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Determination of the reference range of whole blood cholinesterase activities in Thai postmortem cases

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ABSTRACT

Background: Whole blood cholinesterase activities are biomarkers for diagnosis of organophosphate and carbamate poisoning. The reference ranges for these biomarkers in Thai people were available for living people. However, there is no baseline data for these biomarkers in Thai postmortem cases.

Objectives: The objective of this study is to study the reference range of whole blood cholinesterase activities in Thai postmortem cases and factors influencing enzyme activities.

Materials and methods: Postmortem blood samples were collected from Thai dead bodies who were 18-60 years old and were sent for medico-legal autopsies at the Department of Forensic Medicine, Siriraj Hospital, Mahidol University between 9th June 2020 and 31st December 2020. Data including gender, age, postmortem interval (PMI) and liver pathology were recorded. Whole blood cholinesterase activities were analyzed by using UV-visible spectrophotometer. Whole blood cholinesterase activities were analyzed using descriptive statistics. Mann-Whitney U test and Kruskal-Wallis H test were also tested for comparison between each factor using statistical significance at p<0.05.

Results: There were 176 subjects recruited in this study (121 males and 55 females). Whole blood cholinesterase activities in all subjects were 3514.32-7771.13 IU/mL and the mean and median values were 6150.27 and 6326.78 IU/mL, respectively. There was significant difference among classified four age groups (p value=0.014). Whole blood cholinesterase activities of two lower age groups (18-30 and 31-40 years old) were significantly lower than the third age group (41-50 years old) (p value=0.027 and 0.005, respectively). Whole blood cholinesterase activities were also significantly related to PMI (p value=0.042). The values from early PMI (0-8 hours) period was significantly lower than the values from the second PMI (8-16 hours) period (p value=0.043). In addition, postmortem cases with fatty change >50% significantly presented lower enzyme activities than those with fatty change <50% (p value=0.042).

Conclusion: Whole blood cholinesterase activities in Thai postmortem cases whose age ranged from 18 to 60 years old were 3514.32-7771.13 IU/mL. Age, PMI and liver pathology were three factors that affect whole blood cholinesterase activities in Thai postmortem cases.

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Introduction

Cholinesterase enzymes (EC 3.1.1.x) are enzymes found in normal human bodies and belong to serine hydrolase superfamilies.[1] They can be categorized into two types including acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BuChE, EC 3.1.1.8). AChE is normally found in erythrocyte, neuro-muscular junction and central nervous system whereas BuChE is mainly found in liver and plasma.[1] Cholinesterase activities can be employed as the biomarker for organophosphate and carbamate poisoning because these two insecticides result in cholinesterase inhibition.[2] Thus, cholinesterase activities should be decreased after the exposure to organophosphate and carbamate insecticides. However, the levels of cholinesterase activities can be affected by several underlying diseases, drugs, malnutrition and genetic variabilities.[1] Thus, the analysis of cholinesterase activities should be carefully interpreted and inter-personal variation and underlying diseases should be also considered.

The analysis of cholinesterase activities can be performed by two methods. The first method is the analysis for whole blood or red blood cell cholinesterase activities which is used for determination of AChE activities.[3] The second method is the analysis for plasma or serum cholinesterase activities which is applied for BuChE activities.[3] Although the determination of AChE activities is preferred for the diagnosis of organophosphate and carbamate poisoning, the analysis of BuChE activities can be performed in some context like the detection of exposure to insecticides or monitoring for clinical recovery.[2,4] However, the detection of BuChE activities may not be suitable for the diagnosis of organophosphate and carbamate poisoning in postmortem cases because of the occurrence of hemolysis in postmortem blood particularly in prolonged postmortem intervals.[5] Thus, the analysis of whole blood cholinesterase activities should be more appropriate for the diagnosis of organophosphate and carbamate poisoning in postmortem cases.

The studies of cholinesterase activities in Thai people from previous publications were conducted in healthy Thai living people who were not exposed to pesticides and the authors reported that means and ranges of AChE activities in healthy Thai people were 3320-5136 IU/mL[6,7] and 3684-6588 IU/mL,[6] respectively. However, there is no information for whole blood cholinesterase activities in normal Thai postmortem cases. Klette KL et al reported that the mean and range of whole blood cholinesterase activities in postmortem cases were 4800 IU/mL and 2000-7400 IU/mL.[8] Postmortem changes may affect whole blood cholinesterase activities in dead bodies due to the effect of postmortem blood hemolysis[5] and the effect of bacterial cholinesterase activities[9] from bacterial translocation into blood stream after death.[10] Therefore, this study aims to determine the baseline reference values of whole blood cholinesterase activities in Thai postmortem cases for the application to the diagnosis of organophosphate and carbamate poisoning in Thai postmortem cases.

