CHAPTER-2

COMBINATION EFFECT OF CHLORPYRIFOS AND LEAD AT THE BIOCHEMICAL LEVEL BY SINGLE AND NONLETHAL HIGH DOSE LEVELS

Introduction

Measurable biochemical alterations in laboratory animals on exposure to environmental chemicals /xenobiotics serve as a tool in the overall assessment of risks and hazards on humans and animals. Acute exposure to pesticides and heavy metals at higher dose levels reveal altered physiological functions in blood and serum/plasma components many a times. Clinical Pathology tests used in toxicology studies usually involve hematological and clinical chemistry parameters. The results of these tests are used to identify general metabolic and pathological processes. The study of the effect of a mixture of chemicals on clinical pathology parameters helps scientists and practitioners to understand the interaction or variability's. Hence, the present study was undertaken to evaluate the effect of a combination of chlorpyrifos (an OP pesticide) and lead (a heavy metal) on hematology and clinical chemistry parameters in the Wistar rats.

Materials and Methods

The clinical pathology estimations were carried out in animals after single dose exposure to chlorpyrifos and lead acetate. On 3rd and 15th days of experimental period, blood was collected from all animals. Animals were anaesthetized using anesthetic ether and blood was collected by ocular sinus

puncture. Nearly 0.5 mL of blood for hematology, approximately 0.5 -1.0 mL for RBC acetylcholine esterase estimation and 2.0 mL for clinical chemistry parameters were collected in respective labeled vials/tubes. Hematology vials containing about 50 µL of 4 % EDTA solution were dried in oven at 40 °C for over night. About 100 µL of EDTA was added to centrifuge tubes for RBC cholinesterase estimation and dried in oven for 2 days.

The number of animals, dose levels and experimental procedure used in the study are mentioned in the chapter 1.

Hematology

Hematological Parameter

Erythrocytes
Hemoglobin
Hematocrit
MCV (Mean Corpuscular Volume)
MCH (Mean Corpuscular Hemoglobin)
MCHC (Mean Corpuscular Hemoglobin Concentration)
Platelets
Clotting Time
Total WBC Count
Differential Leucocyte Count

Instrument Used

Hematological parameters i.e., WBC, RBC, Hb, HCT, MCV, MCH and MCHC were analysed in hematology analyzer, Sysmex K-1000. Sysmex K-1000 counts and sizes the blood cells and platelets using the electronic resistance detection method. Sysmex K 1000 is composed of hydraulic, pneumatic and electronic systems. The hydraulic system performs aspirating, dispensing, diluting, mixing and hemolyzing functions. The electronic system analyses and computes signal from specimens processed by the hydraulic system and obtained results are on printed on the built in printer. The pneumatic

system produces both the constant air pressure and vaccum required for operating valves and moving samples through the hydraulic system. The electronic system controls test sequence and test data checking and performs quality control under microprocessor control (Sysmex Operator's Manual K-1000, 1998).

Principle of Cell Counting and Sizing

The blood cells and platelets are counted by detecting a difference in conductivity between the particles and the diluent in which they are suspended. Its operating principle is as follows.

1) Blood cells are diluted in an electrically conductive diluent. As there are large differences between conductivity or resistance of cells and diluent, the cells can be enumerated in terms of both quantity and volumetric size by detecting and measuring this difference in conductivity.

2) Blood cells suspended in the diluent are aspirated through transducer aperture. Inside and outside of the transducer there are electrodes with constant DC flowing from internal electrode to external electrode. When a blood cell passes through the aperture, the electrical resistance between the electrodes increases causing a change in voltage between the electrodes which is proportional to the resistance change. The volume of the cell passing through the aperture is proportional to this voltage change. The mathematical expression of this occurrence is from Ohm'law:

$$E = I \times R$$

Where, E = voltage, I = current, R= resistance.

Since I is constant, voltage E is proportional to resistance R which is proportional to cell volume.

Hemoglobin Measurement

The hemoglobin is measured in Flow cell by a cyanomethemoglobin method. Surfactant releases Hb by lysing red blood cell membrane. Hemoglobin iron is converted to from the Fe^{2+} to Fe^{3+} state to form methemoglobin, which combines with KCN to produce the stable cyanomethemoglobin. The concentration of the Hb is then measured by light absorbance methods at 540 nm wave length. The Hb value is computed by subtracting blank absorbance from sample absorbance.

Hematocrit

The hematocrit is measured based on the principle that pulse height (voltage change) produced by blood cells passing through the transducer aperture is proportional to cell volume. The hematocrit count is calculated by generating these pulses between two discrimination levels, multiplied by a constant factor involving the dilution ratio, and indicated as the ratio of the total RBC volume in the whole blood. This ratio is expressed as %. The diluent absorbance value is subtracted from the sample absorbance value and the result is computed for Hb measurement.

MCV, MCH and MCHC values are also calculated by the microprocessor (Sysmex Operator's Manual K-1000, 1998).

Mean Corpuscular Volume

The mean corpuscular volume is the calculated volume of the erythrocyte and hematocrit and expressed in fL. The formula used is

$$\text{MCV (fL)} = \frac{\text{hematocrit}}{\text{Erythrocyte count } (10^6/\mu L)} \times 10$$

Mean Corpuscular Hemoglobin

Mean corpuscular hemoglobin concentration is a calculated erythrocyte index of the concentration of hemoglobin by weight in the erythrocyte derived by the formula

$$\text{MCH (pg)} = \frac{\text{Hemoglobin concentration (g/dL)}}{\text{Erythrocyte count } (10^6 \text{ cells}/\mu\text{L})} \times 10$$

Mean Corpuscular Hemoglobin Concentration (MCHC)

Mean corpuscular hemoglobin concentration is a calculated index that expresses the ratio of hemoglobin to hematocrit and the formula used is

$$\text{MCHC (g/dL)} = \frac{\text{Hemoglobin (g/dL)}}{\text{Hematocrit (\%)}} \times 100$$

Clotting Time

After puncturing the orbital sinus, blood was allowed to flow into a 7.5 cm capillary tube and the time required for clotting was recorded manually.

Differential Leucocyte Count

The differential leucocyte count is an important blood test to find the pattern of variation in the proportion of different leucocytes in the peripheral blood. DLC with total leucocyte count give an indication about the nature of response of the body to an injurious agent. Differential lecucosyte count is the percent distribution of various white cells in the peripheral blood.

Procedure for Blood Smear Preparation

One drop of blood was placed near the frosted end of the slide. Smear was drawn by pushing the second slide placed at an angle of 40° to 45°. Smear

was dried at room temperature. Dried slides were arranged on glass rods kept over an enamel tray. Using a pipette, few drops of Leishman's stain was poured over each smear. After 2 - 3 minutes, the stain was diluted by adding double quantity of distilled water and allowed to stand for 20 minutes where after the slides were washed with tap water and air dried (Benjamin, 2001).

Leishman Stain Preparation

Leishaman stain solution was prepared by mixing Leishman powder with Leishman stain solution (supplied by Qualigen Fine Chemicals). After mixing at regular intervals for 48 hours, solution was filtered into amber colored bottle.

Peripheral Smear Examination

Smear was examined for a total of 100 cells following criss-cross movement in oil immersion objective. The leucocytes were identified on the following basis.

