JAMS

JOURNAL OF ASSOCIATED MEDICAL SCIENCES

Volume 52 Number 3 September-December 2019 E-ISSN: 2539-6056

Journal of Associated Medical Sciences

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Publication frequency

Journal of Associated Medical Sciences publishes 3 issues a year

Issue 1: January-April
Issue 2: May-August
Issue 3: September-December

Journal Impact Factor
The journal's 2017 Impact Factor is 0.237

Journal website
Homepage https://www.tci-thaijo.org/index.php/bulletinAMS/index

Journal E-ISSN:
2539-6056

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Faculty of Associated Medical Sciences, Chiang Mai University
110 Inthawaroros Road, Suthep, Muang, Chiang Mai, 50200
Phone 053 935072 Facsimile 053 936042

Journal of Associated Medical Sciences

Vol. 52 No. 3 : September 2019

Content

Journal of Associated Medical Sciences 2019; 52 (3): 150-157

JAMS
Journal of Associated Medical Sciences

Thai-Journal Citation Index Centre (TCI) & ASEAN Citation Index (ACI)

Journal of Associated Medical Sciences

Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index

Prevalence of patellofemoral pain syndrome in young Thai athletes registered in Phitsanulok Provincial Administrative Organization Sports School, Thailand

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ARTICLE INFO

Article history:
Received 22 November 2018
Accepted as revised 8 May 2019
Available online 11 June 2019

Keywords:
Prevalence, patellofemoral pain syndrome, training duration, knee

ABSTRACT

Background: Patellofemoral pain syndrome (PFPS) is one of the most common knee pain diagnoses in sports medicine clinics. The disorder is usually related with sports and activities of daily living and the condition may affect up to 25% of males and females who participate in sporting activities. However, only researchers in Europe, Australia, USA, and a few Asian countries have conducted studies of prevalence of PFPS. There is still a lack of good epidemiological evidence studying incidence or prevalence of PFPS in most countries. PFPS is also often related to overuse so recent changes in activities and changes in frequency, intensity, and duration of training should be considered.

Objectives: Primary aim of the study was to estimate the prevalence of PFPS in young Thai athletes and the secondary aim was to investigate the relationship between PFPS and training duration per week.

Materials and methods: Three hundred and sixty-two young Thai athletes (12-18 years) were recruited in the study. The participants completed a self-reported questionnaire known as "Anterior Knee Pain Scale (AKPS)" for the initial screening process. Participants who provided a score of less than 100 underwent further physical examination for PFPS. Details of their training schedule according to training frequency per week and types of training were given by sports coaches at school.

Results: Three hundred and ten athletes (mean age: 14.8±1.6 years) completed the Anterior Knee Pain Scale (AKPS) questionnaire. There were 51 (35 males and 16 females) out of 310 participants who reported a questionnaire score of less than 100. Nineteen (12 males and 7 males) out of 51 participants presented with PFPS with a greater prevalence in females. However, no significant difference of PFPS prevalence was found between males and females (males: 12/35 = 34%, females: 7/16 = 44%, p=0.521). The overall prevalence of PFPS was 6% (19 out of 310). PFPS was weakly related to longer sports training duration (r=0.115, p=0.042) for the overall population. When genders were considered, PFPS was weakly related to both longer general training duration (r=0.174, p=0.011) and sports training duration (r=0.147, p=0.033) for males.

Conclusion: The overall prevalence of PFPS in young Thai athletes was 6% which was a lower rate compared to previous studies. Sports training duration and sum of both training duration significantly presented weak correlation with PFPS. The results of the current study may have implications for coaches or sports teachers for planning the schedule of sports training duration for the young Thai athletes.

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doi: 10.14456/jams.2019.23
E-ISSN: 2539-6056

Introduction

Patellofemoral pain syndrome (PFPS) is one of the most common knee pain diagnoses in sports medicine clinics.[1-5] Anterior knee pain (AKP) is a symptom that most commonly results from PFPS so the terms AKP and PFPS are often used synonymously to describe the same syndrome.[5,6] The disorder is usually related to sports and activities of daily living and the condition may affect up to 25% of males and females who participate in sporting activities.[7] The major complaint is pain around or behind the patella (retropatellar pain) usually during running, inclined walking, stair climbing, prolonged sitting with the knee in a flexed position, and squatting.[8-11] As a result, a large number of children and adolescents may be restricted in activities or perform submaximally on the sports field.[12] Limitation of physical activities can lead to a negative effect on physical development, motor skill and psychosocial development,[13,14] and can also increase risk of becoming over-weight and obese adults.[15]

Researchers in Europe, Australia, and USA have found that 25% of the general or sporting population present with PFPS.[12,16-19] Callaghan and Selfe conducted a literature review to investigate incidence or prevalence of PFPS in the United Kingdom.[1] Only 40 out of 136 articles cited rate or ratio for incidence or prevalence of PFPS and of these 15 out of 40 papers found a PFPS prevalence of 25% or 1:4 ratio in general population. However, there is still a lack of studies assessing prevalence of PFPS in most countries and Thailand is one country where prevalence of PFPS has not previously been evaluated. Thai National Statistical Office stated that in 2011, 26.1% of the Thai population 11 years old and above participated in exercise and sporting activities. When investigating the exercise participation rate in each age group, it was found that the 11-14-year age group had the highest exercise participation rate which was 60%. The second highest rate was found in the 15-24-year age group at 40%. The 60+ year age group was 23.6% and the 25-59 year age group was the lowest participation rate at 19%.[20] Whilst the young Thai population have a high exercise participation rate and there is a lack of knowledge of the prevalence of PFPS in Thailand.

The anterior knee pain scale (AKPS), also known as Kujala scale, was designed to evaluate patellofemoral pain.[21] It is a self-report questionnaire consisting of 13 items that evaluates subjective responses to specific activities and to assess the symptoms and the level of disability of patients with PFPS. The score of the questionnaire runs from a minimum of 0 to a maximum of 100 points. Lower scores represent greater pain and lack of ability. Participants who have no sign of AKP will have a score of 100.[2,3,21-23] Esculier et al conducted a systematic review of 5 self-reported questionnaires used to assess the level of symptoms and disability in people with PFPS.[2] The questionnaires included Activities of Daily Living Scale (ADLS), International Knee Documentation Committee (IKDC), Lysholm Scale (LS), Functional Index Questionnaire (FIQ) and AKPS. It was found that AKPS presented excellent test-retest reliability (intraclass correlation coefficients (ICC)>0.80) and minimal detectable change was only 9 out of 100 points. AKPS has also been translated into other languages which are Turkish, Chinese, Persian,[2] Dutch,[24] and Thai versions.[25] All translated versions appeared to be reliable and valid similar to the original English version.

Although, the causes of PFPS have still been under investigated,[8,10] in orthopaedic sports medicine, one of the most common reasons is overuse.[26] As PFPS is often related to overuse, recent changes in activities and changes in frequency, intensity, and duration of training should also be considered.[6] Training duration is an extrinsic risk factor for overuse injuries that is believed to also influence PFPS.[27] Taking any history about recent alterations in sporting activities, training program including any changes in the frequency, intensity and duration of training is very important in athletes presenting with PFPS.[6,28] However, there is no evidence demonstrating relationship between PFPS and this extrinsic risk factor, so evidence is required to prove this theory. Therefore, the primary aim of this study was to estimate the prevalence of PFPS in young Thai athletes. The secondary aim was to investigate the relationship between PFPS and training duration per week. It was hypothesised that the prevalence of PFPS in young Thai athletes would be more than 25% and PFPS and training duration per week would be related.

Materials and methods

Participants

This survey study was an observational descriptive research (cross-sectional) that recruited students in Phitsanulok Provincial Administrative Organization Sports School, Thailand. The total number of students enrolled in the school was 362 and ages ranged from 12 and 18 years. Every student in the school trained and engaged in one type of sport differently.

Procedure

The study was approved by the School of Sport & Exercise Sciences Research Ethics and Advisory Group (REAG), University of Kent at Medway (Ethics reference: Prop 53_2015_2016). The AKPS questionnaire was translated from English into Thai by the researcher. The backward translation was performed by a Thai native speaker who can read and understand English fluently. The original and translated English versions were compared. After necessary corrections were made, the final Thai version was obtained. The Thai version of the questionnaire was analysed by the intraclass correlation coefficient (ICC) 95% CI for test-retest reliability. The result showed that the translated version of the AKPS questionnaire presented very strong test-retest reliability of 0.97. The assent form for participants under 16 years, consent form for their guardians, consent form for participants at age 16 to 18, and participant information sheet were also translated into Thai. All participants and their guardians whose children were under 16 gave written informed consent prior to the participation. A total of 362 written informed consents were given. The informed consent forms were divided into 3 types: 1) one for participants under 16 (228 forms provided); 2) one for guardians of participants under 16 (228 forms provided); 3) one for participants

aged between 16 and 18 (134 forms provided).

The initial screening process was that participants completed the Thai version of AKPS questionnaire which was given to the participants at school. They were provided with 1 hour to complete the questionnaire. Participants who provided a score of 100 presenting that they had no pain and disability related to PFPS[23] did not need to go through further assessment. Participants who provided a score of less than 100 underwent further investigation which was a physical examination for PFPS. Details of their training schedule per week according to training duration, frequency, and types(29) were given by sports coaches at school from interviewing. The training consisted of general training and sports training. All participants performed the same general training which was speed, agility, power, and strength training 3 or 4 days a week depending on sports they participated. Sports training was different in each sport with the training including specific skills and game-based training 6 or 7 days a week.

Diagnosis of PFPS

Determining the best tests for PFPS diagnosis is still limited.[5,30,31] In the present study, PFPS was diagnosed following the criteria in the Table 1.

Statistical analysis

Age, weight, and height were expressed as mean±SD and analysed for differences between males and females by Independent T-test. The difference of the questionnaire score between males and females was analysed by independent T-test. The prevalence was calculated as the number of PFPS cases divided by the total number of participants that completed AKPS questionnaire in the study. All the data was normally distributed. The relationship between PFPS and training duration per week was analysed by Pearson correlation (Point biserial). The continuous variable (ratio data) was training hours per week and the dichotomous variable (nominal data) was PFPS.

Results

The total number of students enrolled in the school was 362. Three hundred and forty-one consent forms were returned 1 week after they were handed out. Fifteen out of 341 participants refused to participate in the study, so there was 326 participants willing to participate. However, another 16 participants did not present on the day that the AKPS questionnaires were distributed to the participants at school, so the response rate was 90% (310/341) (Figure 1). A total of 310 questionnaires were completed by 310 participants. The participants were given 1 hour to complete the questionnaires and all the questionnaires were returned to the researcher in the same day, so this gave a return rate of 100%. Baseline characteristics of the participants are shown in Table 2. There were 51 participants who reported a questionnaire score of less than 100. No participants who met any of the criteria for AKP (Table 1) scored the maximum of 100. When gender sub-groups were considered, 35 out of the 213 males (16%) and 16 out of the 97 females (16%) scored less than 100 on the questionnaire (Table 3).

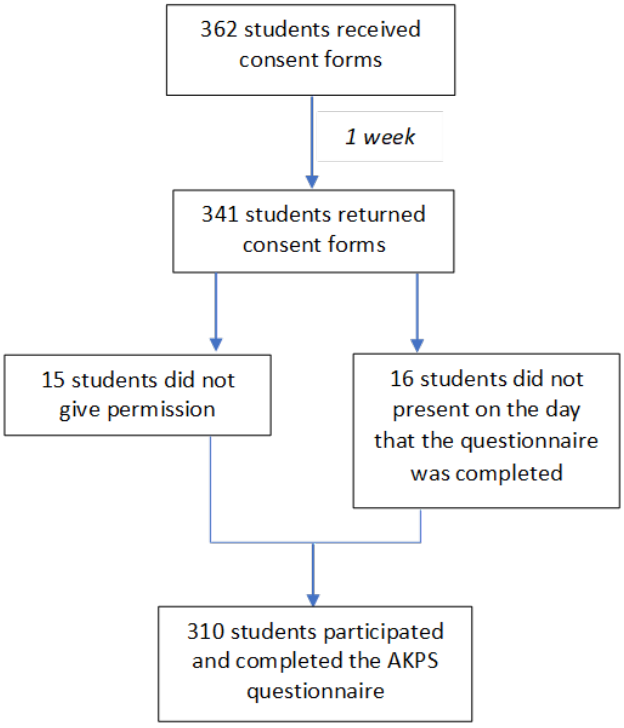

Figure 1 *Flow chart representing the number of participants participating in the study.*

Table 1 PFPS diagnosis criteria.

Criteria	Comment	Study
Pain behind or around the patella following prolonged sitting with knee flexed, rising from sitting, or pain during activities such as ascending or descending stairs, squatting, kneeling, or running	Common in PFPS	Dixit et al., 2007; Crossley et al., 2016; Cook et al., 2010
Tenderness on palpation of medial or lateral retinaculum	Common in PFPS	Dixit et al., 2007
Full range of motion of the knee joint	Common in PFPS	Dixit et al., 2007; Crossley et al., 2016

Table 1 PFPS diagnosis criteria. (continued)

Criteria	Comment	Study
No knee effusion	Sign of articular cartilage injury, chondromalacia patellae, loose bodies, patellofemoral osteo-arthritis	Dixit et al., 2007; Crossley et al., 2016
No locking of the knee joint	As locking suggests a meniscal tear or loose bodies in the joint.	Dixit et al., 2007
No localised pain at the inferior patellar pole	This suggests patellar tendi-nopathy.	Crossley et al., 2016
No localised tenderness and swelling around the tibial tuberosity	This suggests Osgood Schlatter disease.	Crossley et al., 2016
No morning stiffness, involvement of multiple joints or tendons, and joint swelling	This refers to systemic joint disease.	Crossley et al., 2016

Table 2 Baseline characteristics of the participants.

	Male (N = 213)		Female (N = 97)		*p* value	Total (N = 310)	
	Mean±SD	Max:Min	Mean±SD	Max:Min		Mean±SD	Max:Min
Age (year)	14.8±1.6	18:12	14.7±1.7	18:12	0.645	14.76±1.63	18:12
Weight (kg)	52.3±9.4	85:28	51.5±8.6	82:32	0.471	52.01±9.14	85:28
Height (cm)	165.9±9.0	185:136	160.7±6.9	178:145	<0.001*	164.31±8.74	185:136

Notes: *s: second, CI: confidence interval*

Table 3 Number and percentage of participants who had the questionnaire score of 100 and less than 100.

Score	Number of participants (N = 310)	Percentage	Male		Female	
			N = 213	%	N = 97	%
<100	51	16	35	16	16	16
100	259	84	178	84	81	84

Nineteen out of 51 participants (37%) who had a questionnaire score of less than 100 had signs and symptoms commensurate with a diagnosis of PFPS as shown in Table 4. There was no significant difference of average AKP scores between the positive and negative group (p=0.740). When gender sub-groups were considered, 12 out of 35 males (34%) and 7 out of 16 females (44%) had signs and symptoms commensurate with a diagnosis of PFPS indicating a higher prevalence of PFPS in females but a significant difference between these 2 proportions was not found (p=0.521). Overall, the prevalence of PFPS in young athletes registered at Phitsanulok Provincial Administrative Organization Sports School, Thailand who participated in this study was 6% (19 out of 310).

Table 4 Number and percentage of participants whose scores were less than 100 and were diagnosed with or without PFPS.

PFPS	Number of participants (N = 51)	Average AKP score	Percentage	Male		Female	
				N = 35	%	N = 16	%
Positive	19	81.47	37	12	34	7	44
Negative	32	82.38	63	23	66	9	56

Participants who performed the same sport had the same training duration and frequency. Football, athletics, and futsal had the same general training duration which was 6 hours per week while volley ball was 8 hours per week. Football, volleyball, and futsal had longer sports training duration which was 10.5 hours per week compared to athletics that was 9 hours per week. PFPS was weakly correlated with longer sports training duration (r=0.107, p=0.042) and sum of both training duration (r=0.122, p=0.031). Significant correlations were not found between PFPS and general training duration, age, weight, and height (Table 5).

Table 5 Correlations between PFPS and general training duration, sports training duration, age, weight, and height.

	General training duration (hours/week)	Sports training duration (hours/week)	Age (year)	Weight (kg)	Height (cm)
PFPS r	0.107	0.115	-0.070	-0.034	-0.065
***p* value**	0.061	0.042*	0.217	0.549	0.257

** Correlation is significant at 0.05*

When the gender sub-groups were analysed, PFPS was found to be weakly related to longer general training duration (r=0.174, *p*=0.011) and sports training duration in males (r=0.147, *p*=0.033) whilst age, weight, and height were not associated with PFPS. There were no significant correlations between any variables in females (Table 6).

Table 6 Correlations between PFPS and general training duration, sports training duration, age, weight, and height.

	General training duration (hours/week)		Sports training duration (hours/week)		Age (year)		Weight (kg)		Height (cm)	
	M	F	M	F	M	F	M	F	M	F
PFPS r	0.174	-0.001	0.147	0.068	-0.107	0.002	-0.016	-0.071	-0.082	0.000
***p* value**	0.011*	0.995	0.033*	0.506	0.118	0.983	0.822	0.489	0.232	0.998

** Correlation is significant at 0.05*

Discussion and Conclusion

The prevalence of PFPS in young athletes registered at Phitsanulok Provincial Administrative Organization Sports School, Thailand was found to be 6% (19/310) with a higher prevalence in females. The initial hypothesis that prevalence of PFPS would be higher than 25% was therefore rejected. The prevalence in the present study was found to be lower than reported in previous studies. Evidence from the Europe, USA, and Australia has reported levels of prevalence of PFPS of 25% for general or sporting populations.[1] Barber Foss et al found that AKP was present in 26.6% of adolescent female athletes screened over 3 years[23] while Roush and Curtis stated that the estimated prevalence of AKP in 18-35-year-old females was 12%.[5] Nejati et al also investigated prevalence of PFPS in Iranian female athletes and it was found that the prevalence was 16.74%.[4] Callaghan and Selfe stated that most PFPS prevalence studies recruited university athletes, competitive athletes, and male military.[1] A possible reason for the low prevalence of PFPS found in this present study may be that these young Thai athletes were still at the beginning level of sports training and competition compared to those in the previous studies, so their training schedule and intensity may not be as high as competitive athletes or from the military. However, children and adolescents are still at risk of lower extremity injuries. A previous study characterized sport injuries and verify related factors with injuries in children (aged up to 12 years) and adolescents (aged 12-18 years). Duration of training (years) and weekly hours of practice were observed and it was found that both duration of training and hours of practice were associated with injuries among adolescents as injured athletes presented with higher duration of training and weekly hours of practice.[32] Table 4 shows that 32 of 51 participants presented with the questionnaire score of less than 100 but were not diagnosed with PFPS. AKPS questionnaire was not only developed to respond to six activities associated with AKP but also symptoms such as inability to weight bear through the affected limb, swelling, abnormal patellar movement, muscle atrophy, and knee flexion limitation.[33] It is possible that these 32 participants may have presented with these symptoms.

The prevalence of PFPS in the present study was found to be higher in the female participants compared with the male participants but a significant difference was not found. However, the result of females presenting with higher PFPS prevalence supports the knowledge that PFPS occurs more frequently in females compared with males.[3-5,12,23,34] Boling et al examined the association between gender differences and prevalence and incidence of PFPS in 1525 participants from the United States Naval Academy (USNA).[34] After following for 2.5 years, they found the prevalence of PFPS in females and males was 15.3% and 12.3% respectively. The incidence rate in females was 33/1000 person-years whilst 15/1000 person-years was found in males. Similarly, Phillips and Coetsee investigated the incidence of AKP in 11-17-years-olds school males and females.[12] Their results showed that AKP was common among children between 11-17 years with a peak during 12-15 years in females. There are anatomical and biomechanical factors that may lead to a higher prevalence of PFPS in the females compared to the males.[34] One of those factors is the difference in quadriceps angle (Q-angle)[34] as females have larger Q-angles than males[35] and a greater Q-angle is a risk factor for PFPS.[36] Theoretically, a greater Q-angle increases the lateral pull of the quadriceps muscle and potentiates patellofemoral joint disorders.[35,37] Lower extremity muscle strength is believed to be another risk factor for PFPS.[38,39] Females have been reported to be significantly weaker

than males on measurements of hip abduction, hip extension, hip lateral rotation, and quadriceps strength.[38,39] This muscle weakness places the females at a higher risk of joint pain and injuries, including PFPS.[12] The current study only investigated point prevalence of PFPS and period prevalence and incidence rate were not investigated. For a chronic condition such as PFPS, the manifestation is often intermittent. As a result, point prevalence, based on a single assessment at one point of time, is likely to underestimate the prevalence of PFPS in the Thai athletes.[1,5] As period prevalence and incidence rates include a specific period of time,[1,5] reporting period prevalence or incidence rates helps to normalise for the time factor. Nevertheless, point prevalence is useful to find if a number of cases increase or decrease the next time that another point prevalence is investigated.[1,5]

The secondary aim of this study was to investigate the relationship between PFPS and training duration per week. The second hypothesis of PFPS being significantly related to training duration per week was only partially supported. There were no significant correlations between PFPS and general training duration, age, weight, and height for the overall group of participants. However, PFPS was found to be significantly related (r=0.107, *p*=0.061) to longer sports training duration which supported the second hypothesis. This finding supports the literature that PFPS is more common among physically active population.[1-5,12,40,41] In individuals with PFPS, training errors such as changes in frequency, intensity, and duration of training can contribute to PFPS.[6] Importantly, when gender sub-groups were considered, the male participants presented with significant correlations between PFPS and both general training duration and sports training duration whilst there were no significant correlations between PFPS and other variables. A possible reason that longer general and sports training duration were significantly associated with PFPS in the male group but were not in the female group may be a small sample size of the females as both genders had the same training duration (general training duration: 6-8 hours per week, sports training duration 9-10.5 hours per week). In the present study, there were 97 females competing AKPS questionnaire and only 7 females were diagnosed with PFPS. When summing up general and sports training duration, a significant relationship with PFPS was also found. The result supports the study of Vanderlei et al who found that duration of training and weekly hours of practice were associated with intrinsic and extrinsic risk factors for lower extremity injuries in adolescent aged 12-18 years.[32] However, all correlations found in this study were weak correlations. Age, weight, and height were not significantly correlated with PFPS in both genders. There were no significant differences of age (*p*=0.645) and weight (*p*=0.471) between male and female participants in the present study but a significant difference of height was found (*p*=0.000) (Table 2). However, this significant difference of height should have no effect on the result as Pappas and Wong-Tom (2012)[41] conducted a systematic review on risk factors and prospective predictors for PFPS and concluded that anthropometric variables including age, weight, and height were not associated with PFPS. However, as the PFPS cases diagnosed in the present study was only a small number

There were several limitations of the current study, firstly the data collection only took place at one school and this limits the generalisation of the findings to a general Thai population of this age group. Secondly, there was a few PFPS cases diagnosed in the present study. This may be the reason that correlations between PFPS and general and sports training duration were not found in females and may lead to type II error. Thirdly, the current study was a point prevalence study which a specific period of time was not included. Future studies of prevalence of PFPS in other groups of Thai population or in other schools with the same age range are required to clarify if the results will be similar to the present study. Statistical power calculation for the sample size is also required to reduce type II error. Period prevalence and incidence rates should be considered to normalise the time factor.

Conclusion

The overall prevalence of PFPS in young Thai athletes was found to be 6%, which is lower than previously reported levels of prevalence. Female participants presented with a higher overall prevalence of PFPS than their male counterparts, but no significant difference was found. PFPS was significantly related to longer sports training duration in the overall population. However, PFPS was only significantly related to longer general training duration and sports training duration in the males. Even though the correlations were found weak, the results of the current study may provide implications for coaches or sports teachers for planning the schedule of sports training duration.

Acknowledgements

This research was supported by School of Sport and Exercise Sciences, University of Kent, United Kingdom with cooperation from Phitsanulok Provincial Administrative Organization Sports School, Thailand for the recruitment of participants.

Conflict of interest

The authors report no conflicts of interest in this research.

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Journal of Associated Medical Sciences 2019; 52 (3): 158-162

JAMS
Journal of Associated Medical Sciences

Thai-Journal Citation Index Centre (TCI) & ASEAN Citation Index (ACI)

Journal of Associated Medical Sciences

Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index

Thermostable serine protease inhibitor from Death cap (*Amanita phalloides*)

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ARTICLE INFO

Article history:
Received 14 November 2018
Accepted as revised 7 May 2019
Available online 20 June 2019

Keywords:
Protease inhibitor, trypsin, *Amanita phalloid*, thermostable inhibitor

ABSTRACT

Background: Protease inhibitor plays an important role in many biological processes in an organism, its selective binding toward protease potentially tuning down some specific biological processes such as enzymatic catalysis regulation, protein signaling as well as protein clearance in order to accomplish homeostasis. Several protein-based protease inhibitors have been isolated and identified, the majority are directed toward serine protease.

