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RESEARCH ARTICLE

EVALUATION OF CHLORPYRIFOS INDUCED PROTEIN METABOLIC CHANGES WITH AN EMPHASIS IN DIFFERENT TISSUES OF MICE

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ABSTRACT

The literature pertaining to "pesticide protein interaction" contributes immensely to the field of pesticide biochemistry. The pesticides are found to be alter the structural and soluble proteins by causing biochemical changes in the cell. In the present study Healthy adult mice of same age (100±10 days) and weight (75±10 g) were divided into four groups having ten animals each. The second, third and fourth groups of animals were termed as experimental animals, To the animals of second group single dose of pesticide (i.e. on 1st day) was administered orally by gavage method. To the third group of animals double doses were given i.e. on 1st and 3rd day. Similarly multiple doses i.e., 1st, 3rd, 5th and 7th day were given to the fourth group of animals. The first group of animals was considered as controls. The experimental Mice exposed to chlorpyrifos showed statistically significant (P<0.01) decrease of total protein content, significant (P<0.01) increase of free amino acid (FAA) content, significant (P<0.01) increased of Aspartate aminotransferase(AST) activity and significant (P<0.01) increase of Alanine aminotransferase(AlAT) activity in heart, liver, kidney and muscle respectively. The activities was dose and time dependent manner in chlorpyrifos treated mice.

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INTRODUCTION

Proteins are the most abundant organic compounds and constitute a major part of the body dry weight (10-12 kg in adults). They perform a wide variety of static and dynamic functions. About half of the body protein is present in the supportive tissue while other half is intracellular. Proteins are large organic compounds made of amino acids arranged in a linear chain and joined together by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. Proteins are the ubiquitous macro molecules in the biological system and are derivatives of high molecular weight polypeptides (Murray *et al.,* 1988). They constitute about one by fifth of an animal body on wet weight basis (Swaminathan, 1983). The concentration of proteins on tissue is a balance between the rate of their synthesis and degradation (Schmike, 1974). Proteins have important activities, including catalysis of metabolic reactions and transport of vitamins, minerals, oxygen and fuels. Some proteins make up the structure of tissues, while others function in nerve transmission, muscle contraction, cell motility and still others in blood clotting and immunologic defenses, as hormones and regulatory molecules. (Swaminathan, 1983). Proteins involve in cellular architecture, metabolic replications, enzyme mechanism. These are hydrolyzed to amino acids in the body which are further metabolized by incorporation in to proteins or deamination or oxidation of amino acids (Murray *et al.,* 2007).

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Proteins play a dual role as a building material and as a source of energy for the organism. Proteins not only serve as fuel to yield energy but also play a vital role in the structural and functional characteristics of the living organisms. Proteins are also involved in the maintenance of osmotic balance, storage of some particular elements, enzymes to catalyze the biochemical reactions, hormones for regulation of biochemical activities, oxygen carrier (Respiratory pigments, like haemoglobin, haemocyanin, pinnaglobin etc.,) transport of lipids (Lipoproteins) (Lehninger and Albert 1988). Protein, the chief organic macromolecule for all aspects of cellular structure and function, is expected to react first upon pesticide exposure. Pesticides alter the buffering system of the intracellular environment very rapidly. Pesticides impair protein metabolism leading perhaps into disarray of functional and structural status of the cell (Shukla *et al.,* 1998). The reason for the decrement of protein is that tissue protein might be metabolized to produce glucose by the process of gluconeogenesis and glucose is utilized for energy production during stress condition by pesticide (Jayantha Rao, 1982) and it may be cause for lower amounts of protein in experimental tissues. Total protein content decreased in non-target vertebrate fauna after pesticide treatment indicating pesticide produced changes in the biochemical systems of non-target organisms. Proteins play an important role in the life of all living organisms. Pesticides alter protein synthesis by proteolysis or protein hydrolysis (Sivaiah, 2006; Usha Rani, 2010).

Amino acids are called as building blocks; these are major components of proteins. Specificity of protein molecule is due to the number and sequence of amino acids. Most remarkable is that cells can produce with stricklingly different proteins and activity by joining the same 20 amino acids in many different combinations and sequences (Nelson and Cox, 2005). The physiological state of the cell is depending upon its free amino acid pool (Vani, 1991). The protein constituents of plasma include a number of enzymes. Some of which are clinical diagnostic importance eg. amylase, lipase, phosphatases, amino transferases and glycolytic enzymes (Latner, 1975). Amino acids normally serve as substrates for the synthesis of the body's own proteins, rather than as source of energy. However, during a prolonged fast, or after illness or injury, proteins are degraded and the constituent amino acids are converted into glucose (Baynes and Dominiczak, 2005). Amino acids may not only act as precursors for the synthesis of essential proteins, but also contribute towards gluconeogenesis, glycogenesis and keto acid synthesis (Murray *et al.,* 2007). Free amino acids are known to act as osmotic and ionic agents (Jurss, 1980) in establishing/maintaining ionic equilibrium between external and internal media.

Liver is exclusive site for the metabolism of several amino acids and the free amino acid content of liver is known to change during physiological and pathological conditions (Schreier, 1962). Free amino acids are considered to act as a connecting link between protein and carbohydrate metabolism (Murray *et al.,* 2007). Skeletal muscle is the major site of amino acid deposition and also the target of many metabolic disorders. Free amino acids also plays an important role in the maintenance of osmotic pressure in the cells (Munro, 1970). Any abnormality or stress in the protein or amino acid metabolism will have its own consequence in the tissue towards the catabolic products like ammonia (Kamakshi Sundari, 1990).

The disease of a particular organ also causes serious disruption in the normal amino acid metabolism (Munro, 1983). Several enzymes, called aminotransferases (or transaminases) are capable of removing the amino group from most amino acids and producing the corresponding α-keto acid. Aminotransferase enzymes use pyridoxal phosphate, a cofactor derived from the vitamin B_6 (pyridoxine, as a key component is their catalytic mechanism (Baynes and Dominiczak, 2005). In the catabolism of amino acids, aminotransferases play a dominant role. These are the key enzymes of nitrogen metabolism and in energy mobilization (Calabrese *et al.,* 1977). Aspartate and alanine aminotransferases are present both in mitochondria and cytosolic fractions of animal (Walton and Cowey, 1982).

Transaminases are important enzymes in animal metabolism which are intimately associated with amino acid synthesis and lysis. Among these, aspartate and alanine transaminases (AST and AlAT) are widely distributed in the cells of all animals. The AST catalyses the inter conversion of aspartic acid and α-ketoglutaric acid to oxalo acetic acid and glutamic acid. While AlAT catalyses the inter conversion of alanine and α-ketoglutaric acid to pyruvic acid and glutamic acid. The enzyme glutamate dehydrogenase plays a significant role in the catabolism of amino acids. It catalyses the reversible oxidative deamination of glutamate to α-ketoglutarate and ammonia with pyridine nucleotide (NAD or NADP) as coenzyme. All these enzymes functions as a link between protein and carbohydrate metabolisms and the net out come is incorporation of keto acids into the TCA cycle. There is much evidence for the alteration in the activities of these enzymes to a variety of environmental and physiological conditions (Knox and Greengard, 1965). In protein metabolism aminotransferases catalyzes the transfer of the amino group from an amino acid to a keto acid, new amino and keto acids are formed in the process (Murray *et al.,* 2007). The activities of these aminotransferases were shown to be altered in tissues under several pathological conditions (Paul *et al.,* 1984). Elevated AST and AlAT activities can be considered as an index of gluconeogenesis (Knox and Greengard, 1965; Murray *et al.,* 2007). Several workers also observed alterations in AST, AlAT activities under pesticide stress (Swamy *et al.,* 1992; Sivaiah, 2006; Madhava Rao, 2007).

