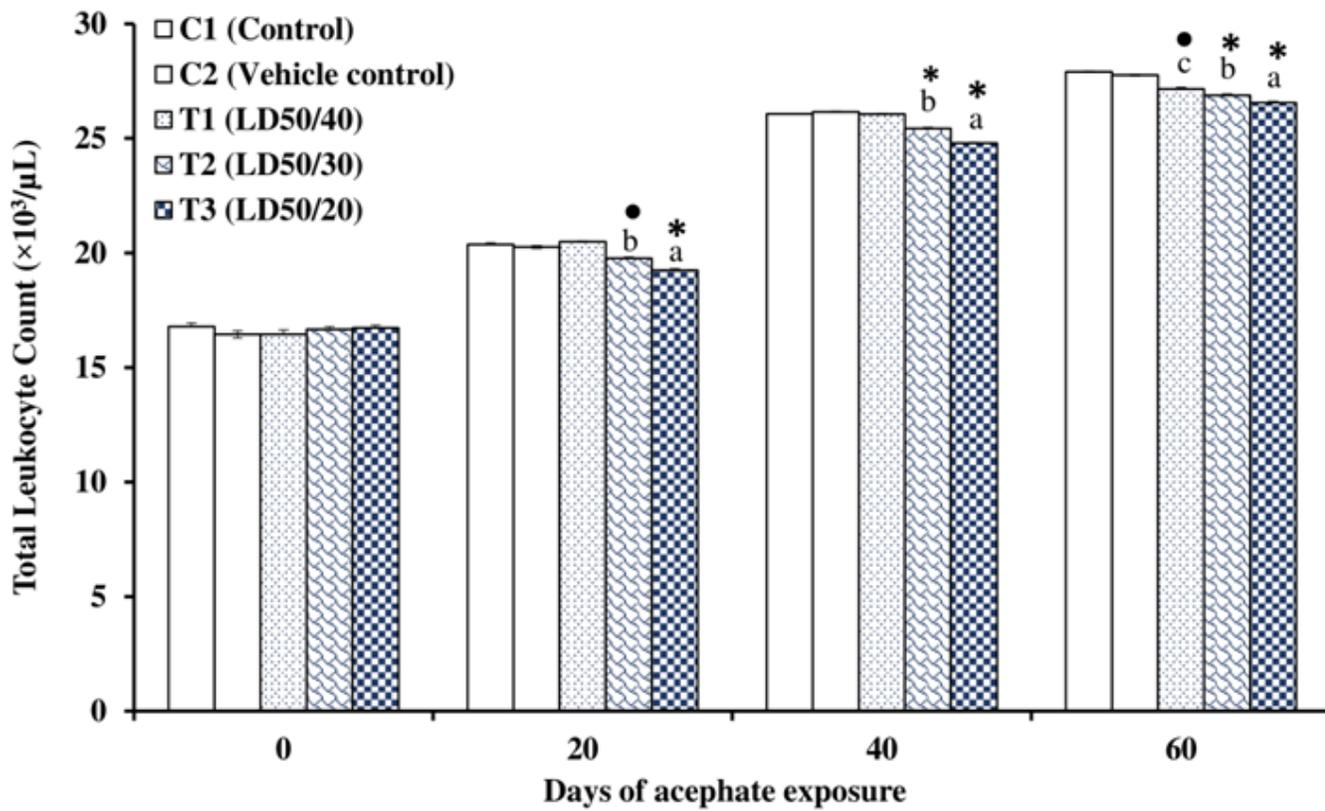


Figure 2

Trends of Total Leukocyte Count (TLC) in cockerels upon daily exposure to acephate dissolved in peanut oil over 60 days. Each bar is a mean \pm SEM of 10 cockerels. Bars with different alphabets display the mean difference at level 0.05 while bars with bullet point (\bullet) and asterisk ($*$) vary significantly ($p < 0.05$) and strongly significantly ($p < 0.01$) in Tukey's HSD post-hoc tests, respectively.