Materials and methods

Study subjects

Blood samples were collected from Thai medico-legal cases who were sent for medico-legal autopsies at Department of Forensic Medicine, Siriraj Hospital, Mahidol University between 9th June 2020 and 31st December 2020. Inclusion criteria for this study were Thai postmortem cases who were between 18 and 60 years old with no histories of any underlying diseases and had declared postmortem intervals not greater than 24 hours (no signs of decomposition). Exclusion criteria consisted of 5 types including:

1. Dead bodies who were agriculturists
2. Dead bodies who were suspicious for organophosphate or carbamate poisoning
3. Dead bodies who had the history of hospital admission greater than 24 hours
4. Dead bodies who had signs of sepsis from medical records or autopsy findings
5. Dead bodies who were given for cholinesterase inhibitor drugs.

This study was approved by the Siriraj Institutional Review Board, Faculty of Medicine, Siriraj Hospital, Mahidol University (Certificate of Approval No. Si 389/2020).

Sample collection

Blood samples were taken from femoral vein access during autopsy procedures. Blood samples approximately 7-10 mL were collected into blood tubes without any anticoagulants. Then, blood samples were transferred to keep in the refrigerator at 4 °C. Whole blood cholinesterase activities were analyzed by using UV-visible spectrophotometer in the next day. Before blood sample was analyzed, the blood tube was gently overturned back and forth around 8-10 times. Next, the blood tube was slightly inclined and liquid blood sample was slowly taken out approximately 2-3 mL into the other tube which was sufficient for analysis. Any blood clot inside the blood tube was avoided during blood pipetting to ensure that liquid blood form was transferred. Then, transferred blood sample was mixed using blood vortex mixer to obtain blood homogeneity for analysis. The analysis was performed in duplicated and the mean values were determined and recorded as whole blood cholinesterase activities for blood samples.

Subject data including gender, age, postmortem interval (PMI), liver pathology and cause of death were recorded for each blood sample for statistical analysis. The age in this study was classified into four categories: 18-30, 31-40, 41-50 and 51-60 years old. PMI was categorized into three groups: 0-8, 8-16 and 16-24 hours. Liver pathology could be defined based on gross and microscopic findings and then divided into two groups: liver with fatty change less than 50% and liver with fatty change more than 50% (Figure 1).

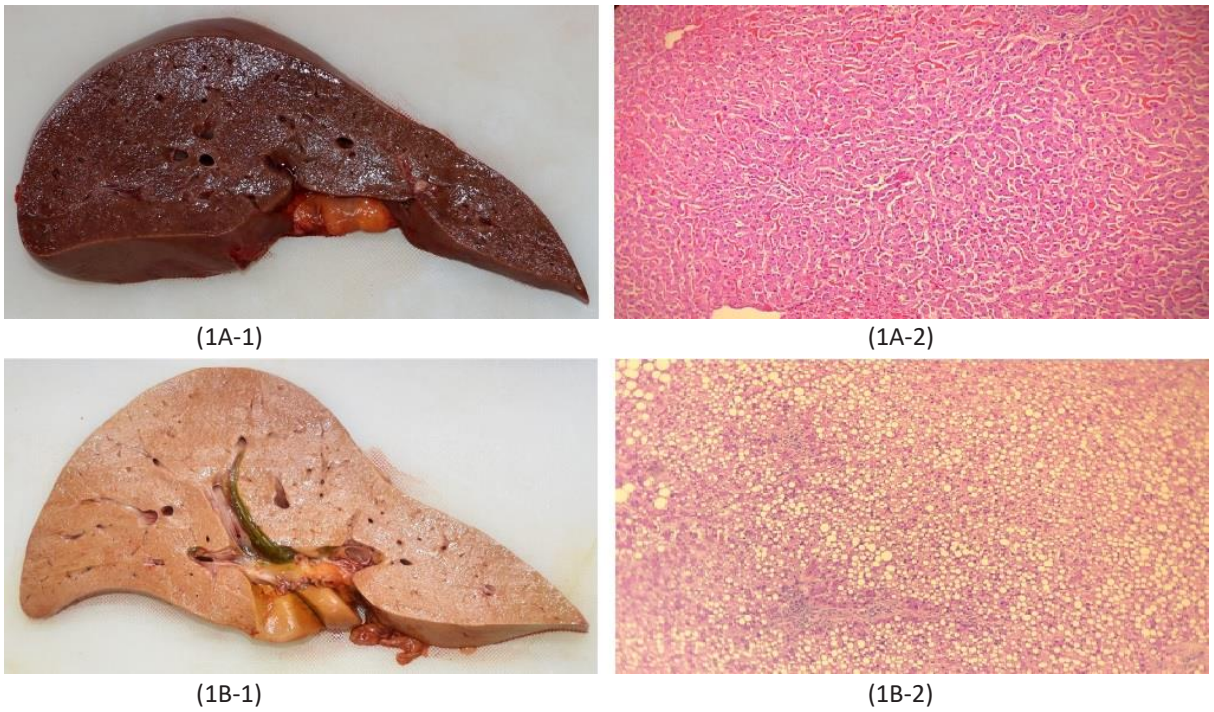

Figure 1. *Gross and microscopic findings for liver pathology. 1A: fatty change <50%, 1A-1: gross findings, 1A-2: microscopic findings, 1B: fatty change >50%, 1B-1: gross findings, 1B-2: microscopic findings.*