The protein metabolic profiles are studied in different pesticide poisoned mice and rats i.e., in mice with azadirachtin and monocrotophos (Sivaiah, 2006). In rats with endosulfan (Choudhary and Joshi, 2002), persistent contaminants–organ chlorines (Wade *et al.,* 2002), novel phosphorothionate (RPR-V) (Rahman and Siddiqui, 2006), lindane and endosulfan (Azhar Baig, 2007), imidacloprid (Kishandar, 2007), chlorpyrifos (Rajendra Prasad, 2007), cypermethrin (Sukanya, 2007) and acephate (Rajeswari, 2008). Pesticide poisoning on protein metabolic profiles were tested in various experimental animals such as crabs. in frogs (Madhava Rao, 2007), in fresh water fishes (Begum, 2007), in birds (Jayasree *et al.,* 2003; Garg *et al.,* 2004; Siddiqui 2004), in buffalo calves (Singh *et al.,* 1999) and in goats (Kaur *et al.,* 2000). The present investigation gives a brief understating on the effect of pesticides on protein metabolism of mice. However, the reports in this account were made mostly by taking a few parameters of protein metabolism in some selected organs of mice exposed to pesticide sublethal doses. Hence, an attempt is made in the present study to document the effect of chlorpyrifos on some aspects of protein metabolism in the

organs of Mice, on exposure to the sublethal doses of chlorpyrifos.

Experimental Design

Species: Mice

Pesticide: Chlorpyrifos Technical (95.30%) was obtained from Nagarjuna Agri. Chem Limited, Ravulapalem Mandal, East Godavari District, A.P., India.

Concentration selected: Tenth fold ($1/10^{th}$) lower concentration of LD_{50} was selected for sublethal treatment to the experimental mice.

Course of study: Single, double and multiple doses with 48 hours interval.

Route of administration: Oral

Tissues selected: Heart, liver, kidney, muscle, intestine, testes and blood.

Pesticide stock solution: Stock solution of chlorpyrifos was prepared in acetone. Working pesticide test solutions were prepared by diluting the stock solution with distilled water.

Selection of sublethal treatment to the experimental model: As the acute oral LD_{50} value of chlorpyrifos was determined, tenth fold lower ($1/10^{th}$) concentration was selected as sublethal to study the effect of chlorpyrifos. Healthy adult mice of same age (100±10 days) and weight (75±10 g) were divided into four groups having ten animals each. The second, third and fourth groups of animals were termed as experimental animals. To the animals of second group single dose of pesticide (i.e. on 1^{st} day) was administered orally by gavage method. To the third group of animals double doses were given i.e. on 1^{st} and 3^{rd} day. Similarly multiple doses i.e., 1^{st}, 3^{rd}, 5^{th} and 7^{th} day were given to the fourth group of animals. The first group of animals was considered as controls.

Isolation of tissues: The control and experimental animals after the stipulated period (i.e. on 9^{th} day) were sacrificed and the tissues were isolated, cleaned in physiological saline and processed immediately for microscopic analysis. The tissues were also quickly isolated under ice cold conditions and stored in deep freezer at -80ºC for biochemical analysis.

Experimental animals: Healthy wistar strain mice of the same age group 100±10 days and weight 75±10 grams were selected as experimental animals for the present study. The mice were collected from Indian Institute of Science (I.I.Sc.), Bangalore. Prior to experimentation the animals were acclimatized according to the instructions given by Behringer (1973).

Maintenance of animals: The mice were maintained at laboratory conditions in the animal house at 25±2°C with a photoperiod of 12hrs light and 12hrs darkness throughout the course of the present study. The mice were fed with standard pellet diet supplied by Sai Durga feeds and foods, Bangalore and water *ad libitum*.

Pesticide selected: Chlorpyrifos, an organophosphate insecticide was selected for the present investigation. Chlorpyrifos O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate with 95.30% purity was used as the test chemical for the present study. Technical grade chlorpyrifos was obtained from Nagarjuna Agri. Chem Limited, Ravulapalem Mandal, East Godavari District, A.P., India. Chlorpyrifos has a wide applicability and safety compared to other compounds of its class. Hence this pesticide was selected for the present study.

Biochemical investigations of protein metabolism:

Estimation of Total proteins: The total protein content was estimated by the method of Lowry et al. (1951). 2% homogenates were prepared in 10 % TCA and centrifuged at 1000xg for 15 minutes. The supernatant was discarded and the residue was dissolved in a known amount of 1N sodium hydroxide. From this 0.2ml was taken and 4 ml of alkaline copper reagent and 0.4ml of folin phenol reagent (1:1 folin phenol: distilled water) was added. The contents were allowed to stand for 30 minutes at room temperature and the developed color was read at 600 nm in a spectrophotometer against a reagent blank. The amount of total proteins present in the sample was calculated by using bovine albumin standard and the values were expressed as mg/g wet weight of tissue.

Estimation of Free amino acids (FAA)*:* Free amino acids content was estimated by the method of Moore and Stein (1954) as described by Colowick and Kaplan (1957). 5% homogenates of different tissues were prepared in 10% TCA and centrifuged for 15 minutes at 1000xg. To 0.25ml of the supernatant 2ml of ninhydrin reagent was added and kept in boiling water bath for 6.5 minutes and then cooled. The contents were made up to 10ml with distilled water. The intensity of the color developed was read at 570nm in a spectrophotometer against a reagent blank. The total free amino acids content was expressed as μmoles of tyrosine equivalents /g wet weight of the tissue.

Estimation of Aspartate aminotransferase (AST): The activity of aspartate aminotransferase (AST) was assayed by the colorimetric method of Reitman and Frankel (1957) as described by Bergmeyer and Bernt (1965). 2 % w/v tissue homogenates of the selected tissues were prepared in 0.25 M ice cold sucrose solution. The homogenates were centrifuged at 1000xg for 15 minutes and supernatant was used for the enzyme assay. The incubation mixture of 2.0 ml contained 100 μ moles of phosphate buffer ($Na_2HPO_4 + NaH_2PO_4$) (pH 7.4), 100μmoles of L-aspartatic acid, 2 μ moles of α-keto glutarate and 0.5 ml of supernatant as enzyme source. After incubation for 30 minutes at 37°C, the reaction was stopped by the addition of 1 ml of ketone reagent (0.001 M, 2,4-dinitrophenyl hydrazine solution in 1 N HCl) and the contents were allowed to stay at laboratory temperature for 20 minutes. After 20 minutes of 10 ml of 0.4 N NaOH was added. The developed color was read at 545 nm in a spectrophotometer against a reagent blank. The enzyme activity was expressed as μ moles of pyruvate formed / mg protein / hr.

Estimation of Alanine aminotransferase (AlAT): The activity of alanine aminotransferase (AlAT) was assayed by the colorimetric method of **Reitman and Frankel (1957)** as described by **Bergmeyer and Bernt (1965)**.The incubation mixture of 2 ml contained 100 μ moles of DL-alanine, 100 μ moles of phosphate buffer (pH 7.4), 2 μ moles of α-

ketoglutarate and 0.5 ml of the supernatant of the homogenate 2% w/v prepared in 0.25 M ice-cold sucrose solution, as enzyme source. The reaction mixture was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 1.0 ml of 2, 4-dinitrophenyl hydrazine solution prepared in 1 N HCl (ketone reagent). The color was developed by the addition of NaOH as described above for AST. The optical density was measured at 545 nm in a spectrophotometer against a reagent blank. The enzyme activity was expressed as μ moles of pyruvate formed / mg protein / hr.

