CHAPTER 2

Acetylcholinesterase and Butyrylcholinesterase activity

2.1 Review of Literature

Acetylcholinesterase (AChE) (EC 3.1.1.7) belonging to the family cholinesterase (ChE) is a specialized carboxylic ester that hydrolyse the breakdown of acetylcholine into choline and acetate after activation of acetylcholine receptors at postsynaptic membrane (Massoulié et al., 1993). Butyrylcholinesterase (BuChE) (EC 3.1.1.8) which is a pseudocholinesterase also hydrolyses acetylcholine and serves to terminate synaptic transmission thus preventing continuous nerve firing at nerve endings. Hence it is essential for normal functioning of central and peripheral nervous system. Butyrylcholinesterase (BuChE) is found in plasma but its physiological function in blood is unknown (Costa et al., 2005).

Organophosphorus and carbamate pesticides have been known to be specific inhibitors of acetylcholinesterase catalytic activity (Hobbiger, 1961). Erythrocyte AChE inhibition serves as a good biomarker of exposure to organophosphate pesticides in field studies with human population. Organophosphate and carbamate pesticides are widely used for pest control (Meerdink, 1989). However, they exert their effect on non-target organisms as well (Lionetto et al., 2003, 2004; Calisi et al., 2009, 2011). Organophosphate and carbamate compounds are capable of infiltrating through the soil into surface water because of their water solubility (Bondarenko et al., 2004). These are more toxic to human beings compared to organochlorines if misused (Wilson and Tisdell, 2001). The organophosphate and carbamate residues cannot be detected easily by chemical analysis because of their short life but their products of environmental degradation can be very harmful retaining acetylcholinesterase activity (Pehkonen and Zhang, 2002).

2.1.1 Cholinesterase activity in agricultural and farm workers

Workers engaged in agriculture and farms get exposed to synthetic agricultural chemicals while working in the fields. Innes et al. (1990) assayed plasma cholinesterase activity in order to screen 44 farm workers spraying organophosphate pesticides. The entire group had a moderately depressed cholinesterase activity while seven (7) out of 44 farm workers, had reduced cholinesterase activity. The mean body mass of these 7 farm workers was lower than that of the others which also lowered the body mass indices. When these 7 workers were removed from spraying for 6 weeks, the cholinesterase activities were increased significantly. The other individuals of the group had similar values of cholinesterase activity. López-Carillo and López-Cervantes (1993) have reported a significant difference in the median activity levels of serum cholinesterase in agricultural workers compared to age and sex matched control groups. Cholinesterase activity was significantly decreased at the beginning and end of the day's work. They suggested that it can be due to an interaction between the type of work and age of workers. Younger workers had a greater decrease in ChE activity as they were engaged in more dangerous activities. In a study by Ciesielski et al. (1994), the North Carolina migrant farm workers were shown to have significantly lower cholinesterase activity than those of non-farm workers. The effects of acephate (AP), cadmium (Cd), methamidophos (MAP), maleic hydrazide (MH), and nicotine (NI) on the activities of the erythrocyte enzymes δ-aminolevulinic acid dehydratase (ALAD), superoxide dismutase (SOD), and plasma cholinesterase (ChE) was determined on the farm workers since they are exposed to a combination of synthetic agricultural chemicals (Dowla et al., 1996). The studies revealed that the enzyme activities were significantly inhibited by a wide concentration range of the agricultural chemicals acephate, methamidophos, cadmium, maleic hydrazide and nicotine (Dowla et al., 1996). It was also found that erythrocyte superoxide dismutase

was most sensitive to these chemicals, while plasma cholinesterase was not inhibited by maleic hydrazide and nicotine (Dowla et al., 1996). In another study, Tinoco-Ojanguren and Halperin (1998) reported the inhibition of erythrocyte cholinesterase by OP pesticides in three communities and control subjects of Mexico. Adverse health effects existed among the poorest communities. Significantly, lower AChE activity has been reported by Rendógn von Osten et al. (2004) in farmers from 4 rural communities of Campeche (Mexico) compared to the mean activity of control group. Their regression analysis indicated that carbofuran was the dependent variable most related to moderate AChE inhibition.

Remor et al. (2009) observed a significant decrease in butyrylcholinesterase (BuChE) activity in farm workers from Rio Grande do Sul (Brazil) compared to the control group. Hematological parameters, MN frequencies and lipid profile did not show any significant difference between the occupationally exposed farm workers and the control, however comet assay revealed higher damage index and damage frequency. Remor et al. (2009) suggested the use of personal protective equipment to prevent contamination.

Similarly, Jintana et al. (2009) measured acetylcholinesterase and butyrylcholinesterase activities in individuals exposed to organophosphate pesticides and control subjects, selecting the high and low exposure periods. The exposed group had significantly lower enzyme activity than control subjects. Also a statistically significant decrease in acetylcholinesterase and butyrylcholinesterase activity was observed in the high exposure period compared to low exposure period. Chakraborty et al. (2009) studied the effect of long term exposure to cholinesterase inhibiting pesticides (organophosphate and carbamate) on the respiratory system of agricultural workers and have shown that the

decreased acetylcholinesterase activity was positively associated with respiratory symptoms.

Recently AChE activity was assayed by Singh et al. (2012) in the blood samples of workers involved in pesticide spraying and control subjects from Delhi, India and reported a significant decrease in AChE activity in workers compared to controls with a positive correlation between the AChE activity and the age and duration of exposure. Similarly, Dhalla and Sharma (2013) showed a positive correlation between serum cholinesterase activity and years of exposure and between body mass index (BMI) and serum cholinesterase inhibition in Bathinda district, Punjab. They observed that the younger population had a significant reduction in serum cholinesterase activity. Crane and workers measured the activity of AChE and BuChE in the blood samples of adolescent agricultural workers exposed to chlorpyrifos and control subjects in Egypt and showed a depressed BuChE activity in both the exposed and controls, but the decrease was significant in exposed group than control. The depression in enzyme activity persisted for 4-7 weeks post pesticide application which may be due to the fact that the non-exposed (control) groups may be receiving environmental chlorpyrifos exposure (Crane et al., 2013).