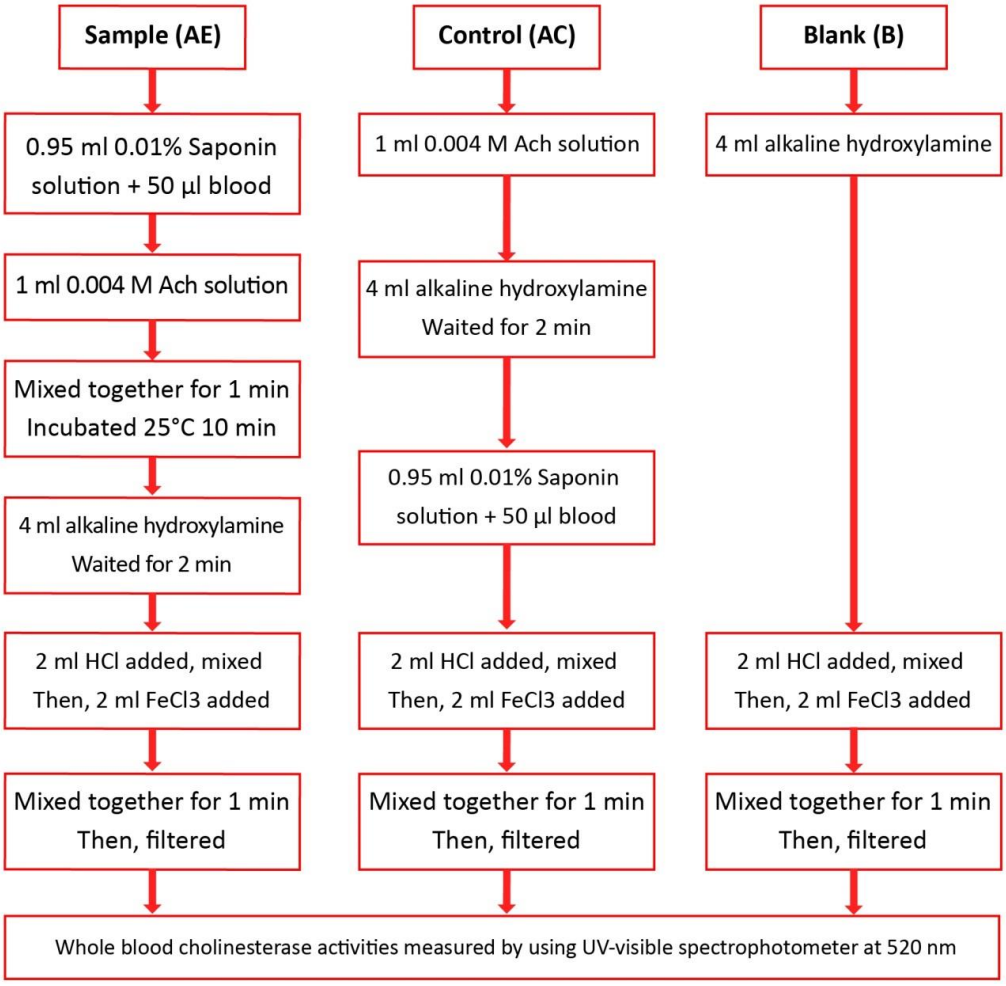

Figure 2. *Experimental steps for the analysis of whole blood cholinesterase activities*[3].

Chemicals and reagents

Acetylcholine chloride (Ach) 99%, sodium acetate AR grade, glacial acetic acid AR grade, disodium hydrogen phosphate pentahydrate AR grade, potassium dihydrogen phosphate anhydrous AR grade, sodium hydroxide (NaOH) AR grade, hydrochloric acid (HCl) 37%, ferric chloride ($FeCl_3$) anhydrous 98%, hydroxylamine hydrochloride 99%, saponin permeating solution (0.5% w/v solution in phosphate buffer solution) and Whatman filter paper number 41 with diameter 110 millimeters (mm) were purchased from U&V Holding (Thailand) Co., Ltd. Deionized water was generated from Merck Millipore Direct-Q® 3 UV-R Water Purification System.

Sample preparation for the analysis of whole blood cholinesterase activities[3]

The analysis of whole blood cholinesterase activities was performed in three sample groups for each blood sample including sample group (AE), control group (AC) and blank group (B). Experimental steps in these three sample groups were described in Figure 2.

After all sample groups were filtered by Whatman paper, all sample groups were taken into quartz cuvette with path length 10 mm. Then, all three sample groups were measured for whole blood cholinesterase activities using the Agilent Cary 8454 UV-visible spectrophotometer at 520 nanometers (nm) within 10 minutes.