Statistical treatment of the data: The mean, standard deviation (SD), percent change and one – way analysis of variance (ANOVA) (Steel and Torrle, 1960) were performed using the SPSS package programming techniques on "Intel Core 2Duo Processor" personnel computer. Probability values less than 0.05 were considered significant. (Snedecor and Cochran, 1968).

RESULTS

Total Protein content: The results of total protein content of the control and experimental mice under chlorpyrifos are given in (Table.1 and Figure.1). The experimental Mice exposed to chlorpyrifos showed statistically significant ($P<0.01$) decrease of total protein content in heart, liver, kidney and muscle respectively. The decrease in total protein content was dose and time dependent manner in chlorpyrifos treated Mice. In experimental conditions the tissues have shown decreased total protein content in liver (58.06%) followed by muscle (41.97%), kidney (39.01%), and heart (1.23%) in multiple doses. The maximum decrease was observed in multiple doses followed by double and single dose chlorpyrifos treated mice. The lyotrophic series of total protein content decrement in multiple doses chlorpyrifos treated mice is as follows:

Liver > Muscle > Kidney > Heart

Free amino acids:(FAA): The results of total free amino acid content of the control and experimental mice under the study are given in (Table 2 and Figure 2).The experimental mice exposed to chlorpyrifos showed statistically significant ($P<0.01$) increase of free amino acid content in heart, liver, kidney and muscle respectively. The increase in free amino acid content was dose and time dependent manner in chlorpyrifos treated mice. In experimental conditions the tissues have shown increased free amino acid content in kidney (101.20%) followed by heart (74.24%), muscle (70.00%) and liver (66.16%), in multiple doses. The maximum increase was observed in multiple doses followed by double and single dose chlorpyrifos treated mice. The lyotrophic series of total free amino acid content increment in multiple doses chlorpyrifos treated mice is as follows:

Kidney > Heart > Muscle >Liver

Aspartate aminotransferase: (AST)

The results of aspartate aminotransferase activity in the control and experimental mice under the study are given in (Table 3. and Figure 3).The experimental rats exposed to chlorpyrifos showed statistically significant ($P<0.01$) increased of aspartate aminotransferase activity in heart, liver, kidney and muscle respectively.

The increase in aspartate aminotransferase activity was dose and time dependent manner in chlorpyrifos treated mice.In experimental conditions the tissues have shown increased aspartate aminotransferase activity in heart (35.11%) followed by muscle (30.13%), kidney (25.54%) and liver (11.17%) in multiple doses. The maximum increase was observed in multiple doses followed by double and single dose chlorpyrifos treated mice. The lyotrophic series of aspartate aminotransferase activity increment in multiple doses chlorpyrifos treated mice is as follows:

Heart > Muscle > Kidney> Liver

Alanine aminotransferase: (AlAT): The results of alanine aminotransferase activity in the control and experimental mice under the study are given in (Table 4 and Figure 4). The experimental rats exposed to chlorpyrifos showed statistically significant ($P<0.01$) increase of alanine aminotransferase activity in heart, liver, kidney and muscle respectively. The increase in alanine aminotransferase activity was dose and time dependent manner in chlorpyrifos treated mice. In experimental conditions the tissues have shown increased alanine aminotransferase activity in kidney (48.31%) followed by muscle (35.86%), heart (27.10%) and liver (23.72%) in multiple doses. The maximum increase was observed in multiple doses followed by double and single dose chlorpyrifos treated mice. The lyotrophic series of alanine aminotransferase activity increment in multiple doses chlorpyrifos treated mice is as follows:

Kidney > Muscle > Heart > Liver

DISCUSSION

Total protein content: Catabolism of proteins and amino acids make a major contribution to the total energy production in mice. Proteins being involved in the architecture and physiology of the cell, they seem to occupy a key role in cell metabolism (Murray *et al.,* 2007). The depletion of total protein content observed in this investigation (Table 1 and Figure 1) can be correlated to this fact. Bradbury *et al.* (1987) pointed out that the decreased protein content might also be attributed to the destruction or necrosis of cellular function and consequent impairment in protein synthetic machinery. Protein depletion in tissues may constitute a physiological mechanism and may play a role of compensatory mechanism under chlorpyrifos stress, to provide intermediates to the Krebs cycle. It has also been reported that this trend of proteins was to enhance osmolarity to compensate osmoregulatory problems encountered due to the leakage of ions and other essential molecules during pyrethroid toxicity (Rafat Yasmees, 1986).

Proteins being the most important organic constituents of organs, their role of them in the compensatory mechanisms of an animal can be accepted during stress conditions (Singaraju *et al.,* 1991). Shifts in proteins may ultimately lead to alterations in the entire protein metabolism of animals. In the present study, effects of chlorpyrifos on the protein metabolism of the tissues of the rats exhibited dose and time dependent changes in all the tissues exposed to the sublethal doses of chlorpyrifos indicating the breakdown of these proteins due to the pesticide toxic stress. Generally the breakdown of proteins dominates over synthesis under enhanced proteolytic activity (Murray *et al.,* 2007). It is evident in the present study that the breakdown of proteins is

associated with the steep elevation in protease activity and free amino acid levels in the organs of the rats exposed to the sub lethal doses of chlorpyrifos (single, double and multiple dose). These results are in agreement with the earlier report of Ravider *et al.* (1988) who demonstrated a similar situation in *Clarius batrachus* exposed to decis. Mommsen and Walsh (1992) have been estimated that oxidation of nitrogenous substances may account for 41 and 85% of total energy production from proteins and amino acid, respectively. Klassan (1991) reported that the depletion of protein suggests increased proteolysis and possible utilization of the products of their degradation for metabolic purposes. They may be fed into TCA cycle through aminotransferase system to cope up with excess demand of energy during the elimination of toxicants from the body. The depletion of protein level induces to diversification of energy to meet the impending energy demands during the toxic stress (Jagadeesan and Mathivanan, 1999).

The decrease in protein content may also be due to reduced generation of energy during Chlorpyrifos toxicity. It is well established that the maintenance of structural integrity in a highly organized state requires a continuous supply of energy. If this is impaired, the structure breaks down and the proteins can be partially denatured and can thus become more accessible to the action of proteolytic enzymes (Murray *et al.,* 2007). Decreased serum total protein is due to lowered synthesis of albumin in liver and a rise in globulins (mostly α-globulins) is due to prolonged exposures of OP pesticides (Subbotina and Belonozhko, 1968). The decrease in blood protein may be due to loss of protein either by reduced protein synthesis or increased proteolytic activity or degradation (Shakoori *et al.,* 1990). Rivarola and Balegno (1991) reported the reduction in protein content in animals treated with pesticides could be attributed to changes in protein and free amino acid metabolism and their synthesis in liver. Thaker and Garg (1993) studied biochemical changes in male WHL chicks following long term daily oral administration of endosulfan and malathion. Endosulfan was administered at 1.21 and 2.42 mg/kg body weight and malathion at 9.04 and 18.08 mg/kg body weight. Both pesticides caused decrease in serum total protein in the treated birds.