Lymphocytes: Two forms observed were a) large lymphocytes b) small lymphocytes. Large lymphocytes have abundant clear pale blue cytoplasm and a large round blue-purple colored nucleus. Small lymphocytes as name suggests are comparatively small, has very little blue cytoplasm and the nucleus is dark, and round. The rat lymphocytes varied in size from 5 – 10 µm.

Neutrophil: The neutrophil contains a dark blue-purple colored nucleus band or ring structure within a clear to pale pink colored cytoplasm.

Monocytes: The nucleus is kidney or horse shoe shaped.

Eosinophil: The eosinophil is a relatively large cell with bright pink to red stained cytoplasmic granules and a pale blue-colored polymorphic nucleus or in the shape of 8.

Basophil: Basophils are seldom observed in the peripheral circulation. The nucleus is not seen easily due to the presence of large, round, deep blue granules.

Clinical Chemistry

Clinical chemistry plays important role in Toxicology to assess toxicity of chemicals. The clinical chemistry tests performed in conventional toxicology studies generate information concerning carbohydrate, lipid, and protein metabolism, renal function, liver function, hepatocyte injury, and electrolyte balance. The choice of tests which are employed in clinical chemistry is to some extent influenced by the guidelines for hazard safety assessment of the various regulatory authorities. As the liver and kidneys are frequently the target organs in toxicity studies, parameters pertaining to them can be used to detect damage to these organs (Hall, 1992).

Procedure

About 2 mL of blood was collected in centrifuge tubes. After 20-30 minutes of collection, centrifuge tubes were placed in a Centrifuge. Serum was separated using micropipette after running the centrifuge at 2500 – 3000 rpm for 20 minutes. Separated serum was transferred to clean labeled vials/sample cups.

Instrument Used

Hitachi 902 was used for analysis of clinical chemistry parameters except sodium, potassium and chloride. The latter parameters were analysed in Rapid Chem 744. Hitachi 902 is a fully automated instrument. Instrument

was calibrated using calibrator for automated systems provided by the manufacturer of reagents. Apart from calibration, control samples i.e., Precinorm and Precipath supplied by manufacturer were also checked before analyzing the test samples (Hitachi Instruction Manual, 1996).

The studied clinical chemistry parameters were as follows.

Glucose	ALT
Protein	AST
Albumin	ALP
Globulin	ChE (serum)
Cholesterol	ChE (RBC)
Blood urea nitrogen (BUN)	Sodium
Urea	Potassium
Creatinine	Chloride
Calcium	
Phosphorus	

Glucose

Test Principle

The modified GOD-POD method was used (Trinder, 1969) by enzymatic colorimetric assay.

Glucose is oxidized by glucose oxidase to gluconolactone in the presence of atmospheric oxygen. The resultant hydrogrn peroxide oxidizes 4-aminophenazone and phenol to 4-(p-benzo-quinone-monoimino)-phenazone in the presence of peroxidase (POD). The color intensity of the red dye is directly proportional to the glucose concentration.

$$\text{Glucose} + O_2 + H_2O \xrightarrow[\text{POD}]{\text{GOD}} \text{gluconolactone} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{phenol} \longrightarrow \text{4-[p-benzoquinone-monoimino]-phenazone} + 4H_2O.$$

Total Protein

Test principle

Total protein is a measure of all the different proteins in plasma with the exception of those that are consumed for clot formation such as fibrinogen and the clotting factors The principle is based on Biuret Method (Flack and Wollen, 1984).The peptide bonds of protein react with copper II ions in alkaline solution to form blue – violet complex, each copper ion complexing with 5 or 6 peptide bonds. Tartarate is added as a stabilizer whilst iodide is used to prevent auto-reduction of the alkaline copper complex. The color formed is proportional to the protein concentration (Biuret Method, ERBA 2002).

Albumin

Test Principle

The principle is based on the methodology of Doumas *et al.* (1972). Albumin binds with Bromocresol green (BCG) at pH 4.2 causing a shift in absorbance of the yellow BCG dye. The blue green color formed is proportional to the concentration of albumin present, when measured photometrically between 580-630 nm with maximum absorbance at 625 nm.

Globulin

Globulin was detected by subtracting albumin from total protein.

Cholesterol

Test Principle

The method used is a modified Roeschlau *et al.* (1974) method. The estimation of cholesterol involves the following enzyme catalyzed reactions.

$$\text{Cholesterol ester} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol + Fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase}} 4H_2O + \text{Quinoneimine}$$

The absorbance of chromophore (Quinoneimine) is directly proportional to the cholesterol concentration in the sample.

Blood Urea Nitrogen (BUN) and Urea

Test principle

Urea/BUN assay is based on Talke and Schubert (1965) method and has been optimized for analyzers that permit kinetic (fixed time) measurements by UV kinetic assay.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_4 + CO_2$$

The ammonia formed then reacts with α-ketoglutarate and NADH in the presence of GLDH to yield glutamate and NAD^+.

$$\alpha\text{-ketoglutarate} + NH_4 + \text{NADH} \longrightarrow \text{glutamate} + NAD^+ + H_2O$$

The decrease in absorbance due to consumption of NADH is measured kinetically.

Creatinine

Test Principle

The method is based on Jaffe reaction as described by Popper *et al.* (1937) and Seelig and Wust (1969) and, modified by Bartel's (1972) kinetic colorimetric Assay.

Creatinine + picric acid $\xrightarrow{\text{alkaline solution}}$ creatnine-picric acid complex

In alkaline solution, creatinine forms a yellow –orange complex with picrate. The color intensity is directly proportional to the creatinine concentration.

Bilirubin

Test Principle

The colorimetric assay is based on Wahlefeld *et al.* (1972) in which a detergent is used to accelerate the reaction and prevent the precipitation of protein. The diazo reagent used is 2, 5- dichlorophenyl diazonium tetrafluoroborate, which under acidic conditions couples very rapidly with bilirubin.

Bilirubin + diazonium ion $\longrightarrow$ Azobilirubin

Calcium

Test Principle

The colorimetric assay is based on Gindler *et al.* (1972).

Calcium + o-cresolphthalein complexone $\xrightarrow{\text{alkaline solution}}$ calcium-o-cresolphthalein complex

The color intensity of the complex formed is directly proportional to the calcium concentration.

Phosphorus

Test Principle The method is based on Henry, (1974). Inorganic phosphate forms an ammonium phosphomolybdate complex having the formula $[(NH_4)_3 \{Po_4 (MoO_3)_{12}\}]$ with ammonium molybdate in the presence of sulfuric acid. The complex is determined photometrically in the UV region (340 nm).

Sodium, Potassium and Chloride

Detected by Rapid Chem 744 and its Test Principle

The Rapid Chem 744, the electrolyte analyzer measures sodium, potassium and chloride in biological fluids using ion selective electrolyte technology. The flow through sodium sensor uses selective membrane tubing specially formulated to be sensitive to sodium ions. The potassium and chloride sensor employ similar designs with appropriate selective membrane materials. The potential of each sensor is measured relative to fixed stable voltage established by the double junction silver/silver chloride reference sensor. An ion selective sensor develops a voltage that varies with the concentration of the ion to which it responds.