Blank group was used for zero adjustment in measurement. Next, control group (AC) and sample group (AE) were measured against blank group to obtain cholinesterase activities. Then, whole blood cholinesterase activities were calculated by using equation 1 as described below

Whole blood cholinesterase activities (IU/mL) = [4-(4AE/AC)]x2000 IU/mL (**Equation 1**)

The analysis was performed in duplicate for each blood sample. Next, %coefficient of variation (%CV) was determined for these two results. Acceptable %CV in this study should not be greater than 15% based on method validation guidelines. Then, these two results were calculated for the mean value of each blood sample and this mean value was recorded and used for the statistical analysis.

External quality control from proficiency testing (PT) samples

Two external quality control samples that were supplied as PT samples were used for verification of this laboratory method and these two samples were kindly supported by the Department of Medical Sciences. These two samples were analyzed for whole blood cholinesterase activities. The results from these two PT samples were compared with the results from the Department of Medical Sciences. All results obtained from the laboratory method should be in good agreement with acceptable criteria for the application to postmortem blood samples.

Statistical analysis

The data for whole blood cholinesterase activities were analyzed using IBM SPSS® Statistics for Window version 26. Descriptive statistics including mean, median, and standard deviation (SD) were analyzed. Normality test was performed using Kolmogorov-Smirnov test. After normality test, data set in this study did not meet the criteria of normal distribution and the equality of variances. Thus, the Mann-Whitney U test and Kruskal-Wallis H test were performed for data comparison where it was appropriate. Statistical significance was set at p value <0.05.

Results

The results of two PT samples compared with the results from all participant laboratories reported in the certificate from the Department of Medical Sciences were shown in Table 1.

Table 1 The results of PT samples compared with the results from the Department of Medical Sciences.

PT sample	Results from all laboratories participated in PT schemes (N = 6) (IU/mL)	Results from this study (IU/mL)
PT NIH/AChE 1/63	2847±791	3338.34
	(Median Xi = 2839, MAD = 338)	(Criterion=1.48)
PT NIH/AChE 2/63	2440±663	3095.99
	(Median Xi=2474, MAD=420)	(Criterion=1.48)

According to the certificate, the Department of Medical Sciences employed median absolute deviation (MAD) method to analyze the results and the acceptable criterion was:

$$\text{Criterion: } \frac{|Xi\text{-}median\ (xi)|}{MAD} < 5$$

When the results from this study were applied for the criterion, it was found that all of these two results were in acceptable ranges. Thus, this method was able to apply for postmortem blood samples.

There were 176 blood samples recruited for the analysis of whole blood cholinesterase activities in this study. The subjects were 121 males (68.75%) and 55 females (31.25%). The mean ages of male and female subjects were 45 and 46 years old, respectively. Whole blood cholinesterase activities in all subjects ranged from 3514.32 to 7771.13 IU/mL and the mean and median values were 6150.27 and 6326.78 IU/mL, respectively. The range, mean and median of whole blood cholinesterase activities in male and female subjects were shown in Table 2. Data for whole blood cholinesterase activities were present in histogram chart as shown in Figure 3.

Table 2 Whole blood cholinesterase activities in all subjects classified by gender.

Sex	N	Range (IU/mL)	Mean±SD (IU/mL)	Median (IU/mL)
Female	55	3642.52-7693.23	6153.86±1098.14	6305.92
Male	121	3514.32-7771.13	6148.64±991.56	6350.23
Total	176	3514.32-7771.13	6150.27±1022.89	6326.78

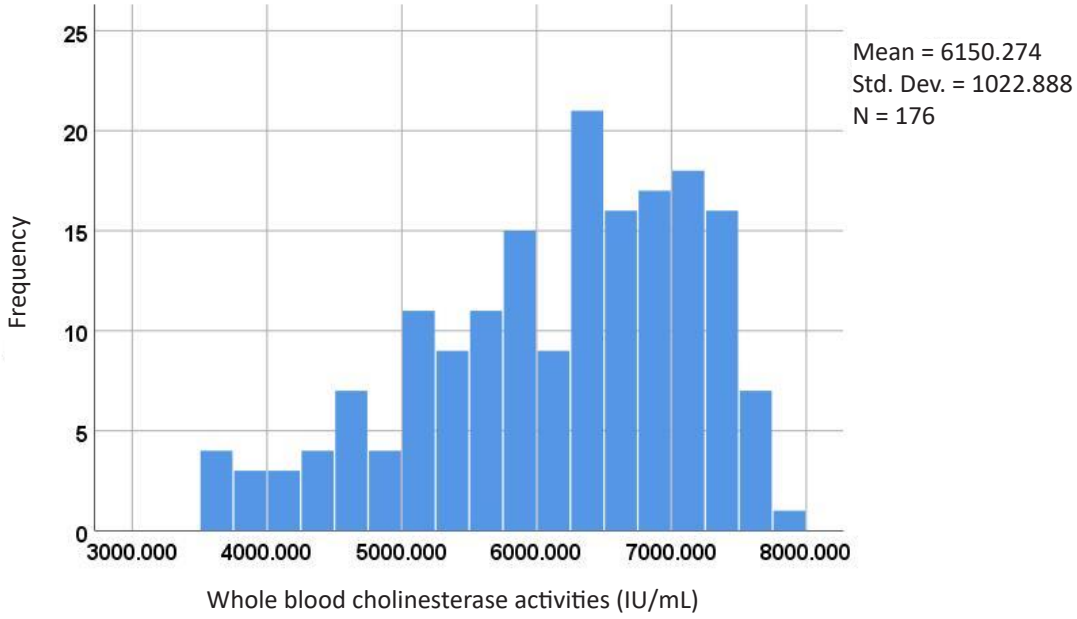

Figure 3. *Histogram of whole blood cholinesterase activities in this study.*

Comparison of whole blood cholinesterase activities between Thai male and female subjects was analyzed by using Mann-Whitney U test and it was found that the values in male subjects were not significantly different from those in female subjects (*p* value=0.812) as shown in Figure 4.