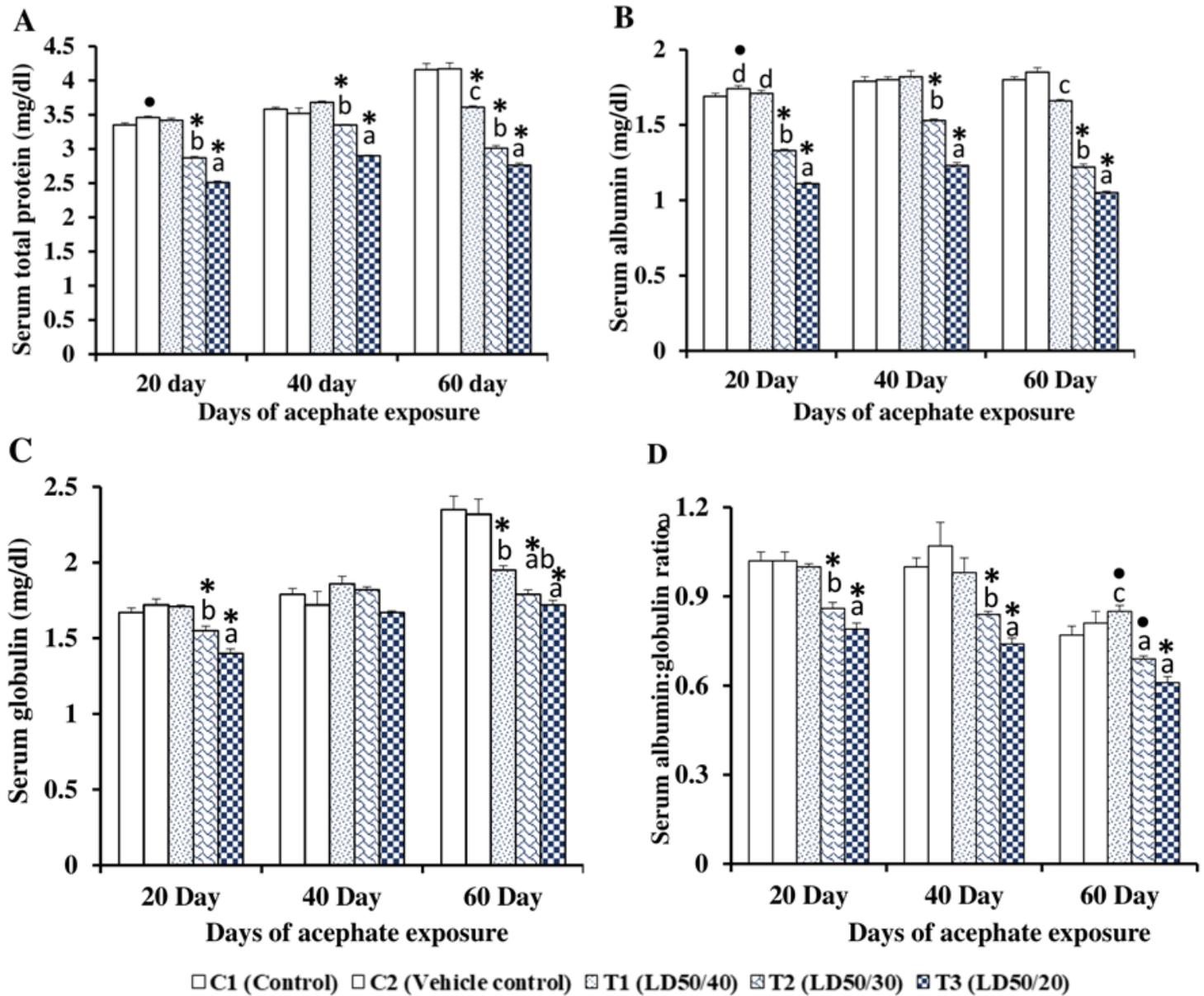


Figure 3

Trends of (A) serum total protein, (B) albumin, (C) globulin, and (D) albumin: globulin ratio in White Leghorn cockerels upon daily exposure to acephate dissolved in peanut oil over 60 days. Each bar is a mean \pm SEM of 10 cockerels. Bars with different alphabet display the mean difference is significant at the level 0.05 whereas bars with bullet point (\bullet) and asterisk (*) vary significantly ($p < 0.05$) and strongly significantly ($p < 0.01$) respectively in Tukey's HSD post-hoc tests.