A comparative method of measurement is utilized. First the analyzer measures the potentials developed when the sample is positioned in the sensors. Next, calibrant A is positioned in the sensors. The difference in the two potentials is related logarithmically to the concentration of sodium, potassium or chloride ions in the sample divided by their respective concentration in the calibrant solution. Since the difference in potentials and the concentration of the sodium, potassium or chloride ions in the calibrant solution are known, the computer can calculate the concentration of the ions in the sample solution in accordance with the Nernst equation.

$E - E^\circ = S \log [C_j (X) / C_j (S)]$

E= ISE potential developed in sample solution

E° = ISE potential developed in standard solution

S = Sensor slope calculated during calibration

$C_j (X)$ = Concentration of ion "i " in the sample

$C_j (S)$ = Concentration of ion "i " in the standard solution

S = the slope, is determined during calibration using calibrants A and B, which have known levels of sodium, potassium or chloride (Rapid Chem 744, 2002).

Alanine Aminotransferase (ALT/SGPT)

Alanine aminotransferase belongs to the group of transaminases which catalyze the conversion of amino acids to the corresponding α-keto acids via the transfer of amino groups: they also catalyze the reverse process.

Test Principle

UV kinetic test is based on the IFCC (1986) reference method.

$$\alpha\text{-ketoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{L-glutamate} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^+$$

The rate of decrease in NADH is directly proportional to the rate of formation of pyruvate and thus the ALT activity.

Aspartate Aminotransferase (AST/SGOT)

AST belongs to transaminases, which catalyze the interconversion of amino acids and α-ketoacids by transfer of amino groups.

Test Principle

UV kinetic test is based on the IFCC (1986) reference method.

$$\alpha\text{-ketoglutarate} + \text{L-aspartatee} \xrightarrow{\text{AST}} \text{L-glutamate+ oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+$$

The rate of the photometrically determined NADH decrease is directly proportional to the rate of formation of oxaloacetate and thus the AST activity.

Alkaline Phosphate (ALP)

Test Principle

The colorimetric assay is based on Deutschen Gesellschaft fur klinische Chemie (1972) method.

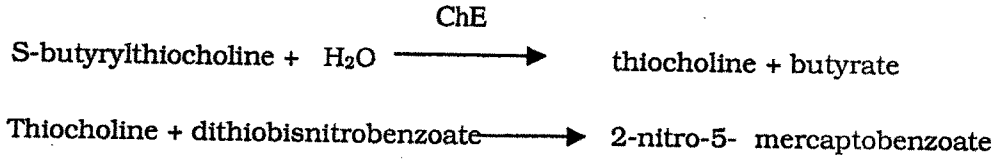
$$\text{p-nitrophenyl phosphate} + H_2O \xrightarrow[Mg^{2+}]{\text{ALP}} \text{Phosphate} + \text{p-nitrophenol}$$

In the absence of magnesium and zinc ions, p-nitrophenyl phosphate is hydrolyzed by phosphatases to form phosphate and p-nitrophenol. The p-nitrophenol released is proportional to the ALP activity.

Serum Cholinesterase (Pseudocholinesterase)

Test Principle

The colorimetric assay is based on Knedel and Bottger (1967). Cholinesterase catalyzes the hydrolysis of butrylthiocholine iodide to thiocholine iodide and butyrate. Thiocholine iodide reacts with 5,5-dithiobisnitrobenzoic acid (DTNB) to form the yellow product, 2-nitro-5-mercaptobenzoate.The rate of formation of 2-nitro-5-mercaptobenzoate is proportional to the ChE activity.

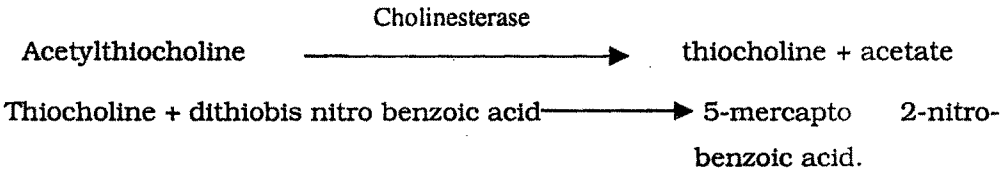


RBC Acetylcholinesterase (True cholinesterase)

Exposure to organophosphate and carbomate pesticides can be monitored by measuring plasma cholinesterase. However, measuring erythrocyte acetyl cholinesterase in conjunction with the former is important for monitoring exposure to these class of pesticides, as plasma cholinesterase and acetylcholinesterase activities may be inhibited by different degrees and for differing lengths of time after exposure. Following method was used based on the colorimetric procedure of Ellman *et al.* (1961) which is accurate and can be done rapidly.

Test Principle

The substrate acetylthiocholine is split into acetate and thiocholine by the esterase and the thio group of thiocholine reacts with dithiobis nitro benzoic acid (Ellman's reagent) to form yellow colored 5-mercapto 2-nitro benzoic acid complex. The increase in color intensity which is proportional to the cholinesterase activity can be monitored kinetically between 400-420 nm.



Procedure

Approximately 1 mL of blood is collected in EDTA coated tubes. After 30 minutes, they are centrifuged in cold at 4000 rpm for 10 minutes. The plasma volume was noted and then aspirated. Equal volume of 0.9% saline

was added to test tubes and then centrifuged. Saline was removed and the step (saline treatment) was repeated for 3 times. Cell suspension was prepared by adding equal volume of saline. 10 µL of cell suspension was added to 0.5 mL of Triton-x-100 [0.01% prepared in phosphate buffer, 0.1M, pH 8.0) and hemolysed. The hemolysate was taken in as the sample for acetylcholinesterase measurement in Hitachi autoanalyser following below mentioned condition.

Reagent (R1)- 350µL of 50 mM Phosphate buffer pH 8.0 containing 3.0 m mol /L dithiobis nitro benzoic acid.

Reagent (R2) – Acetylthiocholine iodide (156 m mol/L) solution

R1: Volume: 350 µL

R2: Volume: 70 µL

Sample Volume: 5 µL

Wavelength: 660 – 415

The values obtained will be multiplied by the dilution factor 50.

Evaluation of Data

Statistical evaluations were performed using validated statistical software (Developed by jai research Foundation). All the parameters characterized by continuous data were subjected to Bartlett's test to meet the homogeneity of variance before conducting Analysis of Variance (ANOVA) and Dunnett' s t-test . Where the data did not meet the homogeneity of variance, Student's t-test was performed to calculate significance. The significance was calculated at 5% ($P \leq 0.05$) and 1% ($P \leq 0.01$) level.

RESULTS

Clinical pathology evaluations include hematology and clinical chemistry parameters and were carried out at 14 days after a single dose exposure of chlorpyrifos and lead and their combination in rats. In addition, serum and RBC cholinesterase assays were performed after two days of single dose exposure. The results of the study are as follows.

Haematology

Males

No statistically significant differences were observed in hematological analysis of treatment group animals as compared to control group animals performed on day 15. However, in males, slight reduction in RBC, Hb and HCT was observed in treatment group of animals. The percent reduction of the RBC count in groups 3, 4, 6 and 7 animals was 6.1 %. Hb was reduced by 6.9, 4.2, 7.7, 5.6, 11.0 and 6.9 % in groups 2, 3, 4, 5, 6 and 7 respectively. The mean hemtaocrit (HCT) value of groups 2, 4, 5, 6 and 7 was reduced by 5.4, 7.1, 4.9, 10.4 and 7.1 respectively. A parallel reduction in mean values of RBC, Hb and HCT was observed in group 6 animals only (Table 1).

