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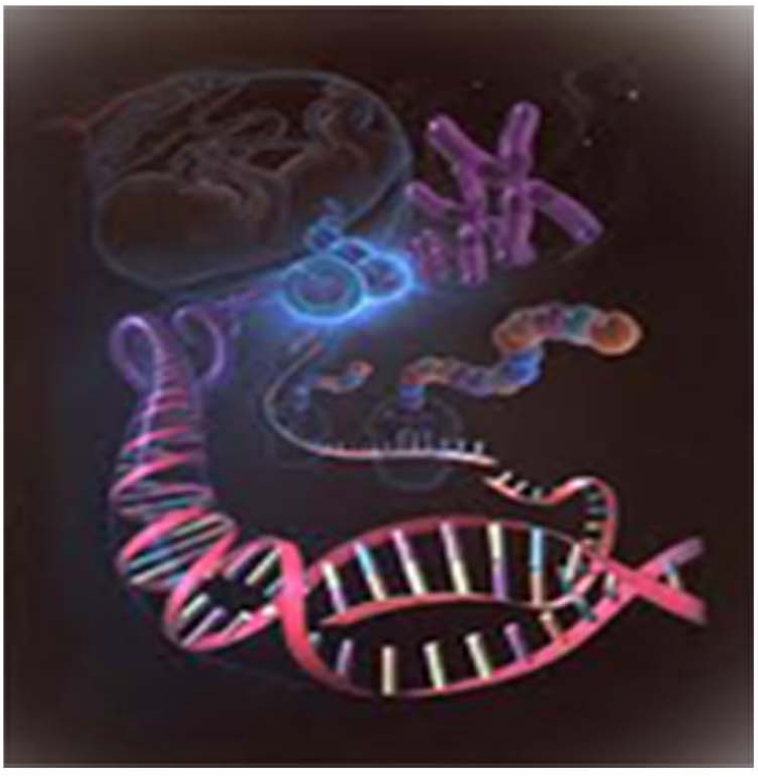

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Research Paper

EFFECT OF COMMERCIALLY AVAILABLE INSECTICIDE ON ANTIOXIDANT ENZYMES DURING CHICK EMBRYONIC DEVELOPMENT

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Organophosphorus Insecticides (OPIs) may induce oxidative stress leading to generation of Reactive Oxygen Species (ROS) and alteration in antioxidant system. In the present study toxic effect of organophosphate insecticide "chlorpyrifos" (100 µg) was estimated in chick embryo in eight day old fertilized eggs. After four days of rest the antioxidant defense system of brain, liver and heart of embryos were estimated by activity of Superoxide Dismutase (SOD), catalase (CAT) and glutathione peroxide (GPx) enzymes. The results showed significantly weight loss of liver and increased levels of CAT and GPx activity compared to their respective controls in the liver tissues of chlorpyrifos treated embryos. Activity of SOD enzyme was lower down in liver and brain tissues due to chlorpyrifos treatment. It may be concluded that *in vivo* administration of chlorpyrifos significantly changed antioxidant enzyme activity, suggesting that ROS may be involved in the toxicity of chlorpyrifos.

Keywords: OPIs, Antioxidant enzymes, ROS, chlorpyrifos, SOD, CAT, GPx

INTRODUCTION

Chlorpyrifos [O,O-Diethyl O-(3,5,6-trichloro-pyridin-2-yl phosphorothioate] as an organo-phosphate (OP), is one of the most widely used insecticides in agriculture to control wide range of insects and arthropods in agriculture (Giesy *et al*., 1999). Chlorpyrifos is also applied to the soil surrounding or beneath buildings as protection against termites including chicken houses (Barron and Woodburn, 1995; Leidy *et al*., 1991). OPIs are generally considered to be neurotoxic and are considered to act as inhibitors of neuronal acetylcholinesterase (AChE) [EC 3.1.1.7] activity. Due to this overstimulation of cholinergic receptors take place by excess acetylcholine (ACh) (Smegal, 2000; Gupta, 2001). It has been suggested previously that several pesticides also exert their biological effect through electrophoretic attack of cellular constituents with simultaneous generation of Reactive Oxygen Species (ROS) (Gultekin, 2000). OPIs also induced oxidative stress leading to generation of ROS and alteration in antioxidant system (Alhifi M, 2010). The organisms possess antioxidant defence systems, inc-

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luding antioxidant enzymes such as superoxide dismutase (SOD) [EC 1.15.1.1], catalase (CAT) [EC 1.11.1.6] and glutathione peroxidase (GPx) [EC 1.11.1.9], to deal with the ROS generated in response to both external and internal stimuli (Mates *et al.,* 1999). There is a balance between the activities of these antioxidants and the level of ROS that is essential for the survival of organisms and their health (Alhifi, 2010). An unbalanced production of ROS plays an important role in the pathogenesis of number of diseases such as ischemia/reperfusion injury, atherosclerosis, neurodegenerative disease, cancer, sepsis and aging (Mates *et al*., 1999; McCord and Fridovich, 1988).