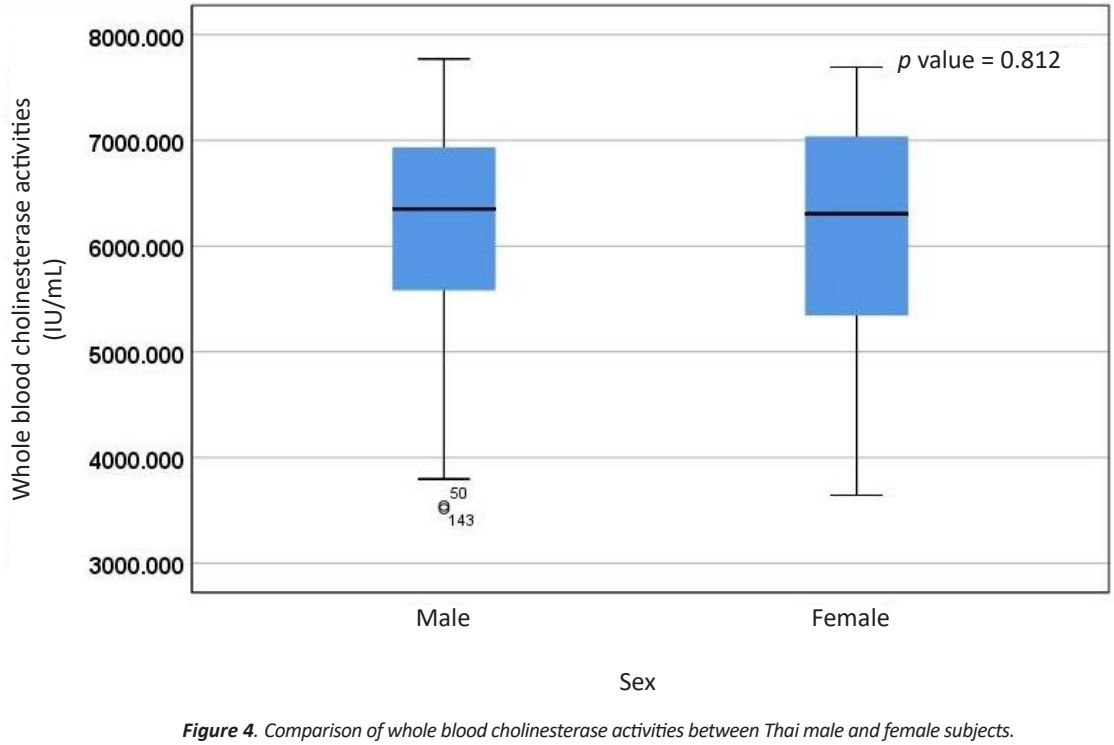

Figure 4. *Comparison of whole blood cholinesterase activities between Thai male and female subjects.*

Whole blood cholinesterase activities in four age groups classified in this study were shown in Table 3. Then, the comparison of whole blood cholinesterase activities among these four age groups was analyzed by using Kruskal-Wallis H test and it was found that there was significant difference among these four age groups (*p* value=0.014) as shown in Figure 5. When the comparison between each group was considered, it was found that the values in the first two lower age groups (18-30 and 31-40 years old) were significantly different from the values in the third age group (41-50 years old) (*p* value=0.027 and 0.005, respectively). In addition, the values in the second age group (31-40 years old) were also significantly different from the values in the fourth age group (51-60 years old) (*p* value=0.022).

Table 3 Whole blood cholinesterase activities in four age groups.

Age (years old)	N	Range (IU/mL)	Mean±SD (IU/mL)	Median (IU/mL)
18-30	48	3514.32–7600.95	6306.91±988.49	6528.72
31-40	43	4510.02–7771.13	6475.01±854.66	6636.21
41-50	41	3542.62–7502.21	5835.67±1060.30	5945.70
51-60	44	3642.52–7693.23	5955.20±1079.24	6144.62
Total	176	3514.32-7771.13	6150.27±1022.89	6326.78