Females

Hematological parameters of treatment group females were comparable to control group females (Table 2).

Clinical Chemistry

Males

Significant increase in the mean values of glucose was observed in groups 6 and 7 animals as compared to control group animals on day 15 (Table 4;

Figure 5). The mean values of sodium in groups 3, 4, 5, 6 and 7, phosphorus in group 6 and chloride in group 5 were significantly increased as compared to control group animals (Table 4; Figure 3).

On day 3, the serum and RBC cholinesterases in groups 5 and 7 and serum cholinesterase in group 4 animals were significantly decreased as compared to control group animals. The percent reduction in serum cholinesterase was 15.1, 44.2, 66.1 and 74.6 % in groups 2, 4, 5 and 7 animals, respectively. The percent reduction in RBC cholinesterase was 11.2, 23.0, 63.8 and 80.9 % in groups 2, 4, 5 and 7 animals respectively (Table 3; Figure 1).

On day 15, mean values of serum cholinesterase of treatment group of animals were comparable to control group animals. However, slight reduction i.e., 20.7% in group 5 and 22.5% in group 7 was observed in RBC cholinesterase as compared to control group animals (Table 4; Figure 1).

Other parameters such as total protein, albumin, globulin, cholesterol, bilirubin, creatinine, BUN, urea, calcium, potassium, ALT, AST, ALP of treatment group animals were very much comparable to control group animals (Table 4).

Females

The mean values of sodium in groups 4, 5, 6 and 7 animals were significantly increased as compared to control group animals. In addition, the mean value of chloride in group 7 was also increased (Table 5; Figure 4).

On day 3, the mean values of serum and RBC cholinesterases in groups 5 and 7 significantly decreased as compared to control group animals. The percent reduction in serum cholinesterase was 62.2 and 78.8 % in groups 5 and 7 animals, respectively. The percent reduction of RBC cholinesterase

was 69.5 and 86.4 % in groups 5 and 7 animals, respectively. A slight reduction also observed in the mean values of serum and RBC cholinesterases in groups 2 and 4 animals. The percent reduction of serum cholinesterase was 5.2 % and 8.1 % and that of RBC cholinesterase 9.7 % and 20.8 % in groups 2 and 4 animals respectively (Table 3; Figure 2).

Like males, on day 15, mean values of serum cholinesterase of treatment group females were comparable to control group females. However, slight reduction i.e., 12.2 % in group 5 and 17.7 % in group 7 was observed in RBC cholinesterase as compared to control group animals (Table 5).

Remaining parameters of treatment group animals were comparable to control group animals.

Table 1

Hematology – Group Mean Values

Dose: G1- 0; G2 (CPF) - 5; G3 (LA) - 100; G4 (CPF+LA) - 5+100; G5 (CPF) - 50; G6 (LA) - 1000; G7 (CPF+LA) - 50+1000 mg/kg body weight/day

Sex : Male **Period : 15th Day**

Parameter/Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
WBC (10^3/µL)	Mean	12.1	15.5	14.4	15.3	12.6	13.1	11.5
	SD	2.86	2.47	6.10	4.84	1.38	1.90	1.82
RBC (10^6/µL)	Mean	6.6	6.5	6.2	6.2	6.5	6.2	6.2
	SD	0.35	0.46	0.22	0.34	0.32	0.24	0.26
Hb (g/dL)	Mean	14.2	13.2	13.6	13.1	13.4	12.6	13.2
	SD	0.77	0.89	0.46	1.15	0.95	0.92	0.47
HCT (%)	Mean	41.1	38.9	41.7	38.2	39.2	36.8	38.2
	SD	3.65	1.54	2.02	2.68	2.96	2.75	1.66
MCV (fL)	Mean	62.5	60.3	67.0	61.5	60.8	59.5	60.2
	SD	7.69	3.67	4.62	6.48	6.83	4.35	2.54
MCH (pg)	Mean	21.5	20.4	22.0	21.1	20.7	20.4	20.9
	SD	0.31	1.81	0.65	1.94	1.79	1.61	1.07
MCHC (g/dL)	Mean	34.7	33.8	33.2	34.4	34.2	34.3	34.6
	SD	3.51	1.85	1.91	2.34	1.48	1.98	1.13
Platelet (10^3/µL)	Mean	912.2	869.2	687.0	783.8	861.6	935.0	916.2
	SD	122.52	261.27	193.01	225.79	248.37	295.60	282.97
Clotting Time (seconds)	Mean	138.0	120.0	102.0	108.0	120.0	126.0	102.0
	SD	40.25	36.74	16.43	26.83	42.43	32.86	16.43
Lymphocyte (%)	Mean	84.8	80.6	88.0	86.4	79.0	82.0	83.4
	SD	4.49	4.39	5.00	3.21	8.00	5.83	10.64
Neutrphil (%)	Mean	12.60	16.00	10.60	11.20	18.60	16.0	15.40
	SD	3.21	3.87	4.45	1.92	7.33	5.43	3.09
Monocyte (%)	Mean	1.4	1.0	0.8	0.8	0.8	1.0	0.8
	SD	0.34	0.22	0.24	0.35	0.25	0.31	0.24
Eosinophil (%)	Mean	1.20	2.20	0.60	1.60	1.60	0.80	0.40
	SD	0.30	0.90	0.25	0.52	0.52	0.30	0.22
Basophil (%)	Mean	0.00	0.2	0.0	0.0	0.0	0.2	0.00
	SD	0.00	0.05	0.00	0.00	0.00	0.05	0.00

Key : N= Number of animals

Table 2

Hematology – Group Mean Values

Dose: G1- 0; G2 (CPF) - 5; G3 (LA) - 100; G4 (CPF+LA) - 5+100; G5 (CPF) - 50; G6 (LA) - 1000; G7 (CPF+LA) - 50+1000 mg/kg body weight/day