Cypermethrin-treated sheep and suggested that the decline in serum protein was mainly due to the decrease in albumin rather than globulin fraction (Yousef *et al.,* 1998). Das and Mukherjee (2000) studied the effect of exposure to sub lethal concentrations of the organophosphate pesticide, quinalphos (1.12, 0.22 mg/l) on biochemical parameters of muscle and enzyme activities in brain, liver and kidney of fish (*Labeo rohita*). They found that after 15, 30 and 45 days quinalphos decreased the muscle protein over a period of 45 days. Kaur *et al.* (2000) investigated the toxic effect of chlorpyrifos following repeated oral administration for 28 days on certain blood biochemical profiles and tissues /organs in goats. Blood samples were collected on days 0, 7, 14, 21, and 28 post chlorpyrifos administration to study the total proteins. It produced significant increase in total proteins level. Singh *et al.* (2001) studied the hemato-biochemical profiles in cockerels following prolong feeding of fenvalerate medicated ration at the rate of 4000 ppm. It was found that the fenvalerate significantly decreased total serum protein level. Shah and Gupta (2001) conducted sub acute toxicity studies of permethrin in young albino male mice. Daily oral administration of permethrin at the rate of 24-120 mg/kg for 30 days showed non-significant changes in the level of total proteins. Lynch *et al.* (2003) reported decreased total protein content in mice serum exposed to inhaled dimethylformamide for 13 weeks. Patil *et al.* (2003) found decrease in serum total protein and albumin levels during exposure of various pesticides on sprayers of grape garden. Garg *et al.* (2004) studied the hemato-biochemical and immuno-patho-physiological changes following feeding of broiler chicks with 20 ppm fenvalerate, 2 ppm monocrotophos and 2 ppm endosulfan. Four groups of broiler birds (30 each) were fed poultry mash without (control) or mixed with pesticides for 8 weeks. Serum globulin level was decreased in all treated groups compared to control, but not the serum albumin. Siddiqui (2004) studied toxicological and immunological effects of sub acute exposure of cockerels to imidacloprid and quinalphos in WLH cockerels. Total protein was decreased in all the pesticide treated birds after 14 days of treatment where as total globulin was decreased only in quinalphos treated groups. Pesticide treatment caused significant reduction in total serum protein in the broiler chicks (Garg *et al.,* 2004). Thus it is possible that the rough endoplasmic reticulum of osteoblasts is adversely altered due to low dose feeding of different pesticides in broiler chicks causing lower synthesis of bone matrix and diminished appositional bone growth.

Acephate affects the serum total proteins, serum total albumin as compared to control white leghorn cockerels, indicating significant damage to vital organs and interference with protein metabolism (Tripathi and Verma, 2007). Chlorpyrifos caused significant decreases in total protein contents in the treated mice; similarly, several authors reported decreased total protein contents in different animal models under pesticidal toxicity, such as in fishes treated with atrazine (Prasad *et al.,* 1991), frogs treated with cypermethrin and permethrin (Khan *et al.,* 2003) and azadirachtin (Madhava Rao, 2007), carbofuran (Begum, 2004), cypermethrin (Philip and Rajasree, 1996; David *et al.,* 2004; Begum, 2007), fenvalerate (Tripathi and Verma, 2004) and quinalphos (Das and Mukherjee, 2000), in *Procellio dilatatus* treated with parathion (Ribeiro *et al.,*2001), in reptiles treated with permethrin and neem fractions pyrethroid and organophosphate, in birds treated with acephate (Tripathi *et al.,* 2007), in mice treated with monocrotophos (Madhavee Latha, 2006) and azadirachtin and monocrotophos (Sivaiah, 2006), in rats treated with monocrotophos mancozeb (Baligar and Kaliwal, 2001), chlorpyrifos (Rajendra Prasad, 2007), cypermethrin (Sukanya, 2007; and Nagarjuna, 2007), imidacloprid (Kishandar, 2007) and acephate (Rajeswari, 2008).

There is an increased evidence of pesticide protein interaction, which relevant to the mode of action of insecticide (Casida *et al.,* 1983). The changes in biochemical constituents caused by pesticide toxicity at sublethal level affects the nutritive value of fish, which is a protein-rich food (Deoray and Wagh, 1991; Suhasini *et al.* (2006) reported decreased protein content in liver tissue of mice exposed to hexachlorophene. Decreased protein content was observed in tissues of *Tilapia mossambica* exposed to sodium selenite (Samson Raju *et al.* 2006). John Sushma *et al.* (2006) reported decreased protein content in tissues of mice exposed to aluminum acetate. Increase in total protein and albumin may be the result of hypertrophic changes in the liver in response to complex mixture of persistent contaminants and the proliferation of endoplasmic reticulum implied by the obvious cellular hypertrophy and increased cytochrome P_{450} activity.

Table 1. Changes in total protein content (mg/gm wet wt of tissue) in different tissues of control and chlorpyrifos treated mice. Values in parentheses indicate percent change over control

Name of the tissue	Control	Single Dose	Double Dose	Multiple Dose
Heart				
Mean	80.0515	70.084	68.016	65.072
SD	±1.1882	±1.0517	±1.1784	±1.1967
PC		(-1.2361)***	(-4.9138)***	(-1.2361)***
Liver				
Mean	140.25	131.17	128.06	115.26
SD	±1.4106	±1.1290	±1.1014	±1.0371
PC		(-46.7241)*	(-48.9416)*	(-58.0681)*
Kidney				
Mean	111.33	99.292	92.16	80.504
SD	±1.2503	±1.2140	± 1.2412	±1.2105
PC		(-22.1428)*	(-28.5490)*	(-39.0188)*
Muscle				
Mean	120.24	105.117	98.85	94.109
SD	±1.1600	±1.1219	±1.1736	±1.3306
PC		(-32.8173)*	(-38.0294)*	(-41.9723)*

All the values are mean ± SD of six individual observations.
SD – Standard Deviation.
PC – Percent change over control.
* Significant P<0.001
** Significant P<0.05
*** Significant P<0.01

Table 2. Changes in free amino acid content (µ moles of tyrosine/gm wet wt of tissue) in different tissues of control and chlorpyrifos treated mice. Values in parentheses indicate percent change over control.

Name of the tissue	Control	Single Dose	Double Dose	Multiple Dose
Heart				
Mean	48.703	54.902	56.538	59.877
SD	±1.1790	±1.2613	±2.2285	±1.7264
PC		(64.0251)	(67.3843)	(74.2401)
Liver				
Mean	62.784	69.863	74.887	80.96
SD	±1.6440	±1.6970	±1.7312	±1.7080
PC		(48.4911)	(56.4932)	(66.1660)
Kidney	39.906			
Mean	±1.7177	44.89014	40.818	56.31
SD		±1.6963	±1.5677	±1.333
PC		(72.5837)	(62.3793)	(101.2006)
Muscle	56.96			
Mean	±1.4063	65.34	69.247	72.32
SD		±1.3232	±1.1391	±1.1939
PC		(57.7520)	(64.6112)	(70.0062)

All the values are mean ± SD of six individual observations.
SD – Standard Deviation.
PC – Percent change over control.
All are significant at P<0.001