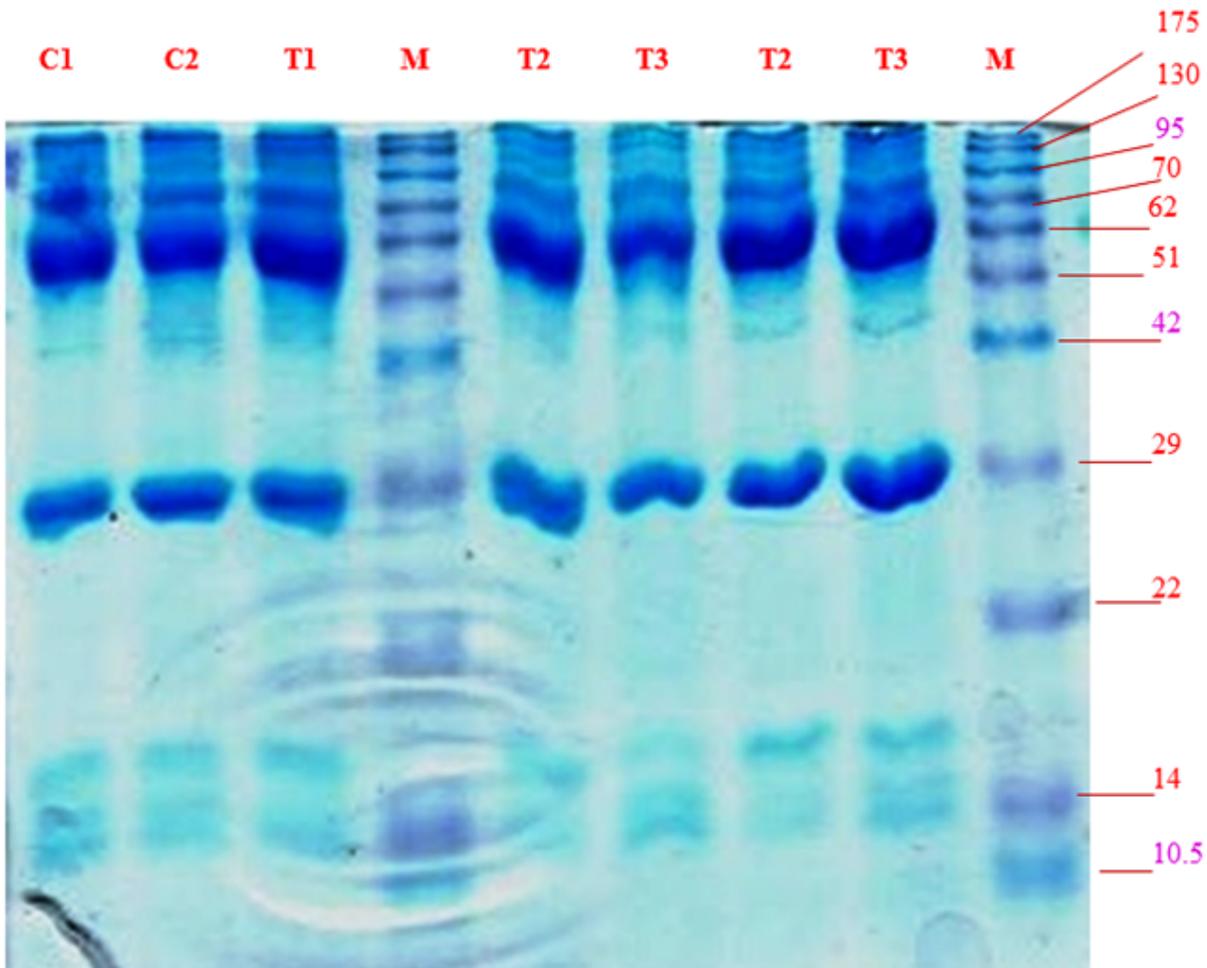


Figure 4

SDS Polyacrylamide-Gel Electrophoresis (SDS-PAGE) of serum protein obtained from cockerels of the White Leghorn cockerels after 60 days of oral acephate exposure dissolved in peanut oil with 12 % resolving and 5 % stacking gel electrophoresis. Lane 4 and 9: marker; Lane 1: plain control; Lane 2: vehicle control; Lane 3: 21.3 mg / kg acephate serum protein band; Lane 5 and 7: 28.4 mg / kg acephate serum protein band and Lane 6 and 8: serum protein band pattern on acephate exposure at 42.6 mg/kg body weight. After exposure to 28.4 mg/kg and 42.6 mg/kg acephate, chicken serum proteins were separated into 9 and 10 MW bands ranging from 15.61-173.95 to 14.65-171.96 KDa, respectively. One different fraction of protein (16.48 KDa) was observed in the serum protein of Acephate exposed chicks at an extremely toxic dose (T3, 42.6 mg/kg) of up to 60 days.

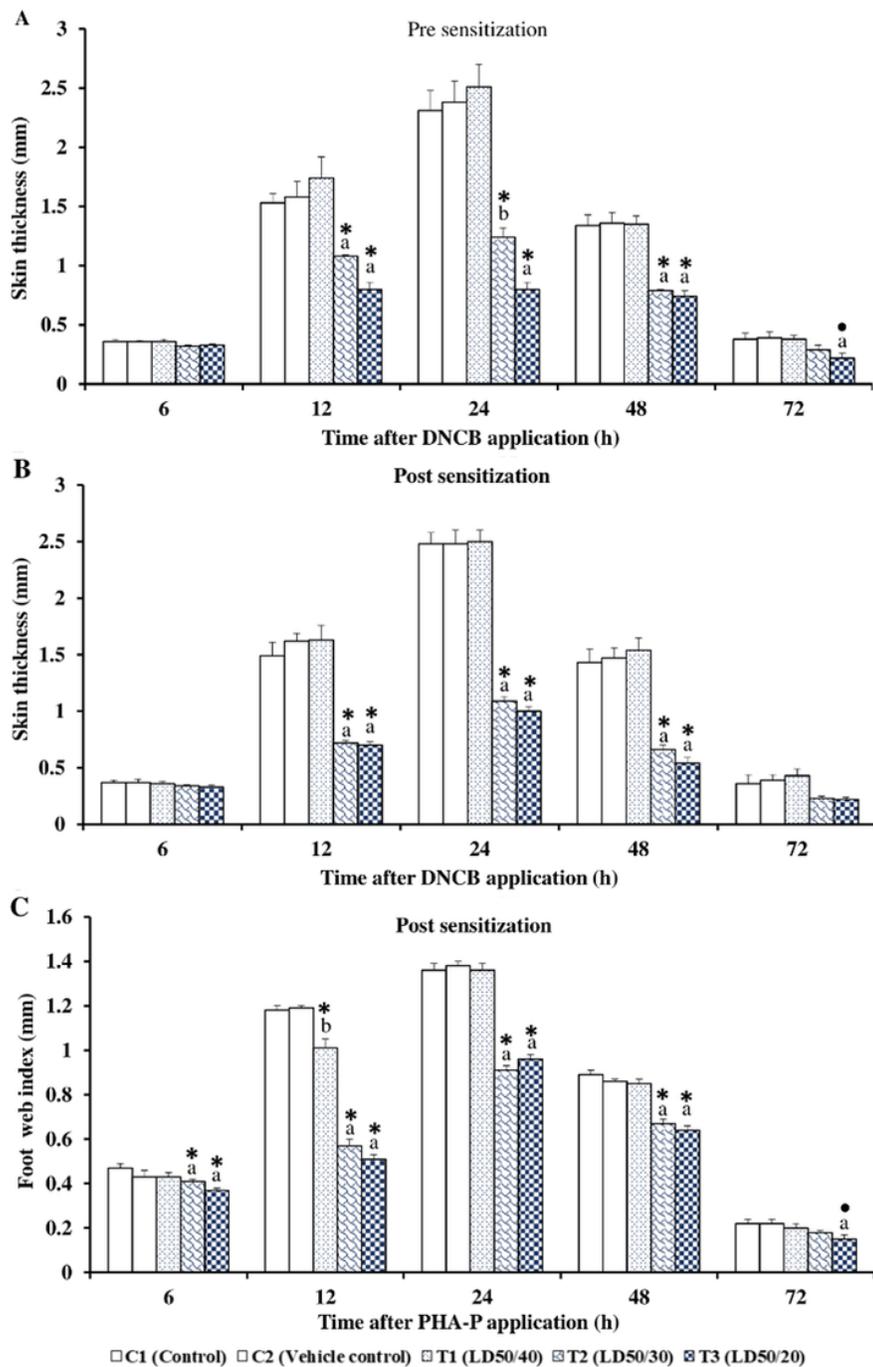


Figure 5

(A and B) Trends of delayed type hypersensitivity reaction in white leghorn cockerels exposed to acephate dissolved in peanut oil upto 60 days, (A) after pre-challenge and (B) post-challenge sensitization with DNCB dye. Bars with distinctive letter set display the mean difference is significant for the level 0.05 while bars with bullet point (•) and asterisk (*) vary significantly ($p < 0.05$) and strongly significantly ($p < 0.01$) respectively in Tukey's HSD post-hoc tests. C. Delayed type hypersensitivity response to PHA-P in white leghorn cockerels following repeated exposure to acephate dissolved in peanut oil over 60 days. Each bar reflects the mean \pm SEM of the foot web index in mm ($n = 10$). Bars with distinctive letter set display the mean difference is

significant for the level 0.05 whereas bars with bullet point (•) and asterisk (*) vary significantly ($p < 0.05$) and strongly significantly ($p < 0.01$) respectively in Tukey's HSD post-hoc tests.