Sex : Female **Period : 15th Day**

Parameter/Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
WBC ($10^3/\mu L$)	Mean	13.8	14.2	11.8	17.3	12.6	18.3	10.9
	SD	2.63	3.95	2.04	4.25	3.04	4.35	1.97
RBC ($10^6/\mu L$)	Mean	5.9	5.9	6.2	6.1	5.9	6.1	6.1
	SD	0.50	0.40	0.42	0.46	0.90	0.43	0.28
Hb (g/dL)	Mean	13.2	13.0	13.8	13.4	13.2	13.7	13.9
	SD	0.62	1.03	1.11	1.29	0.94	1.31	0.75
HCT (%)	Mean	37.0	38.4	37.3	38.0	39.0	37.9	38.7
	SD	2.09	2.68	2.75	3.23	3.78	3.86	313
MCV (fL)	Mean	62.9	65.7	60.5	61.9	66.1	61.7	63.0
	SD	4.05	5.38	3.95	4.44	6.19	2.01	5.29
MCH (pg)	Mean	22.5	22.3	22.5	21.7	22.4	22.4	22.7
	SD	1.68	2.10	1.14	0.65	1.68	0.86	0.75
MCHC (g/dL)	Mean	35.8	34.0	37.1	35.2	34.0	36.3	36.1
	SD	2.75	3.60	1.06	2.14	2.11	1.45	2.30
Platelet ($10^3/\mu L$)	Mean	588.2	770.2	856.0	671.8	794.8	751.8	868.6
	SD	160.42	118.65	125.10	43.83	244.91	146.87	206.71
Clotting Time (seconds)	Mean	96.0	120.0	90.0	126.0	114.0	96.0	126.0
	SD	13.42	16.74	12.0	15.1	13.42	13.42	15.1
Lymphocyte (%)	Mean	84.0	86.6	83.4	81.8	86.2	85.2	83.4
	SD	3.00	4.28	1.82	5.81	3.56	4.55	6.02
Neutrphil (%)	Mean	14.4	9.2	15.8	17.0	12.0	13.6	13.8
	SD	4.16	1.92	1.48	5.96	3.81	4.62	3.42
Monocyte (%)	Mean	0.2	0.8	0.2	0.4	0.8	0.6	0.8
	SD	0.05	0.30	0.05	0.15	0.25	0.25	0.35
Eosinophil (%)	Mean	1.6	1.4	0.6	0.8	1.0	0.6	2.0
	SD	0.52	0.64	0.25	0.30	0.05	0.15	0.55
Basophil (%)	Mean	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Key : N= Number of animals

Table 3

Cholinesterase – Group Mean Values

Dose: G1- 0; G2 (CPF) - 5; G3 (LA) - 100; G4 (CPF+LA) - 5+100; G5 (CPF) - 50; G6 (LA) - 1000; G7 (CPF+LA) - 50+1000 mg/kg body weight/day

Sex : Male **Period : 3rd Day**

Parameter/ Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
Serum ChE (IU/L)	Mean	394.6	335.2	309.8	220.0**	133.8**	337.0	100.2**
	SD	70.15	70.81	29.73	33.83	45.46	86.13	22.25
RBC ChE (IU/L)	Mean	1520.0	1350.0	1470.0	1170.0	550.0**	1490.0	290.0**
	SD	228.08	251.78	240.22	262.07	202.08	230.22	114.02

Sex : Female **Period : 3rd Day**

Parameter/ Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
Serum ChE (IU/L)	Mean	372.8	353.4	388.4	342.6	141.0**	449.8	79.2**
	SD	71.92	28.48	33.4	54.98	74.89	93.96	11.82
RBC ChE (IU/L)	Mean	1540.0	1390.0	1540.0	1220.0	470.0**	1270.0	210.0**
	SD	292.40	216.22	286.57	246.48	182.35	192.35	96.18

Key : N= Number of animals

**= significant at 1% level ($p \leq 0.01$)

Table 4

Clinical Chemistry – Group Mean Values

Dose: G1- 0; G2 (CPF) - 5; G3 (LA) - 100; G4 (CPF+LA) - 5+100; G5 (CPF) - 50; G6 (LA) - 1000; G7 (CPF+LA) - 50+1000 mg/kg body weight/day

Sex : Male **Period : 15th Day**

Parameter/Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
Glucose	Mean	74.4	78.4	72.1	84.1	86.6	120.5**	102.0*
	SD	9.13	10.23	6.70	10.46	11.60	11.70	10.29
Total Protein (g/dL)	Mean	8.1	8.2	8.6	8.2	8.2	8.1	8.4
	SD	0.34	0.42	0.19	0.31	0.36	0.85	0.43
Albumin (g/dL)	Mean	3.83	3.75	3.70	3.79	3.78	3.70	3.64
	SD	0.13	0.19	0.15	0.16	0.20	0.35	0.38
Globulin (mg/dL)	Mean	4.3	4.5	4.9	4.3	4.4	4.4	4.8
	SD	0.36	0.26	0.30	0.28	0.22	0.53	0.77
Cholesterol (mg/dL)	Mean	78.9	73.0	77.2	72.0	76.7	68.0	65.5
	SD	8.63	5.61	4.67	10.04	7.70	15.39	7.18
Total Bilirubin (mg/dL)	Mean	0.23	0.25	0.25	0.23	0.23	0.22	0.23
	SD	0.045	0.031	0.022	0.027	0.036	0.036	0.018
BUN (mg/dL)	Mean	18.75	20.09	20.75	17.87	18.46	20.82	20.64
	SD	3.43	3.31	2.15	4.03	1.60	4.69	3.36
Urea (mg/dL)	Mean	40.2	43.2	43.8	38.6	39.6	44.8	45.2
	SD	7.46	7.01	4.09	8.65	3.65	10.14	7.73
Creatinine (mg/dL)	Mean	0.21	0.22	0.23	0.18	0.23	0.22	0.20
	SD	0.022	0.038	0.031	0.022	0.045	0.046	0.034
Calcium (mg/dL)	Mean	11.2	11.1	10.9	11.1	10.9	11.0	10.5
	SD	0.51	0.46	0.17	0.24	0.48	0.44	0.20
Phosphorus (mg/dL)	Mean	10.5	10.2	10.3	10.6	10.8	12.0*	10.5
	SD	0.58	0.88	0.58	0.42	0.52	0.81	0.45
Sodium (mEq/L)	Mean	147.1	148.2	150.0*	151.8**	150.5*	150.6**	151.1**
	SD	1.23	1.28	0.91	0.58	1.14	1.0	0.69
Potassium (mEq/L)	Mean	4.66	4.67	4.50	4.50	4.72	5.03	4.73
	SD	0.151	0.542	0.454	0.583	0.807	0.283	0.381
Chloride (mEq/L)	Mean	109.0	109.1	109.9	111.6	112.0*	111.6	111.0
	SD	1.26	1.46	1.32	1.21	1.16	1.08	1.35

Key : N= Number of animals

* = significant at 5% level ($p \leq 0.05$); **= significant at 1% level ($p \leq 0.01$)

Table 4 (continued)

Sex : Male | **Period : 15th Day**

Parameter/ Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
ALT (IU/L)	Mean	55.5	56.0	53.2	47.9	46.2	51.6	54.5
	SD	8.68	9.02	17.12	7.16	6.16	12.64	6.34
AST (IU/L)	Mean	209.8	210.4	237.3	192.5	199.5	185.3	192.9
	SD	25.40	23.84	19.02	15.64	36.87	9.27	14.98
ALP (IU/L)	Mean	341.8	304.9	302.1	406.0	257.3	344.6	448.7
	SD	61.49	41.54	51.88	137.57	32.25	87.72	56.89
Serum ChE (IU/L)	Mean	337.4	376.6	308.8	245.4	277.8	299.6	331.4
	SD	82.38	49.13	43.68	15.31	73.61	82.21	23.10
RBC ChE (IU/L)	Mean	1330.0	1270.0	1200.0	1130.0	1050.0	1320.0	1030.0
	SD	251.5	201.9	187.1	292.8	275.8	235.7	241.4

Key : N= Number of animals

* = significant at 5% level ($p \leq 0.05$); **= significant at 1% level ($p \leq 0.01$)