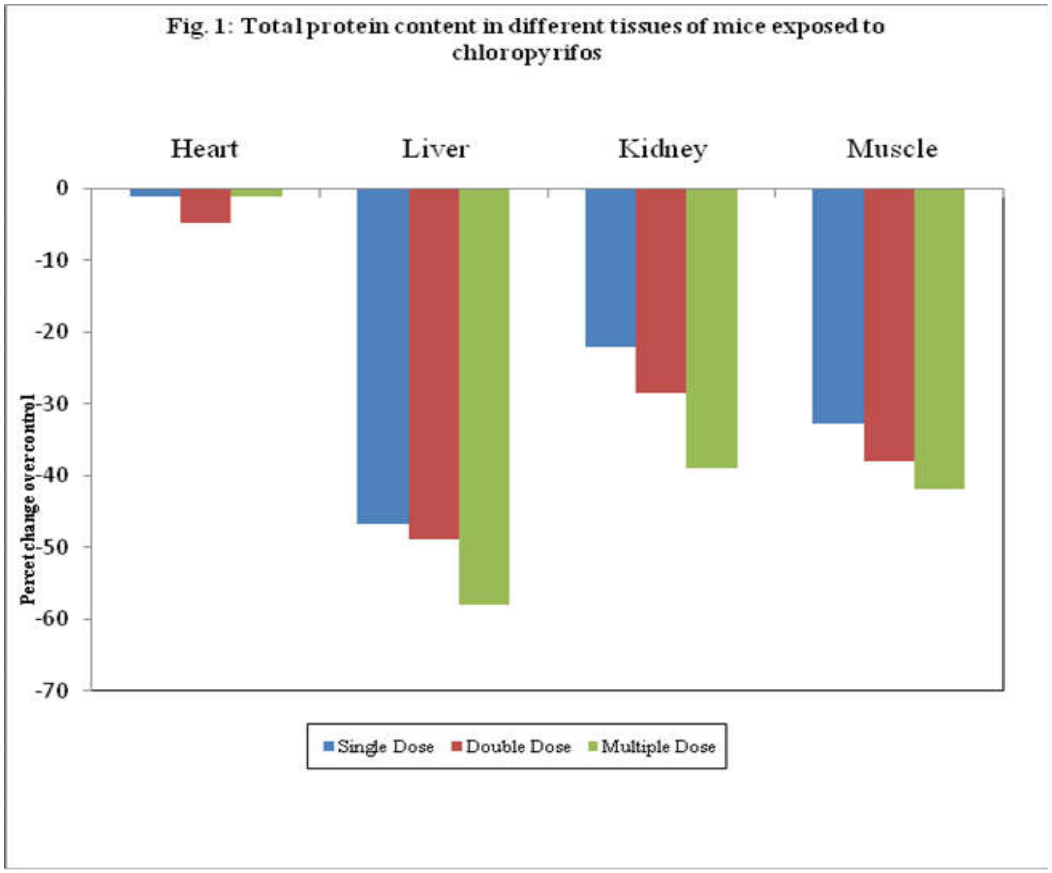
Table 3. Changes in aspartate aminotransferase (µ moles of pyruvate formed/mg protein/hr) levels in different tissues of control and chlorpyrifos treated mice. Values in parentheses indicate percent change over control.

Name of the tissue	Control	Single Dose	Double Dose	Multiple Dose
Heart				
Mean	0.4152	0.4337	0.5587	0.561
SD	±0.14946	±0.1585	±0.0947	±0.0889
PC		(4.4556)***	(34.5616)*	(35.1156)*
Liver				
Mean	1.4135	1.5638	1.5461	1.5715
SD	±0.7196	±0.7576	±0.6706	±0.7327
PC		(10.6331)**	(9.3809)***	(11.1779)**
Kidney				
Mean	0.20366	0.2236	0.2398	0.2556
SD	±0.00766	±0.003983	±0.002787	±0.003011
PC		(9.8231)*	(17.7799)*	(25.540)*
Muscle				
Mean	0.3507	0.4342	0.4462	0.502
SD	±0.1192	±0.1153	±0.0813	±0.1041
PC		(23.8095)*	(27.2312)*	(30.1394)*

Table 4. Changes in alanine aminotransferase (µ moles of pyruvate formed/mg protein/hr) levels in different tissues of control and chlorpyrifos treated mice. Values in parentheses indicate percent change over control

Name of the tissue	Control	Single Dose	Double Dose	Multiple Dose
Heart				
Mean	0.5102	0.5302	0.6082	0.6485
SD	±0.13021	±0.1184	±0.1074	±0.0905
PC		(3.9200)***	(33.3202)*	(27.1070)*
Liver				
Mean	1.500	1.6098	1.7622	1.8558
SD	±0.1870	±0.5795	±0.6364	±0.6951
PC		(7.32)*	(17.48)*	(23.72)*
Kidney				
Mean	0.350	0.400	0.452	0.5191
SD	±0.0188	±0.001414	±0.0781	±0.1151
PC		(14.285)**	(29.142)*	(48.314)**
Muscle				
Mean	0.5314	0.5964	0.6414	0.722
SD	±0.0928	±0.1084	±0.1082	±0.1052
PC		(12.2318)**	(20.7000)*	(35.8675)*

All the values are mean ± SD of six individual observations.
SD – Standard Deviation.
PC – Percent change over control.
* Significant P<0.001
** Significant P<0.05
*** Significant P<0.01



Alternatively, increased serum protein and albumin levels could indicate mild dehydration that is consistent with negative water balance seen in mice treated with a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), similar to the total dose administered to the high dose animals over the 70-day treatment of period (Wade *et al.* (2002). Garg *et al.* (1997) reported mice pre-treated with fluvalinate exhibited a dose-dependent increase in relative liver weights and total liver proteins. The alterations in protein metabolism were also observed at low dose (single dose) as the stress also prevailed at low dose (single dose) exposure more changes were observed in multiple dose cypermethrin treated mice (Nagarjuna, 2007). Protein profile alterations were observed in acephate an organophosphate treated albino rats (Rajeswari, 2008). The data presented on total protein content indicates that a significant decrease occurs in total protein content in all tissues under chlorpyrifos toxicity in dose and time dependent manner. Decrease in total protein content suggests its metabolic utilization under chlorpyrifos toxic stress. It is clear from the foregoing account that overall decrease in total protein with abnormal rise in free amino acid pool might be explained in terms of accelerated proteolytic activity observed in different tissues during chlorpyrifos toxicity.

Fig: 2. Free amino acid content in different tissues of mice exposed to chlorphyrifos