Some previous studies have shown that OPIs poisoning is associated with enhanced lipid peroxidation with reduced Glutathione levels and elevated antioxidant status and increased oxidative stress (Vidyasagar *et al*., 2004, Rastogi *et al*., 2009). ROS are produced by the univalent reaction of dioxygen to superoxide anion ($\bullet O_2^-$), which turn into H_2O_2 and O_2 through the reaction catalyzed by SOD. Otherwise, it may generate a highly reactive free hydroxyl radical (•OH) via Fenton reaction, which is strongly believed to be responsible of oxidative damage (Gultekin, 2000). ROS may react with the basic cellular constituent's proteins, lipids, RNA, and DNA and induce lesions of the liver (Guillouzo, 1998). The liver is the principal organ involved in the biotransformation of exogenous substances, with its capa-city to convert hydrophobic substances into water soluble products that can be secreted readily from the body. A series of complex cellular processes are involved in the uptake, biotransformation and elimination of potentially toxic compounds that finally induced liver damage *in vivo*. Cells are protected against oxidative stress by an interacting network of antioxidant enzymes (Davies, 1995). The superoxide released during stress processes, is first converted to hydrogen peroxide (H_2O_2) and then further reduced to give water. This detoxification pathway is the result of multiple enzymes, with SOD catalysing the first step and then CAT and various peroxidases (like GPx) removing hydrogen peroxide (Sies, 1997).

In modern farming and food production, non-approved use of pesticides increasingly contributes to environmental pollution. Chlorpyrifos is one of the OPIs commonly used in Bilaspur (Chhattisgarh) by local farmers in farming and food production. Present study was conducted to evaluate the toxic effect of commercially available chlorpyrifos on the various antioxidant enzymes SOD, CAT and GPx in liver, brain and heart during chick embryo development.

MATERIALS AND METHODS

Chemicals: Commercially available insecticides formulation was purchased from a local pesticide vendor (manufactured by Excel Crop Care Limited, Mumbai, India), which constituted of chlorpyrifos (20%). Dilution was made in autoclaved double distilled water. The final concentration of chlorpyrifos was 100 µg/100µL.

Experimental Animal: Eight day-old fertilized chicken eggs were obtained from the local hatchery. All eggs were cleaned with 70 % ethanol to remove external contamination and blotted dry.

Experimental Design: The eggs were divided into two groups (control and experimental) of four each (n=4). Control group received 100 µL of vehicle (autoclaved distilled water) and experimental group received 100 µL of 100 µg of chlorpyrifos into the air sac of egg (Blankenship *et al*., 2003). After each injection the hole is sealed with cello tape. The eggs were incubated in an

automated humid incubator and set at a temperature of 37±0.5 °C and a humidity of 60-80%. The eggs were turned manually every 3 h.

Biochemical Study: On day 12 after administration of the insecticide, the embryos were removed from their shells, washed with Phosphate Buffer Saline (PBS), weighted and dissected for isolation of the brain, liver and heart. The brain, liver and heart were taken out, washed with PBS, weighted and homogenized in cold phosphate buffer, pH 7.5, in a ratio of 1:5 w/v. The homogenization was performed with the use of the Teflon Homogenizer in ice-cold water bath. The homogenates were centrifuged at 10,000 rpm for 15 min at 0-4 °C and the supernatants were used for the total protein and enzymatic assay.

Total Protein Estimation

Total protein estimation was performing by Bradford Assay method. Add 0.1 mL of each of the homogenate to separate microfuge tubes and add 0.9 mL of Coomassie Brilliant Blue (Bradford reagent) to each tube and mix well. Read the absorbance at 595 nm (Bradford, 1976).