Per cent Reduction of Cholinesterase Enzymes – Male						
Group	Day 3				Day 15	
	Group 2	Group 4	Group 5	Group 7	Group 5	Group 7
Serum	15.1	44.2	66.1	74.6	0	0
RBC	11.2	23.0	63.8	80.9	20.7	22.5

Table 5

Clinical Chemistry – Group Mean Values

Dose: G1- 0; G2 (CPF) - 5; G3 (LA) - 100; G4 (CPF+LA) - 5+100; G5 (CPF) - 50; G6 (LA) - 1000; G7 (CPF+LA) - 50+1000 mg/kg body weight/day

Sex : Female **Period : 15th Day**

Parameter/Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
Glucose	Mean	96.5	84.4	73.1	83.4	95.3	87.0	93.1
	SD	15.23	12.83	17.30	7.98	8.25	25.90	24.71
Total Protein (g/dL)	Mean	8.3	8.0	8.2	8.3	8.8	8.7	8.3
	SD	0.34	0.24	0.38	0.55	0.35	0.16	0.37
Albumin (g/dL)	Mean	3.9	4.1	4.0	3.9	4.1	4.0	3.7
	SD	0.22	0.19	0.16	0.24	0.14	0.23	0.33
Globulin (mg/dL)	Mean	3.9	4.0	4.2	4.4	4.7	4.7	4.6
	SD	0.30	0.32	0.26	0.48	0.33	0.26	0.20
Cholesterol (mg/dL)	Mean	77.9	88.5	92.7	84.2	87.3	88.3	89.2
	SD	7.79	11.33	12.39	14.32	22.48	9.30	5.43
Total Bilirubin (mg/dL)	Mean	0.12	0.12	0.10	0.12	0.12	0.11	0.10
	SD	0.04	0.02	0.04	0.08	0.03	0.04	0.01
BUN (mg/dL)	Mean	19.1	17.6	19.3	20.3	20.4	18.3	16.9
	SD	2.42	3.03	3.02	1.38	3.54	2.38	4.55
Urea (mg/dL)	Mean	40.8	37.6	41.2	43.4	43.6	39.2	36.2
	SD	5.36	6.43	6.54	3.05	7.64	5.36	9.58
Creatinine (mg/dL)	Mean	0.22	0.25	0.23	0.24	0.26	0.24	0.21
	SD	0.033	0.036	0.034	0.036	0.025	0.031	0.038
Calcium (mg/dL)	Mean	10.7	10.9	10.8	11.1	11.2	11.1	11.0
	SD	0.31	0.35	0.33	0.36	0.25	0.37	0.17
Phosphorus (mg/dL)	Mean	9.8	9.7	9.9	10.0	10.7	9.4	10.2
	SD	0.50	0.39	0.72	0.39	1.00	0.67	0.45
Sodium (mEq/L)	Mean	142.5	143.6	143.7	146.0**	145.5*	146.2**	146.2**
	SD	0.96	1.02	1.15	1.23	1.27	1.26	1.12
Potassium (mEq/L)	Mean	3.8	4.0	4.4	4.0	4.2	3.8	4.5
	SD	0.80	0.30	0.37	0.31	0.66	0.48	0.37
Chloride (mEq/L)	Mean	104.5	105.1	105.8	107.1	107.0	107.1	107.4*
	SD	1.25	1.97	0.95	2.50	2.15	2.28	2.33

Key : N= Number of animals

* = significant at 5% level ($p \leq 0.05$); **= significant at 1% level ($p \leq 0.01$)

Table 5 (continued)

Sex : Female **Period : 15th Day**

Parameter/Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
ALT (IU/L)	Mean	45.0	45.2	44.2	47.8	42.1	38.2	49.3
	SD	5.17	5.62	6.30	3.68	4.40	6.37	4.27
AST (IU/L)	Mean	208.0	186.3	190.3	189.0	177.7	186.7	193.6
	SD	31.76	19.06	11.04	22.56	35.67	37.40	40.38
ALP (IU/L)	Mean	367.3	348.8	271.5	366.1	314.8	300.0	349.8
	SD	101.16	124.65	78.06	70.89	68.45	109.34	117.54
Serum ChE (IU/L)	Mean	514.8	581.8	567.2	672.2	610.2	726.6	535.4
	SD	176.51	134.34	166.79	123.46	133.44	133.62	165.78
RBC ChE (IU/L)	Mean	1470.0	1400.0	1360.0	1330.0	1290.0	1560.0	1210.0
	SD	313.45	393.7	253.48	277.49	224.72	330.8	339.55

Key : N= Number of animals

* = significant at 5% level ($p \leq 0.05$); **= significant at 1% level ($p \leq 0.01$)

Per cent Reduction of Cholinesterase Enzymes – Female						
Group	Day 3				Day 15	
	Group 2	Group 4	Group 5	Group 7	Group 5	Group 7
Serum	5.2	8.1	62.2	78.8	0	35.2
RBC	9.7	20.8	69.5	86.4	12.2	17.6