Objectives: This study aimed to find a potential protease inhibitor from the local Northern Thailand Death cap (*Amanita phalloides*) together with biochemical characterization of its general properties.

Materials and methods: Death cap extract collected from Phayao Province, Thailand was initially performed trypsin inhibitory activity assay using BSA and alzoalbumin as substrates. Detection of its inhibition activity was assessed by SDS-PAGE and spectrophotometry. Additionally, molecular size was observed by filtering the extract through 3 kDa molecular cut off membrane. Finally, hydrophobic property was verified by passing the filtrate through phenyl sepharose column chromatography.

Results: Death cap contained serine protease inhibitory activity. Molecular size of inhibitor was suspected to be less than 3 kDa. Hydrophobic property of this inhibitor was observed. Interestingly, its inhibitory property retained after heat inactivation at 100 °C for 10 min.

Conclusion: A novel heat-tolerant inhibitor from the water extract of Death cap was characterized to be a small peptide with hydrophobic property, which could be used as a new peptide protease inhibitor targeting to serine protease that benefit for agricultural and medical field.

Introduction

Protease inhibitors are substances that interact with target proteases and abolish protease activities. The inhibitors with native origin are ultimately significant for regulating proteolysis activities in living cells, which are vitally essential.[1,2] In addition to the functions in living cells, these inhibitors have been applied for a variety of applications; in medical field, agriculture and biotechnology.[3,4] Large numbers of protease inhibitors were discovered in a wide range of living organisms including animals, plants and microorganisms.[5] Protease inhibitors can be classified into several classes based on targeted amino acid on their binding pockets such as cysteine protease inhibitors, serine protease inhibitors.[1] Prominently, serine protease inhibitors have shown to be an interesting group of the inhibitors. Their importance role in many biological processes such as regulation of proteolysis reaction in blood coagulation and implication in tumor suppression were established.[6,7] However, only a few serine protease inhibitor have been found in mushrooms.[8]

Death cap (*Amanita phalloides*) is a well-known mushroom which contains toxic substances capable of conferring death to consumers and found extensively in

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doi: 10.14456/jams.2019.24
E-ISSN: 2539-6056

both Thailand and other parts of the world. It is classified as ectomycorhiza fungi whose growth symbiotically associated to numerous kinds of trees in the deciduous dipterocarp and pine-dipterocarp forest such as pine, chestnut and deciduous wood tree. Normally appearance of its growth is observed around the root of trees when suitable humidity and temperature exists especially in the raining season. Death cap is morphologically considered the condense form of fruiting body with the distinctive colors such as pale green, yellow or light brown. In Thailand, known as "hed Ra-ngok-hin", it constitutes white gills and stem part without hole compared to "an edible mushroom". [9,10]

This mushroom has been reported to cause death in victims following liver failure. Most research of this mushroom has targeted on RNA polymerase inhibitors which block protein synthesis especially in the liver.[11] However, there are several substances in the mushroom not properly characterization.

The present study aimed to find a new source of serine protease inhibitor from Death cap together with the characterization of this new protease inhibitor such as the hydrophobicity by the phenyl sepharose column chromatography and the molecular mass by molecular cut off.

Materials and methods

Materials

Fruiting bodies of Death cap were collected from Rongkamlaung Forest Park in Phayao Province, Thailand. Centricon was from Merck, Germany. Phenyl-SepharoseCL-4B was from GE Healthcare, Sweden. Trypsin, Azoalbumin, Bovine serum albumin (BSA) and Phenylmethylsulfonyl fluoride (PMSF) purchased from Sigma, USA. All other chemicals were analytical grade.

Mushroom extraction

A 30 gm of the fresh fruiting body of mushroom was grounded with 90 ml of distilled water at room temperature. The homogenate was centrifuged at 10,000xg for 10 min at room temperature. Supernatant was determined for protein content by Lowry method using BSA as standard protein[12] and collected as a crude water extract.

Determination of protease inhibitor activity using SDS-PAGE

A reaction mixture of 30 µL contained 12 µL of 1 mg/mL of BSA solution, 6 µL of distilled water, 10 µL of crude water extract or PMSF as positive control and 2 µL of 1 mg/mL of trypsin (Sigma) solution. Reaction was started by adding the trypsin solution and incubated 37 ºC for 30 min. It was stopped by adding 6 µL of 5x SDS buffer, immediately heated at 100 ºC for 5 min and then centrifuged at 10,000 xg for 5 min. Supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). [13]

Protease inhibitor assay by spectrophotometric method

Method was modified from Tomarelli [14] as follows, 1000 µL reaction composed of 858 µL of "0.5% (w/v) sodium bicarbonate, pH7.4", 100 µL of 1.25% (w/v) Azoalbumin, 40 µL of distilled water or testing sample (mushroom extract etc.) or PMSF as positive control and 2 µL of 1 mg/mL of trypsin solution. Reaction was started by adding the trypsin solution and incubate 37 ºC for 20 min. Then 300 µL of the reaction mixture was taken, immediately mixed with 600 µl of 5% TCA and 450 µL of 0.1M sodium hydroxide was then added. After mixing, the solution mixture was centrifuged at 10,000 xg for 5 min. The supernatant was measured for absorption at 440 nm. To determine the effect of heat, the mushroom extract was incubated for 10 min at 0, 30, 60, or 100 °C before adding to the reaction mixture.

Separation by molecular filtration

Mushroom extract (96 mg protein) was loaded into 3-kDa-cutoff Ultracel-3K molecular filtration device and centrifuged at 10000 xg at room temperature until the retentate decreased to minimal volume. The filtrate was collected. The retentate was adjusted to original volume using 50mM Tris-HCl buffer, pH 8.3. Both the filtrate and the retentate were determined for protein contents and tested for protease inhibitor activity using the spectrophotometric method.

Phenyl Sepharose column chromatography

Phenyl Sepharose CL-4B was washed with distilled water to avoid the preservative agent and broken beads. Sediment bead was used to load on 12x1 mL glass column which washed and equilibrated with 10 volume of 50 mM potassium phosphate buffer, pH 6.5 containing 1M ammonium sulfate. The filtrate (26 mg protein) obtained from molecular filtration was pre-equilibrated with 50 mM potassium phosphate buffer, pH 6.5 containing 1M ammonium sulfate with 1:1 (v/v)ratio before loaded on to the column and then left until sample absorbed into a gel completely. Unbound fraction was collected by washing with 50 mM potassium phosphate buffer, pH 6.5 containing 1M ammonium sulfate 80 mL at 1 mL/min flow rate and Fractions of 2 mL were collected until no absorbance at 280nm was observed. Bound substances were eluted by a stepwise method using 50 mM potassium phosphate buffer, pH 6.5 containing 0.5M ammonium sulfate 60 mL to elute weaker binding peptide and then by 50 mM potassium phosphate buffer, pH 6.5 pH 60 mL to elute stronger binding peptide, the flow through was monitored at 1 mL/min. Fractions of 2 mL were collected until no absorbance at 280 nm was observed. Each peak fraction was pooled, determined for protein content, and for protease inhibiting activity using the spectrophotometric method.

Results

Protease inhibition activity of the crude water extract from Death cap fruiting body

Death cap fruiting body 30 gm was initially extracted with water and obtained 96 mg protein crude water extract which was then assessed for the protease inhibition activity using SDS-PAGE (Figure 1), BSA band at 60 kD was disappeared by trypsin (Lane 5) while the positive control; Phenylmethylsulfonyl fluoride (PMSF) and different concentrations of the crude water extract decreased the activity of the enzyme resulting in the visualized BSA band (Lane 6-8).

Thermo stability of the protease inhibitor

The crude water extract was incubated separately at 0, 30, 60 or 100 °C for 10 min before performing protease inhibition assay by the spectrophotometric method. The result showed that the protease inhibitor in the crude water extract is thermo stable as observed by the retaining protease inhibition activity (20% inhibition) after incubation at 100 °C for 10 min. (Figure 2).

Partial purification of the protease inhibitor

Separation by molecular filtration, the filtrate containing 26 mg protein (27% yield) was obtained from 96 mg protein of the crude water extract. After passing through hydrophobic phenyl sepharose column, an unbound peak (UB) and two bound peaks (B1, B2) were obtained (Figure 3). B1 peak collected from fractions 16-18 contained 5.65 mg protein (5.8% yield) while B2 peak collected from fractions 27-29 had 3.5 mg protein (3.6% yield). All collected peaks were tested for their inhibitions to trypsin. Peak B1 showed the strongest activity with 30% inhibition (Figure 4).

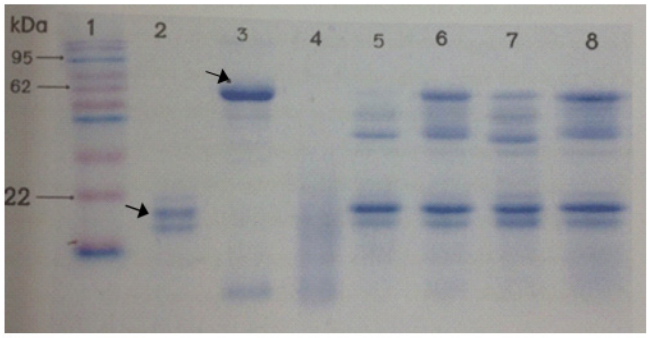

Figure 1. *Detection of protease inhibition activity using SDS-PAGE. Inhibition of trypsin activity was demonstrated on SDS-PAGE (12% polyacrylamide) using BSA as substrate of the enzyme. Protein bands were visualized by Coomassie Brilliant Blue staining. Lane 1: Standard protein maker, Lane 2: trypsin, Lane 3: BSA, Lane 4: Crude water extract (64 ug), Lane 5: BSA plus trypsin, Lane 6: BSA plus trypsin plus 1mM PMSF, Lane 7: BSA plus trypsin plus crude water extract (32 µg), Lane 8: BSA plus trypsin plus crude water extract (64 µg). Position of BSA and trypsin band are indicated by arrow head.*

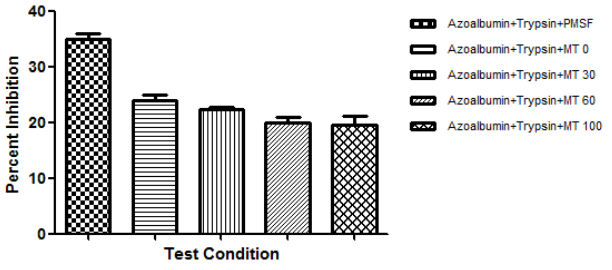

Figure 2. *Thermostability of the protease inhibition factor. Mushroom extract was divided into four parts and then incubated at 0, 30, 60 or 100 °C for 10 min (MT0, MT30, MT60 and MT100, respectively) before using for the protease inhibition assay.*

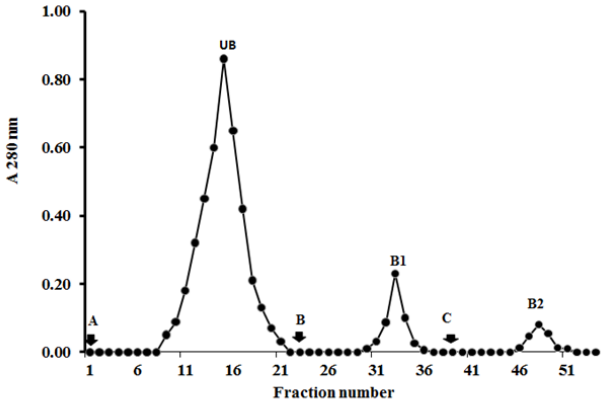

Figure 3. *Phenyl-sepharose column chromatogram of molecular filtrate fraction. Filtrate obtained from molecular filtration was subjected to phenyl sepharose column chromatography following the collection of 2 mL per fraction. Protein concentration of each fraction was determined by monitoring the absorbance at 280 nm. A, B and C indicate the addition of each buffer solution. Different buffers were performed at different stage of elution steps as A: 50 mM potassium phosphate buffer, pH 6.5 containing 1M ammonium sulfate, B: 50 mM potassium phosphate buffer, pH 6.5 containing 0.5M ammonium sulfate and C: 50mM potassium phosphate buffer, pH 6.5. UB means the peak collected from unbound fractions. B_1 and B_2 mean the peaks collected from bound fractions eluted by the buffer solution as indicated in the picture.*

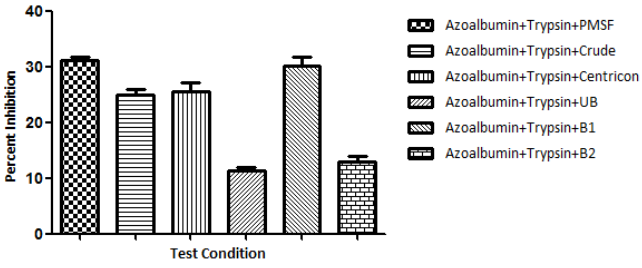

Figure 4. *Inhibition activity of the the purified fractions. Crude water extract, filtrate obtain from molecular filtration (Centricon), unbound fraction and bound fractions were tested for their inhibition of trypsin activity.*

Discussion and Conclusion

Death cap causes the death of people who unintentionally ingest this mushroom which contains toxic substances known as amatoxins and phyllotoxins. Among these toxic substances, the most dangerous substances are amanitins that cause liver failure by inhibition of RNA polymerase II.[15] This mushroom also contains other toxic substances including toxophallin, phallolysin.[16] We are interested to find new protease inhibitor from the Death cap as it contains a varieties of toxic substances.[17] In addition, protease inhibitors have been characterized from different mushrooms.[2,18,19] In this study, the preliminary characterization was assessed by conducting reactions comprising trypsin as serine protease, BSA as protease substrates and PMSF as the positive control for serine protease in accompany with mushroom extracts to observe the potential serine protease inhibitor. The results of SDS-PAGE analysis show that the mushroom crude water extract significantly inhibited

trypsin activity in the concentration dependent manner (Figure 1). Additionally, the spectrophotometric method observing the result of trypsin catalyzed azoalbumin together with its thermostable property confirmed the existence of the inhibitor (Figure 2). In previous report, trypsin inhibitor from *Pleurotus floridanus* was reported to be thermostable up to 80 °C.[19] This study showed the presence of protease inhibitor in Death cap with exceptionally high stability toward heat inactivation (Figure2). Thermal stability of this protease inhibitor is plausibly applied to various applications in the scientific area and industries. This inhibitor is believed to be a small molecule as its passing through 3 kD molecular weight cut off membrane. Stronger inhibition activity was obtained after the partial purification using phenyl sepharose column chromatography. The result also indicates some hydrophobic area on the surface of the inhibitor molecule since it can bind to the column (Figure 3 and Figure 4). Further study will be conducted to find specificity, the molecular structure as well as other properties of this protease inhibitor. This new finding might be applied as the reported protease inhibitor that worked against diseases such as malaria, colds, flu, dengue, cancer, hypertension, Alzheimer as well as AID [4]

Acknowledgements

Authors acknowledges the financial support from the faculty of Medical Sciences, Phayao University, and also deeply indebted to Prof. Dr. Nison Sattayasai for materials supplied, valuable suggestion,and paper proof reading work.

Conflict of interest

The authors declare no conflict of interest.

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Journal of Associated Medical Sciences 2019; 52 (3): 163-169

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Comparison of renal biomarkers in obesity with early renal dysfunction

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ARTICLE INFO

Article history:
Received 22 March 2019
Accepted as revised 24 June 2019
Available online 19 July 2019

Keywords:
Obesity, early renal dysfunction, cystatin C, NGAL, KIM-1

ABSTRACT

Background: Obesity is an important risk factor of chronic kidney disease. Screening and diagnosis of the disease at the early stage are beneficial for risk control and therapeutic management.

Objectives: To assess the relationship among renal biomarkers levels and obesity parameters in obese people with early renal dysfunction.

Materials and methods: Subjects were 180 volunteers, aged 35-60 years, who participated in a health survey at the time of periodic checkup. They were divided into 3 groups using body mass index (BMI) and into 2 groups based on estimated glomerular filtration rate (eGFR); normal kidney function (NKF) and early renal disease (ERD). Serum cystatin C, urinary kidney injury molecule (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) were measured.

Results: The results show that eGFR values of NKF and ERD group were 105.02±8.34 and 79.90±10.76 mL/min/1.73m^2, respectively. The ERD group with decreased eGFR, had significantly increased serum creatinine levels (p<0.001), but decreased KIM-1 in the obesity group. Serum cystatin C significantly increased in ERD (p<0.001) and was negatively associated with obesity parameters but negatively correlated with eGFR. Urinary KIM-1 trended to decrease in ERD, but not significant (p>0.05) compared to normal kidney function group. In addition, urinary NGAL was positively correlated with eGFR. Multivariable adjusted OR (95% confidence interval) for ERD in obese group was 3.8 (1.2-11.9) for cystatin C (p for trend=0.024). This association was more announced in older subjects (≥45 years).

Conclusion: Cystatin C could be used for monitoring normal kidney function as well as early renal injury in obese population. This could be implemented in screening, risk regulation and management of kidney diseases at the early stage.

Introduction

Overweight and obesity are considered a serious health problem worldwide. In Thailand, the prevalence of obesity increased by 10-25% during past 10 years.[1] Obesity can be assessed by using the body mass index (BMI), waist circumference (WC) and waist to hip ratio (WHR). Previous studies have shown that obesity is high risk factor for chronic kidney disease. Obesity results in hypertension and type 2 diabetes, which is the leading cause of kidney failure and end-stage kidney disease.[2] Data from The Framingham Offspring Cohort and the Hypertension Detection and Follow-Up Program indicate that increased BMI is associated with progression to chronic kidney disease (CKD).[3] Obesity has been reported to cause kidney failure even in people with no history of diabetes and hypertension.[4]

In obese people, high level of free fatty acids (FFAs)

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doi: 10.14456/jams.2019.25
E-ISSN: 2539-6056

are found, which causes endothelial dysfunction.[5] This leads to an increase in vascular permeability and albumin leakage from the glomerular endothelial cells into the urine.[6] There is evidence that higher FFAs correlate with microalbuminuria in obese people.[7] In addition, BMI was found to correlate with the glomerular filtration rate (GFR) and could predict early renal dysfunction (ERD) in obese individuals.[8]

Glomerular Filtration Rate (GFR) is regarded as the more precise for determining kidney function; however, it is labor-intensive and very expensive. The most commonly used method is serum creatinine for estimated GFR (eGFR). However, there are many factors affecting creatinine level such as muscle mass, protein intake, gender and age. Therefore, sensitive and early biomarkers are needed to establish for detection early renal abnormalities such as cystatin C, Kidney Injury Molecule-1 (KIM-1), and Neutrophil Gelatinase-Associated Lipocalin (NGAL).

Cystatin C is a low molecular weight protein produced in the constant level from all types of nucleated cells. It is freely filtered by the glomerular membrane and reabsorbed almost entirely in the proximal tubules.[9] The cohort studies in type 1 and type 2 diabetes and diabetic nephropathy, it was found that a higher stage by eGFRcyst than eGFRcreat had a significantly higher risk of end-stage renal disease (ESRD) than those with concordant staging in all three cohorts.[10]

Neutrophil gelatinase-associated lipocalin (NGAL) or lipocalin2 that is expressed at low levels in several human tissues. It is rapidly released from renal tubular cells in response to various insults of the kidney.[11] The studies in patients with CKD due to glomerulonephritis shows that the average concentration of NGAL in urine of patients with chronic renal failure is higher than in the control group. In addition, it was found that the concentration of NGAL in urine is related to the concentration of creatinine in serum.[12]

Another very well characterized urinary biomarker is kidney injury molecule (KIM-1). It is a transmembrane type 1 glycoprotein expressed at very low levels in the normal kidney. It is markedly upregulated in proximal tubules following renal ischemic or toxic injury.[13] The results of experimental and clinical studies show that increased urinary KIM-1 levels are related to KIM-1 expression, indicating that KIM-1 is a biomarker for tubular interstitial pathology.[14]

Most of these renal biomarkers were studied in diabetic nephropathy but there is no information in obese people with ERD. Considering these important gaps, the aim of the present study was to assess the relationship of Cystatin C, NGAL and KIM-1 levels among obese people with early renal dysfunction (ERD) and those with normal kidney function (NKF). In addition, obesity parameters (BMI, WC, and WHR) were also determined the correlation with renal biomarkers (serum creatinine, cystatin C, eGFR (CKD-EPI), urine albumin/creatinine ratio (UACR), NGAL and KIM-1) in order to identify ERD in people with obesity

Materials and methods

Study population

This study protocol was approved by the Human Ethics Committee of Thammasat University, Thailand (COA No.019/2559). The participants were selected from volunteers in Thailand between March 2018 and September 2018. All participants fulfilled the following inclusion criteria: between 35 and 60 years of age and do not use of drugs that affect the function of the liver and kidneys such as acetaminophen, ascorbic acid, cefazolin, chlorothiazide, cimitidine, dexamethasone, nitrofurantoin, ibuprofen, and furosemide. Participants who had hypertension, diabetes, liver and kidney disease were excluded from the current analyses. We included 180 participants and divided into 6 subgroups according to body mass index (BMI) and kidney function. The participants were classified as: (1) normal weight with normal kidney function (NKF) (2) normal weight with ERD (3) overweight with NKF (4) overweight with ERD (5) obesity with NKF, and (6) obesity with ERD. BMI was calculated as weight in kilograms divided by the square of height in meters (normal weight; 18-22.9 kg/m^2, overweight; 23.0-24.9 kg/m^2, obesity; 25 kg/m^2) and ERD subgroups were classified as eGFR; 30-89 or urine albumin to creatinine ratio (UACR); 30-300 mg/gm.

Anthropometric and biochemical measurement

Anthropometric measurements were performed, including weight (Wt), height (Ht) and waist (WC) and hip (HC) circumferences. Wt was measured to the nearest 0.1 kg using a digital scale, and Ht was measured to the nearest centimeter in the standing position. WC was measured midway between the bottom of the rib cage and iliac crest, and HC was measured as the maximum circumference of the buttocks. The waist-to-hip ratio (WHR) was calculated as the WC divided by the HC.

Systolic and diastolic blood pressure (BP) were measured with an automated sphygmomanometer (BP103iII, Omron Health Care Co. Ltd., Kyoto, Japan). Venous blood was drawn into a vacuum tube and centrifuged to separate the serum. Creatinine was measured by enzymatic method using a Beckman Coulter UniCel DXC 800 Synchron automated chemistry analyzer (Beckman Coulter, Inc., Atlanta, USA). This assay maintains a range from 0.2 to 25.0 mg/dL and intra-assay and interassay coefficients of variation of 0.6% to 1.5% and 1.8% to 3.0%, respectively. Albumin was measured by automated analyzer using Bromocresol green (BCG) method with a range from 1.5 to 6.0 gm/dL and intra-assay and interassay coefficients of variation less than 3.0%. Microalbumin was measured by turbidimetry immunoassay with linearity from 0.5-30 mg/dL. Serum cystatin C level was analyzed by immunoassay using an ARCHITECT c8000 automated (Abbott, Abbott Park, Illinois, USA). eGFR were calculated by using the CKD-EPI formula, the most widely-used equations for estimating GFR in adults. The CKD-EPI equation, expressed as a single equation, is: $GFR = 141 * \min(Scr/\kappa,1)^{\alpha} * \max(Scr/\kappa, 1)^{-1.209} * 0.993^{Age} * 1.018$ [if female] * 1.159 [if black]

(Scr is serum creatinine (mg/dL), κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males,

min indicates the minimum of Scr/κ or 1, and max indicates the maximum of Scr/κ or 1.)[15]

Renal biomarkers measurements

Renal biomarkers including urinary KIM-1 and NGAL were measured by ELISA assays using a Human TIM/KIM-1 ELISA kit (Cat.No. DKM 100, R&D systems, Minneapolis, MN, USA). NGAL was measured with a Human NGAL ELISA kit (Cat.No. DLCN 20, R&D systems, Minneapolis, MN, USA) according to the manufacture's protocols. All biomarkers concentration was performed in duplicate and adjusted for urinary creatinine concentration.

Statistical analysis

All analyses and calculations were performed using SPSS statistical ver.18.0 (IBM Co., Armonk, New York, USA). Data are expressed as numbers and percentages. Continuous variables were expressed as mean±standard deviation (SD). The Kruskal-Wallis test was used to compare continuous variables between groups. Differences variables among the six groups were determined using a generalized linear model (Duncan's test of multiple comparisons). Multivariable-adjusted logistic regression analysis was conducted to determine odds ratios (ORs) and 95% confidence interval (CIs) for the evaluation risk of ERD. The *p* values of <0.05 were considered statistically significant.