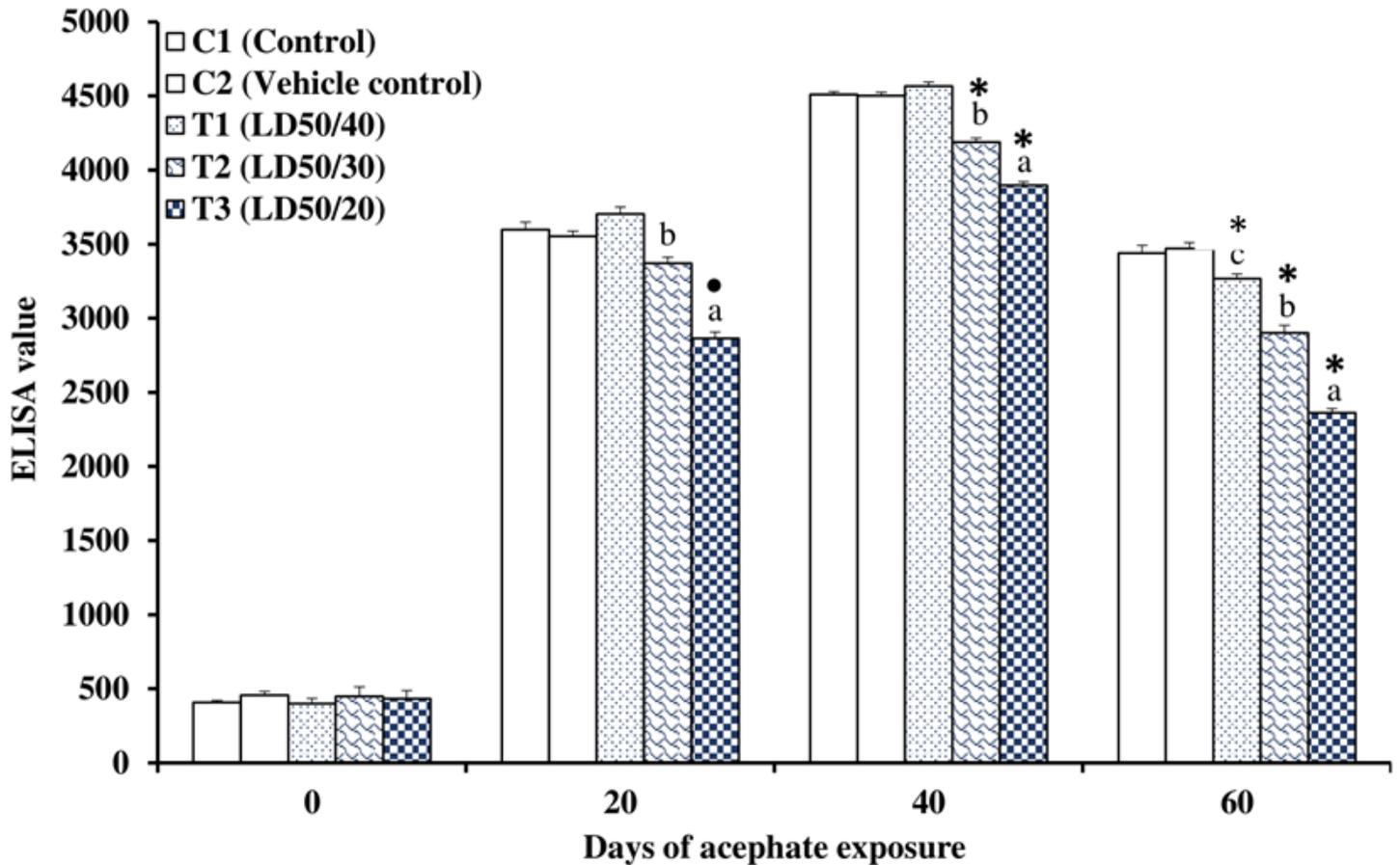


Figure 6

Trends of antibody response in white leghorn cockerels to RD-F following repeated acephate exposure dissolved in peanut oil upto 60 days. Each bar reflects the mean \pm SEM of ELISA values ($n = 10$). Bars with distinctive letter set display the mean difference is significant at the level 0.05 while bars with bullet point (•) and asterisk (*) vary significantly ($p < 0.05$) and strongly significantly ($p < 0.01$) respectively in Tukey's HSD post-hoc tests.