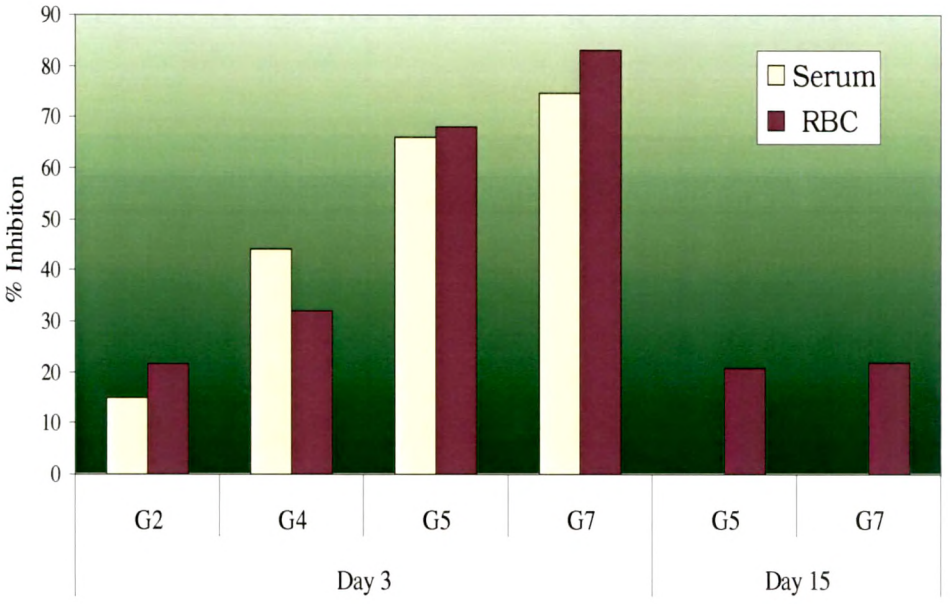


Figure 1. Percent Inhibition of Cholinesterases (Serum and RBC) – Males

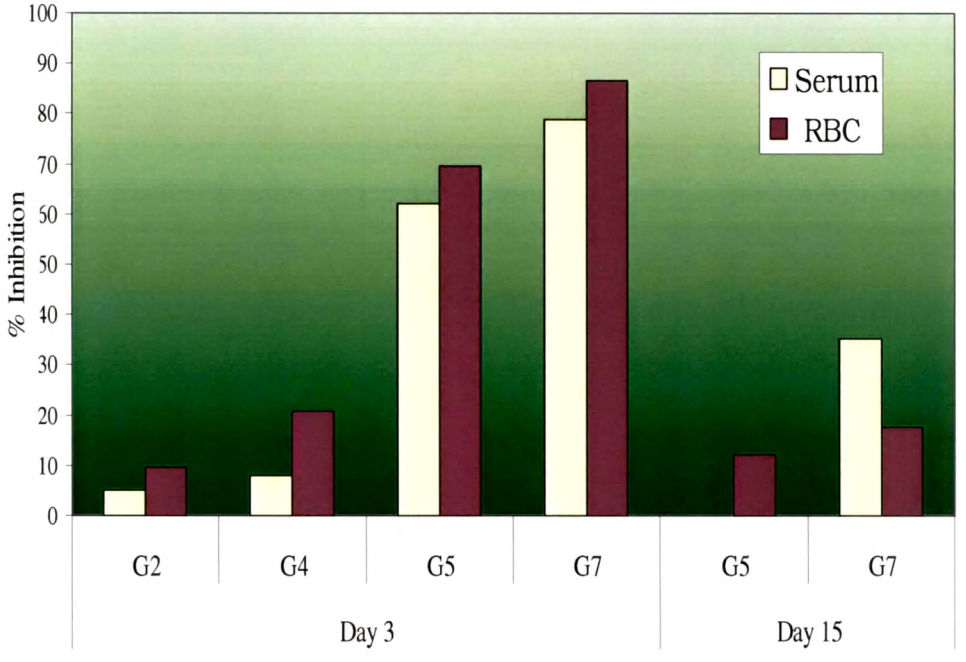


Figure 2. Percent Inhibition of Cholinesterases (Serum and RBC) – Females

G1- 0; **G2** (CP) - 5; **G3** (LA) - 100; **G4** (CP+LA) - 5+100; **G5** (CP) - 50; **G6** (LA) - 1000; **G7** (CP+LA) - 50+1000 mg/kg

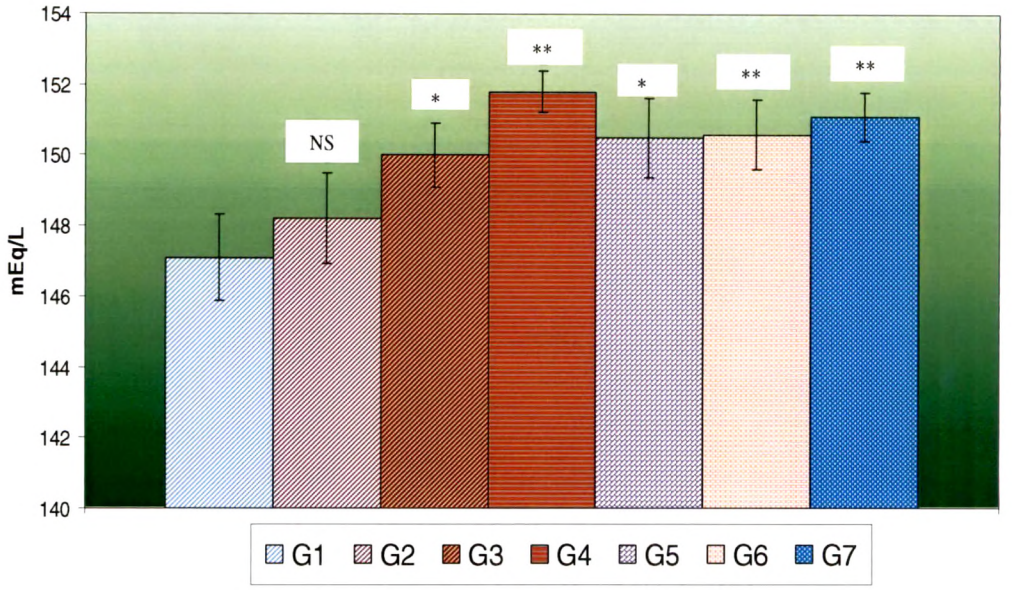


Figure 3. Sodium Values: Males – on day 15.

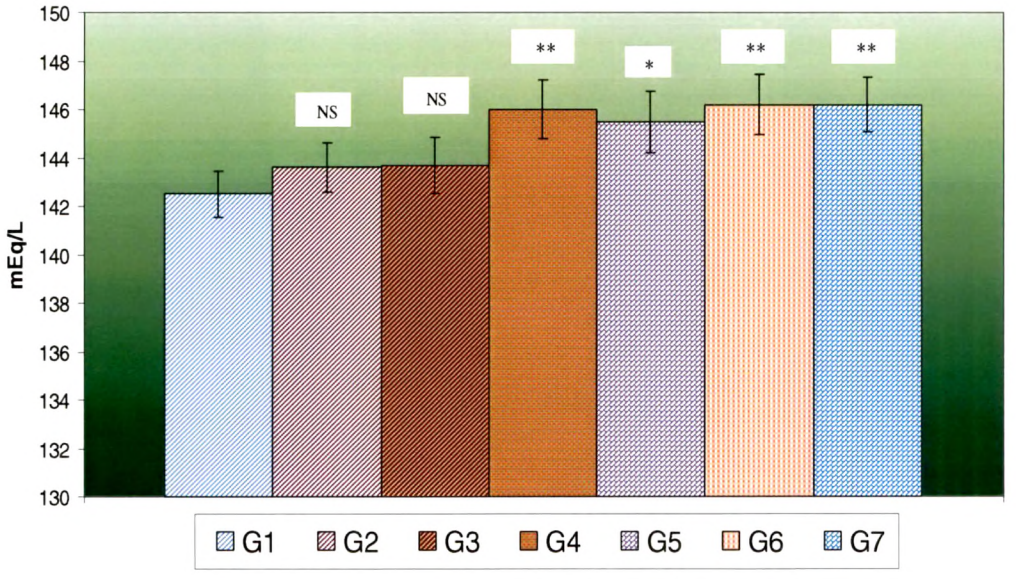


Figure 4. Sodium Values: Females – on day 15.

Key : * = significant at 5% level ($p \leq 0.05$), ** = significant at 1% level ($p \leq 0.01$), NS = Not statistically significant

G1- 0; **G2** (CP) - 5; **G3** (LA) - 100; **G4** (CP+LA) - 5+100; **G5** (CP) - 50; **G6** (LA) - 1000; **G7** (CP+LA) - 50+1000 mg/kg