Results

Characteristic of participants

Characteristics of study participants according to renal function are shown in Table 1. Participants were assigned to two groups according to the renal function. Ninety people were assigned to normal kidney function group (NKF; eGFR >90 or UACR <30 mg/gm) and another ninety people were designated to early renal dysfunction (ERD; eGFR 30-89 or UACR 30-300 mg/gm). ERD group had the statistically significant higher mean age, systolic BP, and diastolic BP, creatinine. On the other hand, ERD group showed the statistically significant lower eGFR level than the NKF group (p<0.001). Other characteristics including BMI, WC, WHR, and UACR were not different among the two groups.

Table 1 Baseline characteristics of study population in NKF and ERD groups.

Variables	NKF Group (n=90)	ERD Group (n=90)	*p* value
Male/Female (%)	15.6/84.4	26.7/73.3	0.069
Age (years)	43.52±5.93	49.99±6.51	<0.001*
BMI (kg/m^2)	24.70±3.88	24.36±3.19	0.971
Systolic BP (mmHg)	116.28±12.46	124.49±13.61	<0.001*
Diastolic BP (mmHg)	75.89±9.34	81.32±11.40	<0.001*
WC (cm)	82.56±10.14	84.37±9.00	0.162
WHR	0.83±0.08	0.86±0.06	0.046*
Creatinine (mg/dL)	0.71±0.11	0.93±0.17	<0.001*
eGFR (mL/min/1.73m^2)	105.02±8.34	79.90±10.76	<0.001*
UACR (mg/gm)	6.72±4.28	13.97±32.96	0.209
KIM-1 (µg/gCr)	0.81±0.51	0.57±0.53	0.003*
NGAL (µg/gCr)	16.95±22.08	15.08±18.12	0.121
Cystatin C (mg/L)	0.82±0.11	0.93±0.13	<0.001*
eGFRcys (mL/min/1.73m^2)	100.69±12.87	85.88±13.98	<0.001*

Note: *NKF: Normal kidney function, ERD: Early renal dysfunction, Data are presented as mean±standard deviation. Asterisks indicate significant differences between groups using student t-test at p value <0.05.*

Level of renal biomarkers and factors associated with obesity parameter and renal function

Comparison of study parameters in NKF and ERD groups showed in Table 2. Participants were categorized to six subgroups according to the renal function and obesity parameters. The mean difference in diastolic BP and WC were statistically significant among the six groups using Kruskal-Wallis test (p=0.003 and 0.001). Renal biomarkers; KIM-1, NGAL and cystatin C were compared among the six groups. In NKF groups, it was found that the BMI was different among the study population. Systolic BP (p=0.001), diastolic BP (p=0.005), WC (p<0.001), WHR (p=0.029), KIM-1 (p=0.011), cystatin C (p=0.013) and eGFRcys (p=0.021) were statistical significantly different. The post-hoc comparison showed the BMI was significantly different in all three groups (p<0.001). Systolic BP and diastolic BP in obesity were higher than in the overweight group and normal weight group respectively. It indicated that obesity was a significant factor contributed to elevate of systolic and diastolic BP. WC was significantly different in all three groups (p<0.001). WC of obesity group was higher than the overweight and normal weight group, respectively. WHR of obesity group was increased significantly from the normal weight group (p=0.011), but there was no difference between the normal weight and the overweight group. Renal biomarkers, including KIM-1, cystatin C and eGFRcys are different in normal weight compared to obesity (p=0.002, 0.004 and 0.005 respectively). However, the values did not differ between normal weight and overweight group. The result demonstrated that obese people with normal kidney function had KIM-1, cystatin C and eGFRcys levels higher than those with normal weight. For ERD group, it was found that the BMI was different among the study population. Diastolic BP, WC, cystatin C and eGFRcys were statistically significant different. So, this showed that BMI in ERD group was determined according to the criteria. The obesity group has a higher significantly diastolic BP than the overweight group (p=0.008) and in the normal weight group (p=0.002). The WC in normal weight was lower significantly than the overweight (p<0.001) and obesity (p<0.001). But there was no difference of WC between the overweight and obesity groups. The mean differences in cystatin C were statistically significant; 1.01±0.16 mg/L in obesity, 0.88±0.11 mg/L in overweight, and 0.91±0.07 mg/L in normal weight with ERD. The eGFRcys in obesity was lower significantly than overweight (p<0.001) and normal weight (p=0.001). The value in other renal biomarker parameters were also evaluated but did not show significantly difference according to group.

Table 2 Comparative study in NKF and ERD groups.

Variables	NKF Groups (n=90)				ERD Groups (n=90)			
	Normal weight (n=30)	Overweight (n=30)	Obesity (n=30)	*p* value	Normal weight (n=30)	Overweight (n=30)	Obesity (n=30)	*p* value
Age (years)	42±6.38	45.07±6.30	43.50±4.73	0.053	49.51±6.81	49.43±7.07	51.03±5.64	0.673
BMI (kg/m^2)	21.21±1.05	23.93±0.57	28.96±3.57	<0.001†	21.01±1.34	24.11±0.56	27.95±2.01	<0.001†
Systolic BP (mmHg)	111.47±14.60	114.87±10.33	122.50±9.53	0.001§¶	120.57±15.01	124.10±11.56	128.80±13.21	0.094
Diastolic BP (mmHg)	74.07±10.45	74.03±8.55	79.57±8.01	0.005§¶	79.20±9.08	77.70±9.31	87.07±13.33	0.003§¶
WC (cm)	73.93±7.08	82.33±5.51	91.40±8.79	<0.001†	77.28±7.49	85.90±6.55	89.93±7.95	<0.001‡§
WHR	0.80±0.08	0.85±0.08	0.85±0.06	0.029§	0.84±0.08	0.87±0.05	0.87±0.06	0.077
Creatinine (mg/dL)	0.70±0.08	0.69±0.10	0.73±0.15	0.477	0.89±0.11	0.91±0.17	0.98±0.22	0.292
eGFR (mL/min/1.73m^2)	106.00±8.59	104.93±7.57	104.13±8.97	0.604	81.37±5.77	82.33±10.33	76.00±13.82	0.060
UACR (mg/gm)	6.12±2.83	6.19±3.06	7.83±6.07	0.816	0.53±0.48	13.65±25.15	20.83±50.79	0.099
KIM-1 (µg/gCr)	0.61±0.40	0.74±0.49	0.95±0.53	0.011§	0.43±0.36	0.56±0.53	0.69±0.62	0.413
NGAL (µg/gCr)	20.08±34.87	17.43±12.88	13.35±9.49	0.436	18.44±17.29	12.58±10.21	14.22±24.19	0.153
Cystatin C (mg/L)	0.77±0.11	0.82±0.10	0.85±0.10	0.013§	0.91±0.07	0.88±0.11	1.01±0.16	<0.001§¶
eGFRcys (mL/min/1.73m^2)	105.63±12.80	99.57±12.65	96.87±11.94	0.021§	87.33±9.98	92.30±12.85	78.00±15.04	<0.001§¶

Note: *NKF: Normal kidney function, ERD: Early renal dysfunction, Data are presented as mean±standard deviation, †The difference in Normal weight, overweight and obesity, ‡The difference is between normal weight and overweight. §The difference is between normal weight and obesity, ¶The difference is between overweight and obesity.*

Association between renal biomarkers and obesity parameters

Table 3 displays the correlations of obesity parameters and eGFR with renal biomarkers. It was found that BMI were positively correlated with KIM-1 and Cystatin C (r=0.227 and 0.211 respectively). Cystatin C concentration showed a positive correlation with obesity parameters (BMI, WC, WHR). A weak negative correlation between NGAL concentrations, WC (r=-0.237), and WHR (r=-0.253) were observed in this study.

Multiple logistic regression analysis was also used to investigate the independent factors for predicting ERD adjusting for BMI, WC, and other factors related to ERD in obesity, such as age; systolic BP; diastolic BP; cystatin C; and eGFRcys (Table 4). As shown in Table 4, the probability of ERD in obesity were higher in the study population with older and increased cystatin C level. The OR and 95%CI for risk of ERD in obesity significantly increased with increasing age (OR=6.5; 95%CI=2.0-21.0; *p* for trend=0.002) and increasing cystatin C greater than 0.875 mg/L (OR=3.8; 95%CI=1.2-11.9; P for trend=0.024). There were no interactions between blood pressure, eGFRcys, and the other independent factors.

Table 3 Correlations of obesity parameter and eGFR with renal biomarkers.

Characteristics	BMI		WC		WHR		eGFR	
	r	*p* value	r	*p* value	r	*p*-value	r	*p* value
Creatinine (mg/dl)	0.036	0.633	0.279	<0.001*	0.346	<0.001*	-0.856	<0.001*
eGFRCre(mL/min/1.73m^2)	-0.039	0.607	-0.191	0.010*	-0.216	0.004*	-	-
UACR (mg/gm)	0.065	0.384	-0.040	0.597	-0.098	0.192	-0.045	0.552
KIM-1 (µg/gCr)	0.227	0.004*	0.142	0.072	-0.101	0.200	0.222	0.004*
NGAL (µg/gCr)	-0.098	0.191	-0.237	0.001*	-0.253	0.001*	0.173	0.020*
Cystatin C (mg/L)	0.211	0.004*	0.317	<0.001*	0.223	0.003*	-0.612	<0.001*
eGFRcys (mL/min/1.73m^2)	-0.200	0.007	-0.270	<0.001*	-0.167	0.025	0.650	<0.001*

r: Correlation Coefficient

Table 4 Adjusted odds ratio from multiple logistic regression analysis for evaluating risk of ERD.

Variables	B	S.E.	Wald	Adjusted odds ratio (95%CI)	*p* value
Age					
35-45 years				1	
46-55 years	1.126	0.386	8.491	3.1 (1.4-6.5)	0.004*
>55 years	1.881	0.594	10.016	6.5 (2.0-21.0)	0.002*
Systolic BP					
<130 mmHg				1	
≥130 mmHg	0.156	0.516	0.091	1.2 (0.4-3.2)	0.763
Diastolic BP					
<85 mmHg				1	
≥85 mmHg	0.443	0.489	0.821	1.6 (0.6-4.0)	0.365
Cystatin C					
<0.875 mg/L				1	
>0.875 mg/L	1.327	0.586	5.131	3.8 (1.2-11.9)	0.024*
eGFRcys					
<90 mL/min/1.73m^2				1	
<90 mL/min/1.73m^2	0.399	0.604	0.437	1.5 (0.5-4.8)	0.509

Chi-square (Omnibus Tests of Model Coefficients) = 60.96, df = 6, Sig. = 0.00 -2 Log likelihood = 188.57, Cox&Snell R2 = 0.287, Nagelkerke R2 = 0.383

Discussion and Conclusion

The present study constructed a model investigating the risk of ERD in obese people from three renal biomarkers; KIM-1, NGAL, and cystatin C. Due to the cystatin C is increasingly recognized as a more sensitive and therefore more useful marker for predicting ERD risk in obesity population. Recently, obesity and high blood pressure has been proposed as an independent risk factor for the leading causes of CKD and ESRD.[16, 17] Being obese or overweight leads to elevations in serum cystatin C levels.[18] Based on pathophysiology, the effects of weight elevation might be due to decrease in hyperfiltration, high filtration pressure, inflammation, and oxidative stress, as well as increase in nitric oxide and proteinuria.[19]

Microalbuminuria and eGFR currently used for CKD and ESRD has significant limitations. Microalbuminuria is suggested to be a sign of early glomerular damage. The occurrence of microalbuminuria in type 1 diabetes can already be associated with diabetic nephropathy lesions. On the other hand, decrease of GFR may not be an adequate indicator for early diagnosis. In addition, many studies have explored the relationship between renal biomarkers and renal failure. Creatinine, eGFR, and cystatin C are known to lead to an increase in the incidence of end-stage renal disease (ESRD).[20, 21]

Our study revealed that ERD subjects had lower urinary KIM-1 and NGAL values compared to NKF subjects, but that there was no difference level in urinary NGAL. In addition, we found that urinary cystatin C is significantly elevated in ERD subjects without increased urinary albumin-creatinine ratio when compared to NFK. In another study, it was also demonstrated that ERD subject had increased cystatin C and highly correlated with eGFR when compared to control subjects.[22, 23] Recent evidence supports that elevated waist circumference (WC) and body mass index (BMI) are among the most important factors leading to obesity. In addition, obesity is associated with hypertension and related to kidney dysfunction. Our results showed that cystatin C level were correlated with BMI, diastolic BP, and decrease eGFR level. Also, we found that the obese with ERD group had a higher value of cystatin C than the obese with NKF group. Some studies investigating the relationship between cystatin C and obesity parameters showed that urinary cystatin C level is related to BMI, WC, WHR,[18, 19, 20] results which support our current work. In our study, urinary NGAL and KIM-1 were not significantly increased in obese people with ERD subjects. To the best of our knowledge, NGAL and KIM-1 have not previously been studied in obese subjects who have ERD condition.

In multiple logistic regression analysis, the present study is concordant with the majority of previously published work, the CREDIT study (Chronic Renal Disease in Turkey) demonstrated a risk of CKD among 30-year-olds-people increased from 1.45-2.18 times for increase every 10 years of age. In addition, cystatin C was significantly associated with ERD among older subjects (>45 years) (p=0.004).[23] The association between the risk of ERD and each parameter is shown in Table 4. We observed lower odds ratio of high blood pressure and low eGFR levels in the models.

We acknowledge that the current study has some limitations. First, this is a cross-sectional study and the sample number was low. Therefore, there is a need for larger prospective studies to confirm the results and reduce the possibility of confounding variables. Second, renal injury biomarkers should be investigated in long term perspective. Despite these limitations, this is the first study to investigate the association of kidney markers and obesity parameters in Thai population from survey study. This information could be implemented in screening, risk regulation and management of kidney diseases at the early stage.

Conclusion

Obesity has the great influence on renal function and it can cause renal alterations and kidney disease. The results of this study revealed that age, high systolic and diastolic BP, elevated creatinine and cystatin C level, and the decline in eGFR associated with the occurrence of ERD. Creatinine and cystatin C levels were significantly associated with eGFR. Cystatin-C could be a promising early biomarker of kidney disease in obese people. Further prospective studies should be conducted to explore the clinical usefulness of cystatin C in the early renal dysfunction in obese people.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

This study was supported by a capacity building program for new researcher 2018 from National Research Council of Thailand (NRCT). and research grant of Faculty of Allied Health, Sciences, Thammasat University.

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Journal of Associated Medical Sciences 2019; 52 (3): 170-174

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Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index

Qualitative and quantitative assessment of hydroquinone in skin whitening cosmetics in Pathum Thani Province, Thailand

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ARTICLE INFO

Article history:
Received 1 March 2019
Accepted as revised 25 June 2019
Available online 15 August 2019

Keywords:
Hydroquinone, skin whitening cosmetic, hydroquinone test kit-2, TLC, HPLC

ABSTRACT

Background: Hydroquinone has been used for decades as a skin bleaching agent, but it has unfavorable effects including contact dermatitis and ochronosis. Therefore, it got prohibited by the Ministry of Public Health of Thailand. However, some cosmetics companies are still illegally using hydroquinone as a cosmetic ingredient that can affect on consumer health.

Objectives: To assess the hydroquinone contamination in skin whitening cosmetic products in Pathum Thani Province, Thailand.

Materials and methods: Fifty skin whitening cosmetic samples were collected from local markets in Lak Hok Subdistrict, Muang District, Pathum Thani Province, Thailand. Screening test for hydroquinone contamination was performed by using hydroquinone test kit-2 from Department of Medical Science, Ministry of Public Health. All samples were confirmed by using Thin-layer Chromatography (TLC) technique. The hydroquinone concentration was estimated by using High-Performance Liquid Chromatography (HPLC) technique.

Results: Nine of the 50 samples showed a positive result in hydroquinone test kit-2, but only 3 of the 9 positive samples found hydroquinone contamination based on TLC and HPLC analysis. The concentration of hydroquinone was 0.0005, 0.0009, and 0.0016%.

Conclusion: Six percent of skin whitening cosmetics in local markets of Lak Hok Subdistrict, Muang District, Pathum Thani Province found hydroquinone contamination. Therefore, consumer should concern about their safety and checked the manufacture's information prior making a purchase decision.

Introduction

White skin is considered to be the highest beauty standard in Thailand. This attitude was influenced by commercial advertisements which divides and excludes dark skin people as the group of "Marginal ones".[1] This perception has encouraged most women to engage in skin bleaching. Skin whitening cosmetic products became a priority for women to bleach their skins. Most of these cosmetic products contain different kinds of depigmentation agents such as retinoid, alpha-and beta-hydroxy acids, ascorbic acid, arbutin, hydroquinone, and derivative mercury. Some agents (especially hydroquinone) are harmful chemicals and can affect on health.[2]

Hydroquinone (HQ) is one of most effective skin bleaching agent by inhibits tyrosinase (key enzyme responsible for melanin production) synthesis and tyrosinase activity, as well as destructs melanocytes.[3,4] In the past, hydroquinone was allowed to use as an ingredient of the blemish cream at a concentration of less than 2%.[5] Later on, it was found that it has numerous unfavorable effects after long-term application, including irritative dermatitis, contact dermatitis, and ochronosis.[6,7] Therefore, its use in cosmetics got prohibited

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doi: 10.14456/jams.2019.28
E-ISSN: 2539-6056

and been so since 1996.[8] However, the hydroquinone contamination in skin whitening cosmetics is still being reported, indicating that unsafe product is remain distributed in a several areas of Thailand.[9-12]

There are several techniques to assess hydroquinone contamination in a cosmetic product including using hydroquinone test kit, thin layer chromatography (TLC), and high-performance liquid chromatography (HPLC).[11,13,14] These techniques have different characteristics for hydroquinone assessment such as the hydroquinone test kit and TLC provide only qualitative results, as well as hydroquinone test kit can give a false positive when presence of some agents in cosmetic product such as sodium sulfite, sodium metabisulfite, alpha-todopherol, and ascorbic acid.[10] Whereas HPLC technique can provide qualitative result, but it takes a long time with high cost for the analysis. Therefore, concerning to the accuracy of hydroquinone assessment as well as saving time and cost, the qualitative and quantitative analysis of hydroquinone contamination in skin whitening cosmetics in this study will be started with screening test by using hydroquinone test kit-2, and then confirmed by using TLC method. Hydroquinone concentration in a positive sample was estimated by using HPLC method.

Materials and methods

Sample collection

Fifty skin whitening cosmetic samples consisting 45 creams and 5 lotions were collected from local markets in Lak Hok Subdistrict, Muang District, Pathum Thani Province, Thailand. The main intention of the present study was focused on a cosmetic product that advertised "skin whitening effect, depigmentation, and anti-melasma" on the label in combination with giving false labeling such as not mentioning applicant (name and addressee of manufacturer) and/or not mentioning the registration number.

Screening test for hydroquinone by hydroquinone test kit-2

The hydroquinone test kit-2 was obtained from Department of Medical Science, Ministry of Public Health, Thailand. The detection limit was 0.006% w/w in cream, and 0.014% w/w in lotion. The reagent for hydroquinone assessment was freshly prepared before the test started. About 0.2 mg of each skin whitening cream or 2 mL of skin whitening lotion were placed into a palette, and 2 mL of the reagent was added and mixed. The color of mixture was observed within 30 ms, if the color of the mixture turned green to dark blue color (positive result) indicating that hydroquinone may be contaminated in this sample.

Preparation of sample solution for TLC and HPLC analysis

The skin whitening cosmetic sample was prepared as described previously.[13] Briefly, two grams of each sample was accurately weighed in a 25 mL beaker. Fifteen mL of 96% (v/v) ethanol was added and mixed on the water bath at 60°C for 10 min. A mixture was cooled in an ice bath until the separation of fats occurred, and then filtered by using Whatman Filter Paper No.1. The filtrate was collected for TLC and HPLC analysis. The same procedure was repeated for all samples.

TLC analysis

Preparation of standard solution

The standard solution was prepared by dissolving 0.5 gm of hydroquinone in small volume of 96% (v/v) ethanol in 25 mL volumetric flask and the resulting volume was made up to 25 mL.[13]

Chromatographic method

TLC plates (8x4 cm) were made on 0.25 mm thick silica gel 90G (Merck, Germany). A mixture of chloroform:ethyl acetate (3:1) was used as mobile phase. The standard and duplicate of each sample solution were spotted onto the start line of the TLC plate followed by placed into the separating jar containing mobile phase. The plate was developed at room temperature to the height of approximate 8 cm from the start line. After drying or spraying with 0.2% ethanolic dichlorofluorescein, the plate was visually examined under UV light at 254 nm and 366 nm, respectively.[13] The retention factor (Rf) value were calculated by the equation:

$$R_f = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

Identification of the hydroquinone in each sample was done by comparison of its Rf value with those of the standard

HPLC analysis

Preparation of standard solutions

Stock solutions of concentration 1, 10, 20, 30, 40, and 50 mg/L were prepared by dissolving 0.5 mL HPLC-grade methanol and the resulting volume was made up to 1 mL with the solvent for the mobile phase (MtOH:H_2O; 45:55 v/v).[13]

Chromatographic method

HPLC analysis was performed on a modular Shimadzu LC-10 system (Shimadzu, Japan) equipped with a LC-10AD pump, a CTO-10A column oven, SPD-M20A UV-DAD detector, a CBM-10A interface and a LC-10 Workstation. Reverse phase chromatography analysis was achieved by an innersil-ODS-3 column (5 µm particle size, 250x4.6 mm i.d.) with a mobile phase consisting of a mixture of methanol and water, and employing gradient elution (from 45:55 to 85:15, v/v), volume ejection was 20 µL, and UV detection was at 295 nm.[13] Each sample was identified by comparison of its retention time and UV absorption spectrum with standards under the same conditions. Quantification of hydroquinone in each sample was done by the measurement of integrated peak area and the content was calculated using the calibration curve by plotting peak area against concentration of the respective standard sample. Each quantification was carried out in triplicate.

Results and Discussion

Hydroquinone was considered to be as one of the most effective skin bleaching agent, but it has numerous unfavorable effects after long-term application. Therefore, it is prohibited to be added in cosmetic products.[8] However, some cosmetic companies are still illegally using hydroquinone as a cosmetic ingredient that can affect on consumer health.

Therefore, in the present study focuses on the assessment of hydroquinone contamination in a skin whitening cosmetic products in local markets of Lak Hok Subdistrict, Muang District, Pathum Thani Province, Thailand by using a combination of hydroquinone test kit-2, TLC, and HPLC technique. A screening test of hydroquinone contamination by using hydroquinone test kit-2 showed a positive result in 9 of 50 (18%) skin whitening cosmetic samples, including sample no. 6, 14, 22, 28, 32, 42, 45, 49, and 50 (Table 1). However, hydroquinone test kit-2 can give a false negative when the concentration of hydroquinone was lower than the detection limit (0.06% w/w in cream and 0.014 % w/w in lotion), and give a false positive when presence of some agents such as sodium sulfite, sodium metabisulfite, alpha-todopherol, and ascorbic acid. Therefore, the hydroquinone contamination in all samples was confirmed by using TLC technique. The R_f value of sample spots was compared with the R_f value of the standard solution of hydroquinone (0.05). Based on TLC results, a negative sample in hydroquinone test kit-2 also showed negative result in TLC analysis. Whereas, only 3 of the 9 (33%) positive samples showed a R_f value of 0.50 like the standard solution, including sample no. 6, 28, and 50 (Table 1, Figure 1). The hydroquinone concentration of the positive samples in TLC analysis was estimated by using HPLC technique. The calibration curve of the standard solution of hydroquinone showed the linearity of the detector over the tested range (1-50 mg/L). The linear regression equation was y =37842x-16537 (R^2 =0.9992). The retention time and UV spectrum of hydroquinone in standard solution and sample also coincided (Figure 2). The concentration of hydroquinone in sample No. 6, 28, and 50 was 0.0016, 0.0005, and 0.0009%, respectively (Table 1). This finding showed that 3 of 50 (6%) whitening cosmetic samples were contaminated with hydroquinone. This result was similar reported by Klinsamut and coworkers in 2013.[10] It is indicated that nowadays unsafe skin whitening cosmetic products are remain distributed in the local markets, and did not get approved yet. In addition, the false positive of hydroquinone test kit-2 found about 67% indicating that although some cosmetic products give a positive result, it didn't truly mean hydroquinone contamination. Therefore, the sample with a positive result in hydroquinone test kit-2 needed to be confirmed by other methods

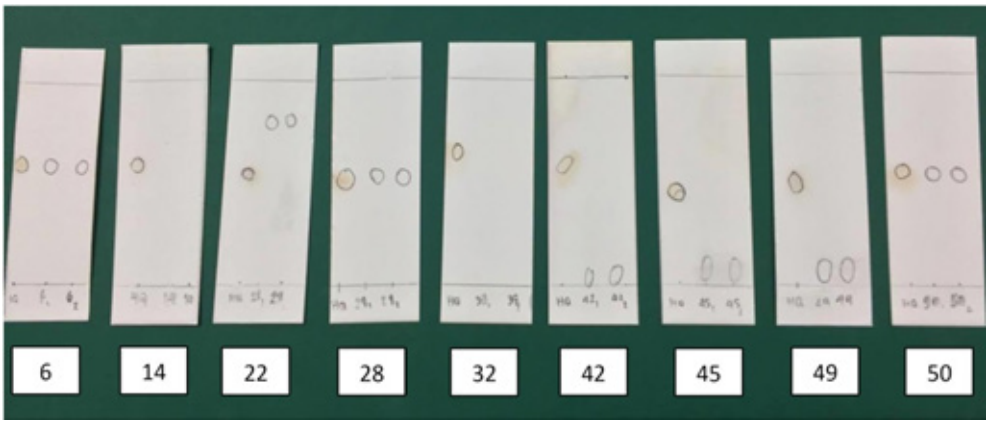

Figure 1*. TLC analysis results of nine positive samples in hydroquinone test kit-2.*

Table 1 Evaluation of hydroquinone by using hydroquinone test kit-2, TLC analysis, and HPLC analysis.