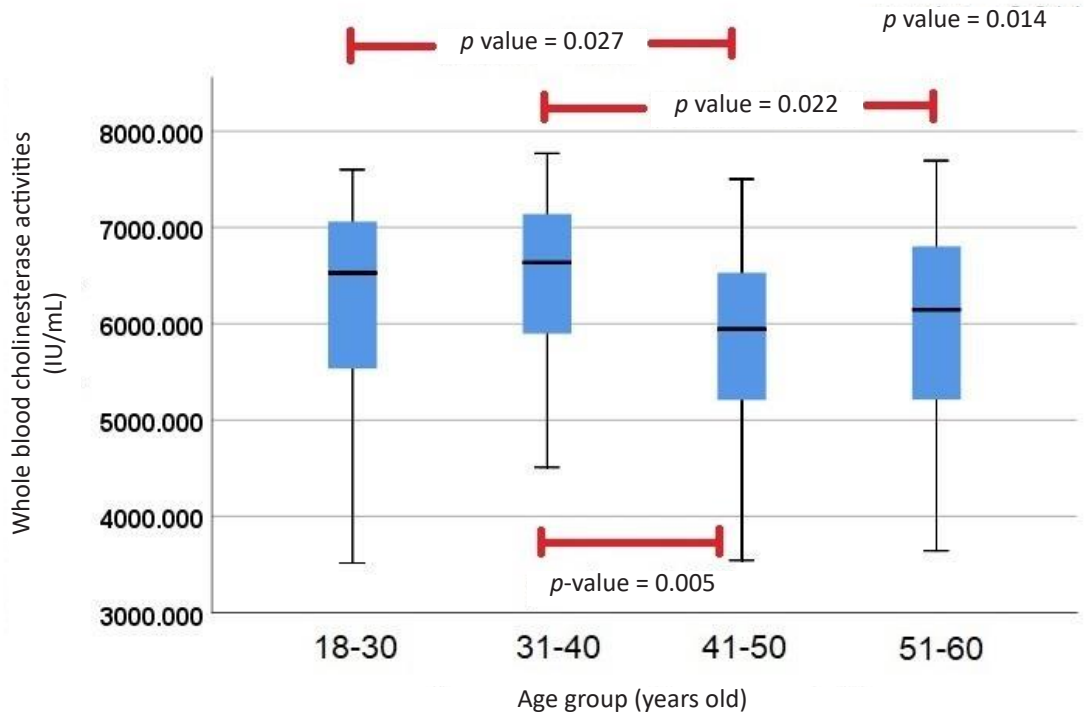

Figure 5. *Comparison of whole blood cholinesterase activities among four age groups.*

Whole blood cholinesterase activities in three PMI groups were shown in table 4. When the analysis by Kruskal-Wallis H test was performed for these three PMI groups, it was found that there was significant difference among these three PMI groups (*p* value=0.042) as shown in figure 6. It was found that the values in the first PMI group (0-8 hours) were lower than the other two groups. The values in the second PMI group were significantly higher than the first PMI group (*p* value=0.043). According to Table 4, the values in the third PMI group were lower than the second PMI group but still higher than the first PMI group. However, this difference did not have statistical significance.

Table 4 Whole blood cholinesterase activities in three PMI groups.

PMI (hours)	N	Range (IU/mL)	Mean±SD (IU/mL)	Median (IU/mL)
0-8	44	3514.32-7568.96	5883.69±1124.99	6003.32
8-16	56	3642.52-7771.13	6413.85±936.88	6538.77
16-24	76	3714.47-7693.23	6110.39±988.38	6340.43
Total	176	3514.32-7771.13	6150.27±1022.89	6326.78