Tobacco field workers were also investigated for blood enzymes, δ-aminolevulinic acid dehydratase (ALAD), superoxide dismutase (SOD) and cholinesterase (ChE) with respect to different duration of exposure to agricultural chemicals. Panemangalore et al. (1999) showed that the ALAD activity was declined by 30% after 1 day and no decline was observed after 30 days of exposure, SOD activity was declined by 30% after 1 day and 50% after 30 days, while plasma ChE activity was declined by 19% after 1 day and 30 days of exposure. A restoration of the activities of all three enzymes to pre-exposure level

was observed during post-exposure period (no tobacco production). Highest respiratory nicotine level was found after 30 days post-exposure. In order to evaluate the toxicity of pesticides at occupational level, a study was conducted to evaluate the activities of cholinesterase, acid phosphatase, β-glucuronidase and paraoxonase in the plasma of plastic greenhouse workers (Hernández et al., 2004). Cholinesterase and paraoxonase activities were shown to be decreased in the pesticide applicators compared to the ones who were not pesticide applicators. The analysis showed that BuChE was significantly correlated with the level of β-glucuronidase and acid phosphatase. β-glucuronidase and acid phosphatase were related to each other and were associated with pesticide exposure in humans. BuChE was significantly decreased in sprayers compared to non sprayers during maximum exposure period. AChE activity did not differ significantly indicating that BuChE activity is more suitable marker than AChE. A higher inhibition of butyrylcholinesterase activity was found to be associated with the paraoxonase B allele (Hernández et al., 2004). In a study, Thetkathuek and workers investigated the biological effect of chlorpyrifos among 53 Thai fruit farm workers by measuring blood and plasma cholinesterase activities and showed a decrease in plasma cholinesterase activity in exposed workers. They also proposed that plasma cholinesterase activity can be used as a biomarker to detect the toxic effect of the chlorpyrifos insecticides (Thetkathuek et al., 2005).

In the pregnant mothers living in areas of pesticide exposure in Argentina, the placental acetylcholinesterase and catalase activities were shown to be significantly associated with organophosphorus pesticide exposure period, whereas glutathione S-transferase was unaffected (Souza et al., 2005). They also reported a positive correlation between newborn head circumference and the environmental exposure to organophosphorus and carbamate pesticides. The study also showed an association between placental

acetylcholinesterase and catalase activity and prenatal exposure to pesticide (Souza et al., 2005).

2.1.2 Cholinesterase activity in case of occupational exposure to metals

It is clear from the above review of literatures that AChE and BuChE inhibition serves as a useful biomarker for organophosphate and carbamate pesticides. Studies have also been conducted to determine the effect of other environmental pollutants on the AChE and BuChE activity. The inhibitory effects of five metal ions namely, nickel, copper, zinc, cadmium and mercury were assayed and all the metals except nickel inhibited acetylcholinesterase activity (Frasco et al., 2005). The neurotoxic effects of lead have been studied by measuring erythrocyte acetylcholinesterase activity, blood pressure and pulse in the workers exposed to lead and engaged in various works in Abeokuta, Nigeria (Ademuyiwa et al., 2007). In their study, acetylcholinesterase activity was inhibited by 39% and 32%, respectively in male and female petrol station attendants, 31% in welders, 38% in painters and 15% in panel beaters. No significant difference was found between blood pressure and pulse compared to the controls in the same study. An inverse linear relationship was obtained between AChE activity and blood lead levels as calculated by Pearson's method. Thus, acetylcholinesterase activity can be used as a biomarker to detect neurotoxicity induced by lead in occupationally exposed subjects (Ademuyiwa et al., 2007).

Excess of certain metals that are required for physiological functions may cause serious damage. The effect of Fe^{2+} and Fe^{3+} ions on human plasma cholinesterase activity was studied by Karami et al. (2010) and showed the suppression of BuChE activity by Fe^{2+} and Fe^{3+}. In an early study Jett et al. (1999) had reported that some polycyclic aromatic

hydrocarbons (PAHs) enhance the inhibitory effect of chlorpyrifos-oxon (CPFO) on acetylcholinesterase activity.

2.1.3 Cholinesterase activity in earthworm exposed to chemical pollutants

Toxicity assessment studies have also been carried out in animals. Earthworm's biomarker response is studied for a better understanding of pesticide contamination. In a study conducted by Capowiez et al. (2003), the effects of sublethal doses of imidacloprid on the earthworm (*Aporrectodea nocturna* and *Allolobophora icterica*) behavior was tested using AChE as a biomarker. It was observed that AChE activity was not affected by the treatment, which could be due to the fact that imidacloprid blocked the AChE receptor: nicotinic acetylcholine receptor instead of the enzyme itself (Capowiez et al., 2003). Acetylcholinesterase activity in the earthworm *Lumbricus terrestris* exposed to chemical pollutants was assessed in either the whole organism or the pre-clitellar and post-clitellar part of the animal (Calisi et al., 2011). Calisi et al. (2011) noticed a decrease of 70% AChE activity in earthworms following methiocarb (a carbamate pesticide) exposure for 14 days. Two ChEs (E1 and E2) are found in earthworm *Eisenia fetida* (Aamodt et al., 2007). *Eisenia fetida* were exposed to clean soil or soil containing chlorpyrifos (240 mg/kg) for 48h. E1 and E2 and the chlorpyrifos content of earthworms were monitored after transfer to clean soil for 12 weeks. E2 could not recover during 84d in clean soil which indicated that this enzyme was irreversibly inhibited by chlorpyrifos and de novo enzyme synthesis did not occur significantly. Thus E2 can serve as potential biomarker for OP insecticide exposure in *Eisenia fetida* (Aamodt et al., 2007). AChE activity in *Eisenia andrei* was inhibited by carbaryl (carbamate compound) in a dose dependent manner *in vitro*. Pure and co-formulated carbaryl exhibited different time and dose-dependent effects when treated *in vivo*, but they caused persistent and significant

inhibition of AChE. Thus, inhibition of AChE can be used as an indicator of pesticide contamination for soil toxicity monitoring (Gambi et al., 2007). Farrukh (2017), studied the chronic effect of endosulfan (organochlorine pesticide) on the AChE and cellulose enzyme of *Eisenia fetida* and reported that the long term exposure to endosulfan can lead to severe and irreparable effect on earthworms.