Superoxide Dismutase Activity

The activity of SOD was measured according to Beauchamp and Fridovich (1971). SOD assay was determined by water-soluble Nitroblue tetrazolium salt (NBT), which produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of NBT reduction by superoxide anion is linearly related to the riboflavin oxidation and is inhibited by SOD (Beauchamp and Fridovich, 1971). The reaction mixture containing: 50 mM potassium phosphate buffer (pH 7.8), 13 mM Methionine, 2 µM Riboflavin, 0.1 mM EDTA, 75 µM NBT and 50 µL of enzyme solution. Make up the volume equal by the adding double distilled water. Set a blank without enzyme and NBT to calibrate the spectrophotometer. Set a control having NBT but no enzyme solution. Expose all the tubes to 300 W bulbs for 15 min. Read the absorbance at 560 nm immediately using spectrophotometer (Shimadzu UV-1800). The 50% inhibition of the reaction between riboflavin and NBT in the presence of methionine is taken as 1 unit SOD (Sadasivam and Manickam, 2009).

Catalase Activity

The ultraviolet light absorption of H_2O_2 solution can be easily measured at 240 nm. On decomposition of H_2O_2 by CAT, the absorbance decrease with time (Luck, 1974). 1mL reaction mixture conta-ining: 0.9 mL of H_2O_2 (10% w/v) and 100 µL enzyme solution. Set blank with phosphate buffer to calibrate the spectrophotometer. Read the absorbance at 240 nm immediately and after 60 s. The time required for the decreasing of the absorbance is used for calculation of the enzyme activity (U/mL) (Sadasivam and Manickam, 2009).

Glutathione Peroxidase Activity

Glutathione peroxidase assay is based on the reaction between leftover glutathione in the following reaction with the Ellman's reagent (DTNB) to form a compound, which absorbs light maximally at 412 nm (Rotruck *et al*., 1973). Reaction mixture containing: 0.4 mL of phosphate buffer, 0.1 mL sodium azide, 0.2 mL reduced glutathione, 0.5 mL of enzyme solution and 0.1 mL H_2O_2 into the test tube. Make up the final volume to 2.0 mL with distilled water. Incubate the tubes at 37 °C for 30 min. Add 0.5 mL of 10% TCA to stop the reaction. Centrifuge the content, save the supernatant and to this add 3.0 mL of disodium hydrogen phosphate and 1.0 mL of DTNB reagent. Set blank with disodium hydrogen

phosphate buffer and DTNB reagent. Read the absorbance at 412 nm and calculate the activity in U/mg (Sadasivam and Manickam, 2009).

RESULTS AND DISCUSSION

Present investigation conducted on chick embryo to evaluate the toxicity of chlorpyrifos at the dose of 100 µg/egg on biochemical parameter of liver, brain and heart tissue. The present studies revealed that chlorpyrifos induce oxidative stress by generating free radicals and altered activity of antioxidant enzymes in chick embryo (Figure 1). Gultekin *et al*. (2000) have reported that chlorpyrifos insecticide significantly induced free radical production in plasma, liver and brain. Similar observations were made in chlorpyrifos and cypermethrin treated fertilized eggs (Uggini *et al*., 2010). This study showed that the total embryo weight and liver weight significantly decrease in the chlorpyrifos treated groups (P<0.05, Table 1 and Figure 2). The total protein concentration in liver tissues also showed significantly decreased in chlorpyrifos treated embryo (P<0.01, Table 2 and Figure 3). Activity of CAT and GPx showed significantly increased levels in chlorpyrifos treated group with respect to controls (P<0.01, Table 2 and Figures 4 and 5). Increased GPx enzymes activity might provide

Figure 1: The Effect of Chlorpyrifos on the Weight of Chick Embryo, Results are Expressed as Mean ± Sem (N=4)