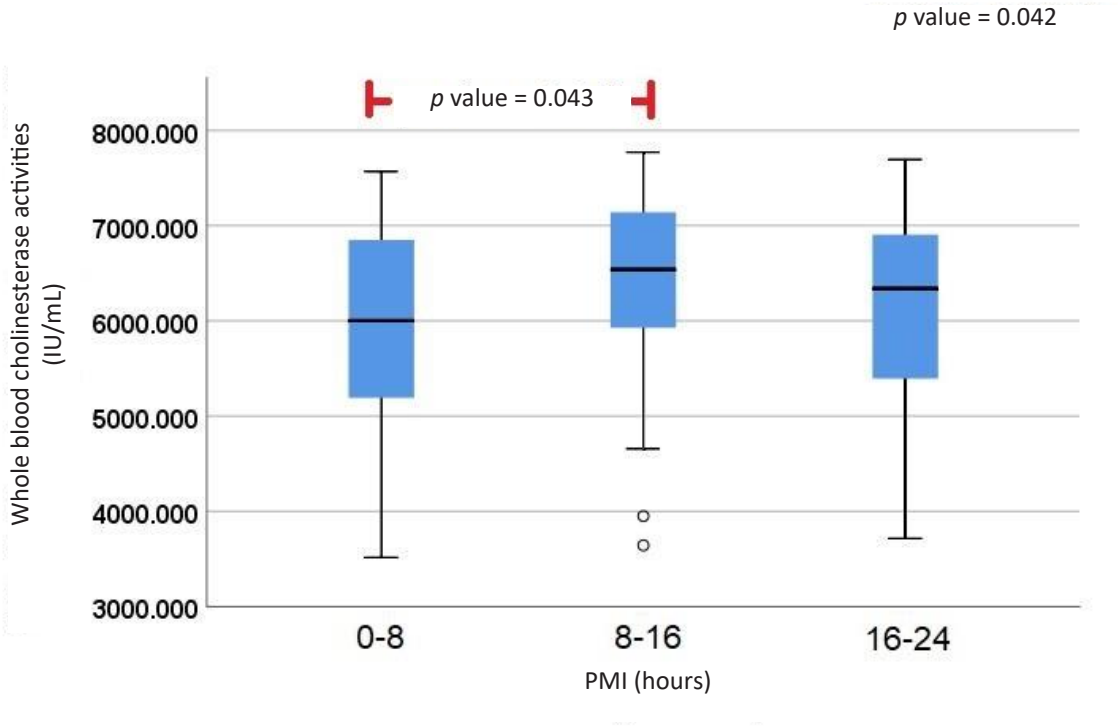

Figure 6. *Comparison of whole blood cholinesterase activities among three PMI groups.*

Whole blood cholinesterase activities in two groups of liver pathology were shown in Table 5. After the analysis with Mann-Whitney U test, it was found that there was significant difference between the group with fatty change less than 50% and the group with fatty change greater than 50% (*p* value=0.042) as shown in Figure 7.

Table 5 Whole blood cholinesterase activities in two groups of liver pathology.

Liver pathology	N	Range (IU/mL)	Mean±SD (IU/mL)	Median (IU/mL)
Fatty change <50%	116	3514.32-7771.13	6269.33±982.94	6368.58
Fatty change >50%	60	3642.52-7493.35	5920.10±1066.99	6168.97
Total	176	3514.32-7771.13	6150.27±1022.89	6326.78