Sample No.	Type	HQ test kit-2	TLC analysis Rf value	HPLC analysis Retention time	HPLC analysis HQ conc. (mg/L)	HQ concentration in sample (% w/w)
6	Cream	+	0.50	4.507	2.14±0.013	0.0016
14	Cream	+	N.D.	-	-	-
22	Cream	+	0.73	-	-	-
28	Lotion	+	0.50	4.536	0.71±0.009	0.0005
32	Lotion	+	N.D.	-	-	-
42	Cream	+	0.96	-	-	-
45	Cream	+	0.91	-	-	-
49	Lotion	+	0.92	-	-	-
50	Cream	+	0.50	4.515	1.18±0.088	0.0009

Notes: HQ; hydroquinone, +; positive result, N.D.; Not detected.

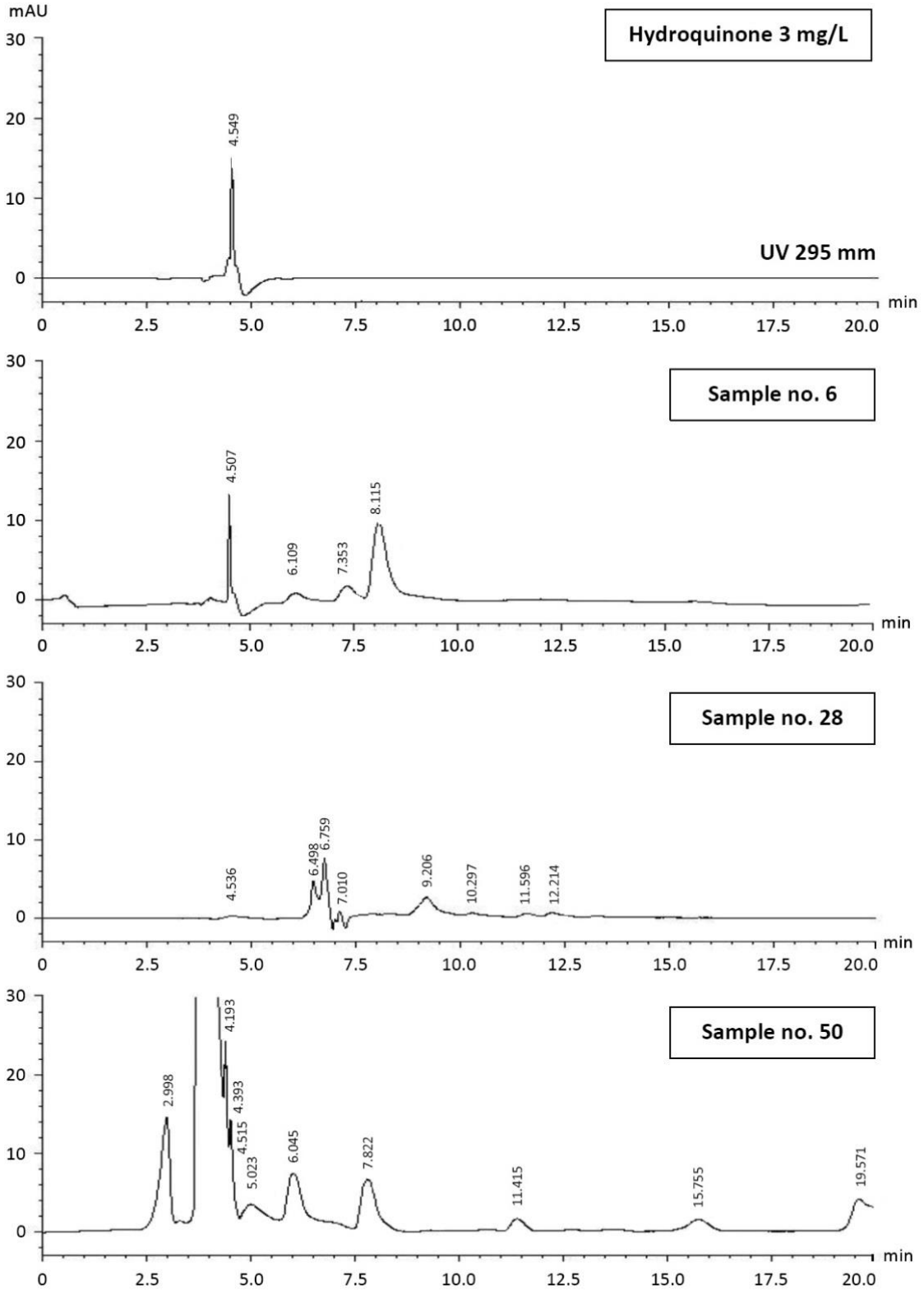

Figure 2. *HPLC chromatogram of the standard of hydroquinone and skin whitening cosmetic samples.*

Conclusion

Nine of the 50 skin whitening cosmetic samples showed a positive result in hydroquinone test kit-2, but only 3 of the 9 positive samples showed hydroquinone contamination when TLC and HPLC analysis were used. We can conclude that about 6% of skin whitening cosmetics in local markets of Lak Hok Subdistrict, Muang District, Pathum Thani Province found hydroquinone contamination. Therefore, the consumer should be concerned about their safety and checked the manufacture's information prior making a purchase decision.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to the Pharmacology and toxicology Unit, Faculty of Science, Rangsit University. The authors also extend thanks to Rujira Podee and Suvanan Saentaweesuk for their technical support. This work was funded by Rangsit University.

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Journal of Associated Medical Sciences 2019; 52 (3): 175-184

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Journal of Associated Medical Sciences

Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index

Identification of CD4 isoforms by two anti-CD4 monoclonal antibodies

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ARTICLE INFO

Article history:
Received 24 May 2019
Accepted as revised 4 July 2019
Available online 19 July 2019

Keywords:
CD4 molecule, CD4 isoform, anti-CD4 monoclonal antibody, hybridoma

ABSTRACT

Background: CD4 isoforms expressed on leukocyte surface have been reported. The function of the CD4 isoforms is, however, unknown. Several studies are conducted aiming to uncover the functions of the CD4 isoforms which will lead to a better understanding of the immune responses.

Objectives: To identify CD4 isoforms expressed on leukocyte surface using two anti-CD4 monoclonal antibodies (mAbs) clones MT4 and MT4/3.

Materials and methods: Anti-CD4 mAbs MT4 and MT4/3 were purified by affinity chromatography. Specificity of the obtained purified mAbs were verified by 293T transfection and cell depletion experiment. Cellular distribution profiles of mAbs MT4 and MT4/3 were determined by immunofluorescence and flow cytometry. Identification of CD4 isoforms was performed by confocal microscopic analysis.

Results: Anti-CD4 mAbs MT4 and MT4/3, generated in our research center, were purified and confirmed their specificity by CD4-DNA transfection and cell depletion experiment. Cellular distribution profiles obtained from mAbs MT4 and MT4/3 were similar to those obtained using standard anti-CD4 mAb. By confocal microscopic analysis, mAbs MT4 and MT4/3 were demonstrated to recognize different CD4 molecule expressed on cell surface.

Conclusion: Anti-CD4 mAbs MT4 and MT4/3 were demonstrated to react with different CD4 isoforms. To the best of our knowledge, this is the first report showing that CD4 isoforms could be determined by specific mAbs. These mAbs will be an important tool for employing in characterization of structure and function of the CD4 isoforms.

Introduction

CD4 is a cell surface glycoprotein expressed on a sub-population of T-lymphocytes named helper T lymphocytes or $CD4^+$ lymphocytes.[1] As was described for $CD4^+$ lymphocytes, CD4 molecule is a 55 kDa glycoprotein. It is a member of the immunoglobulin (Ig) superfamily which contains four-Ig-like extracellular domains, D1-D4.[2-4] The oligomers of CD4 molecules have been described.[2, 5-10] CD4 molecules play very important roles in $CD4^+$ lymphocyte activation. This molecule was demonstrated to stabilize the interaction of TCR on T lymphocytes and peptide-MHC class II complexes on antigen presenting cells (APCs) and mediate intracellular T cell signaling.[2, 11, 12] Upon T cell activation, large multiprotein clusters of CD4, TCR and CD3 molecules are formed and provide a focal point for intracellular signal transduction resulting in T cell activation and induction of the adaptive immunity.[3, 13-17]

In addition to T lymphocyte, CD4 molecules are expressed on several cell types, including monocytes, macrophages, Langerhans cells, dendritic cells, eosinophils, megakaryocytes and mast cells.[3, 18-23] However, the expression of CD4 on non-lymphatic cells is considerably lower than

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doi: 10.14456/jams.2019.26
E-ISSN: 2539-6056

those on $CD4^+$ lymphocytes. It was reported that monocytes express CD4 molecules 10-20 folds less than those expressed on lymphocytes.[24, 25] Regarding to the molecular interaction, it is well documented that, on $CD4^+$ lymphocytes, CD4 function as the receptor for MHC class II, IL-16 and HIV gp 120.[3, 26] However, the function of CD4 molecules on other cells is not yet clearly known. Neither the interaction nor the signaling capability of CD4 on non-T cells is fully revealed.

Monocytes are phagocytes which typically represent approximately 10% of peripheral blood mononuclear cells (PBMCs). In addition to its specific marker CD14, all monocytes express CD4 molecules on their surface.[27, 28] However, the CD4 molecules expressed on $CD4^+$ T lymphocytes and monocytes are significant different.[29] In contrast to the well-documented 55 kD CD4 monomer in lymphocytes, monocytes express two CD4 monomers, i.e. 55 kD and 59 kD isoforms.[29] The 59 kD CD4 isoform appears to be distended through disulfide disruption.[29] The functions of 59 kD isoforms, however, is still unknown. CD4 isoforms expressed on T lymphocytes, nevertheless, is still mystery. Several ongoing researches are conducted to uncover the characteristics and functions of the CD4 isoforms. This will, subsequently, lead to the better understanding of the immune responses.

In the present report, by using our generated anti-CD4 mAbs MT4 and MT4/3, we demonstrated that these mAbs recognized different CD4 molecules expressed on leukocyte surface. The mAbs MT4 and MT4/3 could differentiate CD4 isoforms

Materials and methods

Antibodies, reagents and cell lines

Anti-CD4 mAb clones MT4 and MT4/3,[30, 31] anti -hemoglobin clone Hb1b, anti-phage clone 13M1F, anti-RBCs clone WK3 and FITC labelled anti-hemoglobin Bart's clone PB1 were produced in our laboratory. FITC-conjugated anti-CD4 mAb (clone Leu3a) and BD FACS™ lysing solution were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Anti-CD31 mAb (clone LC/70A) were purchased from DAKO (Glostrup, Denmark). FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (H+L) antibodies was obtained from Merck Millipore (Darmstadt, Germany). Hitrap IgM Sepharose column and Protein G Sepharose column were obtained from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Lipofectamine® 2000 reagent for transfection experiments and goat anti-mouse IgM (μ-chain specific) conjugated with Alexa Flour 488 were obtained from Invitrogen (Carlsbad, CA, USA). Goat anti-mouse IgG (Fc_γ specific) conjugated with Cy™3 was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Ficoll-Hypaque solution (IsoPrep) was purchased from Robbins Scientific Corporation (Sunnyvale, CA, USA). Dynabeads™ M-280 Streptavidin, ProLong™ Gold Antifade Mountant and Hybridoma-Serum Free Media (SFM) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). 7-Amino-actinomycin D (7AAD) was purchased from BioLegend (San Diego, CA, USA).

293T cells were maintained in DMEM containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 40 mg/mL gentamicin and 2.5 mg/mL amphotericin B (10%FBS-DMEM) and cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

Production and purification of anti-CD4 mAb clones MT4 and MT4/3

Anti-CD4 mAb clones MT4 and MT4/3 were generated in our laboratory.[30, 31] To produce the purified anti-CD4 mAbs, the hybridoma producing anti-CD4 mAb clones MT4 and MT4/3 were thawed and cultured in 10%FBS-DMEM at 37 °C in a 5% CO_2 incubator. The hybridoma cells were, then, adapted for culturing in Hybridoma-Serum Free Media (SFM) by gradually increasing the ratio of Hybridoma-SFM to 10%FBS-DMEM from 0:100 to 25:75, 50:50, 75:25 until 100% Hybridoma-SFM. After adaptation, the hybridomas were grown for 1 week in 10 cm dish using SFM system. The culture supernatants of hybridoma culture were then collected and tested for the activity of antibody using indirect immunofluorescence technique. Then, the anti-CD4 mAb clones MT4 and MT4/3 were purified from the collected supernatants.

For purification of mAb MT4 (IgM isotype), the culture supernatant was added to the Hitrap IgM Sepharose column and affinity purified using Aktaprime fraction collector (GE Healthcare Bio-Sciences). The unbound mAbs were washed out and the bound mAbs were eluted using 20 mM sodium phosphate buffer pH 7.5. For purification of mAb MT4/3 (IgG2a isotype), the culture supernatant was added to the protein G Sepharose column. The unbound mAbs were washed out. Then, the bound mAbs were eluted using 0.1M citric acid buffer pH 3.0. The eluates were collected and neutralized with neutralizing buffer (2M Tris-HCl pH 8.0) and dialyzed against PBS for overnight. The concentration of mAbs MT4 and MT4/3 were measured by OD280 reading and stored at -20 °C.

Transfection of 293T cells

For 293T cell transfection, plasmid encoding CD4 protein[31] was transfected into 293T cells using lipofectamine mediated transfection. Lipofectamine 2000 reagent was used for plasmid transfection according to manufacturer instruction (Invitrogen). Briefly, 293T cells ($4x10^5$ cells/well) were plated into 6 wells-plate and incubated at 37 °C in a 5% CO_2 incubator for overnight. The plasmid DNA-liposome complex is prepared at ratio 1 μg of DNA : 7 μL of lipofectamine reagent in DMEM and incubated at room temperature for 5 minutes. The mixture was then slowly added into 293T cells and further incubated at 37 °C in a 5% CO_2 for 72 hours to allow expression of the corresponding proteins. After incubation, transfected cells were collected for further staining with mAbs.

Cell depletion experiment

For mAbs coated magnetic bead preparation, streptavidin superparamagnetic beads (Dynabeads™ M-280 Streptavidin) were washed three times with phosphate buffered saline (PBS). Then, 20 μg of biotinylated mAbs MT4 or MT4/3 in PBS were added into 1 mg of the washed M280 beads and incubated on rotator for 30 minutes at room temperature. Afterward, the beads were washed four times with PBS

containing 0.1% BSA (0.1% BSA-PBS). The mAbs coated M280 beads were adjusted to $8x10^8$ beads/mL with 0.1% BSA-PBS containing 0.02%NaN_3 (1%FCS-PBS-0.02%NaN_3) and stored at 4 °C.

For cell depletion, 5 µL of M280-MT4 or M280-MT4/3 beads in 0.1%BSA-PBS-0.02%NaN_3 were added into 200 µL of K3EDTA blood in 1.5 mL microcentrifuge tube and incubated on a rotator for 30 minutes at room temperature. After incubation, the tubes were placed in magnetic stand and let stand for 5 minutes at room temperature. Then, the non-adherent cells were transferred into new tube. The cell depleted blood was then stained with FITC-conjugated anti-CD4 mAb by lysed whole blood staining. The stained cells were then analyzed by flow cytometer (BD Accuri™ C6; Becton Dickinson). The data were analyzed by FlowJo software.

Peripheral blood mononuclear cells (PBMCs), red blood cells (RBCs), and platelets preparation

For PBMCs preparation, heparinized blood was mixed with PBS at 1:1 ratio. The diluted blood was, then, overlaid onto Ficoll-Hypaque solution and then spun at 400 g, 25 °C for 30 minutes with break-off setting. After centrifugation, the PBMCs were harvested from white ring at the interphase of Ficoll-Hypaque and plasma layer. The cells were counted by hemocytometer and adjusted to $1x10^7$ cells/mL with FACS buffer (1%FCS-PBS-0.02%NaN_3).

For RBC preparation, whole blood samples were washed with FACS buffer for 3 times. Packed RBCs were then adjusted to 0.3% (v/v) cell suspension with FACS buffer.

For platelets preparation, platelets were isolated from ACD blood by centrifugation at 200 g for 20 minutes at 25 °C. Platelet-rich plasma (PRP) fraction was collected and centrifuged at 800 g for 20 minutes at 25 °C to obtain platelet pellet. The obtained platelets were resuspended with Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.90 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 5 mM HEPES, 5 mM Glucose, and 1% BSA) and counted with Rees and Ecker' solution by hemocytometer and adjusted to $1x10^7$ cells/mL with FACS buffer.

Indirect immunofluorescent assay

PBMCs ($5x10^5$ cells/mL; 50 µL), RBCs (0.3% RBCs; 50 µL) or platelets ($5x10^5$ cells/mL; 50 µL) were incubated for 30 minutes at 4 °C with 10% human AB serum to block the non-specific Fc receptor mediated binding of the antibodies. Blocked cells were then incubated for 30 minutes at 4 °C with mAbs MT4 or MT4/3, or isotype matched control antibodies (Hb1b; IgM and 13M1F; IgG2a). Then, the cells were washed twice with FACS buffer and FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (H+L) antibodies were added and incubated for another 30 minutes at 4 °C. After washing, cells were fixed with PBS containing 1% paraformaldehyde. The stained cells were then analyzed by flow cytometer (BD Accuri™ C6 or FACSort; Becton Dickinson). The data were analyzed by FlowJo software.

Lysed whole blood staining

Fifty microliters of K3EDTA blood samples were incubated with anti-CD4 mAbs clone MT4 or MT4/3 for 30 minutes at room temperature. Samples were then washed twice with FACS buffer and incubated with the FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (H+L) antibodies. After 30 minutes incubation, 1 mL of lysing solution (BD FACS™ Lysing Solution; Becton Dickinson) was added and mixed well then let stand at room temperature in dark for 15 minutes for lysis of RBCs. The remained WBCs were washed twice with FACS buffer and final stained with 7-Aminoactinomycin D (7AAD) for 10 minutes to separate WBCs from the remained non-lysed RBCs. The stained cells were then analyzed by flow cytometer (BD Accuri™ C6 or FACSort; Becton Dickinson) and the data were analyzed by FlowJo software.

Confocal microscopic analysis

PBMCs were isolated from heparinized whole blood by Ficoll-Hypague density gradient centrifugation. $1x10^6$ of PBMCs were incubated with mAbs MT4 or MT4/3 or isotype matched control antibodies (Hb1b; IgM and 13M1F; IgG2a) for 30 minutes at 4 °C. Goat anti-mouse IgM (µ-chain specific) conjugated with Alexa Flour 488 (Invitrogen) and goat anti-mouse IgG (Fc_γ specific) conjugated with Cy™3 (Jackson ImmunoResearch) for detecting mAb MT4 or MT4/3, respectively, were added and incubated for 30 minutes. After incubation, the cells were washed twice and fixed with 2% paraformaldehyde and resuspended with 10 µL of PBS.

For slide mounting, ten microliters of stained cells were plated on 0.01% poly-L-lysine pre-coated 10 mm diameter cover slips and incubated for 20 minutes to let the cells adhere on cover slip. 10 µL of ProLong Gold antifade reagent (Thermo Fisher Scientific) were dropped on glass slide. Then, cells coated cover slips were slowly place on droplet of ProLong Gold antifade reagent by preventing air bubbles formation. After let the slide dry for overnight, the cover slips were seal with nail polish and stored at 4 °C until determined by confocal microscope (Zeiss, Thornwood, NY, USA).

Human Ethics

This study was approved by the ethics committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand (AMSEC-61EM-022).

Results and Discussion

Purification of anti-CD4 monoclonal antibody clones MT4 and MT4/3

Two anti-CD4 mAbs, named MT4 (IgM isotype) and MT4/3 (IgG2a isotype), were generated in our research center. In order to characterize the mAbs MT4 and MT4/3 reactivity, the purified form of these mAbs were required. Hybridoma producing anti-CD4 mAb clones MT4 and MT4/3 were adapted to grow in SFM. The antibody containing culture supernatants were subjected for antibody purification using affinity chromatography. The obtained purified mAbs were verified for their purity by SDS-PAGE analysis. As shown in Figure 1, the purified mAbs showed the bands at the expected sizes of IgM for MT4 and IgG for MT4/3, in both reducing and non-reducing conditions. In the purified

mAbs, undesired proteins were observed at very low level. The data indicated that the purified mAbs obtained were suitable to be used in further experiments.

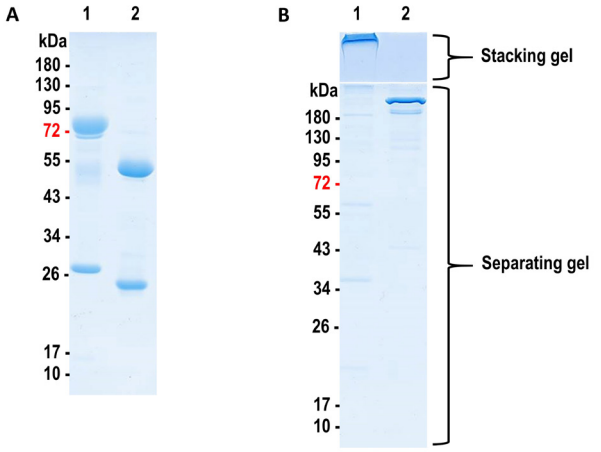

Figure 1. *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified monoclonal antibodies MT4 and MT4/3. Purified mAbs MT4 and MT4/3 were mixed with reducing or non-reducing buffer and boiled at 95 °C for 5 minutes. Electrophoresis was done in a 10% SDS-PAGE gel at 120 V, 25 mA. Gel was stained with Coomassie Brilliant Blue. (A) In reducing conditions, bands at 75 and 27 kDa and 50 and 25 kDa were observed for mAb MT4 (lane 1) and mAb MT4/3 (lane 2), respectively. (B) In non-reducing conditions, >180 kDa bands was observed for MT4 (lane1) and MT4/3 (lane 2). The positions of molecular mass markers in kDa are indicated on the left*

Specificity of anti-CD4 monoclonal antibody clones MT4 and MT4/3

As we aimed to use these mAbs for determination of CD4 molecule expressed on leukocyte surface, the specificity of these mAbs were firstly verified. CD4-DNA[31] was transfected into 293T cells. The transfected cells were then used to determine the specificity of anti-CD4 mAbs MT4 and MT4/3. As shown in Figure 2, both mAbs MT4 and MT4/3 strongly reacted to CD4-transfected cells, but not to untransfected cells. The CD4 transfected cells were confirmed for CD4 molecules expression as they were positive with commercial anti-CD4 mAb (Becton Dickinson) (Figure 2). The results confirmed that the mAbs MT4 and MT4/3 are specific for CD4 molecule.

We further determined whether the mAbs MT4 and MT4/3 recognized the native CD4 molecules expressed on peripheral blood lymphocytes using cell depletion experiment. Mabs MT4 and MT4/3 coated magnetic beads were prepared. The mAb-coated magnetic beads were then used to deplete the correspondence cells from whole blood. The cell depleted blood samples were then subjected for determination of $CD4^+$ lymphocytes by using standard anti-CD4 mAb. As shown in Figure 3, both MT4- and MT4/3-coated magnetic beads could deplete $CD4^+$ lymphocytes from the peripheral blood leukocytes. The results indicated that mAbs MT4 and MT4/3 were specific for CD4 molecules and could react to both recombinant CD4 proteins and native CD4 molecules expressed on cell surface.