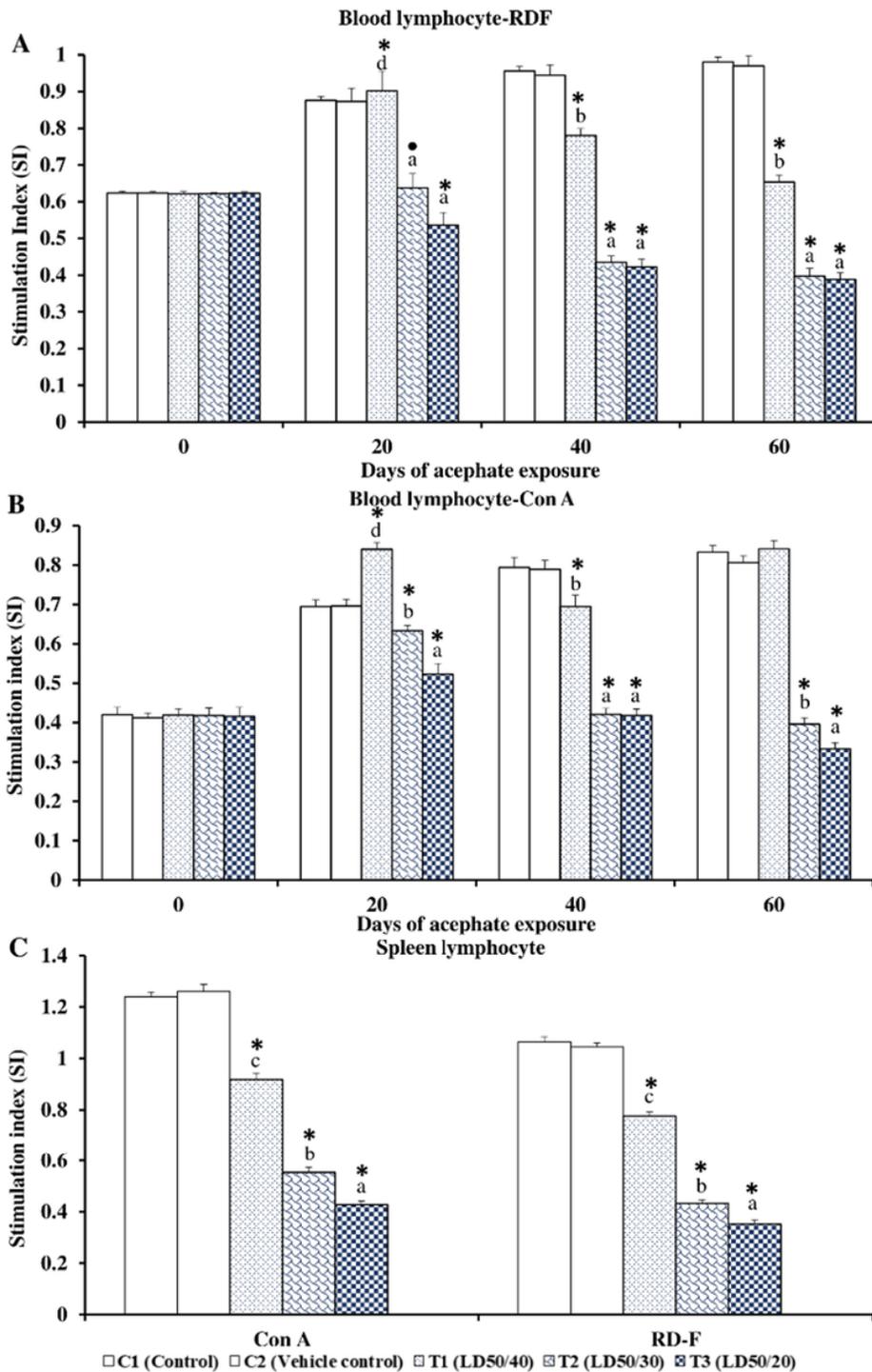


Figure 7

(A and B) Trends of peripheral blood lymphocyte proliferation (Stimulation Index) in white leghorn cockerels following daily exposure to acephate dissolved in peanut oil over 60 days after stimulation with (A) mitogen Con A and (B) RD-F antigen. Bars with distinctive letter set display the mean difference is significant at the level 0.05 while bars with bullet point (•) and asterisk (*) vary significantly ($p < 0.05$) and strongly significantly ($p < 0.01$) respectively in Tukey's HSD post-hoc tests. C. Splenic proliferation in white leghorn cockerels exposed to acephate dissolved in peanut oil over 60 days following stimulation with antigen RD-F and mitogen Con A. Bars with distinctive letter set display the mean difference is significant at the level 0.05 while bars with bullet point

(•) and asterisk (*) vary significantly ($p < 0.05$) and strongly significantly ($p < 0.01$) respectively in Tukey's HSD post-hoc tests.