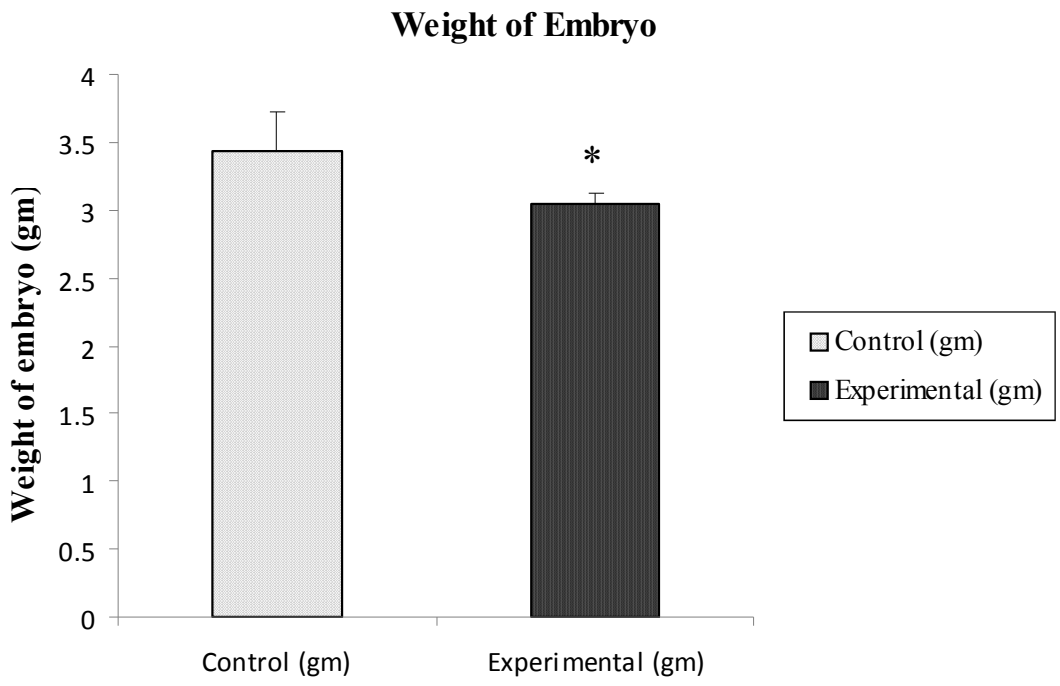
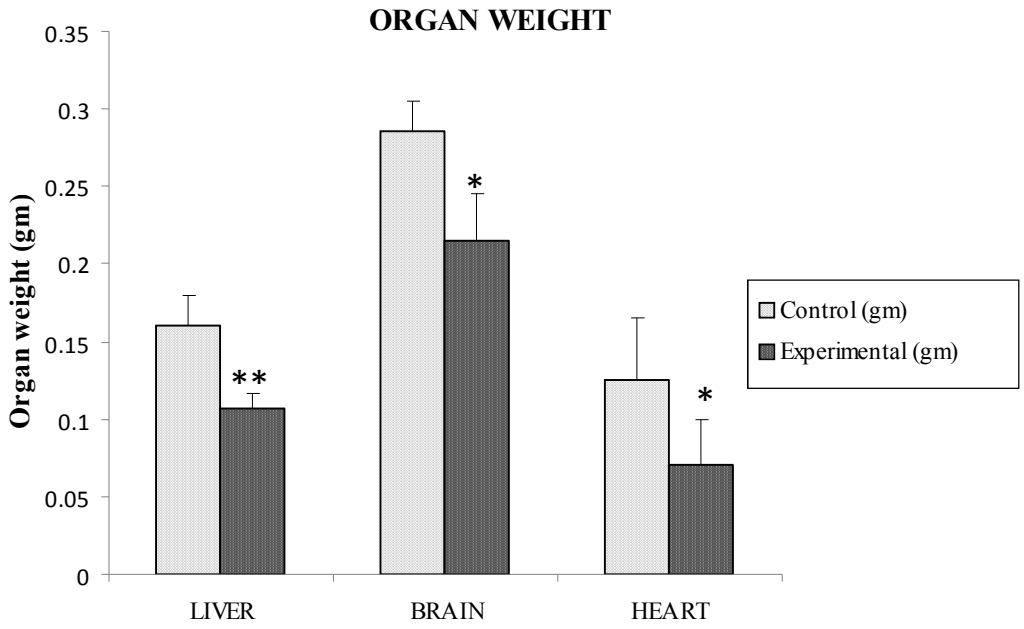


Table 1: Body Weight and Organs Weight of Chlorpyrifos Treated Chick Embryo and Control, Results are Expressed as Mean±SEM (n=4)

S. No.	Parameters	Control (g)	Experiment (g)
1.	Embryo weight	3.441±0.28	3.038±0.09*
2.	Liver weight	0.160±0.02	0.107±0.01**
3.	Brain weight	0.285±0.02	0.215±0.03*
4.	Heart weight	0.125±0.06	0.070±0.03*

Note: *P<0.05 and **P<0.01 is considered as statistically significant.