2.1.4 Cholinesterase activity in aquatic animals exposed to pesticides

Blood cholinesterase is a useful biomarker in case of organophosphate and carbamate poisoning (Hernández et al., 2005; Safi et al., 2005; Souza et al., 2005; Ng et al., 2009; Simoniello et al., 2010). AChE activity has been used as a complementary tool to chemical analysis in two important fish species *Mullus barbatus* and *Trachurus mediterraneus* and in mussels, *Mytilus galloprovincialis* in the industrialized area of Taranto of Salento peninsula (Lionetto et al., 2004). No significant differences in AChE activity was observed in the two fish species compared to the controls, while AChE activity was inhibited in mussels (Lionetto et al., 2004). The effect induced by exposure to chemical pollutants in native marine organisms from a coastal area, in Salento Peninsula (Italy) was studied by the integrated use of acetylcholinesterase (AChE) and antioxidant enzymes (catalase-CAT, glutathione peroxidise-GSH-Px) (Lionetto et al., 2003). *Mytilus galloprovincialis* (a sessile invertebrate) and *Mullus barbatus* (a benthic teleost fish), the two bioindicator species were included in the study at 8 sampling stations. Four sampling stations (non-urbanized) served as controls and the four other stations were exposed to anthropogenic agents. Lionetto et al. noted a significant difference in AChE activity in *M. galloprovincialis* in the sample sites. The reduction of AChE activity observed in two control stations may be due to leaching of pesticides into the sea from the agricultural lands or because of heavy metals. In *M. barbatus* significant

difference in AChE activity among the sample sites were observed. The activity was found to be inversely correlated with liver GSH-Px activity. Catalase activity did not differ significantly among animals sampled from different stations (Lionetto et al., 2003).

The effect of malathion and cadmium on acetylcholinesterase activity in fish *Seriola dumerilli* was studied by exposing the fish to various concentration of malathion for different periods and cadmium for 2 days (Jebali et al., 2006). In brain, acetylcholinesterase was inhibited after 2 and 7 days of exposure to malathion in a dose-dependent manner, while no inhibition was observed after 13 days of exposure. 50 µg/kg body weight of cadmium showed an increase in acetylcholinesterase activity, whereas higher doses, 100 and 250 µg/kg of cadmium revealed a strong dose-dependent inhibition of AChE activity (Jebali et al., 2006). Following cadmium treatment a rapid increase in malathion concentration was seen in liver, which suggested that the hepatic malathion concentration and brain acetylcholinesterase activity can be used as a biomarker to organophosphate and cadmium toxicity in fishes (Jebali et al., 2006). Vioque-Fernández et al. (2007) conducted a study in *Procambarus clarkii* (a species of cambarid freshwater crayfish) at Doñana National Park to assess the inhibitory effect of pesticides on AChE and carboxylesterase activities. The activities were significantly reduced in *P. clarkii* from affected sites compared to the ones from reference sites. It was also proposed that the metals inhibited the esterases too in combination with the pesticides, since high metal concentration was found at rice-growing sites compared to the other affected and reference sites (Vioque-Ferńandez et al., 2007).

2.1.5 Cholinesterase activity in rats and mice.

Tomokuni and Hasegawa (1985), determined the erythrocyte, plasma and brain ChE activities in rats and mice exposed to diazinon and observed that the ChE was most

remarkably inhibited in plasma of mice. Cocaine toxicity is supposed to cause low plasma cholinesterase activity. Cahill-Morasco et al. (1998) tested the cocaine toxicity in Swiss albino mice reared on high protein diet, low protein isocaloric diet and a protein with calorie deficit diet for 3 weeks. After acclimatization for 3 weeks the animals were allowed to feed on these diets for 3 additional weeks. The mice were treated intraperitoneally with a single dose of 75 mg/kg body weight cocaine and were kept on observation for 4 hours to record seizures and death. After 4 hours, ChE activity was measured. The cocaine toxicity was shown to be associated with reduced plasma cholinesterase activity by 4% and 10% in low protein isocaloric diet and protein with calorie deficit diet, respectively whereas the plasma cholinesterase activity remained stable for high protein diet group of mice. Recently, Santos et al. (2013), reported that the activity of plasmatic and erythrocyte ChEs in rats decreased from 29% to 0.5% and from 35.9% to 33% on increasing disulfoton (an organophosphate insecticide) dose from 0-6.6 mg/kg body weight.

2.1.6 Cholinesterase activity and clinical management of patients

Serum AChE level can be a helpful parameter to determine acute OP poisoning. Eddleston et al. (2008) measured BuChE activity and OP pesticide concentrations in the blood samples of patients related to either chlorpyrifos or dimethoate poisoning. Out of 91 patients, 25 died of dimethoate poisoning, whereas 11 out of 208 patients died of chlorpyrifos poisoning. Eddleston et al. found greater OP concentration in deceased patients compared to the ones who survived and suggested that BuChE activity should be interpreted correctly based on the type of the ingested OP.

OP pesticides act as an inhibitor of AChE which results in accumulation of neurotransmitter acetylcholine and continuous nerve firing. This could be treated with an

oxime antidote which reactivates the inhibited acetylcholinesterase and the biochemical effect of acetylcholine can be reversed with atropine (Kwong, 2002). However, certain studies reported that the oxime treatment was dependent on the concentration of OP in plasma and that the effects were minimal at high levels of OP in blood (Finkelstein et al., 1989).