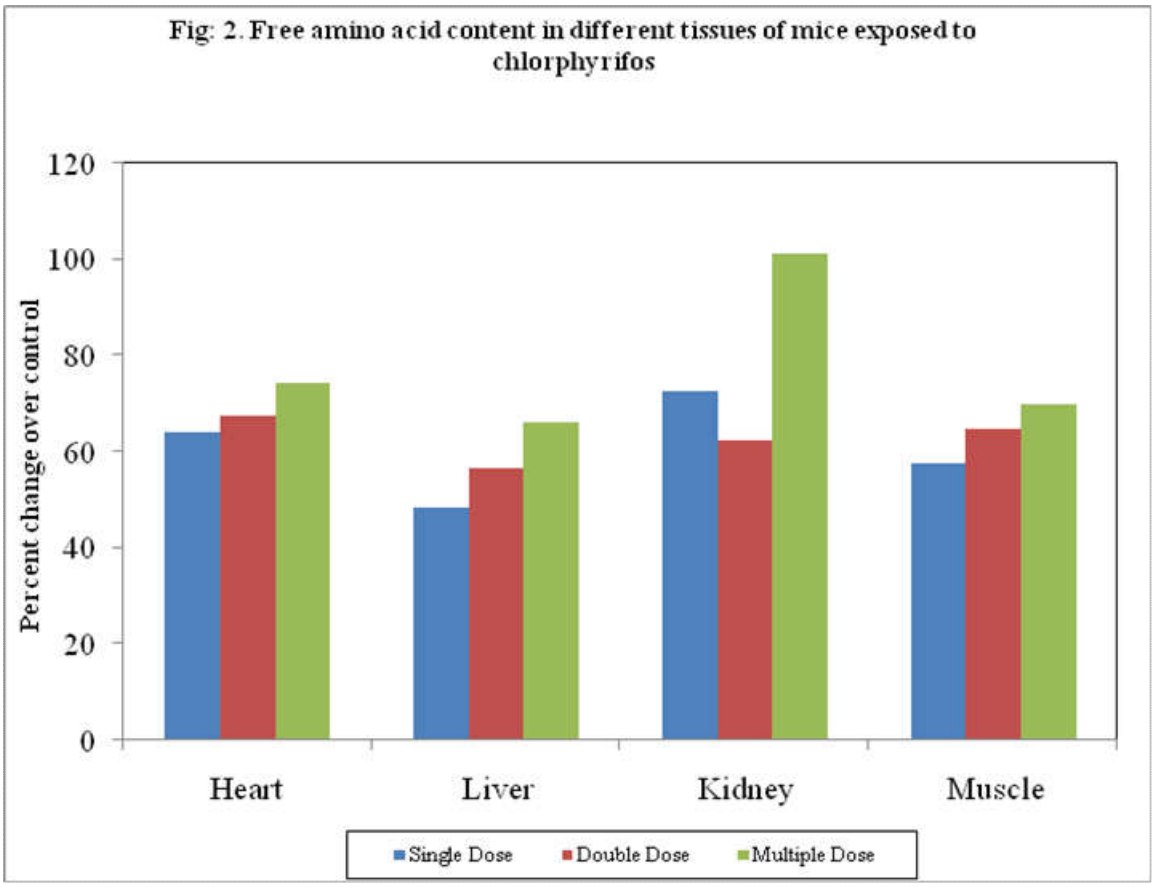
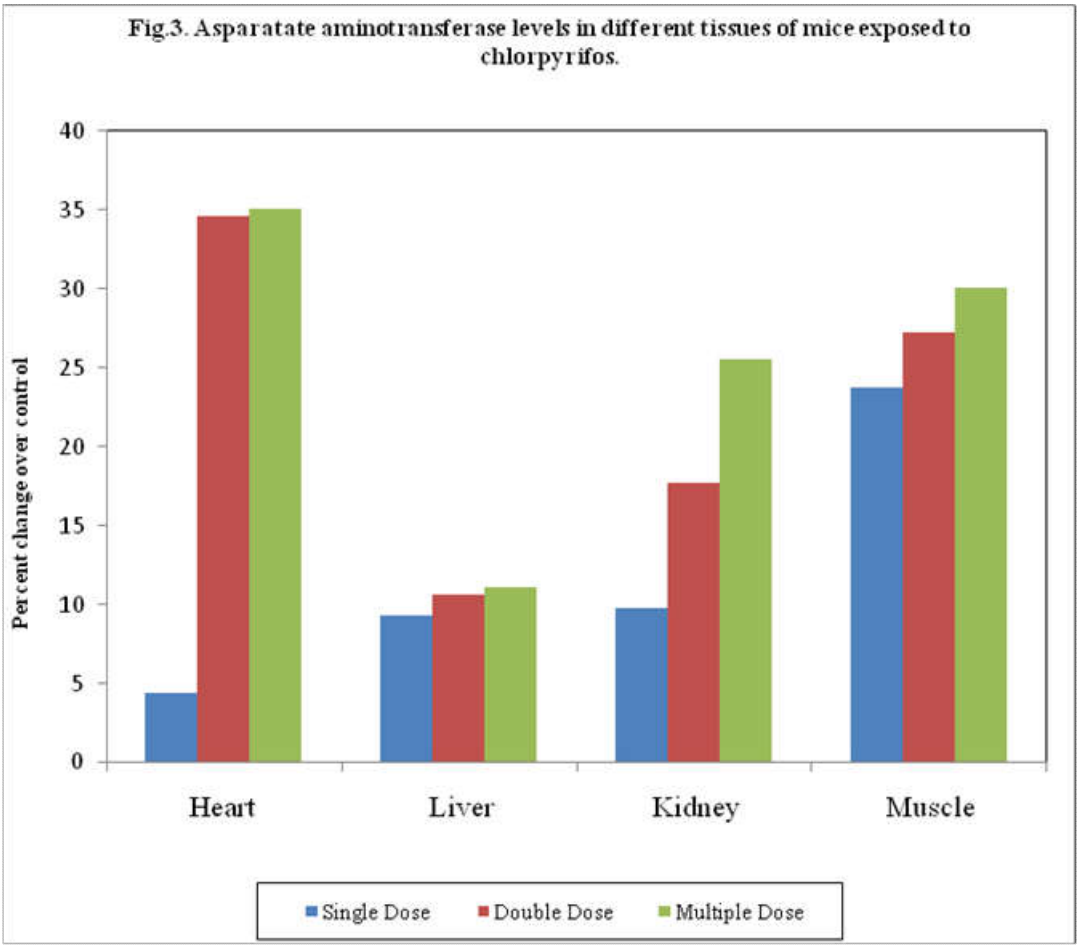
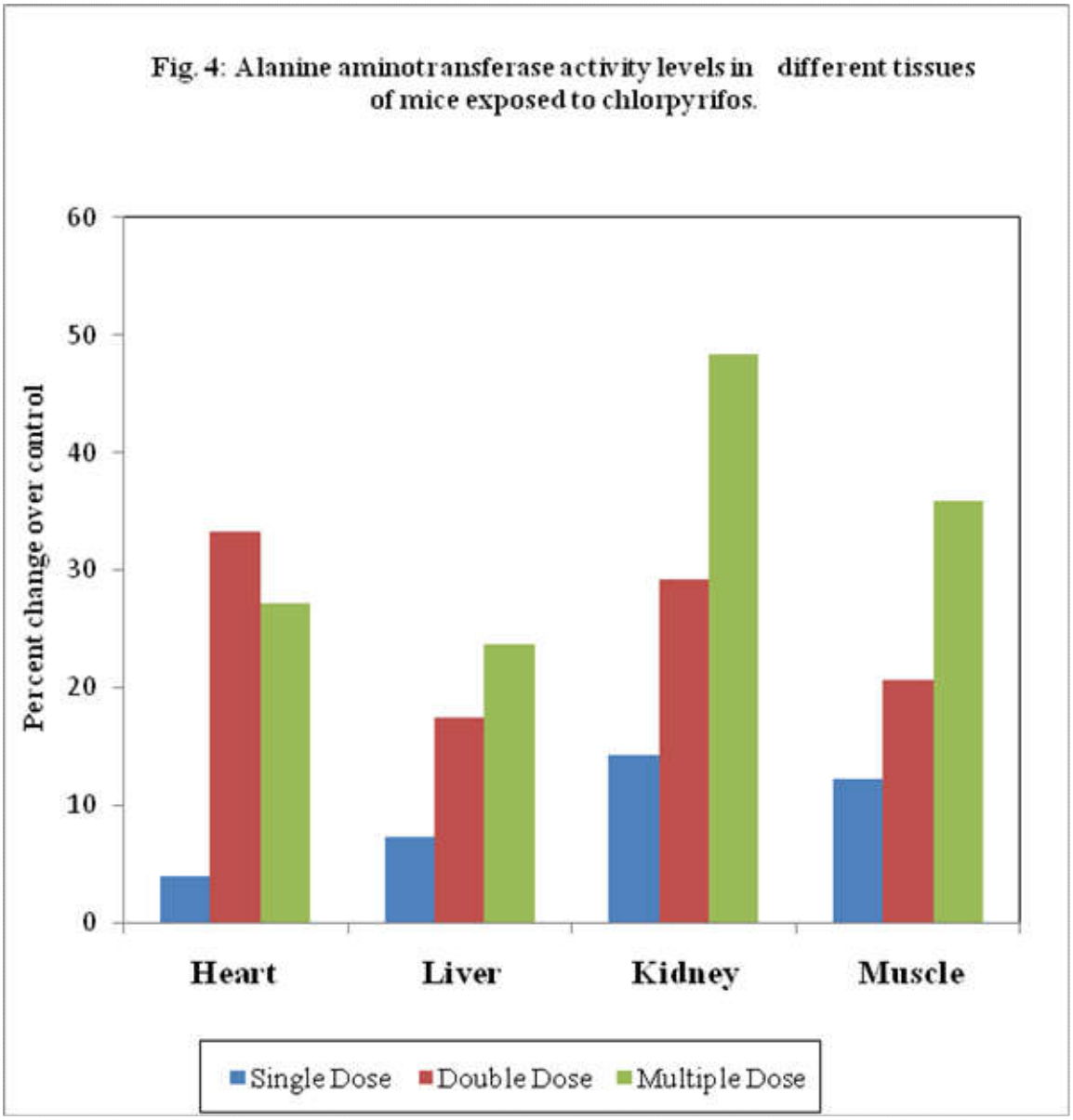


Fig.3. Asparatate aminotransferase levels in different tissues of mice exposed to chlorpyrifos.