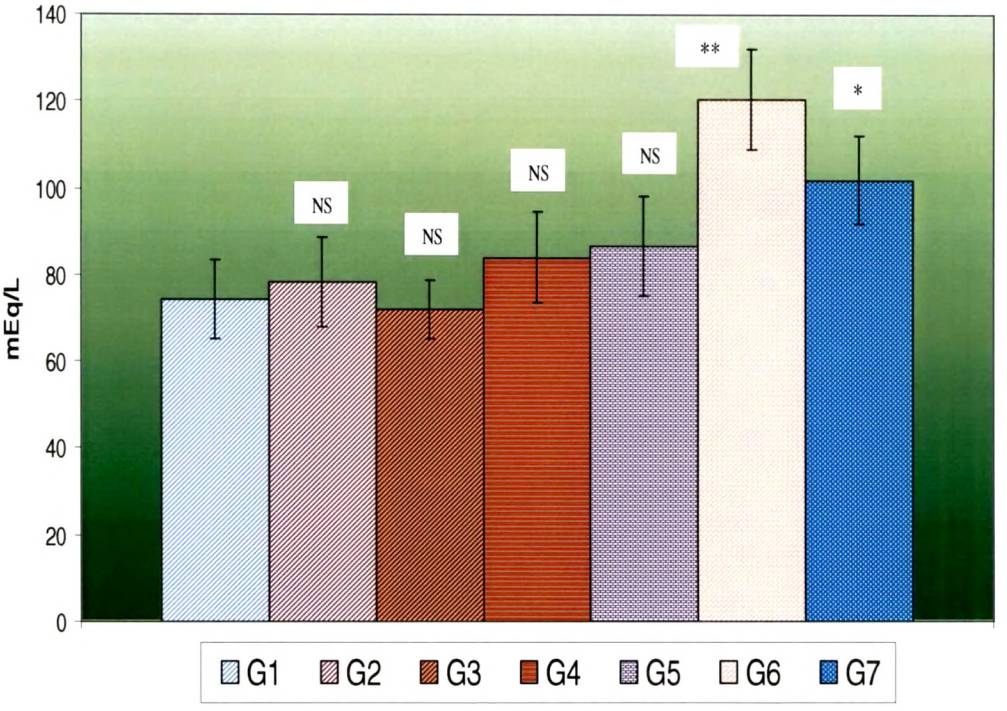


Figure 5. Glucose Values: Males – on day 15.

Key : * = significant at 5% level ($p \leq 0.05$), ** = significant at 1% level ($p \leq 0.01$), NS = Not statistically significant

G1- 0; **G2** (CP) - 5; **G3** (LA) - 100; **G4** (CP+LA) - 5+100; **G5** (CP) - 50; **G6** (LA) - 1000; **G7** (CP+LA) - 50+1000 mg/kg

DISCUSSION

The present study has evaluated the effects of an acute combination of chlorpyrifos and lead acetate on hematology and clinical chemistry parameters of blood.

There seems to be no effect of chlorpyrifos or lead either individually or in combination on hematological parameters except for a slight decrease in RBC count, haemoglobin count and HCT value of high lead group (group 6). This non-significant decrease is understandable in the light of the known anaemic effect of lead (Goyer, 1996). Even no interactive effect is manifested.

On day 3, the serum and RBC cholinesterases in groups 5 and 7 and serum cholinesterase in group 4 animals (males) were significantly decreased (Tables 3, 4 and 5; Figures 1 and 2). It is well known that OP compounds acts by inhibiting enzyme cholinesterase. In combination groups of males i.e., groups 4 and 7, reduction in both serum and RBC cholinesterase was comparatively higher as compared to groups 2 and 5 respectively, i.e., treated with chlorpyrifos alone. Higher reduction in values of cholinesterase observed in combination groups is also comparable with the clinical signs and neurobehavioral effects associated with cholinergic over stimulation. Group 7 animals showed wide range of cholinergic signs and it was clear from the activity levels of both butyrylcholinesterase and RBC acetylcholinestarase.

The slight reduction in RBC cholinesterase observed in groups 5 and 7 animals on day 15 suggest that the enzyme activity has not returned to normalcy completely after 14 days of exposure (Tables 3, 4 and 5; Figures 1

and 2). The recovery of enzyme activity to normal level depends on the dose of chlorpyrifos administered (Verma *et al.*, 2002).

Higher inhibition of both types of cholinesterase enzyme in lead and chlorpyrifos combination groups again may be associated with local activation. Local activation of CPF to CPF oxon and availability of oxon in general circulation contributes to CPF toxicity. Due to formation of chelating complex between metal (lead) and OP (chlorpyrifos), bypassing or escaping of parent chlorpyrifos from the detoxification mechanism of liver may be the probable reason for higher availability in general circulation. It is known that maximum amount of lead accumulates in RBC after absorption from the digestive system. This unique property of lead also may contribute to higher amount of parent CPF in circulation. Hence, these conditions might be providing extrahepatic activation of chlorpyrifos to its oxon and thereby providing additional scope for inhibition of its target enzymes.

In males, mean values of glucose were significantly increased in groups 6 and 7 (Tables 4 and 5; Figure 5). Stevenson *et al.* (1976) have reported effects of subacute and chronic lead treatment on glucose homeostasis and renal cyclic AMP metabolism in rats. Rats treated for short term revealed marked decrease in the insulinogenic index (the ratio of serum immunoreactive insulin to blood glucose concentration) in 15 minutes after the administration of a glucose load. They concluded that increased glucose synthesis as well as suppressed pancreatic function may be responsible for lead-induced disturbances in glucose homeostasis. Apparently, in the present study, higher dose of lead acetate and its combination with chlorpyrifos seems to have a hyperglycemic effect which could be related with poor insulin release/insulin sensitivity.

Mean values of sodium were significantly increased in males of groups 3, 4, 5, 6 and 7 (Table 4; Figure 3). Like males, females also showed increased values of sodium in groups 4, 5, 6 and 7 (Table 5; Figure 4). However, no treatment related variations were observed in BUN, and creatinine at any of the dose levels. The variation in sodium level observed in serum might be associated with functional alteration in the proximal tubules of the nephron after acute exposure. Dysfunction of the proximal tubules after acute exposure is a common finding at higher level of acute lead exposure (ASTDR, 1999).

SUMMARY

The effect of an acute exposure to chlorpyrifos, an organophosphorus insecticide and lead, a heavy metal or a combination of the two on hematological and clinical chemistry parameters has been studied in Wistar rats. The study was conducted using two different dose levels of chlorpyrifos and lead acetate and grouped into seven groups; control (Group 1), chlorpyrifos-5 mg/kg (Group 2), lead acetate- 100 mg/kg (Group 3), chlorpyrifos-5 mg/kg + lead acetate- 100 mg/kg (Group 4), chlorpyrifos-50 mg/kg (group 5), lead acetate-1000 mg/kg (Group[6) and chlorpyrifos-50 mg/kg + lead acetate-1000 mg/kg (Group 7). Hematological and clinical chemistry parameters were evaluated after 14 days of exposure. In addition, serum butryl and RBC cholinesterase were estimated on 3rd day. No treatment related or interactive effects were noticed in hematology values except for a decrease in RBC count, Hb content and HCT values in group 6 males. Significant decrease in both serum and RBC cholinesterase activities were noticed in groups 5 and 7. Lead treated animals showed increased serum sodium levels. Chlorpyrifos in presence of lead acetate increases the inhibitory effect on both serum and RBC cholinesterases and thereby causing manifestation of severe cholinergic symptoms. Since it is known that maximum amount of lead accumulates in RBC after absorption and as chlorpyrifos can form chelating complex in presence of metals, it is likely that chlorpyrifos escapes general metabolic processing within the liver. Hence, this higher availability of parental chlorpyrifos in general circulation and the resultant higher levels of chlorpyrifos oxon in extrahepatic tissues might be responsible for the observed inhibition of cholinesterases.