Different clinical symptoms (vomiting, respiratory distress, lacrimation, abdominal pain) were reported to be associated with different level of reduction of plasma cholinesterase in human. Deceased patients had lowest plasma cholinesterase level and respiratory problems (Prasad et al., 2013). No significant difference was found in the serum acetylcholinesterase activity in patients with severe and mild organophosphate poisoning on the first day, but a significant difference was found in serum acetylcholinesterase activity in patients with non intermediate symptoms on days 1 and 3 than those with intermediate symptoms. No increase in serum acetylcholinesterase was noted on first and last day in patients who died compared to the patients survived (Aygun et al., 2002). In another study related to organophosphate poisoning, Chen et al. (2009) proposed that high mortality rates were associated with cases where the serum cholinesterase activity was not elevated and therefore, serum cholinesterase can play a role in management of patient within 48 hours of OP poisoning.

2.2 Materials and methods

2.2.1 Sampling area

The study was carried out in the tea gardens located in the Terai region of Darjeeling foothill specifically, Upper Bagdogra (26°68'16.73" N, 88°25'83.11" E) and Matigara (26°74'00.82" N, 88°37'83.69" E).

2.2.2 Characteristics of the participants

A total of 225 individuals consisting of 95 (60 males and 35 females) pesticide exposed tea garden workers between 15 and 62 years of age (mean age 35.37 ± 9.48), 60 (43 males and 17 females) pesticide non-exposed, non-smoker and non-alcoholic controls between 22 and 63 years of age (mean age 31.05 ± 9.93), 39 smokers (males) who smoked 6-30 cigarettes/day (different from the exposed ones) between 23 and 60 years of age (mean age 35.36 ± 11.47) and 31 alcoholic males consuming 60-120 ml alcohol/day (different from the exposed ones) between 24 and 55 years of age (mean age 39.94 ± 7.12) were analyzed. The pesticide exposed individuals were involved in either pesticide spraying/mixing (males) or tea leaf plucking (females) for at least 8h/day for 6d/week for at least a year and the work duration ranged from 1 to 30 years, hence were exposed to a mixture of pesticides simultaneously. The workers involved in the study were not found to use any kind of protective measures (gloves, breathing masks, protective goggles, impermeable boots, etc.). Most of the workers had self-reported symptoms like headache, abdominal pain, nausea, watery eyes and vomiting. The male individuals involved in pesticide spraying smoked cigarettes or bidi (a bidi is thin, Indian cigarette filled with tobacco flake) and consumed alcohol too. Few female individuals involved in tea leaf plucking were also found to smoke and all consumed alcohol. The control subjects (non-exposed, non-smokers and non-alcoholic) were from different walks of life who voluntarily participated in the study. Since the pesticide exposed workers smoked cigarettes and consumed alcohol, we have included 39 smokers and 31 alcoholics in our study. Control, smokers and alcoholics with apparently no exposure to genotoxic agents were randomly collected away from the target area (**Table 2.1**).

Personal information such as exposure period, smoking habit, alcohol consumption, drug intake, X-ray exposure, health status and any particular disease was obtained in the form

of a structured questionnaire. The individuals considered as 'smokers' were non-alcoholic and the 'alcoholics' consumed alcohol but did not smoke cigarettes. Individuals under any sort of medication or exposure to X-ray in the past 1 year were excluded from the study.

Table 2.1: Characteristics of the study population.

Variables	Control (N=60)	Tea garden workers (N=95)	Smokers (N=39)	Alcoholics (N=31)
Male	43	60	39	31
Female	17	35	0	0
Height(ft)	5.42 ± 0.26	5.08 ± 0.49	5.49 ± 0.25	5.46 ± 0.19
Weight(kg)	58.36 ± 5.38	47.81 ± 11.25	65.03 ± 9.82	66.37 ± 8.29
Age in years (mean ± SD)	31.05 ± 9.93	35.37 ± 9.48	35.36 ± 11.47	39.94 ± 7.12
Work Duration in years (mean ± SD)	0	14.27 ± 9.48	0	0
Smoking	No	Yes	Yes	No
Alcohol	No	Yes	No	Yes

2.2.3 Sampling procedure

Prior consent was obtained from each individual before the collection of blood. The venous blood was collected by venepuncture using 5ml sterile disposable syringe, transferred immediately in tubes containing EDTA as an anticoagulant and brought to the laboratory. Blood was processed for separation of plasma and erythrocytes for enzyme activity.

2.2.4 Plasma separation

1 ml whole blood was taken in 1.5 ml microcentrifuge tube and centrifuged at 5000 rpm for 3 minutes at 4^{o}C to remove plasma from blood. Supernatant plasma was removed carefully and kept on ice for further assay.

2.2.5 Erythrocyte separation

The erythrocyte pellet was washed twice with 0.9% saline by centrifugation at 1000 rpm for 2 minutes. Erythrocytes were suspended in 0.9% saline corresponding to the initial volume of whole blood for lysis. Hemolysate was prepared by diluting the cell suspension 600 times with 0.1M phosphate buffer, pH 8.0 (0.1M sodium phosphate monobasic, 0.1M sodium phosphate dibasic) and kept for 10 minutes at room temperature.

2.2.6 Measurement of enzyme activities of erythrocyte acetylcholinesterase

Enzyme activities were determined from blood samples following the protocol of Ellman et al. (1961) with slight modification. For the measurement of activity of AChE, 3 ml of hemolysate was pipetted into a quartz cuvette. To it 0.10 ml of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) or Ellman's Reagent (10 mM DTNB, 17.85 mM $NaHCO_3$ in 100 mM phosphate buffer pH 7.0) was added. The cuvette was placed in the spectrophotometer (Rayleigh UV-2601). 0.02 ml of acetylthiocholine solution (75 mmol acetylthiocholine iodide) was added to the cuvette. The blank consisted of hemolysate and DTNB. Change in absorbance was recorded at 60 s interval for 3 minutes at 412 nm. The activities were expressed as micromoles of acetylthiocholine iodide hydrolyzed per min per ml using an extinction coefficient of 13,600/cm/M.