Free amino acids: (FAA): The elevation in free amino acid content in all the tissues in the present investigation (Table 2 and Figure 2) is consistent with the decreased protein level, enhanced protease activity and enhanced transamination during chlorpyrifos exposure of single, double and multiple doses. The alterations in amino acid indicate the condition of the tissue, and their increase or decrease might be considered as the operation of the stress phenomenon at the tissue level (Shakoori *et al.,* 1976). Free amino acids thus increased may be fed into the TCA cycle through aminotransferases, possibly to be utilized for energy production. Presumably the degradation of proteins has led to FAA accumulation. This higher level of FAA can also be attributed to the decreased utilization of amino acids and is also suggestive of its involvement in the maintenance of osmotic and acid base balance (Moorthy *et al,* 1984). Vacuolar degeneration and membrane disruption during hexachlorophene stress were accompanied by manifestation of hepatic dysfunction leading to the abnormal rise FAA levels (Prasad, 1986). Tissue damage, which is due to pesticide action, may also elevate the levels of amino acids (Vijay Joseph, 1989). Amino acids may not only act as precursors for the synthesis of essential proteins, but also contribute towards gluconeogenesis, glycogenesis and keto acid synthesis (Murray *et al.,* 2007).

Free amino acids are known to act as osmotic and ionic agents (Jurss, 1980) in establishing/ maintaining ionic equilibrium between external and internal media. The increase in FAA content is a clear indication of (a) stepped up proteolysis and or (b) fixation of ammonia into keto acids resulting in amino acid synthesis. These two processes in general contribute to the amino acid pool. These amino acids may be utilized for the synthesis of new types of essential proteins and enzymes to cope with the toxic stress conditions to which the animal was exposed (Ogata *et al.,* 197). Amino acids might be fed into the TCA cycle as keto acids by way of transamination, since transaminases are known to be elevated during pesticide intoxication (Kabeer, 1979; Rajeswara Rao *et al.,* 1983). The increase in AST and ALT activities results in the greater production of glutamate, which in turn favors the elevation of GDH activity. The increased glutamate partly aids in meeting the energy demands under toxic stress by entering into the TCA cycle. This links protein metabolism with carbohydrate metabolism. Increased amino acid levels could be partly responsible for the increased GDH activity (Ramanadikshithulu *et al.,* 1976). The elevation in free amino acid levels with the increased exposure periods could indicate the speedy channeling of these bio-molecules for thes synthesis of required proteins and to meet the energy demands by

incorporating into TCA cycle in the form of keto acids through trans-de-amination reactions (Suresh *et al.,* 1991) as evidenced by the gradual increase in AST, ALT activities. In the present study, chlorpyrifos caused significant increases in free amino acid contents in the treated rats; similarly, several authors reported increased free amino acid contents in different animal models under pesticidal toxicity, such as in frogs treated with azadirachtin (Madhava Rao, 2007), in fishes treated with atrazine, carbofuran (Begum, 2004) and cypermethrin (David *et al.,* 2004; Begum, 2007), in mice treated azadirachtin and monocrotophos (Sivaiah, 2006), in rats treated with chlorpyrifos (Rajendra Prasad, 2007), cypermethrin (Sukanya, 2007; and Nagarjuna, 2007), imidacloprid (Kishandar, 2007) and acephate (Rajeswari, 2008). Increased free amino acid content was observed in liver tissue of albino rat exposed to hexachlorophene (Suhasini *et al.* 2006). Samson Raju *et al.* (2006) reported increased free amino acid content in tissues of *Tilapia mossambica* exposed to sodium selenite. John Sushma *et al.* (2006) reported increased free amino acid content in tissues of mice exposed to aluminum acetate. Free amino acid levels were elevated in all the tissues of albino rats exposed to cypermethrin (Nagarjuna, 2007).Elevated levels of free amino acids were observed in different tissues of albino rats exposed to acephate (Rajeswari, 2008). Free amino acids were elevated in all the tissues studied exposed to chlorpyrifos. The elevation in free amino acids was in consonance with the increased proteolytic activity. The elevated free amino acid levels indicate altered protein homeostasis and nitrogen balance due to chlorpyrifos toxicity.

Aspartate and Alanine aminotransferases: (AST and AlAT): Increased levels of AST and AlAT activities offer an excellent support to the above trend (Tables 3 and 4 Figures 3. and 4). This is a clear indication of shunting of amino acids into TCA cycle through oxidative deamination and active transamination. Such a phenomena was necessary to cope up with the energy crisis during chlorpyrifos stress. It has also been suggested that stress conditions in general induce elevation in the transamination pathway (Awasthi *et al.,* 1984). Involvement of alternate pathways like aminotransferase reactions are also possible due to inhibition of oxidative enzymes like succinate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase and cytochrome-c-oxidase, a situation also demonstrated by Ghosh (1989) in *Labeo rohita* under cypermethrin toxicity.

Increased AST and AlAT activities may also be due to disruption of mitochondrial integrity or increased synthesis of enzymes. Increased ammonia content was also shown to be responsible for the increased transaminases activity (Chandra Mohan, 1979). The aminotransferases play a very important role in the animal metabolism in the sense that they are intimately associated with amino acid synthesis and lysis. Aminotransferases play an important role in the utilization of amino acids for the oxidation and/or for gluconeogenesis (Murray *et al.,* 2007). The depletion in energy reserve necessitates enhancement of other alternative mechanisms like aminotransferase reaction to feed the keto acids into the TCA cycle (Kabeer *et al.,* 1978). The aspartate and alanine aminotransferases which function as a strategic link between carbohydrate and protein metabolisms are known to alter under severe pathological conditions (Ramana Rao *et al.,* 1990), hence they are considered as sensitive indicators of stress. The aspartate and alanine aminotransferases are referred as "markers of cell injury" (Loeb, 1982) and are excellent indicators of early hepatic lesions, since they are first to leak out from the cell in the case of injury. Increase in AST and AlAT in different tissues of the rats at different doses of chlorpyrifos indicates the initial effort taken by the animal for raising its energy resources through active transamination and/or for the synthesis new proteins required for detoxification of the toxicant and its disposal. To have an insight into the role of these enzymes in the altered metabolism of pesticide intoxicated rats the activities of both AST and AlAT were investigated in the present experiment. Elevated levels of AST and AlAT indicate the enhanced transamination of amino acids, which may provide keto acids to serve as precursors in the synthesis of essential organic elements. Activities of superoxide dismutase, catalase, glutathione and malonyl-dialdehyde levels in the liver reflect the oxidative status and the serum enzymes like AST, AlAT and ALP represent the functional status of the liver (Manna *et al.,* 2005). Mukhopadhyay *et al.* (1982) reported increased GOT and GPT activities in liver and serum of the carbofuran treated *Clarias batrachus* are compatible with liver damage and growth rate reduction. The increase in transaminases can also be linked to the formation of urea (Ramana Rao and Ramamurthi, 1983). The steady increase in these enzyme activities may be helpful in metabolic compensation and to allow the animal to adapt toxic stress. The increase in AST and AlAT activities may be attributed to cellular damage (Drotman and Lawhorn, 1978), increased plasma membrane permeability (Ramazzotto and Carlin, 1978) or altered metabolism of enzymes (Dinman *et al.,* 1963; Malik *et al.,* 1980). The increased activity of AST and AlAT may be a compensatory mechanism for the impaired mitochondrial oxidation (Riaz Fathima, 1984). Travlos *et al.* (1996) reported an association between treatment-related increases in AlAT and SDH activities and the occurrence of histopathological changes in the rat liver.