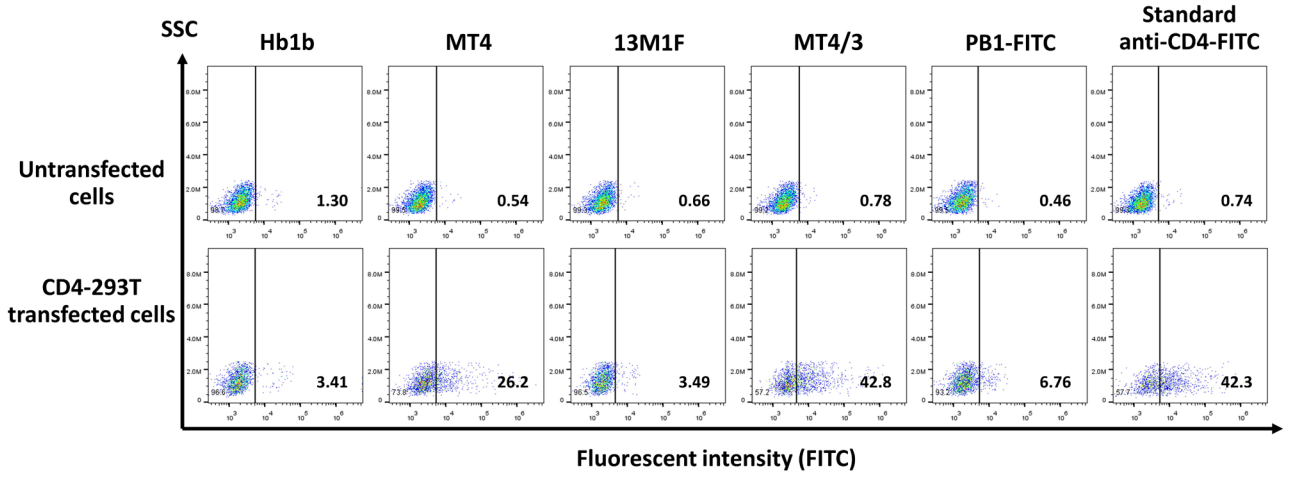

Figure 2. *Immunofluorescence analysis of monoclonal antibodies MT4 and MT4/3 with CD4-DNA transfected cells. The 293T cells were transfected with plasmid encoding CD4 protein (CD4-DNA). The CD4-293T transfected or untransfected cells were stained with mAbs MT4, MT4/3, or isotype matched control mAbs (Hb1b; IgM and 13M1F; IgG2a) by indirect immunofluorescence staining and analyzed by flow cytometer. In addition, the CD4-293T transfected cells were confirmed for expression of CD4 molecules by staining with standard anti-CD4 mAb labeled with FITC (standard anti-CD4-FITC) or isotype matched control mAb (PB1-FITC) and analyzed by flow cytometer.*

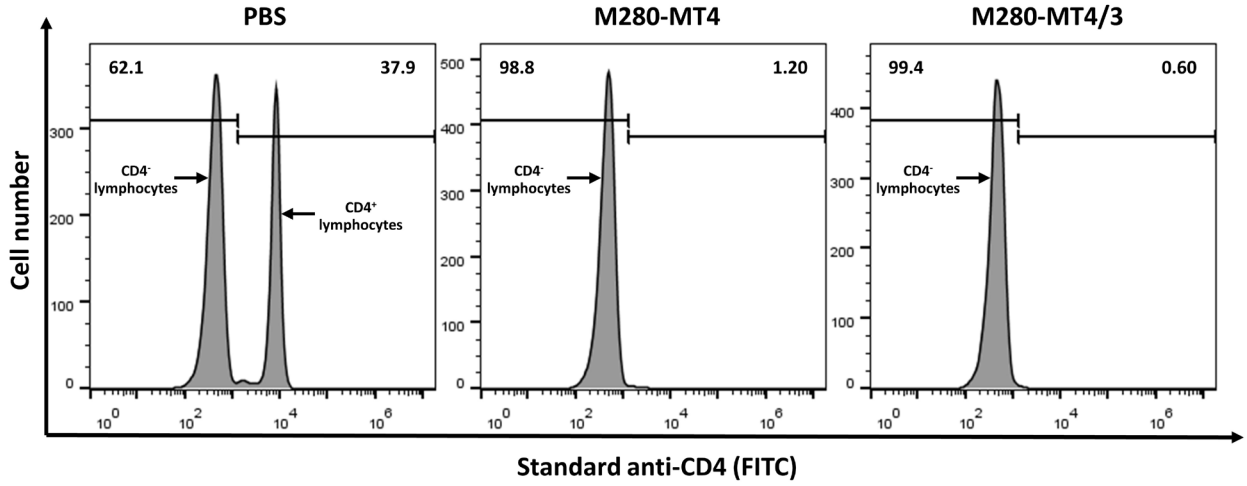

Figure 3. *Determination of the specificity of monoclonal antibodies MT4 and MT4/3 by cell depletion. CD4+ cells in whole blood samples were depleted by mAb MT4-coated magnetic beads (M280-MT4) or mAb MT4/3-coated magnetic beads (M280-MT4/3) or PBS. The CD4+ cells depleted whole blood samples were stained with standard anti-CD4-FITC and 7-AAD by lysed whole blood staining and analyzed by flow cytometer. The lymphocyte population was gated according to their granularity (SSC) and 7-AAD staining. The percentage of CD4+ cells and CD4- cells (as indicated) in the CD4⁺ cells depleted whole blood by using PBS (control), M280-MT4 or M280-MT4/3 are shown in histograms.*

Reactivity of mAbs MT4 and MT4/3 with peripheral blood leukocytes

The reactivity of mAbs MT4 and MT4/3 with various peripheral blood leukocytes were determined. By lysed whole blood staining, the mAbs MT4 and MT4/3 (but not isotype matched control mAbs) reacted to a population of lymphocytes (Figure 4). The number of MT4⁺ lymphocytes and MT4/3⁺ lymphocytes were similar to those obtained by using standard anti-CD4 mAb (n=10) (Table 1).

In monocyte staining, the mAbs MT4 and MT4/3 reacted to monocyte population (n=10), but in different patterns (Figure 5). In all subjects, the mAb MT4 showed weaker reactivity compare to mAb MT4/3. It is worth noting that monocytes in some subjects (for example; sample No. 10) were negative with mAb MT4 at all mAb concentrations tested but were positive with mAb MT4/3 (Figure 5). The saturated concentration of the mAbs used was demonstrated to be 20 µg/mL or 40 µg/mL depend on individual blood samples (Figure 5). These results indicated that the different reactivity pattern between mAbs MT4 and MT4/3 with monocytes was not affected by insufficient antibodies as at concentration of 80 µg/mL the mAb MT4 still showed negative reactivity with monocytes. The results are in accordance to our previous reports.[30, 32]

The reactivity of mAbs MT4 and MT4/3 with red blood cells and platelets were also determined. In all subjects tested (n=5), RBCs and platelets were negative with mAbs MT4 and MT4/3 (Figure 6A and B).

The reactivities of mAbs MT4 and MT4/3, as was compared to the standard anti-CD4 mAb and the reported CD4 expression profiles,[33, 34] confirmed that the both mAbs MT4 and MT4/3 are anti-CD4 mAbs.

Table 1 Percentage of CD4⁺ lymphocytes determined by monoclonal antibodies MT4, MT4/3 or FITC labeled standard anti-CD4 mAb. Ten different whole blood samples were stained with mAbs MT4, MT4/3, or FITC labeled standard anti-CD4 mAb by lysed whole blood staining. The percentages of CD4⁺ cells in lymphocyte population are shown.

Sample Number	% Positive cells		
	Standard anti-CD4 mAb	mAb MT4	mAb MT4/3
1	29.4	29.8	31.2
2	41.8	38.4	40.8
3	32.0	32.2	35.4
4	34.5	35.3	33.8
5	41.5	38.9	39.4
6	39.8	34.5	37.6
7	45.4	43.9	44.6
8	39.7	39.2	39.1
9	39.0	37.0	37.2
10	38.0	32.5	36.9

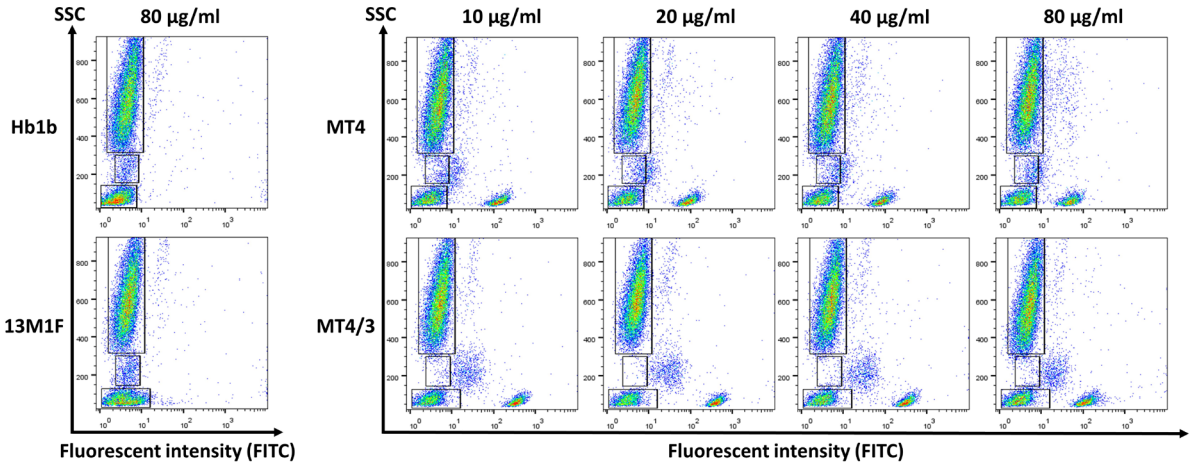

Figure 4. *Immunofluorescence analysis of the reactivity of monoclonal antibodies MT4 and MT4/3 with peripheral blood leukocytes. Whole blood samples were stained with various concentrations of mAbs MT4, MT4/3, or isotype matched control mAbs (Hb1b; IgM and 13M1F; IgG2a) and 7-AAD by lysed whole blood staining. The leukocyte population was gated according to their granularity (SSC) and 7-AAD staining. Granularity (SSC) and FITC fluorescence were plotted to show the reactivity of mAbs MT4 and MT4/3 at concentration of 10, 20, 40, or 80 µg/mL to each leukocyte population. The reactivity of isotype matched control mAbs (Hb1b and 13M1F) at 80 ug/mL are shown and marked by rectangles. One subject is shown as representative of 10 studied subjects.*

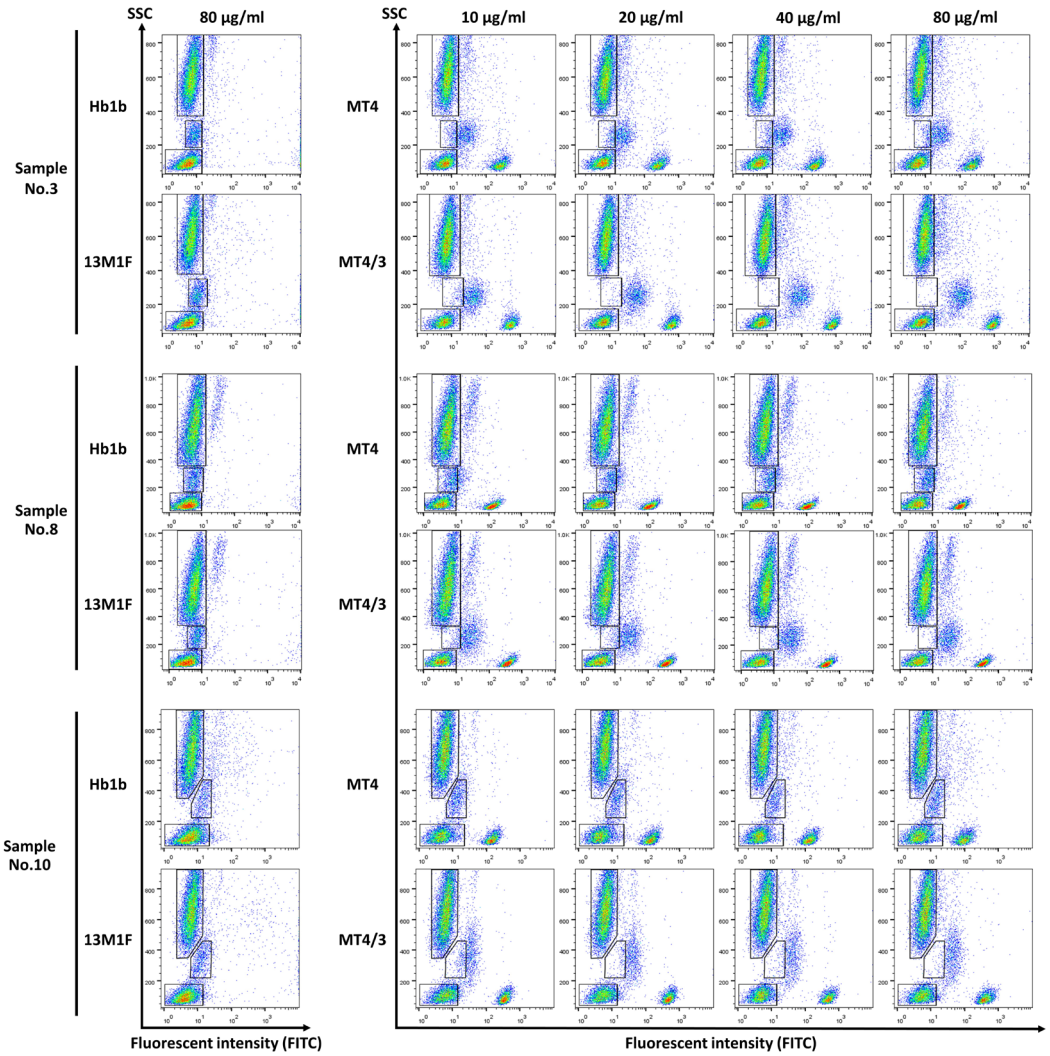

Figure 5. *The reactivity of monoclonal antibodies MT4 and MT4/3 with peripheral blood leukocytes of three study subjects. Whole blood samples were stained with mAbs MT4, MT4/3, or isotype matched control mAbs (Hb1b; IgM and 13M1F; IgG2a) and 7-AAD as was described in Figure 4. The leukocyte population was gated according to their granularity (SSC) and 7-AAD staining. Granularity (SSC) and FITC fluorescence were plotted to show the different reactivity of mAbs MT4 and MT4/3 to monocyte population of different blood samples. The mAb MT4 shows positive, weak positive, and negative reactivities with monocytes from sample No. 3, No. 8, and No. 10, respectively. Whereas, mAb MT4/3 shows positive reactivity with monocytes from all tested samples at any concentrations. The fluorescence intensities of isotype matched control mAbs for all cell populations are shown and marked by rectangles. Three subjects are shown as representative of 10 studied subjects.*

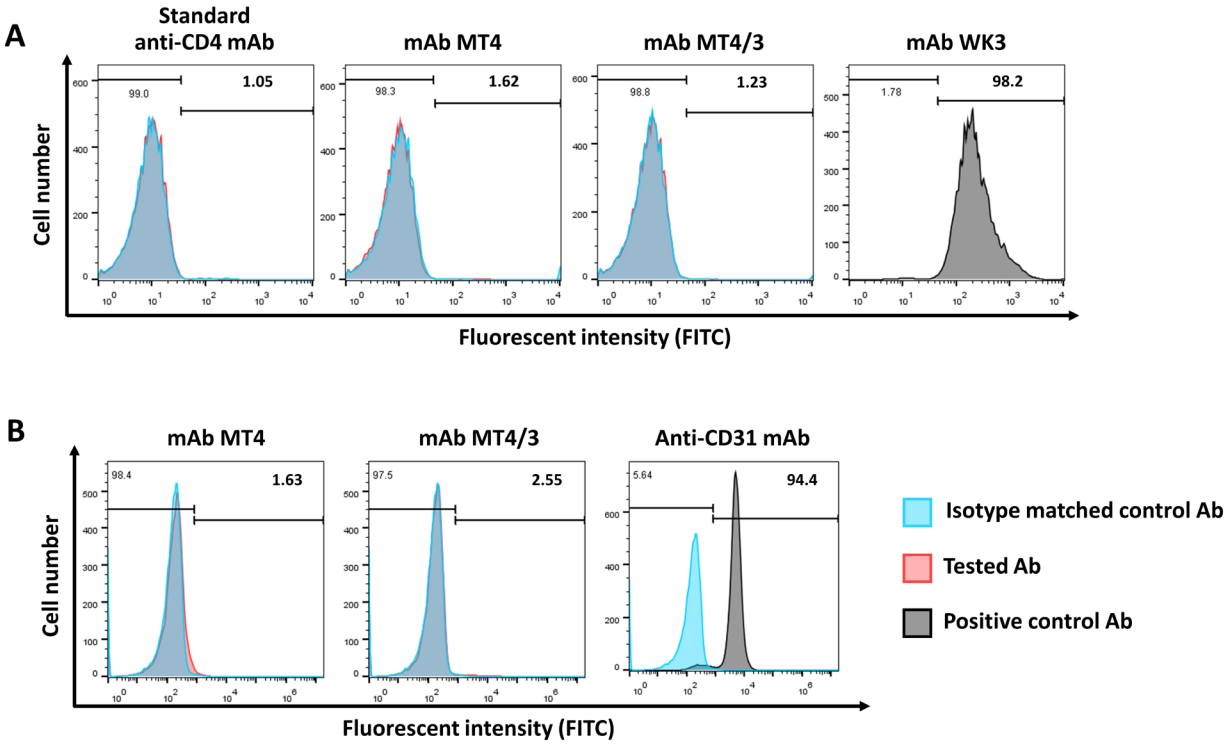

Figure 6. *Immunofluorescence analysis of the reactivity of monoclonal antibodies MT4 and MT4/3 with red blood cells and platelets. (A) RBCs and platelets (B) were stained with mAbs MT4, MT4/3, isotype matched control mAbs (Hb1b; IgM and 13M1F; IgG2a), standard anti-CD4-FITC, or mAb WK3 (as positive control for RBCs), or anti-CD31 mAb (as positive control for platelets) by indirect immunofluorescence staining and analyzed by flow cytometer. The RBCs or platelets were gated and the cells expressing molecule recognized by the tested mAbs are shown in red histogram. The isotype matched control and positive control mAbs are shown as light blue histograms and grey histogram, respectively.*

Monoclonal antibodies clones MT4 and MT4/3 recognize different CD4 isoforms

CD4 isoforms expressed on leukocyte surface have been reported.[29] As described above, the reactivities of mAbs MT4 and MT4/3 with monocytes were different. We speculated that mAbs MT4 and MT4/3 perhaps recognized different CD4 isoforms. Co-localization of the molecule recognized by mAbs MT4 and MT4/3 were, then, determined by confocal microscopic analysis. It was found that upon staining with mAbs MT4 and MT4/3, co-localization of the two mAbs were observed (Figure 7B and C). The non-colocalized molecules recognized by either mAb MT4 or MT4/3, however, were also detected (Figure 7B and C). Interestingly, cell that expressed only molecules that recognized by mAb MT4 was identified (Figure 7B).

These results demonstrated that, on cell surface, epitopes that recognized by mAbs MT4 and MT4/3 are on the same molecule or on different molecules but they are very closely expressed on cell surface, therefore, caused co-localization. However, some CD4 molecules contain epitopes that can be recognized by either mAb MT4 or MT4/3 and are expressed separately, thus caused non-co-localization. Some cells, nonetheless, expressed CD4 molecules contain only epitope that recognized with mAb MT4. These CD4 molecules are not carried the epitope recognized by mAb MT4/3. Our results indicated that, by mAbs MT4 and MT4/3, CD4 isoforms could be identified.

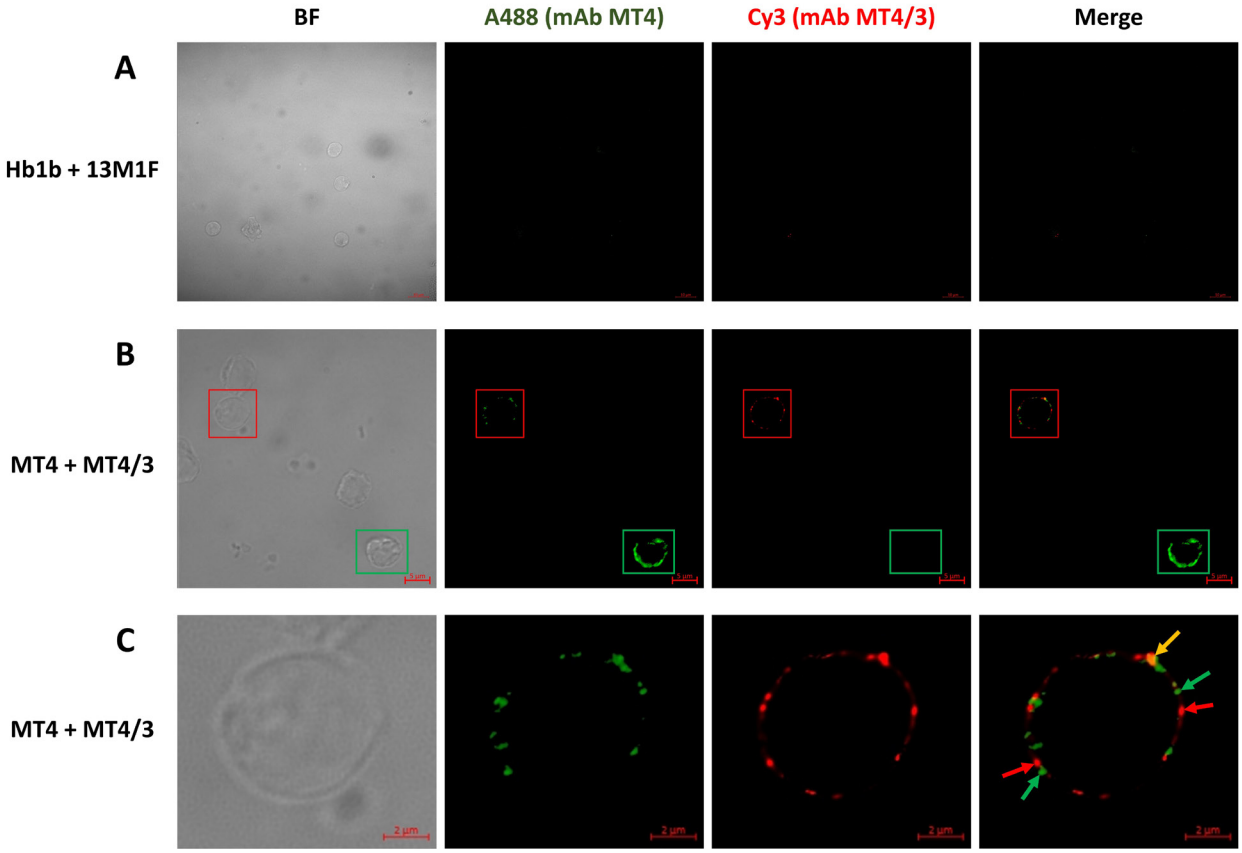

Figure 7. *Confocal microscopic analysis of the reactivity of monoclonal antibodies MT4 and MT4/3. PBMCs were double stained with mAbs MT4 and MT4/3 or Hb1b and 13M1F (isotype matched control mAbs) and following by Alexa Flour 488-anti-mouse IgM (μ-chain specific) and Cy™3-anti-mouse IgG (Fcγ specific) for detecting mAbs MT4 and MT4/3, respectively. The stained cells were analyzed by confocal microscope (100x). The mAbs MT4 and MT4/3 double positive (red rectangle) and MT4 single positive cells (green rectangle) are shown (B) while the isotype matched control mAbs are negative (A). The MT4 and MT4/3 double positive cell (from A, red rectangle) was magnified to observe in detail by ZEN 2.3 software (C). In this cell, the non-colocalized and colocalized cell surface molecules recognized by mAbs MT4 or MT4/3 are demonstrated. Non-colocalization of the molecules recognized by mAbs MT4 or MT4/3 are marked by green and red arrowheads, respectively. The co-localization of the molecules recognized by mAbs MT4 and MT4/3 are marked by yellow arrowhead.*

Discussion

The structure and function of CD4 molecules expressed on $CD4^+$ lymphocytes have been well characterized. However, little is known about CD4 molecules of non-lymphocytic cells. There are some, but few, reports demonstrated that the structure of CD4 molecules on lymphocytes and monocytes were different.[29] Lymphocytes express monomeric CD4, whereas monocytes express dimeric form.[6, 8] Furthermore, it was demonstrated that lymphocytes express a 55 kD monomeric CD4 molecules, while monocytes co-express two monomeric isoforms, 55 and 59 kD species.[29] The 59 kDa isoform was demonstrated as a transition-state, structural -intermediate in the formation of disulfide-linked homodimers.[29] The function of CD4 expressed on lymphocytes and monocytes were also different. Tyrosine kinase of CD4 on lymphocytes and monocytes were dissimilar, in which CD4 of lymphocytes were associated with Lck, but of monocyte were associated with Hck.[26, 29, 35] Heterogeneity in both structure and functions in the CD4 molecules of lymphocytes and monocytes was concluded. The existence of isoforms of CD4 molecules were also postulated.