2.2.7 Measurements of enzyme activities of plasma butyrylcholinesterase

BuChE activity was measured according to the protocol of Ellman et al. (1961) with slight modification. Plasma obtained above was diluted 150 times with phosphate buffer (0.1M pH 8.0) (0.1M sodium phosphate monobasic, 0.1M sodium phosphate dibasic) and kept for 10 minutes at room temperature. 3 ml of diluted plasma was pipetted into a quartz cuvette. To it 0.10 ml of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) or Ellman's Reagent (10 mM DTNB, 17.85 mM $NaHCO_3$ in 100 mM phosphate buffer pH 7.0) was added. The cuvette was placed in the spectrophotometer (Rayleigh UV-2601). 0.02 ml of acetylthiocholine solution (75 mM acetylthiocholine iodide) was added to the cuvette. The blank consisted of diluted plasma and DTNB. Change in absorbance was recorded at 60 s interval for 3 minutes at 412 nm. The activities were expressed as micromoles of acetylthiocholine iodide hydrolyzed per min per ml of plasma using an extinction coefficient of 13,600/cm/M.

2.2.8 Ethical consideration

The study was approved by the Institutional Human Ethics Committee of the University of North Bengal, Siliguri, District- Darjeeling, West Bengal, India [Ref No. Zoo/9114(i)/2015, dated 22 September 2015].

2.2.9 Statistical analysis

A descriptive analysis was performed and data were expressed as mean ± SD. Crosstable of groups versus gender along with Chi-square (χ^2) test for homogeneity has been done. Pearsonian product moment correlation coefficient and test for normality (Shapiro-Wilks test) of the parameters (four groups, sex) have also been done. The departure from the normality has been observed in very few cases. The problems of confounding factor due

to gender in case of control and exposed were resolved by multiple comparisons using the software SPSS version 21 (SPSS Inc., Chicago, IL). The level of significance was considered at 0.001 and 0.05. For testing the hypothesis of differences of means of parameters for AChE and BuChE across the groups and gender were carried out with the help of multivariate analysis of variance (MANOVA), multivariate analysis of covariance (MANCOVA) along with post-hoc (Tukey's test) for multiple comparison through generalised linear model (in case of normality of parameters, equivalent non-parametric Kruskal Wallis test was performed but the results of MANOVA and Kruskal Wallis were almost the same).

2.3 Results

Results showed an AChE activity of 11.81 ± 3.40 (2.74-12.40) µmoles/min/ml and 6.43 ± 1.85 (4.51-21.25) µmoles/min/ml in controls and tea garden workers, respectively. The difference was highly significant at $p \leq 0.001$. The measured AChE activities in smokers and alcohol consumers were 11.04 ± 2.48 (4.32-15.61) µmoles/min/ml and 12.45 ± 2.58 (7.19-17.63), µmoles/min/ml, respectively. The comparison with control did not reveal any significant difference. Similarly, BuChE activity of 4.73 ± 1.84 (1.52-9.58) and 3.50 ± 1.89 (0.08-7.28) µmoles/min/ml were recorded in control and exposed groups, respectively. The difference in the activity was significant at $p \leq 0.001$ (**Table 2.2**). BuChE activity recorded in controls, smokers and alcohol consumers were 4.73 ± 1.84 (1.52-9.58) µmoles/min/ml, 5.06 ± 1.87 (1.86-10.82) µmoles/min/ml and 5.02 ± 1.41 (2.41-7.23) µmoles/min/ml, respectively. The activities were not significantly different.

The activities of AChE and BuChE were also compared among the workers, smokers and alcohol consumers. Almost two fold higher activity of AChE in smokers (11.04 ± 2.48 µmoles/min/ml) and alcohol consumers (12.45 ± 2.58 µmoles/min/ml) than the exposed

workers (6.43 ± 1.85 µmoles/min/ml) was recorded. The difference between AChE activity was found to be highly significant ($p \leq 0.001$). The results showed a BuChE activity of 5.06 ± 1.87 µmoles/min/ml and 5.02 ± 1.41 µmoles/min/ml in smokers and alcohol consumers, respectively which was approximately 1.5 times higher than the exposed workers (3.50 ± 1.89 µmoles/min/ml). The difference was significant ($p \leq 0.001$) (**Table 2.3**).

Since the control and worker population consisted of both males and females, the activity of AChE and BuChE was also analyzed separately in the males as well as in the females. The exposed males showed almost two-fold lower activity of AChE (6.23 ± 1.59 µmoles/min/ml) which is significantly lower than the control males (11.15 ± 2.83 µmoles/min/ml) at $p \leq 0.001$. A two-fold decrease in the AChE activity was recorded in the exposed females (6.78 ± 2.21 µmoles/min/ml) than females in control group (13.47 ± 4.18 µmoles/min/ml). The difference was highly significant ($p \leq 0.001$). When BuChE activity was compared separately in males and females, the activities in the exposed males and females were found to be (3.15 ± 1.95 µmoles/min/ml) and (4.08 ± 1.66 µmoles/min/ml) which were slightly lower than their non-exposed control males (4.57 ± 1.86) and control females (5.14 ± 1.78), respectively however, the differences observed in both the groups were found to be significant at $p \leq 0.001$ (**Table 2.4**).