Increased activities of AST and AlAT are adaptive physiological responses to combat energy demand since a local correlation appears between mitochondrial integrity and transaminase levels (Bonitenko, 1974). As cell viability depends directly on the structure of the membrane, the damage to the cellular membrane was detected by blood enzymes leakage (Jung-HoonJee *et al.,* 2005). It has been generally accepted that the leakage of the cytosolic enzymes correlates well with cellular viability, thus being a useful indicator of membranes damage (Del Raso, 1992). When the liver cell membrane is damaged varieties of enzymes normally located on the cytosol are released into the bloodstream. Their estimation in serum is a useful quantitative marker of the extent and type of hepatocellular damage (Dwivedi *et al.,* 1991; Philip and Rajasree, 1996; El-Gendy *et al.,* 1998; Mitra *et al.,* 1998; Murray *et al.,* 2007). Increased levels of enzymes and other parameters can be caused by structural liver alterations (e.g., cirrhosis) reducing aminotransferase activity, and concomitantly reducing the deamination capacity. A significant increase in the activity of creatine kinase, lactate dehydrogenase and transaminases plasma enzymes indicates stress-based tissue impairment (Svoboda, 2001). Elevated levels of serum aspartate and alanine aminotransferase after oral administration of monocrotophos were observed at the dose rate of 0.5 mg/kg/day for 28 days (Sandhu and Malik, 1988). Damage to liver is known to result in cellular changes in the tissues and alteration in activities of enzymes in liver and serum (Zimmerman, 1990). Significantly increased the SGPT activity in liver and heart and SGOT activity in heart were observed following once daily oral administration of

fenvalerate at the rate of 525.6 mg/kg in broiler chicks for 28 days (Majumder *et al.,* 1994). Fluvalinate produced a significant increase in AST activity and blood urea nitrogen concentrations which are indicative of hepato and nephropathies (Garg *et al.,* 1997). Kaur *et al.* (2000) investigated the toxic effect of chlorpyrifos following repeated oral administration for 28 days on certain blood biochemical profiles and tissues /organs in goats. Blood samples were collected on days 0, 7, 14, 21, and 28 post chlorpyrifos administration to study aspartate and alanine aminotransferase activities. It produced significant increase in aspartate and alanine aminotransferase activities. Singh *et al.* (2001) studied the biochemical profiles in cockerels following prolong feeding of fenvalerate mediated ration at the rate of 4000 ppm. They observed increased the level of serum AST and AlAT in chicks. Jayasree *et al.* (2003) evaluated the mechanism of toxicity due to deltamethrin in poultry. Broiler chicks were fed on ration containing deltamethrin (100 mg/kg) for 6 weeks. Body weights and serobiochemical parameters were evaluated at every 2 week interval. The study revealed significant increase in the activity of aspartate aminotransferase. The liver was the primary site of toxicity in rats and mice exposed to inhaled dimethylformamide for 13 weeks. Centrilobular hepatocellular necrosis seen in exposed rats was accompanied by increased activities of hepatic intracellular enzymes in the serum, and also by increase in relative liver weights. The centrilobular hepatocellular hypertrophy seen in exposed mice was accompanied by increased absolute and relative liver weights (Lynch, 2003). Subchronic dosing of novel phosphorothionate (RPR-V), aspartate and alanine aminotransferases increased significantly in serum and kidney but decreased significantly in liver and lung tissues of rats, indicating hepatotoxicity and necrosis of these tissues (Rahman and Siddiqui, 2003). Patil *et al.* (2003) found increase in serum AST and AlAT levels during exposure of various pesticides on sprayers of grape garden. Siddiqui (2004) found that imidacloprid and quinalphos treated 8 to 10 week old WLH cockerels had increased transaminase activity (SGOT and SGPT) in serum after 14 days of treatment.

Chlorpyrifos caused significant increases in AST and AlAT activities in the treated rats; similarly, several authors reported increased AST and AlAT activities in different animal models under pesticidal toxicity, such as in fishes treated with atrazine (Prasad *et al.,* 1991), in frogs treated with fenvalerate (Sakr and Hanafy, 2002) and azadirachtin (Madhava Rao, 2007), carbofuran (Begum, 2004), cypermethrin (Philip and Rajasree, 1996; David *et al.,* 2004; Jung-Hoon Jee *et al.,* 2005; Velisek *et al.,*2006; Begum, 2007) and fenvalerate (Ananda Raju *et al.,* 2005), in broiler chicks treated with permethrin (Shah and Gupta, 2001) and fluvalinate (Garg *et al.,*2004), in rabbits treated with cypermethrin, in buffalo calves treated with fenvalerate (Singh *et al.,*1999), in mice treated azadirachtin and monocrotophos (Sivaiah, 2006), in rats treated with malathion impurities (Keadtisuke *et al.,* 1990), tetramethrin and sumithion (Abu-El Zahab *et al.,*1993), endosulfan (Choudhary and Joshi, 2002), α-cypermethrin (Manna *et al.,* 2005), deltamethrin (Manna *et al.,* 2005), chlorpyrifos (Rajendra Prasad, 2007), cypermethrin (Sukanya, 2007 and Nagarjuna, 2007), imidacloprid (Kishandar, 2007) and acephate (Rajeswari, 2008) . Suhasini *et al.* (2006) reported increased AST and AlAT activities in liver tissue of albino rat exposed to hexachlorophene. Samson Raju *et al.* (2006) reported decreased AST and AlAT activities in tissues of *Tilapia mossambica* exposed to sodium selenite. John Sushma *et al.,*. (2006) reported increased AST and AlAT activities in tissues of mice exposed to aluminum acetate. Increase in the levels of glucogenic aminotransferases (AST and AlAT) were elevated in all the tissues of albino rats treated with cypermethrin (Nagarjuna, 2007). Increased levels of Aspartate and Alanine amino transferases activity levels were observed in different tissues of albino rats treated with acephate (Rajeswari, 2008). The activity levels of aminotransferases (AST and AlAT) were elevated in all the tissues studied. The elevated AST and AlAT activities indicate the mobilization of free amino acids towards gluconeogenesis to meet the energy demands during chlorpyrifos toxicity. Elevated levels of AST and AlAT indicate the enhanced transamination of amino acids which may provide keto acids to serve as precursors in the synthesis of essential organic constituents. Activation of detoxification mechanisms by utilizing the proteins and their turnover. Even though the decrease in proteins, they may help the animal to fortify the organs for developing resistance to the imposed toxic stress. Decrease in proteins could indicate the turn over of tissue proteins for the synthesis of enzymes necessary for detoxification and degradation of the pesticide. The removal of pesticide from general intracellular environment helps the animal to adapt to such toxic stress. From these observations made in the mice under chlorpyrifos intoxication, it is concluded that the changes are dependent on the dose of pesticide. High dose (multiple doses) caused more damage to the physiological, biochemical activities of the mice. Hence, there appeared an irrecoverable loss to the biochemical integrity of the cells due to chlorpyrifos stress. The alterations in protein metabolism were also observed at low dose (single dose) as the stress also prevailed at low dose (single dose) exposure chlorpyrifos.

Conclusion

In the present investigation chlorpyrifos has altered the some metabolites and enzymes of protein metabolism in experimental mice. The degree of change is directly proportional to the dose of pesticide and time in experimental mice. However, all tissues have shown highly significant changes in all the parameters investigated under multiple doses of chlorpyrifos toxicity.

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