In our research center, two anti-CD4 mAbs, named MT4 and MT4/3, were generated.[30, 31] These mAbs reacted with both recombinant CD4 proteins and native CD4 expressed on leukocytes. The cellular reactivity profiles of mAbs MT4 and MT4/3 were determined and found similar compared to the standard anti-CD4 mAb. However, when analyzed in detail, some differences in the reactivity of mAbs MT4 and MT4/3 were evidenced. In some subjects, monocytes were negative or weakly positive when stained with mAb MT4, but positive with mAb MT4/3 (this study and [32]). As CD4 isoforms have been reported,[29] we asked whether mAbs MT4 and MT4/3 bind to different isoforms. Co-localization, using confocal microscopic analysis, between mAbs MT4 and MT4/3 were conducted. The co-localization of both tested mAbs were found on cell surface suggesting that epitopes recognized by both mAbs may be present on a CD4 molecule. In addition, it can also be presumed that mAbs MT4 and MT4/3 reacted with different epitopes which located in different CD4 isoforms, but these isoforms were closely expressed on cell surface. Interestingly, within any positive cell, there are some CD4 molecules that reacted

with mAb MT4 or MT4/3, but without co-localization. The results indicated that mAbs MT4 and MT4/3 reacted with its epitopes expressed on distinct CD4 isoforms and expressed at different area on the cell surface. Surprisingly, very rare cells were detected positively only with mAb MT4. This observation indicated that this cell expressed only CD4 isoform that recognized by mAb MT4. Taken together, we demonstrated that our generated anti-CD4 mAbs MT4 and MT4/3 reacted with different CD4 isoforms expressed on leukocyte surface. Based on our findings, we postulated that CD4 isoforms are existence and be detected by mAbs MT4 and MT4/3. The molecular mechanism of the uncovered isoforms, however, is still unknown.

In conclusion, we reported here that the CD4 isoforms are existence and could be identified by our generated anti-CD4 mAbs, MT4 and MT4/3. To the best of our knowledge, this kind of anti-CD4 mAb has never been reported. Our anti-CD4 mAbs that can differentiate CD4 isoforms will be a valuable tool for characterization in detail of the structure and function of CD4 isoforms.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

This work was supported by the TRF Senior Research Scholar (Grant number RTA5980007) and was partially supported by Chiang Mai University (Chiang Mai Center of Excellence Project) and the Innovation Hubs of Thailand 4.0 project (in collaboration of Chiang Mai University and Mahidol University). Thanapol Ngamvilaisiriwong obtained Teaching Assistance/Research Assistance (TA/RA) Scholarship supported by Graduate school, Chiang Mai University.

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Journal of Associated Medical Sciences 2019; 52 (3): 185-192

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Maprang seed extracts suppressed chemoresistant properties of breast cancer cells survived from ionizing radiation treatment via the regulation of *ABCB1* genes

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ARTICLE INFO

Article history:
Received 18 April 2019
Accepted as revised 19 July 2019
Available online 19 July 2019

Keywords:
Breast Cancer, Maprang seed extracts, minimal residual disease, multidrug resistant, chemoresistance

ABSTRACT

Background: Minimal Residual Disease (MRD) is a major obstacle for eradication of cancer cells due to therapeutic resistance which results from the expression of multidrug resistant (MDR) proteins. MRD contributes to tumor metastasis and relapse in cancer patients. Therefore, any strategy that can reduce this resistance or eliminate all cancer cells must be one of the main goals for cancer treatment.

Objectives: This study aims to investigate the effect of Maprang Seed Extracts on drug resistance of minimal residual breast cancer cells that have survived radiotherapy.

Materials and methods: The treatment of resistant breast cancer cells MCF-7/IR6 and MCF-7/MPIR6 were established from parental MCF-7 cells having two different conditions for radiation treatment. Chemoresistant phenomenon of MRD was determined by MTT assay. A spectrofluorometric technique was used to determine the cellular drug uptake and the MDR protein-mediated drug efflux. MDR gene expression was confirmed by reverse transcription-polymerase chain reaction (RT-PCR).

Results: MCF-7/IR6 cells have increased resistance to doxorubicin when compared to the parental cells with the descending order of the concentration that inhibiting cell growth by 50% (IC_{50}) as follows: MCF-7/IR6 (IC_{50} =342.95±30.94 nM) > MCF-7/MPIR6 (IC_{50} =282.75±24.64 nM) > MCF-7 (IC_{50} =215.42±23.73 nM), accompanied by a remarkably enhanced expression level of MDR1 genes in MCF-7/IR6 compared with parental MCF-7 cells. For the multidrug protein function, it showed that MCF-7/IR6 cells mediated higher rate of drug efflux out of cells (V_a =0.2308 $nM.s^{-1}$) than MCF-7/MPIR6 (V_a =0.0679 nM.s-1) and the parental MCF-7 cells (V_a =0.0232 $nM.s^{-1}$). MPSEs effectively decreased chemoresistance phenomenon in the MCF-7/IR6 by aberrant MDR gene expression and decreased MDR protein-mediated drug efflux function.

Conclusion: The novel strategy combination of MPSEs and radiotherapy might be one strategy for improving curative effects in breast cancer treatment.

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doi: 10.14456/jams.2019.27
E-ISSN: 2539-6056

Introduction

Breast cancer is the most common cancer found in women around the world. Currently, many advanced strategies in breast cancer treatment are developing in many countries to improve the curative effects and to decrease cancer-related morbidity and mortality. Unfortunately, the goal of breast cancer treatment is still not achievable in many patients because of residual cancer cells called Minimal Residual Disease (MRD), defined as the cellular population that can survive after treatment which can lead to tumor relapse and treatment failure.[1] Mounting evidence suggests that the failure of the treatment associated with the use of cancer stem cells represents a more chemoresistant and radioresistant subpopulation within many cancers.[2-4] Recent studies have shown that tumor radioresistance mechanisms are associated with several signaling pathways including the Epithelial-Mesenchymal Transition (EMT), Adenosine Triphosphate Binding Cassette (ABC) transporters, and phosphatidylinositol 3-kinase/protein kinase B.[5-7]

In cancer patients, radioresistance may occur simultaneously with chemoresistance.[8] Furthermore, resistance to conventional anti-cancer therapies remains a major obstacle in achieving successful treatment, highlighting the urgency of improving tools for eliminating minimal residual disease in order to prevent metastasis and to improve the therapeutic effects in breast cancer patients.

Cancer stem cells (CSCs) are the small number of cells within a tumor that drive the tumor's growth. These cells are capable of self-renewal and differentiations leading to the heterogeneous nature of cancer cells that comprise the tumor.[9, 10] CSCs are now thought to be contributing to drug and radiation resistance in solid tumors. CSCs express specific markers, cells expressing $CD44^+/CD24^-$ and $CD133^+$ on their surface, are thought to be breast cancer stem cells. CSCs in breast cancer also express high levels of drug resistance transporter proteins including P-glycoprotein (P-gp also known as MDR1), Multidrug Resistance-associated Protein 1 (MRP1), and Breast Cancer Resistance Protein (BCRP),[11, 12] which renders them resistant to drug and radiation therapy. Moreover, several studies have demonstrated that ionizing radiation can enrich cancer stem cells *in vitro* and *in vivo*.[13] Therefore, targeting CSCs may be a promising strategy for improving breast cancer treatment.[14, 15]

Alternative therapy by natural products has attracted much attention due to its considerable chemical and biological activity and beneficial potential effects on human health. The anticancer activity research on natural products demonstrated that it can be used for cancer prevention and treatment in combination with current conventional therapies, including chemotherapy and radiotherapy.[16] Maprang Seed Extracts (MPSEs) is the natural product extract from the Thai fruit *Bouea macrophylla* Griffith. It has demonstrated to exhibit anti-proliferative activity against drug-sensitive as well as drug-resistant leukemic and lung cancer cells.[17] Moreover, we demonstrated that MPSEs effectively sensitize breast cancer cells MCF-7 to radiation, and the combination of MPSEs and irradiation increase numbers of cell death in comparison to irradiation alone.[18] However, the benefits of MPSEs in breast cancer treatment related with chemoresistance have rarely been reported.

In the present study, we demonstrated the effects of MPSEs in terms of how they can overcome treatment resistance, especially chemoresistance, in minimal residual breast cancer cells. We performed comparative analysis of the chemoresistance phenomenon in radiation-survived MCF-7 cells (MCF-7/IR6) and MPSEs pretreatment radiation-survived MCF-7 (MCF-7/MPIR6). Our findings demonstrate that MPSEs can be a sensitizer to chemotherapy and may lead to the identification of a new strategy for improving curative effects in breast cancer treatment.

Materials and methods

Chemicals

Doxorubicin (Dox), pirarubicin (THP), (±)-verapamil, MK-571 and novobiocin were purchased from Sigma-Aldrich (Singapore). Maprang Seed Extracts (97% purity by HPLC) were provided by the MSN Nature Solution Ltd., Thailand.

Establishment of resistant breast cancer cell sub-lines and culture conditions

The two resistant breast cancer cell lines were established from the parental cell known as MCF-7 and were used for two different types of treatment. The first sub-line, MCF-7 was exposed to 6 Gy x-rays (Linear Accelerator 6 MV, Primus; Siemens Healthineers, USA) and the surviving cancer cells (MCF-7/IR6) were collected after two months of exposure. The other sub-line known as MCF-7 was pretreated with 50 µg/mL MPSEs for twenty-four hours before being exposed to 6 Gy x-rays. The surviving cancer cells (MCF-7/MPIR6) were collected after four months of exposure. All cell lines were maintained as a monolayer attachment with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 95% air humidified atmosphere and 5% CO_2 at 37°C.

Chemo-resistant pattern determination by MTT assay

Briefly, 5×10^4 cells were seeded per well into 24-well plates and were cultured for twenty-four hours before being treated with various concentrations of doxorubicin. After forty-eight hours of treatment, 1 mg/mL MTT solution was added and was incubated for four hours to form formazan crystals. These crystals were later dissolved by DMSO. The absorbance was measured at 550 nm. Cell viability (%) = $(OD_1/OD_0)\times100$ was calculated, where OD_1 and OD_0 were the absorbance of treated and untreated cancer cells, respectively. The cytotoxicity curve was plotted and the concentration of doxorubicin required for inhibiting 50% of cell growth (IC_{50}) was determined.[19]

MDR gene expression by RT-PCR (Reverse Transcription -Polymerase Chain Reaction)

The total RNA was extracted with an E.Z.N.A.® Total RNA Kit I (OMEGA bio-tek, USA) and reverse transcription reactions were performed using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover kit (TOYOBO, Japan) following the manufacturer's guidelines. PCR was performed in 10 µL of reaction mixture at 40 cycles for *ABCB1* and 30 cycles for *GAPDH, ABCC1*, and *ABCG2*. PCR products were resolved in 1.5% agarose gel that was stained with redsafe™

nucleic acid staining solution. *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control. The primer sequences are listed below.[20]

ABCB1 Forward primer: 5'- CTGGTTTGATGTGCACGATGTTGG - 3'
Reverseprimer:5'-TGCCAAGACCTCTTCAGCTACTG-3'
ABCC1 Forward primer: 5'- CAGATGACACCTCTCAACAAAACC - 3'
Reverse primer: 5'- GCTAGGGCACACACTAGGGCT - 3'
ABCG2 Forward primer: 5'- CATGTTAGGATTGAAGCCAAAGGC - 3'
Reverse primer:5'-TGTGAGATTGACCAACAGACCTGA-3'
GAPDH Forward primer: 5'- CACCATCTTCCAGGAGCGAGATC - 3'
Reverse primer: 5'- GTGGTGCAGGAGGCATTGCTGA - 3'

Cellular drug uptake and the MDR protein-mediated drug efflux function by spectrofluorometry

The rationale and validation of the experiment setup for monitoring the kinetics of pirarubicin uptake and MDR protein- mediated drug efflux function in cancer cells had been widely described.[21, 22] In order to initiate the monitoring process, 2×10^6 cells were incubated in two mL HEPES-Na^+ buffer pH 7.30 in the presence of 10 mM NaN_3 for thirty minutes. The absence of glucose was necessary in order to off-function MDR proteins since there is no energy. The solution was placed in a 1 cm quartz cuvette that was continuously stirred at 37 °C. Next, 1 µM of pirarubicin was added and the fluorescence intensity at 590 nm (excited at 480 nm) was observed as a function of time by using a spectrofluorometer (Perkin Elmer Luminescence Spectrometer LS 50B). The initial rate of pirarubicin uptake was determined as a decrease in fluorescence intensity due to quenching after intercalation between base pairs of DNA. At steady state, 5 mM glucose was added to be the substrate of glycolysis to permit ATP production that led MDR proteins to re-function which can be observed as an increase in fluorescence intensity. The MDR protein-mediated drug efflux function could be determined as the slope of tangent, dF/dt after glucose addition.

To create condition for inhibition, the experiment was performed in the same manner as described above with notable differences. The difference was the addition of a specific inhibitor for MDR proteins to the cuvette five minutes before the addition of 5 mM of glucose. Verapamil, MK-571, and novobicin were used as a specific inhibitor for P-gp, MRP1, and BCRP, respectively.[23-25]

Statistical analysis

The presentation data is shown as a mean±SD. The difference of means was determined by two sample t-test with the *p* value =0.05, 0.01 and 0.001 for the entire data, using OriginPro 2018 software.

Results

MPSEs decrease chemoresistance of MRD

The chemoresistant pattern of MRD was determined by MTT assay. The cell viability at forty-eight hours after being exposed to doxorubicin (Figure 1a) in MCF-7/IR6 cells was highest, followed by MCF-7/MPIR6, with MCF-7 cell having the lowest viability. The IC_{50} value (Figure 1b) was 215±23.73 (RF=1.0), 342.95±30.94 (RF=1.6), and 282.75±24.64 nM (RF=1.3) for MCF-7, MCF-7/IR6, and MCF-7/MPIR6 cells, respectively. The Resistant Factor (RF) was determined by the IC_{50} of an irradiated cell line divided by IC_{50} of the MCF-7 parental cell line. The results show that MCF-7/IR6 cells were more resistant to doxorubicin than MCF-7/MPIR6 and MCF-7 cells. These findings were statistically significant (p<0.05).

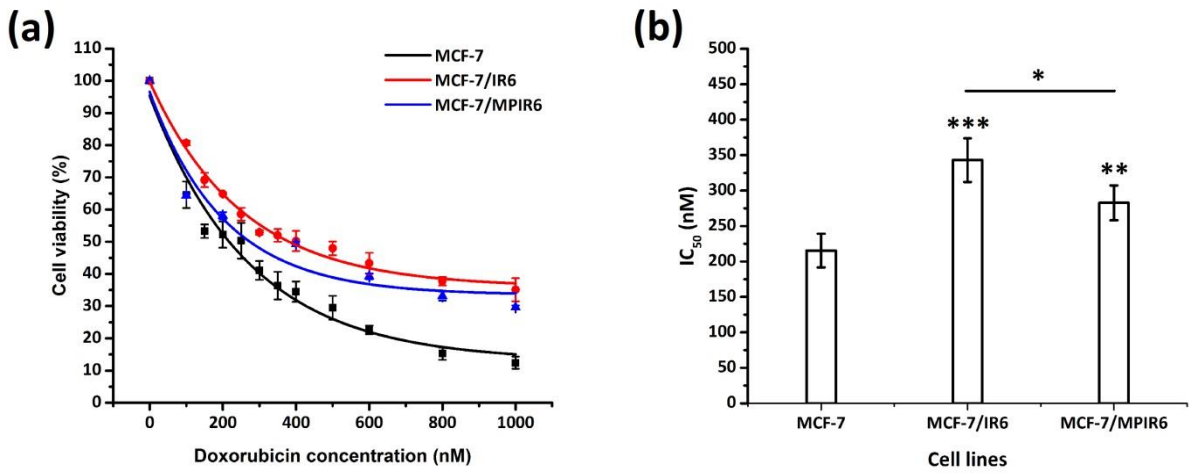

Figure 1. *MPSEs decrease chemoresistance of MRD. (a) Cytotoxicity curve depends on doxorubicin concentration, MCF-7 (black line), MCF-7/IR6 (red line), and MCF-7/MPIR6 (blue line). (b) IC50 value. Data is shown as mean±SD. *p<0.05, **p<0.01 and ***p<0.001.*

MPSEs decrease MDR protein-mediated drug efflux function of MRD

Pirarubicin, a substrate of P-gp, MRP1 and BCRP was used to monitor cellular drug uptake and MDR protein mediated-drug efflux function in MRD breast cancer cells by using spectrofluorometry.[23, 24, 26] The typical pirarubicin uptake and MDR protein-mediated drug efflux is indicated in Figure 2 (a, b, and c). The apparent rate of MDR protein-mediated pirarubicin efflux (V_a) can be determined from the slope of tangent after addition of glucose to the ATP-deprived cells and be calculated by the equation below;

$$V_a = (dF/dt)_t = {}_{glucose} \times (C_T/F_0)$$

Where $(dF/dt)_t = {}_{glucose}$ is the slope of tangent after glucose addition

C_T is the total concentration of pirarubicin (µM)

F_0 is the maximum intensity (a.u.)

As seen in Figure 2c, among the three cell lines, MCF-7/IR6 shows a higher active efflux of pirarubicin than MCF-7/MPIR6 and MCF-7. The efflux rate (V_a) obtained was about 0.0232±0.0001, 0.2308±0.0213 and 0.0678±0.0082 nM. s^{-1} for MCF-7, MCF-7/IR6, and MCF-7/MPIR6 cells, respectively (Figure 2d).

In addition, a specific inhibitor of MDR proteins present in MRD breast cancer (verapamil for P-gp, MK-571 for MRP1, and novobiocin for BCRP) was used to inhibit the MDR protein-mediated drug efflux functioning. MCF-7/IR6 was used as a model to test the inhibition effect of inhibitors because it has the highest potential of drug efflux function. The typical pattern of pirarubicin uptake while using inhibitors is shown in Figure 3a and the decrease in efflux rate was observed as a function of concentration. The efflux rate had indeed decreased. However, when using the two inhibitors (Figure 3b), verapamil and MK-571, there was no noticeable effect for novobiocin. Verapamil and MK-571 can decrease efflux rate about 30% and 20%, respectively at the concentration of 10 µM. MPSEs aberrant multidrug resistance gene expression of MRD

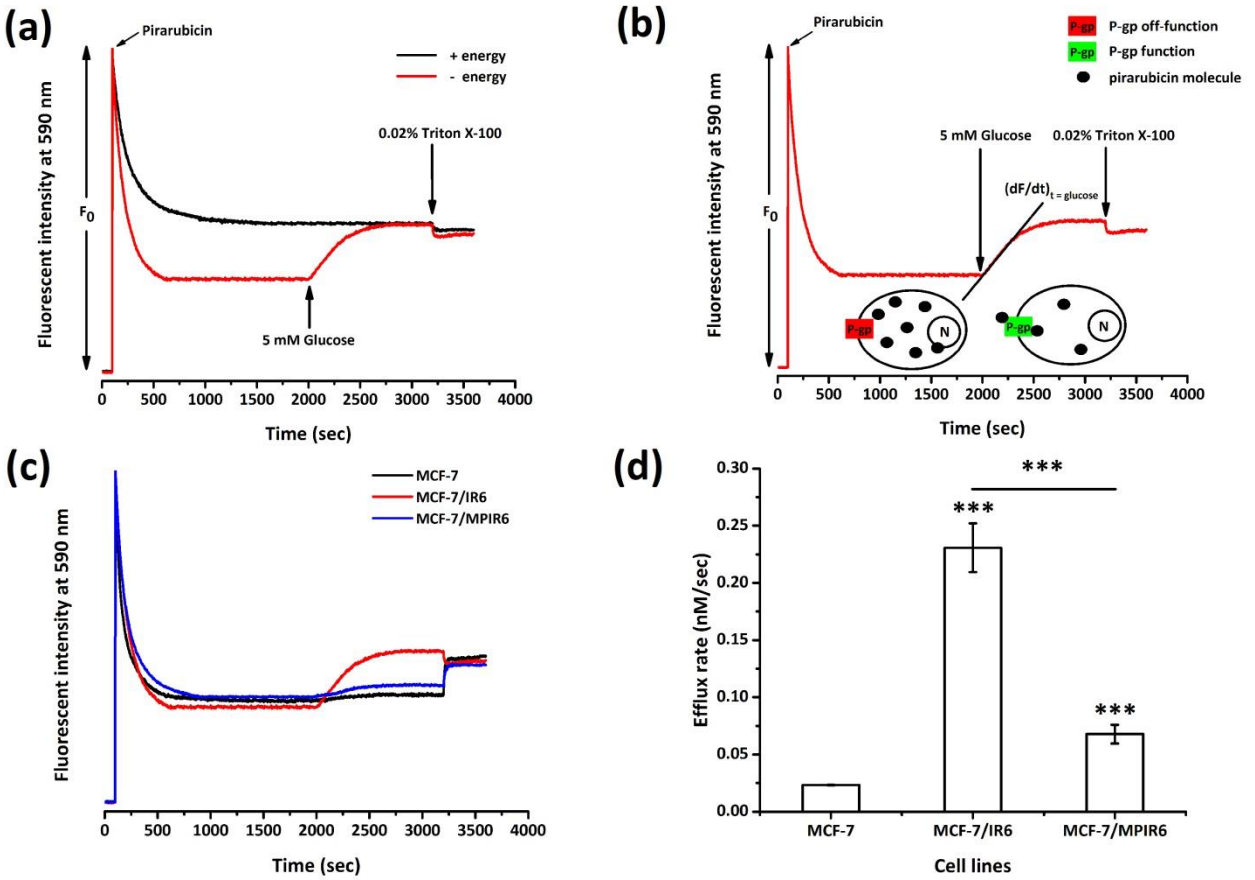

Figure 2. *Typical kinetics pattern of pirarubicin (THP) influx and efflux by resistant breast cancer cell, MCF-7/IR6. (a) Typical pattern of THP uptake by breast cancer cells in a condition with and without energy. F = fluorescent intensity at 590 nm (excited at 480 nm) observed as a function of time. After adding pirarubicin, the maximum intensity (F_{max}) and the C_T (concentration of pirarubicin that add into solution) were yield. F_0 was calculated from F_{max} – background. (b) At steady state, 5 mM glucose was added and the observing of an increasing in fluorescent intensity and slope of tangent, $(dF/dt)t={}_{glucose}$ was monitored. (c) The typical pattern of pirarubicin uptake in breast cancer cell. (d) The MDR protein-mediated drug efflux rate. Data was presented as mean±SD. *p<0.05, **p<0.01, ***p<0.001.*

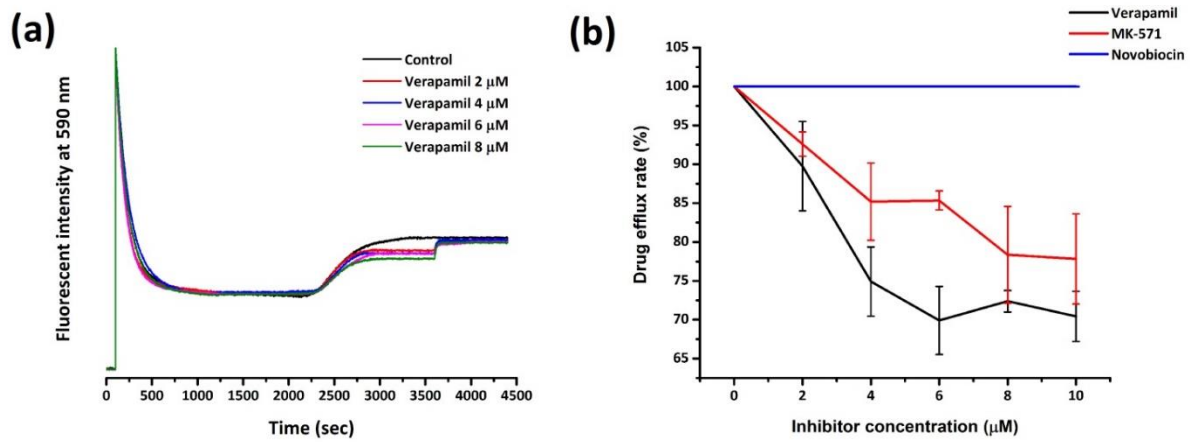

Figure 3. *(a) Typical kinetics of pirarubicin uptake by MCF-7/IR6 cell with verapamil inhibitor. (b) Effect of inhibitors, verapamil (black line), MK-571 (red line), and novobiocin (blue line) on the THP efflux rate of MCF-7/IR6 cell.*

It is well known that ABC transporters play an important role in drug efflux function by utilizing the ATP energy. The ABC transporters which help cancer cells resist against chemotherapeutic drugs are P-gp, MRP1, and BCRP which are encoded as *ABCB1*, *ABCC1*, and *ABCG2* genes, respectively. In this study, we found that two types of MRD (MCF-7/IR6 and MCF-7/MPIR6) were different in MDR gene expression patterns (Figure 4a). We determined that ionizing radiation at 6 Gy can diminish the mRNA expression of *ABCC1* and *ABCG2*, but also found that it modulated increasing expressions of *ABCB1* mRNA when compared with its parental cell (MCF-7). Additionally, the MDR mRNA expression pattern of MCF-7/MPIR6 was similar to that of MCF-7.