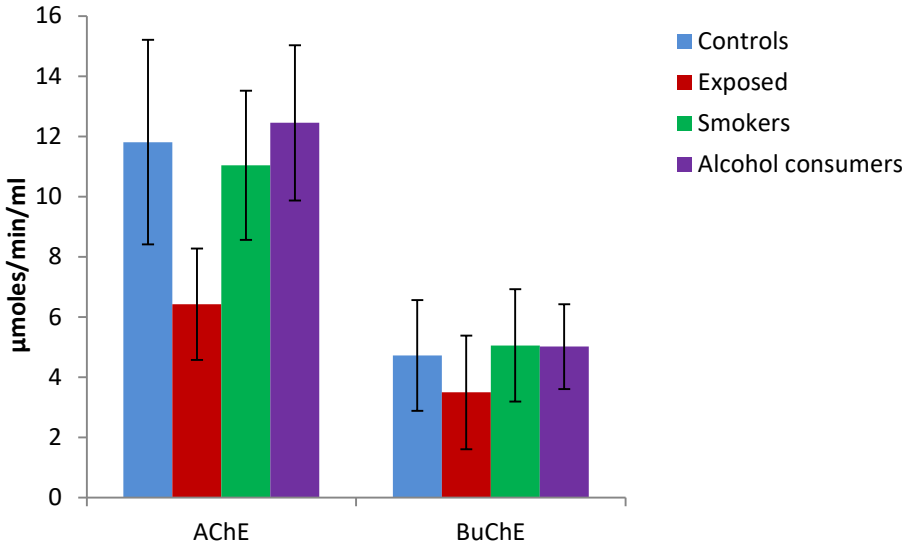
In order to test the effect of gender (sex) in the exposed and control groups, an analysis using MANOVA was also performed. Significant difference existed in the AChE activity when females and males of the controls were compared (13.47 ± 4.18 versus 11.15 ± 2.83 µmoles/min/ml, $p \leq 0.05$). A slight increase in the AChE activity was observed in the exposed females (6.78 ± 2.21 µmoles/min/ml) than the exposed males (6.23 ± 1.59 µmoles/min/ml) however, the difference was non-significant. When BuChE was analysed

gender wise in control females and control males, the activities were 5.14 ± 1.78 and 4.57 ± 1.86 µmoles/min/ml, respectively. The slight difference observed was not significant. Similarly, the BuChE activities were 4.08 ± 1.66 and 3.15 ± 1.95 µmoles/min/ml in exposed females and males, respectively. The analysis showed that the difference was significant at $p \leq 0.05$. The effect of gender (sex) observed in the present study appears to have very little influence on the AChE and BuChE activity, however, a wide range of individual variation exists (**Table 2.4**).

In an attempt to check whether age can be correlated with the activity, the exposed subjects were categorized into 3 groups of 15-30, 31-45 and 46-62 years of age. AChE activity was recorded to be 6.35 ± 1.87, 6.44 ± 1.68 and 6.60 ± 2.11 µmoles/min/ml in the age groups 15-30, 31-45 and 46-62 years, whereas BuChE activities were 3.31 ± 2.06, 3.65 ± 1.53 and 3.68 ± 2.06 in the age groups 15-30, 31-45 and 46-62 years, respectively (**Table 2.5**). Though not significant but reduced cholinesterase (AChE and BuChE) activities was found in the age group 15-30 years.

Table 2.2: Comparison of AChE and BuChE activity between controls, exposed, smokers and alcohol consumers.

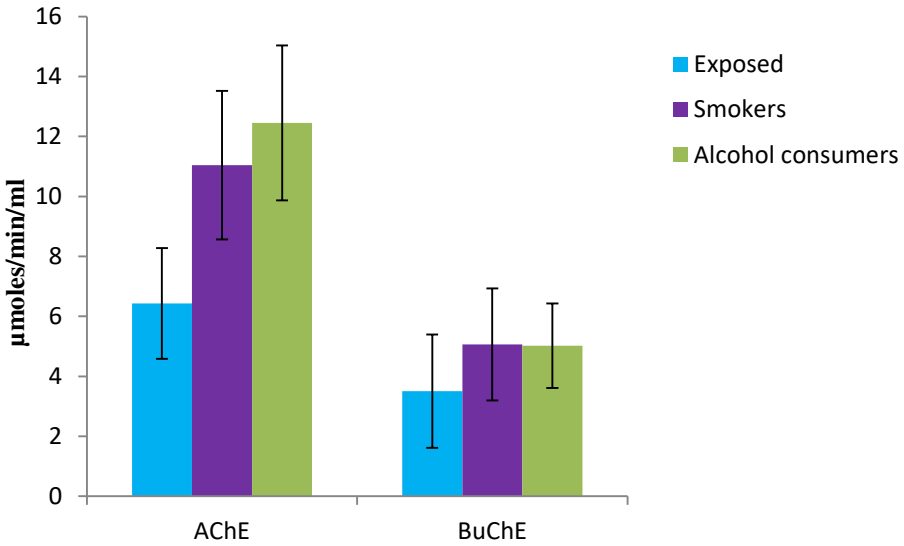
Groups	Enzyme activity	
	AChE	**BuChE**
Controls (N=60)	11.81 ± 3.40	4.73 ± 1.84
Exposed (N=95)	6.43 ± 1.85***	3.50 ± 1.89***
Smokers (N=39)	11.04 ± 2.48	5.06 ± 1.87
Alcohol consumers (N=31)	12.45 ± 2.58	5.02 ± 1.41



Results are expressed as mean ± standard deviation
Activities are measured in µmoles/min/ml
***$p \leq 0.001$ compared with control using MANOVA

Table 2.3: Comparison of AChE and BuChE activity between exposed, smokers and alcohol consumers.

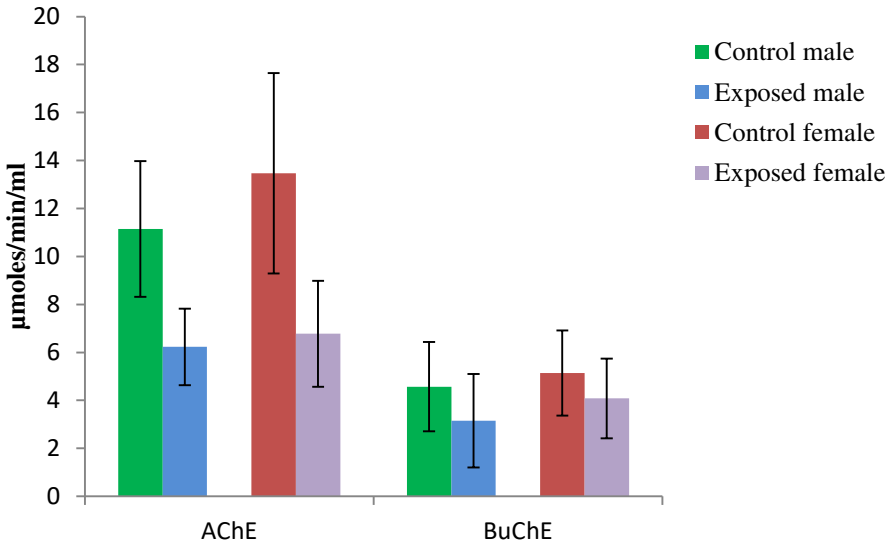
Groups	Enzyme activity	
	AChE	**BuChE**
Exposed (N=95)	6.43 ± 1.85	3.50 ± 1.89
Smokers (N=39)	11.04 ± 2.48***	5.06 ± 1.87***
Alcohol consumers (N=31)	12.45 ± 2.58***	5.02 ± 1.41***