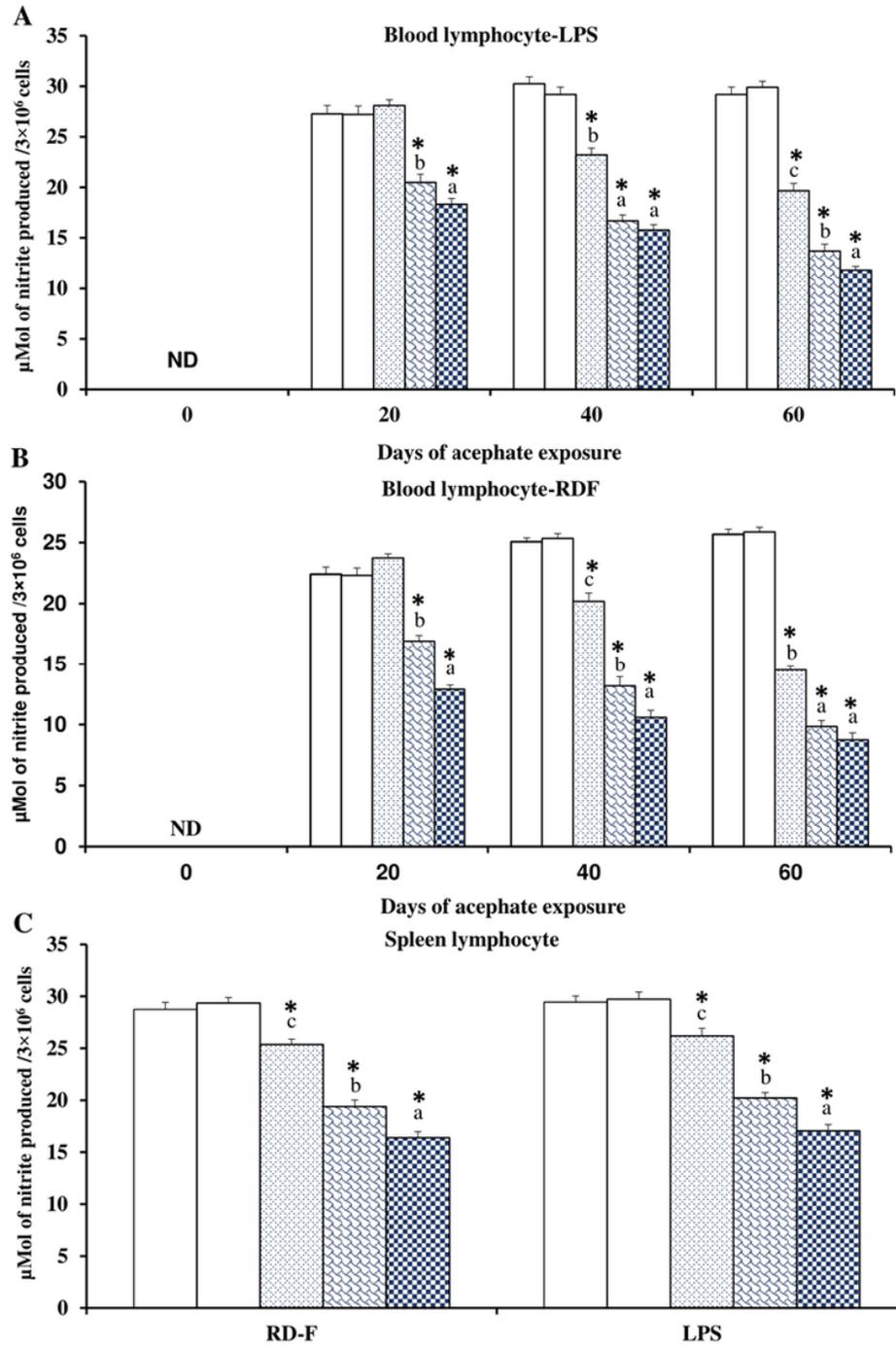


Figure 8

(A and B) Nitrite production by peripheral blood mononuclear cells of white leghorn cockerels following repeated acephate exposure dissolved in peanut oil over 60 days, after stimulation with (A) antigen LPS and (B) RD-F. Bars with distinctive letter set display the mean difference is significant at the level 0.05 while bars with bullet point (•) and asterisk (*) vary significantly ($p < 0.05$) and strongly significantly ($p < 0.01$) respectively in Tukey's HSD post-hoc tests. C. Nitrite production by splenic mononuclear cells of white leghorn cockerels

exposed to acephate dissolved in peanut oil over 60 days after stimulation with antigen RD-F and LPS. Bars with distinctive letter set display the mean difference is significant at the level 0.05 while bars with bullet point (•) and asterisk (*) vary significantly ($p < 0.05$) and strongly significantly ($p < 0.01$) respectively in Tukey's HSD post-hoc tests.

Spleen lymphocytes - ConA

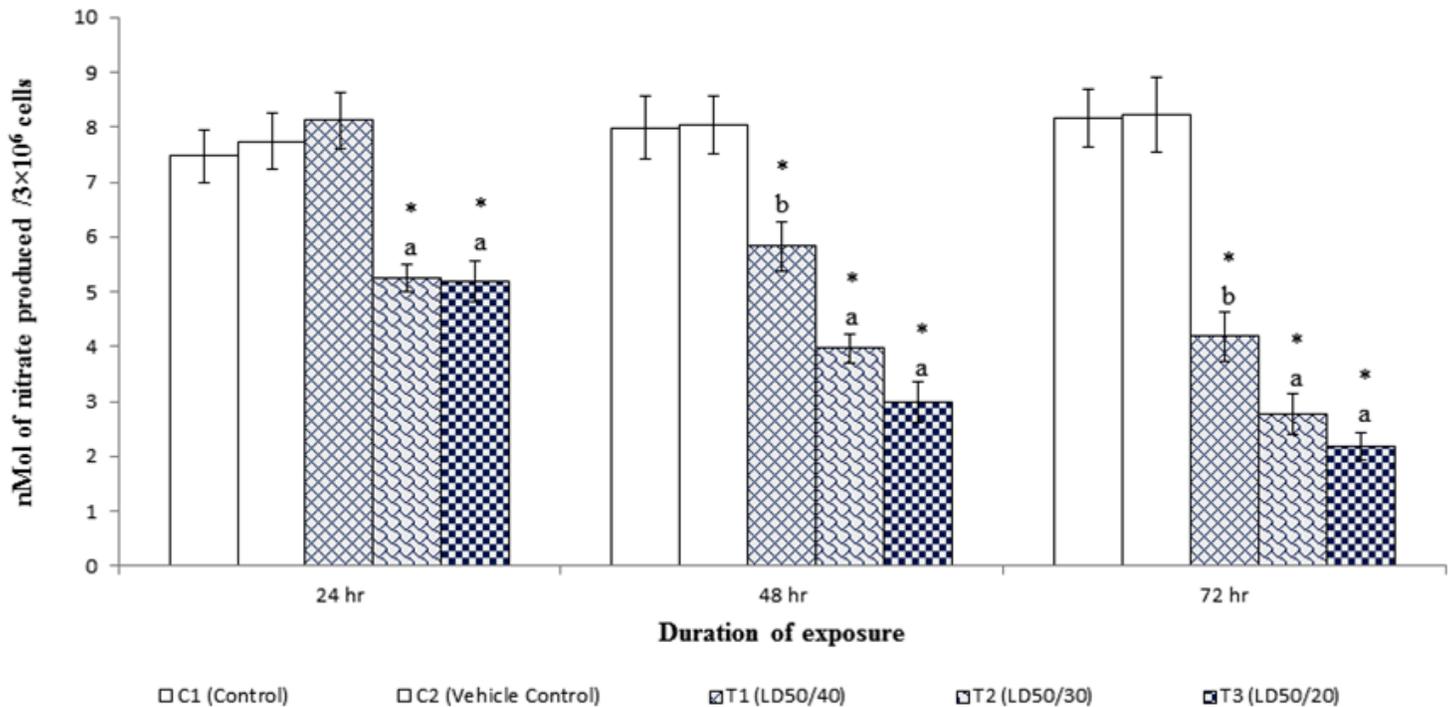


Figure 9

Nitrate production by splenic mononuclear cells of white leghorn cockerels following repeated acephate exposure dissolved in peanut oil over 60 days, after stimulation with mitogen ConA. Bars with distinctive letter set display the mean difference is significant at the level 0.05 while bars with asterisk (*) vary significantly ($p < 0.05$) and (**) strongly significantly ($p < 0.01$) respectively in Tukey's HSD post-hoc tests.