Figure 2: The Effect of Chlorpyrifos on the Weight of Brain, Liver and Heart of Chick Embryo, Results are Expressed As Mean ± SEM (N=4)



protection against chlorpyrifos induced oxidative stress because it is known that peroxidation is reduced by the antioxidant enzymes. Glutathione, a multi-func-tional intracellular non-enzymatic antioxidant is considered to be the main thiol-disulphide redox buffer of the cell and direct

Table 2: Level of Antioxidant Enzymes and Total Protein in Liver, Brain and Heart Tissue of Control and Chlorpyrifos Treated Chick Embryo, Results are Expressed as Mean ± SEM (n=4)

S. No.	Parameters	Control			Experiment		
		Liver	Brain	Heart	Liver	Brain	Heart
1.	SOD (U/mL)	83.42	15.84	59.11	47.51	60.89	49.07
		±4.81	±5.48	±5.75	±7.48**	±4.09**	±8.41
2.	CAT (U/mL)	78.88	56.80	97.36	122.08	109.44	106.18
		±2.14	±9.49	±3.53	±0.48**	±4.55**	±2.77*
3.	GPx(U/mg)	0.394	-	-	0.494	-	-
		±0.007			±0.017**		
4.	Total Protein	1.94	1.99	1.56	1.63	1.76	1.47
	(mg/mL)	±0.08	±0.04	±0.04	±0.13**	±0.11**	±0.02*

Note: *$P<0.05$ and **$P<0.01$ is considered as statistically significant.

Figure 3: The Effect of Chlorpyrifos on the Total Protein Concentration of Brain, Liver and Heart Of Chick Embryo, Results are expressed as mean ± SEM (n=4)

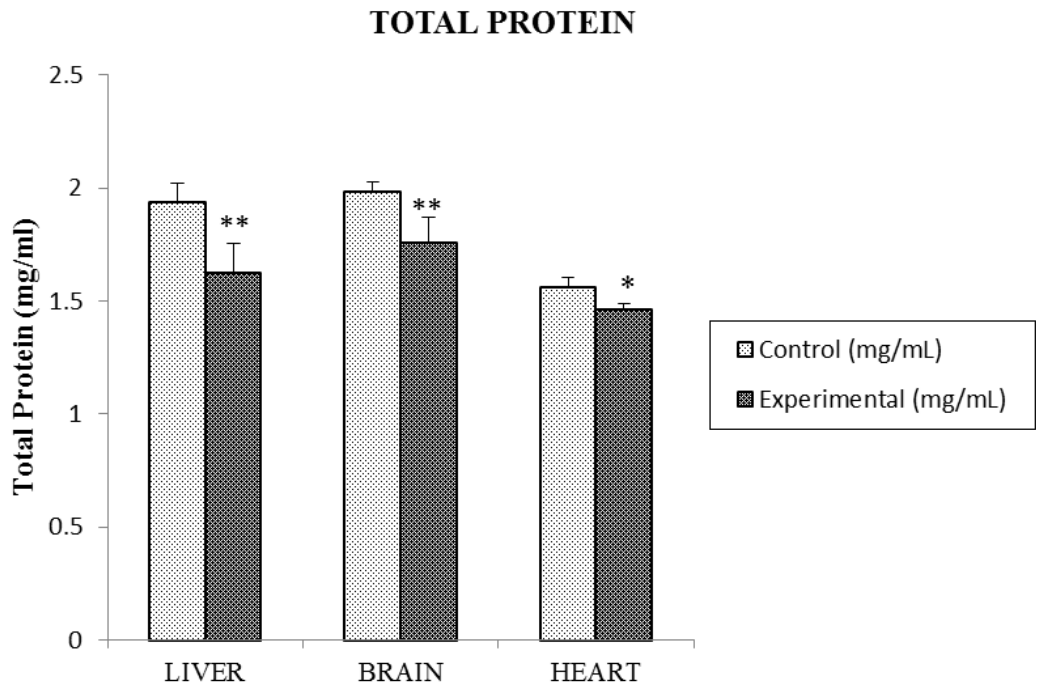


Figure 4: The Effect of Chlorpyrifos on the Activity of CAT in Brain, Liver and Heart Tissue of Chick Embryo, Results are Expressed as Mean ± SEM (n=4)