Results are expressed as mean ± standard deviation
Activities are measured in µmoles/min/ml
***$p \leq 0.001$ compared with exposed using MANOVA

Table 2.4: Comparison of AChE and BuChE activity between males and females of control and exposed groups.

Groups	Enzyme activity	
	AChE	**BuChE**
Controls		
Male (N=43)	11.15 ± 2.83	4.57 ± 1.86
Female (N=17)	13.47 ± 4.18	5.14 ± 1.78
Exposed		
Male (N=60)	6.23 ± 1.59***	3.15 ± 1.95***
Female (N=35)	6.78 ± 2.21***	4.08 ± 1.66***



Results are expressed as mean ± standard deviation
Activities are measured in µmoles/min/ml
***$p \leq 0.001$ compared with control males and females using MANOVA

Table 2.5: AChE and BuChE activity of the exposed group classified according to age.

Age	Enzyme activity	
	AChE	**BuChE**
15-30 (N=45)	6.35 ± 1.87	3.31 ± 2.06
31-45 (N=30)	6.44 ± 1.68	3.65 ± 1.53
46-62 (N=20)	6.60 ± 2.11	3.68 ± 2.06

Results are expressed as mean ± standard deviation
Activities are measured in µmoles/min/ml
compared with individuals of age 15-30 years using MANOVA

2.4 Discussion

Health damages like carcinogenesis (Hagmar et al., 2001), neurotoxicity (Priyadarshi et al., 2000; Alavanja et al., 2004; Kamel and Hoppin, 2004), reproductive and developmental alterations (Hanke and Jurewicz, 2004) and immunological effects are associated with chronic exposure to pesticides (McCauley et al., 2006). The pesticides used in the tea plantations of Terai and Dooars of West Bengal comprise of a heterogeneous mixture of compounds belonging to organophosphates (OP), organochlorines and pyrethroids (Roy et al., 2008; Bishnu et al., 2009; Singh et al., 2015). OP and carbamate pesticides inhibit acetylcholinesterase (AChE), causing accumulation of acetylcholine at peripheral and central cholinergic receptors, leading to overstimulation of the cholinergic system (Altuntas et al., 2002) and subsequent paralysis. The butyrylcholinesterase (BuChE), also known as pseudocholinesterase, is abundant in plasma, liver, smooth muscle and fat cells (Simoniello et al. 2010) and can bind covalently to OPs and carbamates. Tea garden workers are at high risk of exposure to complex mixtures of pesticides. Under these circumstances, it becomes difficult to determine the effect of a single pesticide separately, however the measurement of AChE and BuChE activity can serve as a good biomarker of monitoring exposure to a mixture of pesticides which includes organophosphorus and the carbamates too, and to assess the health risk in the pesticide exposed human populations in the tea gardens of the Terai regions of Darjeeling foothills.

In the present study a population of tea garden workers (sprayers and tea leaf pluckers), non-exposed controls, smokers and alcohol consumers were analyzed. A significantly lower activity of AChE and BuChE in the exposed group indicated the inhibitory effect of organophosphate pesticides present in pesticide mixture (**Table 2.2**) (Tinoco-Ojanguren

and Halperin, 1998; Ali et al., 2008; Remor et al., 2009). Tinoco-Ojanguren and Halperin (1998) have reported the inhibition of erythrocyte cholinesterase by OP pesticides in three communities and control subjects of Mexico. Adverse health effects existed among the poorest communities. A study conducted by Ali et al. (2008) in female Pakistani agricultural workers exposed to pesticides revealed that serum cholinesterase activity was significantly lower in exposed than the unexposed females ($p < 0.001$). Remor et al. (2009) also reported a significant decrease in butyrylcholinesterase (BuChE) activity in farm workers from Rio Grande do Sul (Brazil) compared to the control group. In India, Singh et al. (2007) reported that the organophosphate and pyrethroid pesticides significantly inhibited the AChE and BuChE activities in the sprayer group of mango orchards of Malihabad, Lucknow and showed that these pesticides were neurotoxic. In a study, Vidyasagar et al. (2004) had reported decreased AChE activity with enhanced lipid peroxidation in OP exposed subjects. Similar results have also been shown by other workers (Panemangalore et al., 1999; Abu Mourad, 2005; Hernández et al., 2005; Jintana et al., 2009). Our result is consistent with the above findings. The inhibition may be due to the fact that organophosphate pesticides are capable of disrupting the cholinesterase activity resulting in acetylcholine accumulation in the synapses (Ali et al., 2008).

Moreover, the tea garden workers involved in spraying used knapsack sprayers to apply pesticides without any protective measures (like nose or mouth cover, gloves, impermeable boots nor washing hands or taking bath immediately after pesticide handling), therefore are at high risk of exposure to pesticide. This may enhance the exposure and subsequently decrease the enzyme activities. Various studies have indicated that the amount of exposure is influenced by use of protective equipments (Sivayoganathan et al., 1995; Gomes et al., 1999). In a study, Jintana et al. (2009) have shown a positive association between the use of personal protective equipment and the

AChE activity. Those who did not use personal protective equipment had comparatively lower AChE activity than those using personal protective equipment. However, Ntow et al. (2009) showed that practices such as protective cover, method of pesticide application, direction of pesticide spraying and farmer retry period had no significant association with the ChE activity. It was also shown by Nerilo et al. (2014) that merely washing hands and face after direct pesticide exposure was not effective for minimizing the exposure and the workers who used these procedures were intoxicated more than those who took bath after exposure.