The relative MDR gene expression value is shown in Figure 4b. For *ABCB1* gene, this value was 1.00, 1.18±0.043, and 1.10±0.003 in MCF-7, MCF-7/IR6, and MCF-7/MPIR6, respectively. In addition, this value was 1.00 and 0.98±0.05 in MCF-7 and MCF-7/MPIR6, respectively for *ABCC1* genes. Finally, for *ABCG2* gene, this value was 1.00 and 1.02±0.07 in MCF-7 and MCF-7/MPIR6, respectively.

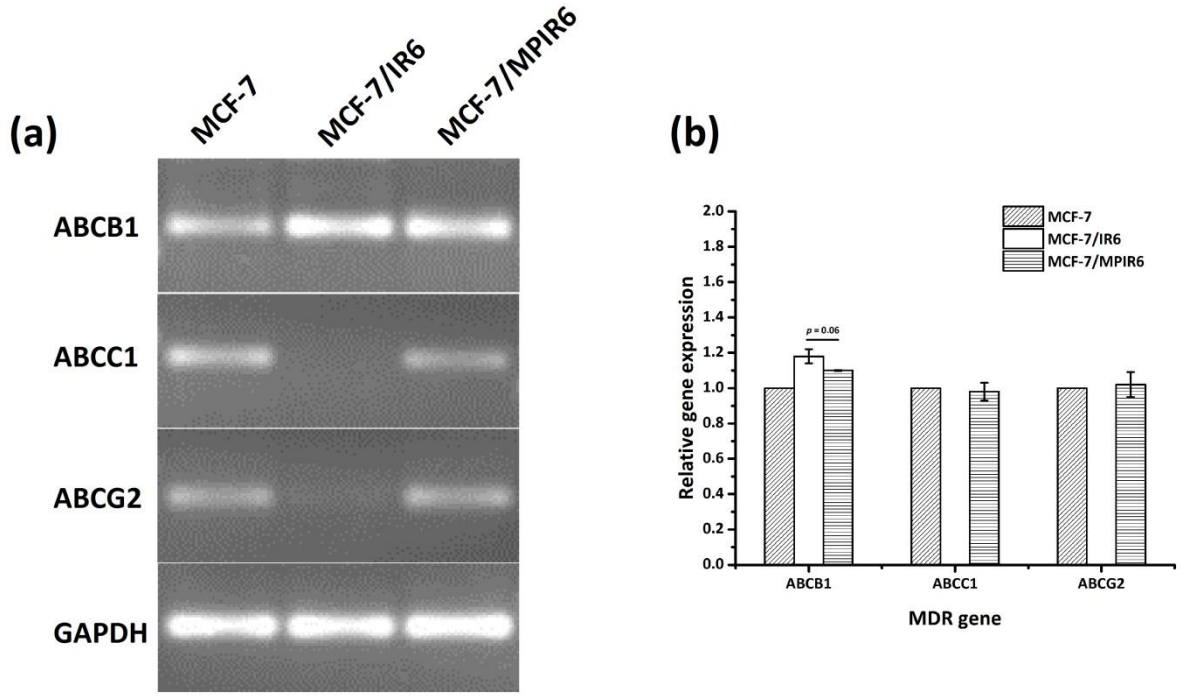

Figure 4. *ABCB1, ABCC1 and ABCG2 mRNA expression by RT-PCR. (a) MDR gene expression pattern of MCF-7, MCF-7/IR6 and MCF-7/MPIR6 cells. (b) Relative MDR gene expression.*

Discussion

Tumor metastasis and recurrence following primary treatment with conventional therapies may develop from the treatment resistant preexisting CSCs such as minimal residual disease or from cancer cells that acquire stemness capacities due to chemo/radiotherapy induced changes in the tumor and microenvironment.[27, 28] In this study, we reported that breast cancer cells MCF-7 that survived ionizing radiation treatment (MCF-7/IR6) have a chemoresistant phenotype by expressing high levels of MDR1 (*ABCB1*), but not MRP1 (*ABCC1*) or BCRP (*ABCG2*). Overexpression of MDR1 was associated with the high activity of P-gp pump and poor response to doxorubicin that is found in MCF-7/IR6 when compared to its parental cells MCF-7. We found that pretreatment of MCF-7 with MPSEs before irradiation has a reversal effect on chemoresistance phenomenon of survived cells (MCF-7/MPIR6). The results detected by RT-PCR indicated that MCF-7/MPIR6 cells decreased the P-gp mRNA (*ABCB1*) expression yet still expresses MRP1 and BCRP mRNA the same way as its MCF-7 parental (Figure 4a). It should be noted that MPSEs can suppress chemoresistance phenomenon of MCF-7/IR6 cells.

Maprang seed extracts (MPSEs) from the Thai fruit *Bouea macrophylla* Griffith exhibits anti-proliferative activity against drug-sensitive as well as drug-resistant leukemic and lung cancer cells. Previous studies suggest that ethanolic extracted MPSEs have more effect on drug-resistant leukemic (K562/adr) and lung (GLC4/adr) cancer cells, although the cells selected exhibited an overexpression of P-gp and MRP1.[17] However, it is unclear whether MPSEs can actually affect drug-resistant cancer cells. In addition, several studies have shown the reversal effects of polyphenol in drug-resistant cell lines, which is a polyphenol suppressed P-gp expression that functions as the main reason for the reversal effect.[29, 30]

We investigated the MDR protein-mediated drug efflux function and found that MCF-7/IR6 has the highest potential drug efflux function, while MCF-7/MPIR6 has a lower rate of extrude drug. In addition, we have investigated the expression level of MDR genes (*ABCB1*, *ABCC1* and *ABCG2*) to confirm MDR proteins function. The MDR protein mediated efflux pump was identified as ATP-binding cassette (ABC) transporters. Among them P-gp is the most important ABC transporter. Notably, another three eminent ABC transporters that were attributed to anticancer drug uptake and efflux such as MRP1, MRP3, and BCRP do not correlate as closely as P-gp with chemoresistance phenotype.[31] We demonstrated that radiation diminished *ABCC1* and *ABCG2*, but increased *ABCB1* expression in MCF-7/IR6 cells. Also, MCF-7/MPIR6 that had completely expressed three types of MDR genes including *ABCB1*, *ABCC1*, and *ABCG2* had lower regulation of *ABCB1* than MCF-7/IR6 coincident with the efficiency of drug efflux function that was significantly lower than that of MCF-7/IR6. Interestingly, we found that the MCF-7/MPIR6 cells that survived from irradiation in the presence of MPSEs show the same genetic profile as parental cells MCF-7 (Figure 4a).

To further confirm multidrug resistance protein function, we used specific inhibitors to test their activity in MCF-7/IR6 cells. Verapamil, a specific inhibitor of P-gp proteins, significantly decreased the drug efflux function of MCF-7/IR6 cells that showed an overexpression of *ABCB1* genes which encoded the expression of P-gp proteins and plays a key role in chemoresistance. Surprisingly, we found that in the presence of specific inhibitors of MRP1 protein (MK-571), MCF-7/IR6 also exhibit decreased drug efflux function. According to the mRNA expression profile we did not observe MRP1 mRNA expression in MCF-7/IR6 cells. It should be noted that ABC transporters such as P-gp, MRP1 and BCRP co-expressed in tumors show a broad and overlapped specificity for substrates and MDR modulators.[32] However, further studies are needed to confirm this underlying mechanism.

Our findings demonstrate that MPSEs could have ability to overcome chemoresistance phenomenon in cancer cells that had survived irradiation treatment. We found that pretreatment with MPSEs before irradiation treatment altered the resistance features of survived-cancer cells after normal treatment and that these phenomena were accompanied by a reversal in P-gp mRNA expression. Furthermore, our results also suggest that breast cancer cells that survived ionizing radiation treatment have a chemoresistant- and radioresistant phenotype.

Conclusion

Maprang Seed Extracts (MPSEs), the natural product extract from the Thai fruit *Bouea macrophylla* Griffith, can suppress chemoresistance properties of breast minimal residual diseases through prevention of ionizing radiation induced upregulation of *ABCB1* gene expression and protein function. Therefore, MPSEs might be used in combination with conventional therapy as a novel strategy for improving the curative effects in breast cancer treatment.

Acknowledgements

The current study was supported by The Graduate School and Faculty of Associated Medical Sciences, Chiang Mai University, Thailand.

Conflicts of interest statement

The authors declare no conflict of interest.

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Journal of Associated Medical Sciences 2019; 52 (3): 193-198

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Effects of low-dose X-rays on the oxidative state, lipid peroxidation and membrane fluidity of human peripheral blood mononuclear cells

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ARTICLE INFO

Article history:
Received 13 May 2019
Accepted as revised 25 July 2019
Available online 14 August 2019

Keywords:
Low-dose X-rays, human peripheral blood mononuclear cell, reactive oxygen species, lipid peroxidation, membrane fluidity, malondialdehyde

ABSTRACT

Background: There is a concern about the effects of low-dose radiation used in medical applications due to risk of biological effects, as these effects have not been fully evaluated.

Objectives: This study aimed to evaluate the effects of low-dose X-ray on the intracellular reactive oxygen species (ROS), lipid peroxidation, membrane fluidity, and cell viability of human peripheral blood mononuclear cells (PBMCs).

Materials and methods: Cells were irradiated using an X-ray generator at the radiation energy of 120 kVp to obtain the absorption dose of 0.05, 0.1 and 0.2 Gy. Fluorescent probe 2'7'-dichlorofluorescein diacetate (DCFH-DA) was used to evaluate intracellular reactive oxygen species of PBMCs. Thiobarbituric acid reactive substances assay (TBARS) was applied to determine malondialdehyde (MDA) level which is an indicative of lipid peroxidation. Membrane fluidity was also determined by fluorescence anisotropy of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Finally, cell viability was determined by resazurin assay.

Results: The instant effects of low-dose X-rays show a significant decrease in ROS level at 0.1 and 0.2 Gy. MDA level per cell of non-irradiated PBMCs was 6.12±1.67 (SD) fmole per cell. There are no significant alteration of MDA level and membrane fluidity from the effects of X-ray at doses up to 0.2 Gy. The cell viability at 72 hours after irradiation at 0.2 Gy shows a significant decreased.

Conclusion: Low-dose of X-rays on human peripheral blood mononuclear cells (PBMCs) shows a significant decrease in the intracellular reactive oxygen species (ROS) (0.1 and 0.2 Gy) and cell dead (0.2 Gy).

Introduction

Due to risk of biological effects from low-dose ionizing radiation used in medical imaging, radiation effects at the subcellular level need to be better evaluated. Intracellular biochemical changes such as damage of DNA, lipids, and proteins can be provoked from ionizing radiation due to direct energy transfer and by free radicals from water radiolysis. High linear energy transfer (LET) radiation can directly damage DNA, while the low LET radiations (i.e. γ-rays, X-rays) can cause damage by oxidative production.[1-6] LET value of diagnostic X-ray is about 3 keV.μm^{-1}.[7] Ionizing radiation is the most important physical stimulus that causes intracellular reactive oxygen species (ROS) formation. In radiation therapy, ROS induces cancer cell death by biochemical changes not only from DNA damage, but also from oxidative membrane damage that causes defects in the cellular mechanism. Membrane damage by ROS occurs as the lipid peroxidation of membrane leads to an altering fluidity and permeability.[2] In high dose X-rays (20-100 Gy), double strand DNA breakage

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doi: 10.14456/jams.2019.29
E-ISSN: 2539-6056

that causes cell damage can be determined.[8] High dose radiation also induces lipid peroxidation and increases the membrane fluidity at the interface between proteins and lipids in cell membranes. The lipid peroxidation can be acquired from unsaturated fatty acid interactions with reactive oxygen species to produce lipid hydroperoxides (LOOH), which then form secondary products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). MDA can induce cellular damage or tissue injury.[9, 10] A study done on high dose radiation (up to 50 Gy) revealed the death of PBMC cells and the increase of membrane fluidity.[11, 12] Despite the fact that radiation induces oxidative stress leading to lipid peroxidation, at very low-doses radiation (0.1 mGy), is contradictory effects on MDA values of different tissues, For example, there are decreases in brain tissue, but increases in lung tissue in experiments done using mice models.[13] Diagnostic radiation doses for general procedures are in range of 0.001-1.5 mGy, while, more special procedures reach up to 10 mGy. Radiation dose of the organ in the beam during CT scanning ranges from 10-100 mGy.[14] In fact, late effects of low-dose radiation of γ-rays were studied by our team in animal models which showed that the effect on genomic instability depended on radiation dose, duration after irradiation and animal type. Our previous data showed no evidence of genomic instability being induced by low-doses.[15-17] DNA is considered to be a principle cellular target for stochastic effects of radiation on cells that occur either directly or indirectly. During irradiation, free radicals are always generated and act as an indirect effect on cellular targets. While DNA damage leads to chromosomal aberrations, gene mutations, and then cell death, defects in other organelles or cellular mechanisms such as mitochondria, endoplasmic reticulum, or plasma membrane can occur, as well. Additionally, reactive oxygen species can also cause cell malfunction.[18-21] Indeed, the interaction of radiation with cell organelles is not a selected target. Aside from DNA, other intracellular targets for determining the early responses to radiation effects should be investigated. Among cellular organelles, plasma membrane is a cellular frontier that involves many cellular mechanisms such as transport controllers, cellular identified markers, immune responses, etc. Therefore, damage to plasma membrane should be a major concern. To clarify effects of radiation in diagnostic procedures, this work focuses on the instant effects occurring at the cellular level of low-dose X-ray (up to 0.2 Gy) that induce reactive oxygen species, lipid peroxidation, membrane fluidity, and cell viability of human peripheral blood mononuclear cells (PBMCs).

Materials and methods

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were collected from buffy coat of healthy donors (age 20-30 years old, blood group O) who had no record of thalassemia and glucose-6-phosphate dehydrogenase deficiency. PBMCs were separated by a gradient density centrifugation technique using ficoll hypaque solution (Lymphoprep™, Norway).[11] Cells were cultivated in an RPMI-1640 medium (Capricorn-scientific, German) supplemented with 10% fetal bovine serum (Capricorn-scientific, German) and 1% penicillin-streptomycin (Capricorn-scientific, German) and they were incubated at 37 °C humidified with 5% CO_2 until time of irradiation.

This study was approved by the Ethical Committee of the Faculty of Associated Medical Sciences, Chiang Mai University (Reference number: AMSEC -62EM-007).

Radiation exposure

Accuracy and linearity of radiation output of X-ray machine (Shimadzu, Collimator type R-20J, Japan) were evaluated first, using a Scintillation detector (Radcal, AGMS-D+, USA). For irradiation, an appropriate amount of PBMCs for each experiment was collected from a culture medium and kept in a volume of 100 µL of phosphate buffer solution (PBS) pH 7.4, then was exposed at 120 kVp and 320 mA.

Measurement of intracellular reactive oxygen species levels

Intracellular reactive oxygen species level in PBMCs was determined by using 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich, USA) as a molecular probe. Irradiated PBMCs (1×10^6 cells) were incubated in 900 µL PBS containing 2 µM of DCFH-DA for 30 minutes. Dichlorofluorescein (DCF) fluorescence intensity representing reactive oxygen species level was measured using a spectrofluorometer (Perkin Elmer, model LS55, USA) at 523 nm when excited at 502 nm.[22]

Measurement of lipid peroxidation

Lipid peroxidation level was measured by using thiobarbituric acid reactive substances assay (TBARS). This method directly measures malondialdehyde (MDA) that is a degradation product of fats as lipid peroxidation. Irradiated PBMCs ($4x10^6$ cells) were incubated at 80 °C for 1 hr in a solution containing 400 µL PBS and 500 µL of working reagent consisting of 2 $mg.mL^{-1}$ of 2-thiobarbituric acid (TBA; Sigma-Aldrich, Japan) solubilized in a mixture of 50 mM NaOH (RCl labscan, Thailand) and glacial acetic acid (Fisher Scientific, UK) with a ratio of 1:1. Next, this solution was completed by cooling down at 25 °C for 10 minutes. An absorption spectrum of MDA-TBA reaction product was determined by spectrophotometer (Agilent, model 8453, China).[23, 24] MDA standard curve was generated using an MDA standard range of 1 to 100 µM.

Membrane fluidity

Membrane fluidity of PBMCs was observed using fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma-Aldrich, Japan). Irradiated cells ($5x10^5$ cells) in 100 µL of PBS were added into 0.1 µM DPH solution (1,900 µL), and incubated for 10 minutes. Next, the fluorescence intensity at 430 nm (when excited at 350 nm) was determined using a spectrofluorometer. Fluorescence anisotropy (r) value was calculated as follows:[25, 26]

$$r = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2G.I_{VH}}$$

Where I_{VV} : fluorescence intensity of components for vertical/vertical (parallel),

I_{VH} : fluorescence intensity of components for vertical/horizontal (perpendicular),

Correction factor G : ratio of I_{HV}/I_{HH}

Cell viability

Cell viability was determined by fluorescent resazurin sodium salt (Sigma-Aldrich, Japan). Irradiated PBMCs (5×10^4 cells) were cultivated in a 900 µL RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C humidified with 5% CO_2 for 72 hrs. To investigate cell viability, 100 µL of resazurin solution (0.1 $mg.mL^{-1}$) was added and cells were incubated at 37 °C and humidified with 5% CO_2 for 4 hrs. Resazurin fluorescence intensity at 590 nm (exited at 570 nm) that is an indicator of living cells was measured on a spectrofluorometer using well-plate reader.[27] Cell viability was quantified by resazurin fluorescence intensity of samples in terms of the percentage of control.

Statistical analysis

Descriptive data were informed as mean±standard error (SE). Statistical analysis was performed by OrigingPro8 Software. All obtained parameters from a variety of radiation doses were analyzed by One Way ANOVA (Tukey procedure). Significance level was denoted at the alpha value of 0.05.

Results

X-rays output showed a good quality either accuracy or linearity with a dose rate of 11.5 $Gy.min^{-1}$ at 120 kVp and 320 mA. The PBMCs for each experiment was exposed at 0.05, 0.1, and 0.2 Gy. All studies were operated in parallel comparison with the non-irradiated group.

The intracellular reactive oxygen species of PBMCs

The fluorescence intensity levles of DCF revealing intracellular reactive oxygen species (ROS) of irradiated PBMCs at 0.05, 0.1, and 0.2 Gy were evaluated and the percentage of non-irradiated cells was reported. The results showed a significant decrease in ROS level at 0.1 and 0.2 Gy. (Figure 1)

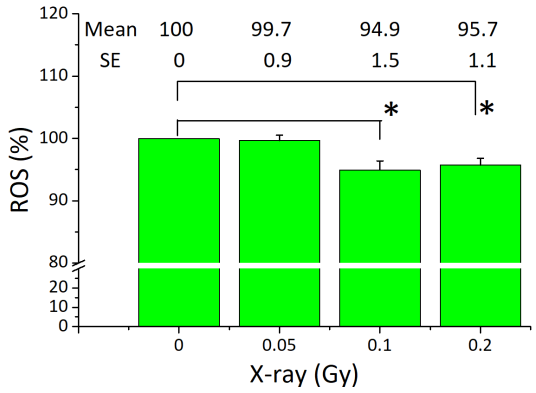

Figure 1. *Percentage of intracellular reactive oxygen species level (ROS) of PBMCs (mean±SE) after irradiation to X-rays dose of 0.05, 0.1 and 0.2 Gy (*p<0.05).*

Lipid peroxidation of PBMCs

Lipid peroxidation of PBMCs was quantitatively measured from optical density of MDA-TBA production at 532 nm. The results demonstrated that intrinsic MDA levels of non-irradiated PBMCs was equal to 6.12±1.67 (SD) fmole per cell. Lipid peroxidation of PBMCs was slightly diminished by the effects of low-dose X-rays, but this was not a significant difference. (Table 1).

Table 1 Malondialdehyde (MDA) levels of PBMCs after the irradiation to X-rays dose of 0.05, 0.1 and 0.2 Gy.

X-ray (Gy)	Mean of MDA (fmole per cell)	SE
0	6.12	0.48
0.05	5.85	0.35
0.1	5.92	0.33
0.2	5.90	0.40

Membrane fluidity of PBMCs

Membrane fluidity of PBMCs taken as an indicator of mobility of membrane lipids was explored by measuring fluorescence anisotropy at 37 °C. Our results showed that anisotropy value at a dose of 0.2 Gy was not significantly altered after irradiation. (Figure 2)

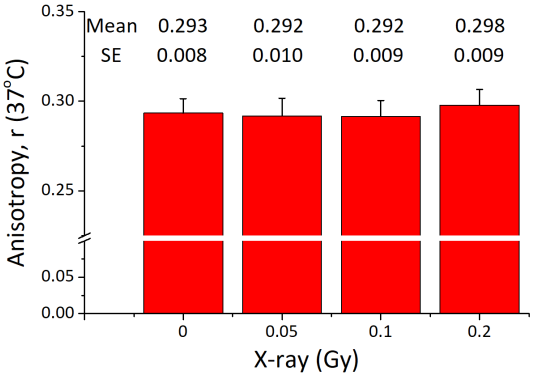

Figure 2. *Fluorescence anisotropy (r) of PBMCs determined at 37 °C after irradiation to X-rays dose of 0.05, 0.1, and 0.2 Gy.*

Cell viability

Effect of low-dose X-rays on cell viability was assessed at 72 hrs after irradiation. Data showed a significant decrease as a function of the increasing absorbed dose. (Figure 3)

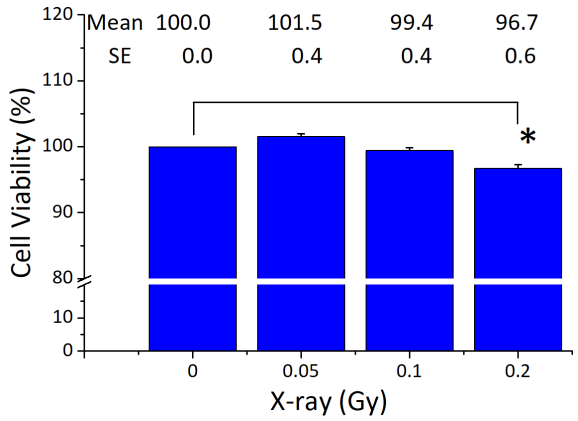

***Figure 3**. Percentage of cell viability of PBMCs after irradiation to X-rays dose of 0.05, 0.1, and 0.2 Gy (*p<0.05).*

Discussion

In this work, normal human peripheral mononuclear cells were used as the cellular model. Intracellular reactive oxygen species (ROS), lipid peroxidation in terms of malondialdehyde (MDA), and membrane fluidity of peripheral blood mononuclear cells (PBMCs) were instantly explored after irradiation by X-ray at 0.05, 0.1, and 0.2 Gy, whereas the cell viability was examined at 72 hours after irradiation. The results indicated a significant decrease in ROS and cell viability and no detectable effect on lipid peroxidation which is also consistent with the absence of influence on membrane fluidity in PBMCs exposed to low-dose X-rays. In normal condition, malondialdehyde is the main lipid peroxidation production that causes free radical such as superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Superoxide radical ($O_2^{\bullet-}$) is catalyzed by superoxide dismutase (SOD) to be hydrogen peroxide (H_2O_2). Then hydrogen peroxide is further catalyzed to produce H_2O.[28, 29] Effects of radiation on oxidant state in living beings is spontaneously regulated depending on the oxidant species, antioxidant mechanism, tissue types, and time. In animal models, superoxide dismutase (SOD) regulate by decreasing its activity at very low-dose of 100 μGy, but continuously increases activity several hours after radiation at doses ranging from 0.05 Gy to 0.2 Gy. This is consistent with our observation in PBMCs and suggests that under low dose X-rays irradiation conditions either the activity or cell concentration of superoxide dismutase increases in order to decrease percentage of cellular irradiation-generated ROS. Alternative mechanisms of cell protection against ROS might be involved since apart from an increase of SOD activity, contradictory results were found in MDA levels.[13, 30] DCFH-DA is a free radical probe that mainly interacts with hydrogen peroxide species (H_2O_2) in intact cell, thus a diminishing of intracellular reactive oxygen species occurs after irradiation which might explain the consequent effects of SOD activity.[31] Similar effects were also observed on normal human lung epithelial cells (HBE135-E6E7) treated with irradiation doses in the range 0.02-0.1 Gy. Moreover, it was observed that X-rays stimulated cell proliferation before appearance of growth inhibition at higher doses of 0.2-3 Gy.[32] Concerning the membrane fluidity, fluorescence anisotropy (r) is observed by fluorescent probe (DPH) that is embedded in the lipophilic part of lipid bilayer. In this work, membrane fluidity did not significantly change from the effects of X-ray at doses up to 0.2 Gy which is in agreement with the results obtained at the same physiological temperature on a previous medical diagnostic X-rays study.[26] The effects of radiation might involve either lipids or membrane proteins that causes membrane rigidity through lipid-protein or protein-protein crosslink.[33, 34] Absence of significant effect on lipid peroxidation under our irradiation conditions is then correlated with the absence of detectable effect on membrane fluidity. This study showed at X-rays doses of 0.05-0.2 Gy no detectable deleterious effects could be observed on cellular membranes. Our results suggest that under the irradiation conditions described the integrity of cellular membranes of PBMCs was conserved.