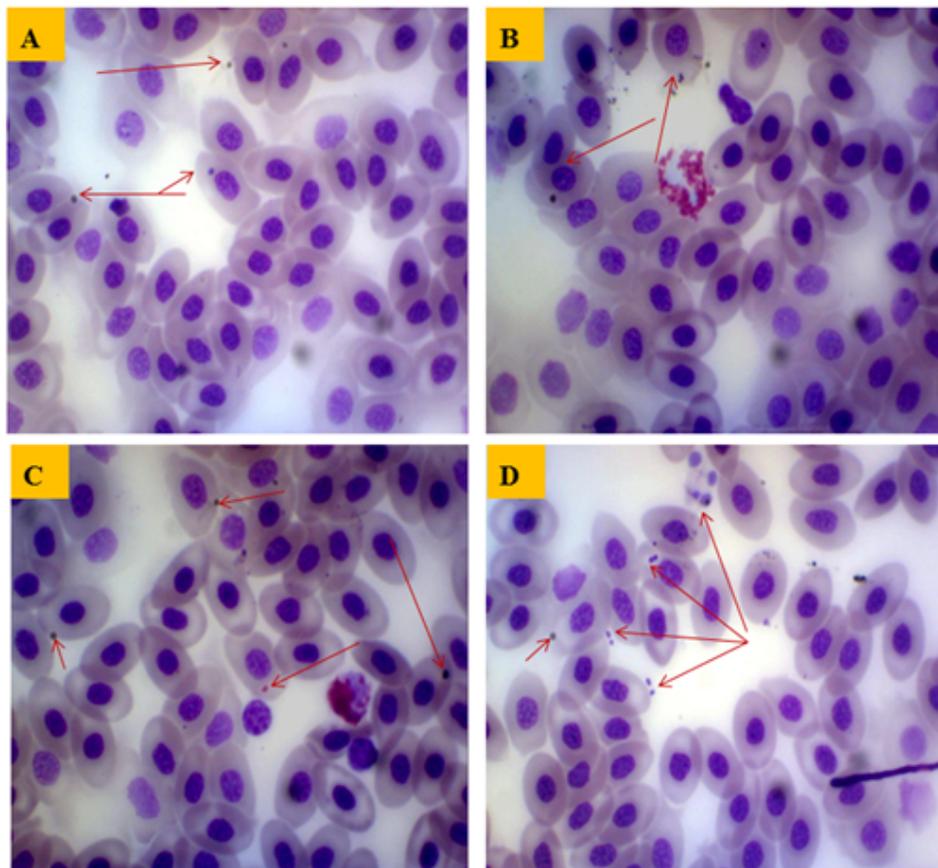


Figure 10

(A-D) Trends of frequency micronuclei formation in cultured splenic lymphocytes of white leghorn cockerels upon daily exposure to acephate dissolved in peanut oil over 60 days. Each bar reflects the mean \pm SEM of frequency micronuclei (n = 10). Bars with distinctive letter set display the mean difference is significant at the level 0.05 while bars with bullet point (\bullet) and asterisk ($*$) vary significantly ($p < 0.05$) and strongly significantly ($p < 0.01$) respectively in Tukey's HSD post-hoc tests.