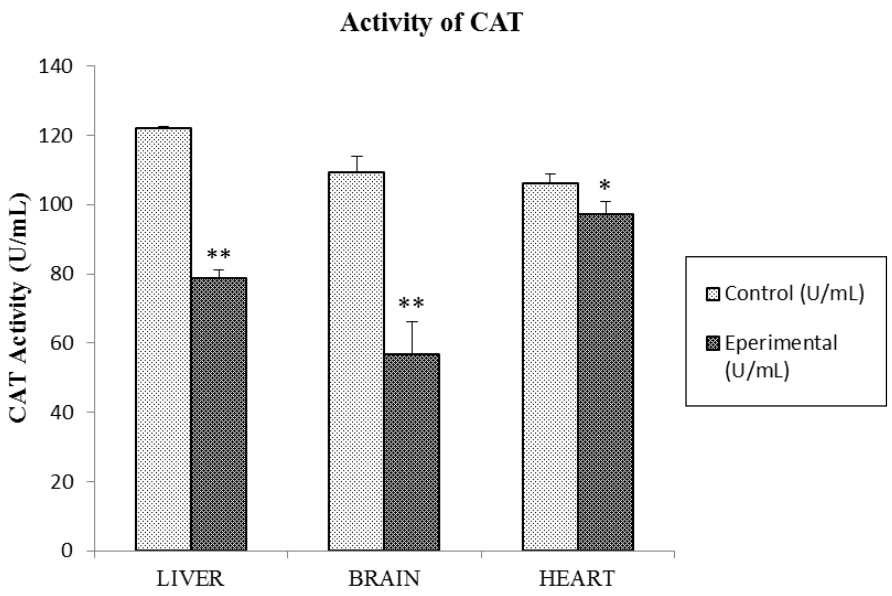


Figure 5: The Effect of Chlorpyrifos on the Activity of GPx in Liver Tissue of Chick Embryo, Results are Expressed as Mean ± SEM (n=4)

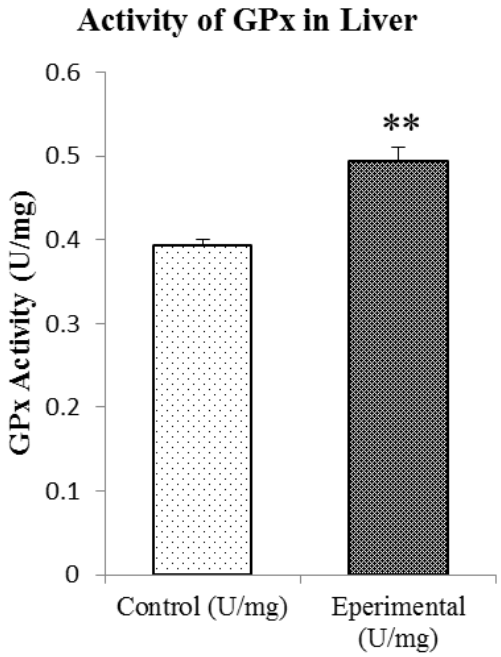
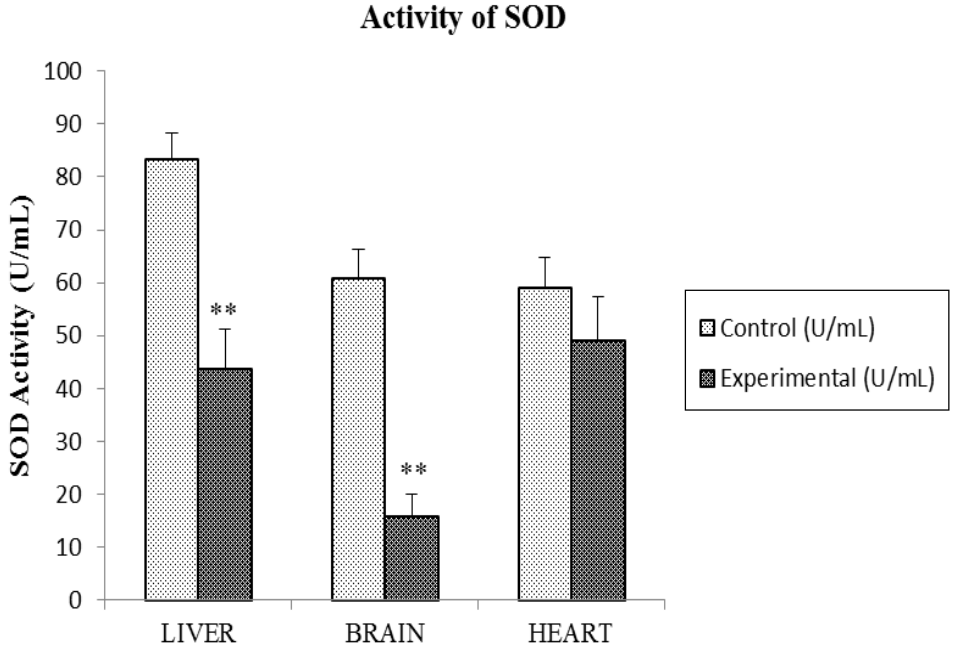