As all the males and few females were found to smoke and consume alcohol, smokers (non-alcoholic and non-exposed) and alcoholics (non-smoker and non-exposed) were included along with the exposed and control group. Nicotine found in tobacco has been shown to exert numerous toxic effects on the central and peripheral nervous system (Nakayama et al., 1993). However, Dowla et al. (1996) have shown that nicotine has no inhibitory effect on plasma cholinesterase activity. Recently, Jintana et al. (2009) have also reported that smoking and alcohol consumption have no effect on the AChE and BuChE activity. In the present study a comparison with smokers and alcohol consumers showed no significant difference in the activity of AChE and BuChE compared to the control group (**Table 2.2**) which corroborates the findings of Dowla et al. (1996) and Jintana et al. (2009). A marked decrease of AChE and BuChE activity in the exposed group compared to the smokers and alcohol consumers strongly suggests that the low enzyme activities in the exposed group were mainly due to pesticide(s) exposure, which includes OPs too (**Table 2.3**).

Inspite of slightly high activities of AChE and BuChE in females compared to males, the sex biased activity differences was significant ($p \leq 0.05$) in the control groups for AChE

and in exposed group for BuChE only ($p \leq 0.05$) (**Table 2.4**). Despite of slight activity differences, a positive correlation between the sexes and enzyme activity was lacking which is also in agreement with the results of Maroni et al. (2000) showing no correlation between sex and the enzyme activity. Maroni and workers had also stated that AChE and BuChE activities may vary due to inter individual differences or due to specific physiopathological conditions in healthy people also. No effect of sex was observed on the mean whole blood ChE activity in a study conducted by Ntow et al. (2009). Moreover, in the present study, the females were only involved in plucking of tea leaves so were exposed to pesticides mainly through skin contact, whereas the males involved in spraying and mixing were more exposed to pesticides both dermally as well as through inhalation. But our results showing higher value of cholinesterase in females compared to males (though not significant in all cases) are in good agreement with the findings of Jintana et al. (2009) who have shown a higher activity of AChE and BuChE in females over high- and low- exposure periods. It has also been suggested that the hormonal and other physiological factors may modify or act as confounding factors on the effect of pesticide(s) exposure in females (Maroni et al., 2000). Moreover, the overall enzyme activity (**Table 2.2**) in a particular group and the gender wise activity (**Table 2.4**) were not different i.e., the enzyme activities were not influenced by a particular sex.

Younger workers had reduced cholinesterase activity in cases where they were engaged in more dangerous activities like they were assigned tasks that bear greater risk of pesticide exposure (López-Carillo and López-Cervantes, 1993; Singhaseni, 1999; Dhalla and Sharma, 2013). In the present study, AChE and BuChE activities were not significantly reduced in any particular age group, however slightly lower ChE activities observed in the 15-30 years age group reflect that these individuals might be engaged in the activities with greater risk (**Table 2.5**). López-Carillo and López-Cervantes (1993) reported the

serum cholinesterase activity to be 4.87, 4.74 and 4.28 KU/l in the age groups <20, 20-29 and 30+, respectively. Our results showing AChE activity of 6.35 ± 1.87, 6.44 ± 1.68, 6.60 ± 2.11 µmoles/min/ml and BuChE activity of 3.31 ± 2.06, 3.65 ± 1.53, 3.68 ± 2.06 µmoles/min/ml in the age groups 15-30, 31-45, 46-62, respectively are in accordance with López-Carillo and López-Cervantes (1993). However, Ntow et al. (2009) in their study reported that work practices that caused high exposures had no significant effect on the ChE activity of the exposed group.

Dose and duration of exposure has been hypothesized to be associated with changes in ChE activity (López-Carillo and López-Cervantes, 1993; Jintana et al., 2009; Dhalla and Sharma, 2013). Also, pesticide toxicity is related to its ability to inhibit acetylcholinesterase (Ecobichon, 2001). AChE is inhibited by OP pesticides in the order of chlorpyrifos > monocrotophos > profenofos > acephate, whereas BuChE is strongly inhibited by malathion, diazinon, chlorpyrifos and dichlorvos (Das et al., 2006; Jintana et al., 2009) suggesting that the sensitivity of the biomarkers can be assessed as per the chemical nature and mode of action of pesticides. The dose and duration of exposure and the sensitivity of biomarkers to the individual pesticide can be assessed only in controlled experiments. It was found that pesticides were applied throughout the year in the form of mixed formulations in the tea gardens; therefore a similar analysis was not possible in the present study.

Chakraborty et al. (2009) reported a 47% decline of AChE activity in the regular sprayers than control and a 25% decline in the occasional sprayers. Jintana et al. (2009) reported 30% and 26% inhibition of AChE and BuChE activity, respectively in individuals exposed to OP pesticides in Thailand. Agricultural workers in Kenya were reported to have 35% inhibition of AChE activity (Ohayo-Mitoko et al., 2000). Our results showing

47% and 26% inhibition of AChE and BuChE activity, respectively in the tea garden workers exposed to pesticide(s) are in concurrence with Ohayo-Mitoko et al. (2000) and Chakraborty et al. (2009). 50.6% lesser activity of ChE in the whole blood was observed in the farmers at Akumadan when they were compared to farmers (controls) from Tono Irrigation project, Ghana (Ntow et al., 2009). The guidelines of WHO for interpretation of erythrocyte AChE measurement state that 20-30% inhibition is an indicator of exposure, 30-50% inhibition is the indicator of hazard and 50% or greater is an indicator of poisoning (WHO, 1986). The percentage of AChE and BuChE inhibition in the exposed worker in the present study indicates exposure to pesticides (including OPs) and health hazards.