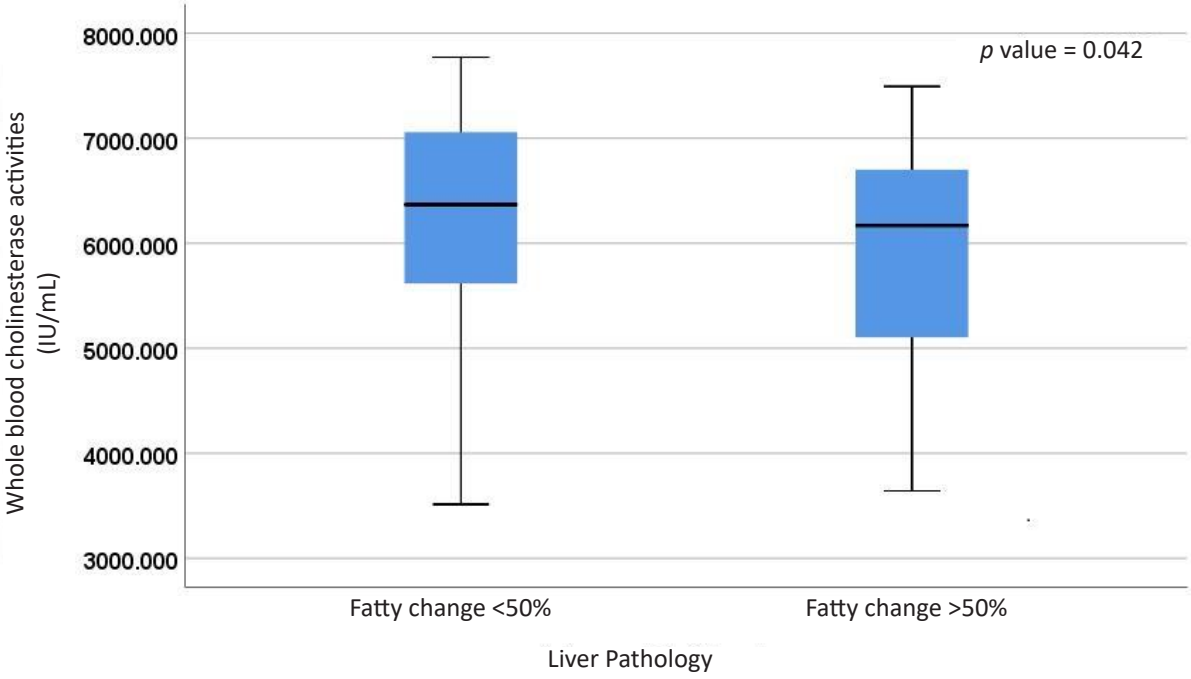

***Figure 7**. Comparison of whole blood cholinesterase activities between two groups of liver pathology.*

Discussion

Whole blood cholinesterase activities in Thai postmortem cases in this study ranged from 3514.32 to 7771.13 IU/mL and the mean and median values were 6150.27 and 6326.78 IU/mL, respectively. These values were slightly higher than AChE activities in healthy Thai living people reported by Singhatong S.[6] This finding might be explained by the effect of bacterial activities following the progression of postmortem change. The process of postmortem change began with early period consisting of cellular death, the reduction of body temperature, loss of adenosine triphosphate (ATP) and circulation cease.[5] This process produced supravital reaction, postmortem cooling, postmortem rigidity and postmortem lividity, respectively[5]. When PMI increased, protein degradation and bacterial translocation due to loss of integrity of intestinal mucosa occurred and these led to secondary flaccidity and transition state before decomposition.[5,10] After 24 hours (1 day), decomposition process was mostly developed in postmortem cases under ambient temperature (20 °C).[5] Putrefaction played an important role in decomposition as a result of bacteria and other microorganisms.[5] According to this process, bacterial translocation from gastro-intestinal (GI) tract into bloodstream occurred after death following increased PMI.[5,10] There were many bacterial strains in GI tract as normal intestinal flora. *Pseudomonas aeruginosa* was reported that it could be detected as normal intestinal flora in GI tract but it had low infectivity because it was unable to attach to normal intestinal epithelium.[11] The previous study showed that some bacterial strains like *Pseudomonas spp*. could produce acetylcholine-hydrolyzing enzymes.[9] Thus, it could be possible that translocation of intestinal flora like *Pseudomonas*. aeruginosa into postmortem blood was responsible for slightly higher cholinesterase activities after death.

This study showed that whole blood cholinesterase activities in male and female subjects were not significantly different and this result was consistent with the previous study.[3] In contrast to gender, this study showed that the younger age groups tended to have greater whole blood cholinesterase activities than the older age groups. Previous studies demonstrated that cholinesterase activities in the brains of younger humans and younger rats were higher than those of older humans and older rats.[12,13] In addition, it was reported that AChE activities in younger age group were higher than older age group although it was not statistically significant.[14] Thus, it might be possible that whole blood cholinesterase activities were age-related in Thai postmortem cases.

This study demonstrated that the trend of whole blood cholinesterase activities changed following increased PMI. In early PMI period (0-8 hours), whole blood cholinesterase activities were lower than those in the next two PMI periods. Whole blood cholinesterase activities were increased significantly in the second PMI period (8-16 hours). Then, whole blood cholinesterase activities declined in the last PMI period (16-24 hours) but the values were still slightly higher than the values in the early PMI period with no statistical significance. This result could be explained by the effect of bacterial translocation and protein denaturation. In the first PMI period (0-8 hours), whole blood cholinesterase activities were not affected by acetylcholine-hydrolyzing enzymes from some bacterial strains[9] due to minor effect of bacterial translocation in early postmortem period. When PMI increased, bacterial translocation significantly

occurred[10] and it might be hypothesized that some bacterial strains like *Pseudomonas aeruginosa* from GI tract could enter the bloodstream and produce cholinesterase activities as mentioned above. When PMI was progressively increased, the secondary flaccidity after rigor mortis occurred because of muscle breakdown and protein denaturation process.[5] Due to the effect of protein denaturation, all human proteins would be denatured and the protein functions should be decreased. Thus, it should be explained that the reduction of whole blood cholinesterase activities in the last PMI group resulted from enzyme denaturation even though the decrease of whole blood cholinesterase activities was not statistically significant. Further study should be conducted in the late PMI period (greater than 24 hours) which would prove the trend of whole blood cholinesterase activities after protein denaturation.

Whole blood cholinesterase activities in the group with fatty change in liver less than 50% significantly higher than those in the group with fatty change in liver more than 50%. AChE was normally synthesized in nervous tissue, nerve fiber, and erythropoiesis.[1] However, AChE was a glycoprotein which contained protein and carbohydrate (10-15% of its molecule).[1] This indicated that the synthesis of AChE required these two nutrients. The previous study reported that the deficiency of some amino acids could be related to fatty change in liver, for example, tryptophan and methionine.[15] AChE had two active sites and one of them was quaternary ammonium binding site for choline.[1] The key amino acid residue in this site was tryptophan.[1] Thus, it might be hypothesized that fatty change greater than 50% in liver affected synthetic capacity of some nutrients and had an effect on whole blood cholinesterase activities.

This study had some limitations. Firstly, there were small proportions of Thai female subjects in this study. Thus, the interpretation that whole blood cholinesterase activities in Thai male and female populations was not different should be carefully performed because there were some studies indicating that gender also affected ChE activities.[14] In addition, whole blood cholinesterase activities in this study were analyzed using the conventional method which did not have AChE extraction process from erythocytes[16] and the previous study stated that the conventional method could suffer from hemoglobin interference which might affect the method accuracy.[1] However, this conventional method consisted of simple analytical steps and more suitable for application in many laboratories. Thus, the analysis of whole blood cholinesterase activities should be carefully interpreted and case information should be also considered.

Conclusion

Whole blood cholinesterase activities in Thai postmortem cases whose age ranged from 18 to 60 years old were 3514.32-7771.13 IU/mL and the mean and median values were 6150.27 and 6326.78 IU/mL, respectively. Age, PMI and liver pathology were three factors that affect whole blood cholinesterase activities in Thai postmortem cases. Thus, these three factors should be considered when the analysis of whole blood cholinesterase activities was performed in Thai postmortem cases.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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