Figure 6: The Effect of Chlorpyrifos on the Activity of SOD on Brain, Liver and Heart Tissue of Chick Embryo, Results are expressed as mean ± SEM (n=4)



involved in the protection ROS damages (Masella *et al*., 2005). Increased CAT activity may be explained by their influence on H_2O_2 as substrate, which is formed in the process of dismutation of superoxide anion radicals (Shaikh *et al*., 1999).

Chlorpyrifos exposed embryos significantly decreased SOD activity in the liver and the brain tissues when compared with control (P<0.01, Table 2 and Figure 6). The decrease in the SOD activity beca-use the efforts of the endogenous antioxidant enzymes to remove the continuously generated free radicals initially increase due to an induction but later enzyme depletion results, resulting in oxidative cell damage (Kalra *et al*., 1994). This observation is in accordance with other studies reported that chlorpyrifos caused significant decrease in SOD activity of erythrocytes of pregnant rats (Zama *et al*., 2007). Altuntas *et al.* (2003) also reported that Phosalone caused decrease in the activities of SOD and CAT, and these effects were seen only at extremely high concentrations of phosalone. The decrease of SOD activity may be occurring by the decreased serum zinc and copper levels (Jyotsna *et al*., 2009).

All the major biomolecules like lipids, proteins, and nucleic acids may be attacked by free radicals, but lipids are probably the most susceptible (Cheeseman and Slater, 1993). As a matter of fact, free radicals can damage DNA and proteins, either through oxidation of DNA bases (primarily guanine via lipid peroxyl or alkoxyl radicals) or through covalent binding to DNA resulting in strand breaks and cross-linking. ROS can also induce oxidation of critical sulfhydryl groups in proteins and DNA, which will alter cellular integrity and function (Azizian *et al*., 2006).

The antioxidant enzyme levels decrease as a result of the consumption of enzymes to neutralize free radicals generated by pesticides (Amer *et al*., 2002). It is well reported that OPIs induce production of ROS and oxidative tissue damage. Generation of oxidative stress and consequent lipid peroxidation by pesticides is also reported in rat and human brain (Ranjbar *et al*., 2002). Moreover, Barlow *et al.* (2005) reported that many widely used agricultural chemicals induce oxidative damage in various systems of the body such as in dopaminergic cells of the brain by modulating the antioxidant defence system. So, the low SOD activity in the brain and heart tissues as compare to its activity in the liver tissue, favours the accumulation of oxygen free radicals which may lead to tissue damage as a result of oxidative binding of key intracellular molecules containing thiol groups and lipid peroxidation of biological membranes, which might be of greatest importance in the cytotoxicity of pesticides (Alhifi, 2010). This also can be eventually responsible for cellular death and developmental defects in these organs reported by organophosphorous insecticides (Alhifi *et al*., 2004).

The results of this study indicated that commercially available chlorpyrifos induces oxidative stress in chick embryo during development due to the generation of free radicals. The liver and brain was the most susceptible organs towards the oxidative stress induced by chlorpyrifos. The liver as high metabolically active organ is capable of removing the ROS at the tested dose. From these result, it can be concluded that *in vivo* administration of chlorpyrifos resulted in the significant changes in antioxidant enzyme activity.

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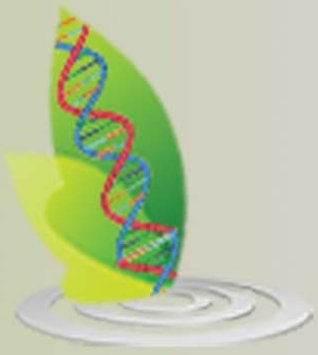

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