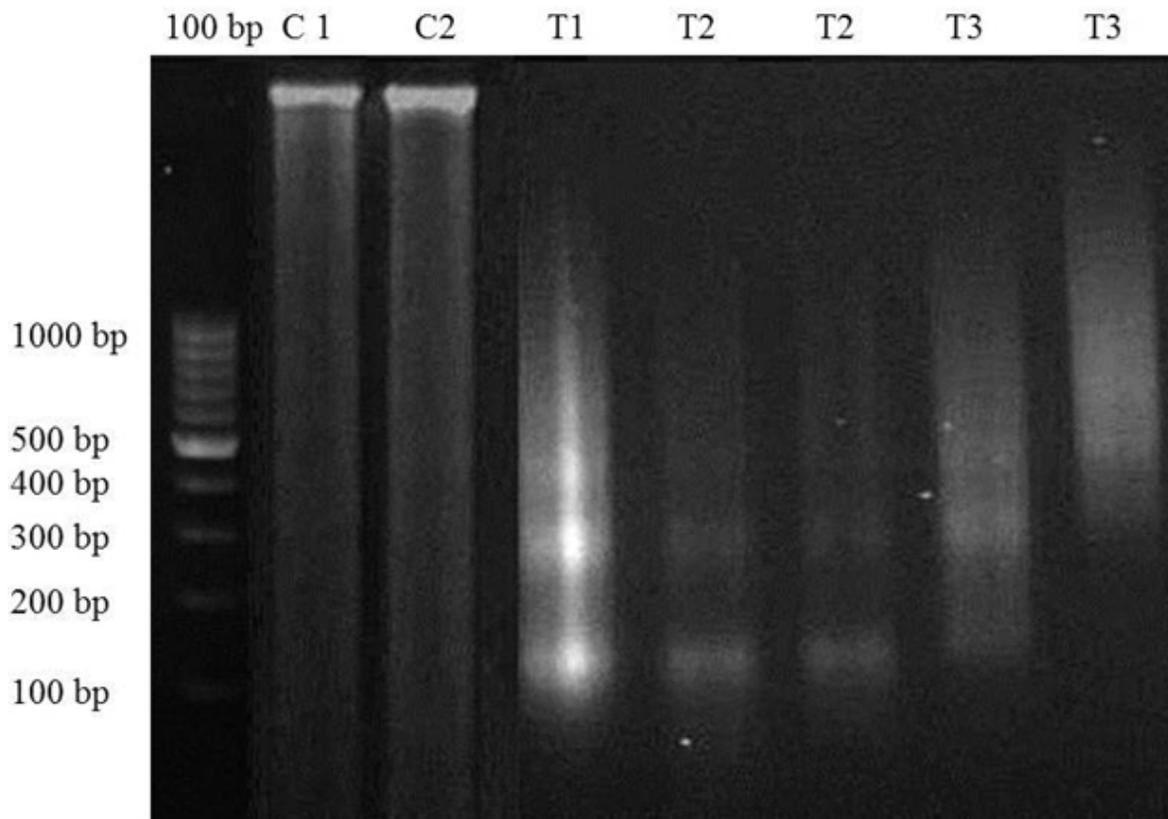


Figure 11

DNA study of representative splenocytes obtained from cockerels of the White Leghorn cockerels after 60 days of oral acephate exposure dissolved in peanut oil with 1 % agarose gel electrophoresis. Lane 1: marker (100 bp); Lane 2: plain control; Lane 3: vehicle control; Lane 4: 21.3 mg / kg acephate ladder; Lane 5 and 6: 28.4 mg / kg acephate ladder and Lane 7 and 8: DNA laddering on acephate exposure at 42.6 mg/kg body weight.

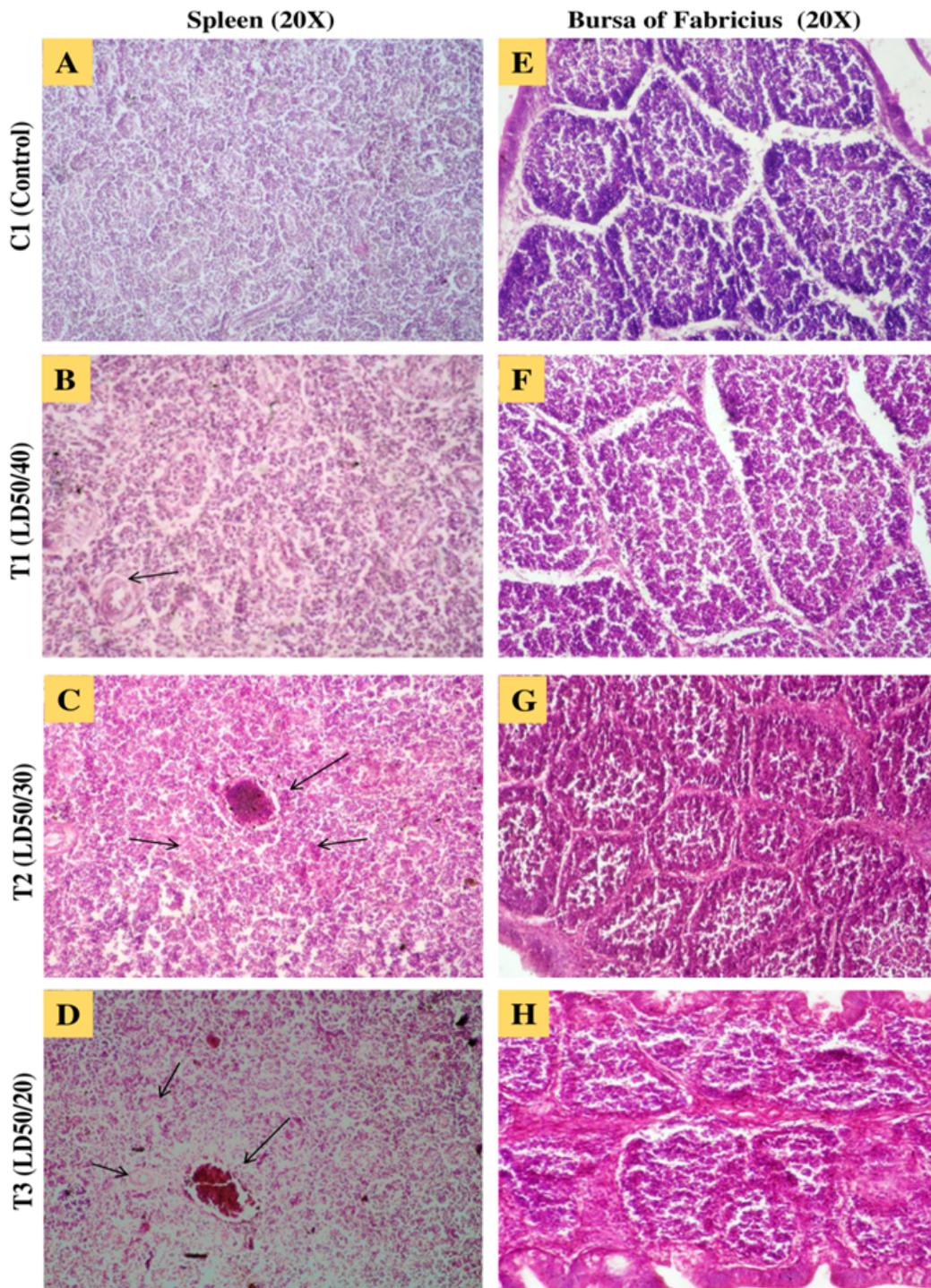


Figure 12

Histopathologic (H&E staining, X 200) appearance of the cockerel spleen (A-D) and Bursa of Fabricius (E-H). A. Photomicrograph of spleen structure showing the normal distribution of lymphocytes and follicles. B. At low toxic dose, there is slight increased cellularity and compartment size especially in marginal zones and follicles. C. At medium toxic dose, there is decreased cellularity and compartment size, congestion (arrow) and mild lymphoid depletion, especially in marginal zones and follicles. D. At extremely toxic doses, there is decreased cellularity and compartment size, congestion (arrow) and severe lymphoid depletion especially in marginal zones and follicles. E. Photomicrograph of bursa of Fabricius showing the normal distribution of lymphocytes,

follicles and stroma F. Lymphoid depletion and mild variation of cortical lymphoid tissue. G. Depletion of lymphoids and moderate variation of cortical follicle tissue and medullary regions, and increased stroma among follicles. H. Severe depletion of follicles with little to no cortex, increased follicle stroma and reduced follicle size.

Supplementary Files

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- [SupplementaryMaterial.docx](#)