Conclusion

In summary, low-dose X-ray (0.05-0.2 Gy) lead to decreases in the intracellular reactive oxygen species (ROS) and cell viability in human peripheral blood mononuclear cells (PBMCs). However, radiation doses at 0.05 and 0.1 Gy altered PBMCs by insignificantly diminishing lipid peroxidation (MDA) and increased membrane fluidity. At 0.2 Gy, contradictory evidence was observed. This might be caused by the regulation of cellular antioxidant mechanism.

Conflicts of interest statement

The authors declare none of conflict of interest.

Acknowledgment

The authors would like to thank the Department of Radiologic Technology, Faculty of Associated Medical Sciences Chiang Mai University, for all facillity support.

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Journal of Associated Medical Sciences 2019; 52 (3): 199-204

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Effect of the *XmnI*-^{G}γ polymorphism on HbE and red blood cell parameters of Hb E carriers with and without SEA-α thalassemia 1

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ARTICLE INFO

Article history:
Received 21 June 2019
Accepted as revised 23 July 2019
Available online 14 August 2019

Keywords:
HbE carrier, SEA-α thalassemia 1 carrier, *XmnI*-^{G}γ polymorphism, RBC indices

ABSTRACT

Background: *XmnI*-^{G}γ polymorphism has been found to be the major *cis*-acting factor responsible to increase γ-globin gene activation and closely linked to HbE. It was expected that the polymorphism might modify HbE and RBC indices in HbE carriers.

Objectives: To determine the effect of *XmnI*-^{G}γ polymorphism on RBC indices and HbE levels in single HbE carriers and double HbE/SEA-α thalassemia 1 carriers.

Materials and methods: Samples comprised 160 EDTA blood collected from routine Hemoglobin Typing Laboratories of Sawan Pracharak Hospital, Nakorn Sawan Province and Lampang Regional Hospital, Lampang Province. *XmnI*-^{G}γ polymorphism was determined by PCR-RFLP. SEA-α thalassemia 1 was genotyped by Gap-PCR. HbE level was determined by cation-exchange HPLC, and RBC indices by automated hematology analyzer. Mann-Whitney U test was computed to analyze the data. *P* value of less than 0.05 is considered to be statistically significant.

Results: The prevalence of *XmnI*-^{G}γ (+/+) was 13.9% and 6.2%, of *XmnI*-^{G}γ (+/-) was 66.0% and 81.3%, and of *XmnI*-^{G}γ (-/-) was 20.1% and 12.5% in single HbE carriers and double HbE/SEA-α thalassemia 1 carriers, respectively. Presence of the *XmnI*-^{G}γ site did not affect HbE levels and RBC indices in both single HbE carriers and double HbE/SEA-α thalassemia 1 carriers.

Conclusion: *XmnI*-^{G}γ polymorphism did not affect RBC indices and HbE levels in HbE carriers. It was not a confounding factor to be concerned when considering RBC parameters in screening for HbE/SEA-α thalassemia 1 double carriers and can be ignored.

Introduction

Hemoglobin E (Hb E) ($\alpha_2\beta^E_2$) is an abnormal hemoglobin commonly found in Thailand with the national prevalence of approximately 13% and 50-60% at Thai-Laos-Kampuchea border.[1] This abnormal hemoglobin is resulted from missense mutation (G to A substitution) at codon 26 of β-globin gene, causing change from glutamic acid to lysine. This missense mutation was found to activate cryptic splice site at codon 25 which reduces normal splicing of messenger RNA (mRNA).[2] Therefore, β^E globin chain is synthesized at a reduced rate, and acts as β^+-thalassemia. Compound heterozygosity of β^E gene and $\beta^{Thalassemia}$ gene results in the life-threatening chronic anemia namely HbE/β-thalassemia.[3] To prevent birth of new patient of HbE/β-thalassemia, detecting HbE in parents is essential.

HbE carrier is an individual having HbE gene or β^E-gene in heterozygous form. Clinically, HbE carrier is asymptomatic.[4] Thus, detecting HbE carrier entirely requires laboratory information. Laboratory tests are conventionally employed

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doi: 10.14456/jams.2019.30
E-ISSN: 2539-6056

for detecting HbE carrier include one-tube osmotic fragility test, red blood cell indices especially MCV, dichlorophenol indophenol precipitation (DCIP) test, cation-exchange high performance liquid chromatography (HPLC), and capillary zone electrophoresis (CZE).[5-7]

Our recent survey showed that beside single or pure HbE carriers, the double HbE/SEA- α thalassemia 1 can also be found in Thailand with the approximate prevalence of 11.0% in pregnant women[8] and 11.2% in general population.[9] SEA-α thalassemia 1 is a severe form of α-thalassemia. Co-existence of SEA-α thalassemia 1 in the HbE carrier reduces MCV, MCH, and HbE to the levels lower than those observed in the single HbE carrier.[10] Our survey, however, had found that anemic status of HbE carriers substantially affected the MCV, MCH, and HbE levels, and different cutoff points were established.[10]

XmnI-$^{G}\gamma$ polymorphism (rs 7482144) is the C-T substitution at nucleotide position -158 in promoter region of $^{G}\gamma$ gene. This polymorphism was shown by twin study to be the major cis factor involved in increased F cell production, accounting for 13% of F cell variance.[11] The presence of thymidine nucleotide (T) instead of wild type cytosine nucleotide (C) at this position creates cutting site for the endonuclease *XmnI*, thus so-called *XmnI*-$^{G}\gamma$ site. The *XmnI*-$^{G}\gamma$ site has been shown to be involved in augmentation of the γ-globin gene expression in normal individuals and in those having erythropoietic stress.[12,13] The presence of *XmnI*-$^{G}\gamma$ site was fairly common in Thai population, attaining the frequency of 24.7% and 2.5% for heterozygote and homozygote, respectively (unpublished data). Survey in Thai HbE/β^{0}-thalassemia patients showed that *XmnI*-$^{G}\gamma$ site was closely linked to β^{E} gene, and mild clinical symptoms.[14] Our recent survey in HbE carriers confirmed this study by showing high prevalence of *XmnI*-$^{G}\gamma$ site and its impact on HbE, HbF, and RBC indices levels.[15] However, the relationship of *XmnI*-$^{G}\gamma$ polymorphism on HbE, HbF, and RBC indices in HbE carrier with SEA-α thalassemia 1, so far, has never been evaluated.

Blood samples

One hundred and sixty EDTA blood samples of HbE carriers were collected from routine Hemoglobin Typing Laboratories at Sawan Pracharuk Hospital, Nakorn Sawan Province, and Lampang Hospital, Lampang Province, Thailand. Blood samples were processed anonymously. The protocol of this study was reviewed and approved by the Research Ethics Committee, Faculty of Associated Medical Sciences, Chiang Mai University (Approved number 103/2556)

Determination of RBC parameters and types plus quantities of hemoglobins

Red blood cell parameters including red blood cell count (M/μL), Hb (g/dL), Hct (%), MCV (fL), MCH (pg), MCHC (gm/dL) and RDW (%) were analyzed by automated blood cell counter (Beckman Coulter, Inc. California, USE). Types and quantities of hemoglobins were determined by cation-exchange HPLC (VARIANT™ Hemoglobin Testing System: BioRad Laboratories, Hercules, CA) (Figure 1).

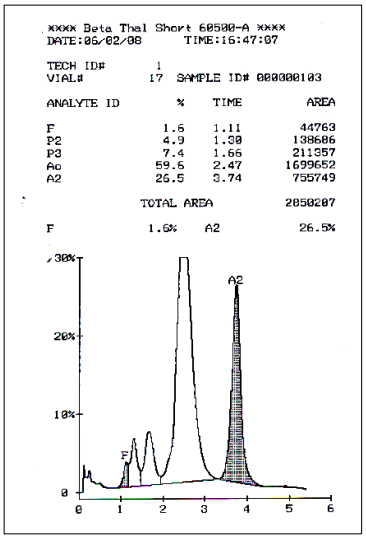

Figure 1. *Hemoglobin pattern and quantities in HbE carrier obtained from the cation-exchange HPLC. HbE is co-eluted with HbA_2 and nominated as "A2". Percentage of 26.5 is a combination of HbA_2 and HbE.*

Preparation of genomic DNA

Genomic DNA was directly prepared from buffy coat using Chelex-100 resin with some modification.[16] Technically, 100 μL of buffy coat was washed consecutively in 1 mL of 0.5% (v/v) Trion X-100 and 1 mL of deionized water (DI). Thereafter, 110 μL of DI and 1-2 drops of 5% (v/v) Chelex-100 suspension (Chelex® 100 Molecular Biology Grade Resin, BioRad Clinical Diagnostics, Hercules, CA) was mixed with pellet and incubated overnight 56°C. The reaction was then heated in boiling water bath for 20 minutes and centrifuged at 12,000g for 1 minute. Finally, supernatant containing genomic DNA was collected and stored at -20°C until use.

Determination of SEA-α thalassemia 1 gene

SEA-α thalassemia 1 gene was determined by Gap-PCR established in our laboratory.[9] This Gap-PCR was able to detect both wild type and SEA deletion in a single reaction with the amplified products sized of 652 bp were wild type and of 762 bp were SEA deletion. Therefore, SEA-α thalassemia 1 carriers had the amplified products of both sizes.

Determination of *XmnI*-$^{G}\gamma$ polymorphism

XmnI-$^{G}\gamma$ polymorphic site (C/T) was determined by PCR-RFLP analysis following the procedure described previously in our laboratory.[15] *XmnI*-$^{G}\gamma$ polymorphism was annotated as *XmnI*-$^{G}\gamma$ (+) for the presence of *XmnI*-$^{G}\gamma$ site ("T" at this point) and as *XmnI*-$^{G}\gamma$ (-) for the absence of *XmnI*-$^{G}\gamma$ site ("C" at this point). By this pattern, *XmnI*-$^{G}\gamma$ (+/+) and (-/-) is indicated homozygote for presence and absence of *XmnI*-$^{G}\gamma$ site, respectively. The heterozygous state of this site would thus have *XmnI*-$^{G}\gamma$ (+/-) genotype.

Statistical analysis

Descriptive statistics, median (min-max), and inferential statistics (Mann-Whitney U test) were analyzed using statistical software. The p value $p<0.05$ was considered statistically significant.

Results

Demographic data

All 160 blood samples were proven to be HbE carriers by cation-exchange HPLC as they all had hemoglobin typing of AE with HbE ranging from 14.1% to 32.5%. All were not anemic as shown by Hb levels >10.0 gm/dL. RBC parameters as well as HbE levels are shown in Table 1. Sixteen samples were found to be double HbE/SEA-α thalassemia 1 carriers, accounting for the prevalence of 10%, while 144 samples were single HbE carriers. Comparing RBC parameters between single HbE carriers and double HbE/SEA-α thalassemia 1 carriers showed that levels of MCV, MCH, and HbE were less in double HbE/SEA-α thalassemia 1 than those in the single HbE carriers (Table 1).

Table 1 RBC parameters and HbE (median, min-max) in all HbE carriers, double HbE/SEA-α thalassemia 1 carriers, and single HbE carriers. The p value of less than 0.05 was considered statistically significant.

RBC parameters	Groups			*p* values
	HbE carriers (n=160)	HbE/SEA-α thalassemia 1 carriers (n=16)	Single HbE carriers (n=144)	
	11.7	11.3	11.7	
Hb (g/dL)	(3.3-25.9)	(6.8-15.3)	(3.3-25.9)	0.325
	35.1	34.0	35.1	
Hct (%)	(9.9-77.7)	(20.4-45.9)	(9.9-77.7)	0.358
	77.3	68.8	77.7	
MCV (fL)	(48.5-114.6)	(48.5-83.5)	(52.0-114.6)	0.001
	25.6	22.6	25.7	
MCH (pg)	(15.0-38.8)	(15.5-27.2)	(15.0-38.8)	0.001
	14.5	15.2	14.5	
RDW (%)	(12.1-43.0)	(14.0-43.0)	(12.1-29.8)	0.424
	27.7	20.1	27.9	
HbE (%)	(14.1-32.5)	(14.1-29.9)	(16.3-32.5)	0.001

Prevalence of *XmnI*-$^{G}\gamma$ site in single Hb E carriers and double Hb E/SEA-α thal 1 carriers

Presence of *XmnI*-$^{G}\gamma$ site (T at nucleotide -158 of $^{G}\gamma$ promoter) found in 150 of 320 chromosomes indicated 0.47 gene frequency. Heterozygote for the presence of this site (*XmnI*-$^{G}\gamma$; +/-) was the most common genotype while homozygote of presence and absence of this site was less common in both single HbE carriers and double HbE/SEA-α thalassemia 1 carriers (Table 2).

Table 2 Prevalence of *XmnI*-$^{G}\gamma$ polymorphism in HbE carriers with and without SEA α-thalassemia 1.

Groups	*XmnI*-$^{G}\gamma$ genotype	Number of cases	Prevalence [%]
HbE/SEA-α thalassemia 1 carriers (n=16)	-/-	2	12.5
	+/-	13	81.3
	+/+	1	6.2
Single HbE carriers (n=144)	-/-	29	20.1
	+/-	95	66.0
	+/+	20	13.9

Comparing of HbE levels and red blood cell parameters in single HbE carriers in the presence and absence of *XmnI*-$^{G}\gamma$ site

Single HbE carriers were divided according to the presence and absence of *XmnI*-$^{G}\gamma$ site. Twenty nine samples were found to be homozygote for absence of the site (*XmnI*-$^{G}\gamma$; -/-). Ninety-five and twenty samples were found to be heterozygote (+/-) and homozygote (+/+) of *XmnI*-$^{G}\gamma$ site, respectively. All red blood cell parameters analyzed and HbE levels were not different among samples with and without *XmnI*-$^{G}\gamma$ site (Table 3). However, trend of low Hb, Hct, HbE, and high MCV, MCH, RDW was observed in group having *XmnI*-$^{G}\gamma$ site (Figure 2).

Table 3 Comparison of RBC parameters and HbE levels (median, min-max) in single HbE carrier without (-/-) and with (+/- and +/+) the ***XmnI***-$^{G}\gamma$ site. The p value of less than 0.05 was considered statistically significant.

RBC parameters	Groups		*p* values
	Single HbE carriers without *XmnI*-$^{G}\gamma$ site (-/-) (n=29)	Single HbE carriers with *XmnI*-$^{G}\gamma$ site (+/-, +/+) (n=115)	
	12.2	11.6	0.237
Hb (g/dL)	(3.3-15.6)	(3.7-25.9)	
	35.9	34.9	0.221
Hct (%)	(9.9-46.2)	(11.1-77.7)	
	76.7	77.9	0.489
MCV (fL)	(57.2-89.3)	(52.0-114.6)	
	25.6	25.8	0.353
MCH (pg)	(16.0-28.8)	(15.0-38.8)	
	14.1	14.5	0.190
RDW (%)	(12.4-21.6)	(12.1-29.8)	
	27.8	27.9	0.696
HbE (%)	(23.3-30.5)	16.3-32.5)	

Comparison of RBC parameters and HbE levels in double HbE/SEA-α thalassemia 1 carriers in the presence and absence of *XmnI*-$^{G}\gamma$ site

Double HbE/SEA-α thalassemia 1 carriers were divided into 2 groups according to the presence and absence of *XmnI*-$^{G}\gamma$ site. Two samples having *XmnI*-$^{G}\gamma$ site in both heterozygous and homozygous form; (+/-, +/+). No difference of both RBC parameters and HbE levels was also observed between these two groups (Table 4). However, trend of low Hb, Hct, HbE, and high MCV, MCH, RDW was observed in group having XmnI-$^{G}\gamma$ site (Figure 2)

Table 4 Comparison of RBC parameters and HbE levels (median, min-max) in double HbE/SEA-α thalassemia 1 carriers without (-/-) and with (+/-, +/+) the *XmnI*-$^{G}\gamma$ site. The p value of less than 0.05 was considered statistically significant.

RBC parameters	Groups		*p* values
	HbE/SEA-α thalassemia 1 without *XmnI*-$^{G}\gamma$ site (-/-) (n=2)	HbE/SEA-α thalassemia 1 with *XmnI*-$^{G}\gamma$ site (+/-, +/+) (n=14)	
Hb (g/dL)	12.2	10.4	0.525
	(12.1-12.3)	(6.8-15.3)	
Hct (%)	36.8	31.1	0.634
	(36.4-37.2)	(20.4-45.9)	
MCV (fL)	68.0	69.0	0.525
	(66.8-69.2)	(48.5-83.5)	
MCH (pg)	22.5	22.6	0.874
	(22.0-23.0)	(15.5-27.2)	
RDW (%)	14.5	15.5	0.203
	(14.0-15.0)	(14.1-43.0)	
HbE (%)	21.8	20.0	0.340
	(21.1-22.5)	(14.1-29.9)	

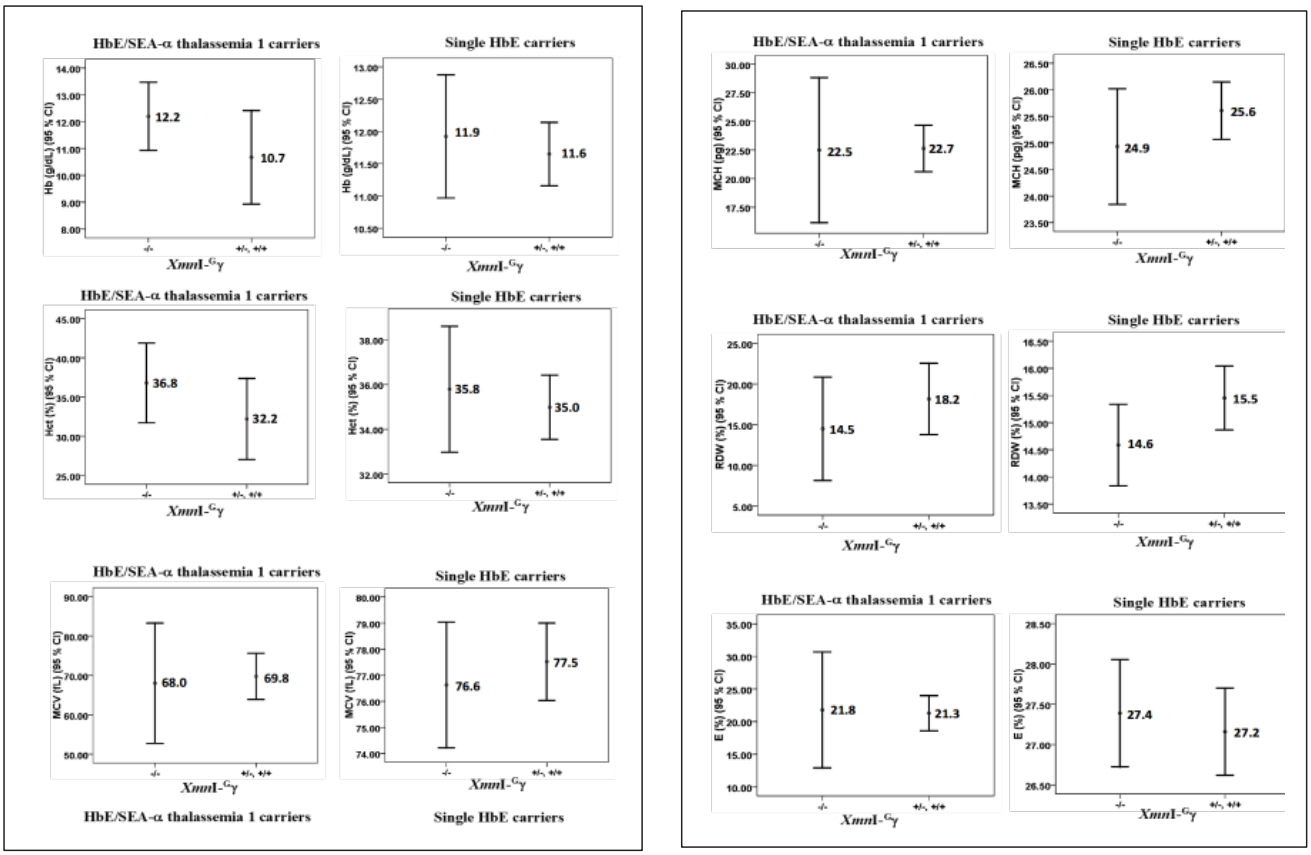

Figure 2. *RBC parameters and HbE levels in single HbE carriers and HbE/SEA-α thalassemia 1 carriers with and without XmnI-$^{G}\gamma$ site. Note that identical relationships of RBC parameters, HbE levels and XmnI-$^{G}\gamma$ polymorphism in single HbE carriers and HbE/SEA-α thalassemia 1 carriers are observed. Numbers at the middle of each bar indicate mean values.*

Discussion

Both HbE and SEA-α thalassemia 1 are common in Thailand and coexisting of these disorders is inevitable. Coexisting of SEA-α thalassemia 1 in HbE carrier causes reduction of MCV, MCH, and HbE levels to the level atypical for traditional or single HbE carrier, *i.e.* HbE carrier without coexisting SEA-α thalassemia 1. This phenomenon has been well described by several authors[8, 17-19] and also by our previous survey.[10]

XmnI-$^{G}\gamma$ site is involved in increased HbF/F cell productions and improvement of clinical phenotype. Our present results showed that RBC indices and HbE levels were not different between samples with or without *XmnI*-$^{G}\gamma$ site in both single HbE carriers and double HbE/SEA-α thalassemia 1 carriers. This meant that *XmnI*-$^{G}\gamma$ site did not have the modifying effect on RBC indices and HbE levels in both groups of HbE carriers. This result was similar to that shown in our previous survey that presence of the *XmnI*-$^{G}\gamma$ site was mildly related to increased MCV, MCH, HbF and lowered HbE levels in HbE carriers, but the levels of these parameters were substantially overlapped.[15] This further confirmed that *XmnI*-$^{G}\gamma$ had the minimal impact on this phenotype in HbE carriers in this cohort. This should be explained by the fact that HbE carrier is a mild form of β-hemoglobinopathy having small degree of erythropoietic stress, which was not appropriate for maximal *XmnI*-$^{G}\gamma$ action.[4, 20]

Although the sample size of double HbE/SEA-α thalassemia 1 carriers and those having no *XmnI*-$^{G}\gamma$ site were quite small, the results of this study should preliminarily establish the conclusion that *XmnI*-$^{G}\gamma$ polymorphism did not have significant effect on all RBC parameters and HbE levels in HbE carriers. Therefore, *XmnI*-$^{G}\gamma$ polymorphism may be ignored in screening for the double carriers of HbE and SEA-α thalassemia 1. Continued study with increased sample size would greatly be invaluable.

Conflicts of interests

Authors declared no conflict of interest.

Acknowledgements

The authors thanks Sawan Pracharak Hospital and Lampang Regional Hospital for providing blood samples. This study was supported by Higher Education Research Promotion, Office of Higher Education Commission of Thailand.

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Journal of Associated Medical Sciences 2019; 52 (3): 205-211

JAMS
Journal of Associated Medical Sciences

Thai-Journal Citation Index Centre (TCI) & ASEAN Citation Index (ACI)

Journal of Associated Medical Sciences

Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index

Improvement of attention and episodic memory functions in community-dwelling elderly with cognitive impairment: A preliminary study using a multimodal method

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ARTICLE INFO

Article history:
Received 13 August 2019
Accepted as revised 23 August 2019
Available online 28 August 2019

Keywords:
Community-dwelling elderly, cognitive impairment, multimodal episodic memory training, multisensory stimulation, mindfulness-based yoga

ABSTRACT

Background: Global geriatric populations will result in increased rates of cognitive decline and dementia; elderly people may suffer from deterioration in cognitive ability, and particularly in attention and memory, that affects their functional independence. Thus, the combination methods of cognitive training are beneficial for enhancing their cognitive abilities and in slowing down the rise in numbers of demented elderly who live with cognitive impairment in the community.

Objectives: To determine the effects of a multimodal episodic memory training (MEMT) protocol on attention and episodic memory performance in community-dwelling elderly with cognitive impairment.

Materials and methods: Participants were the older adults living in community who are the members at the elderly school in the suburban municipal district, Chiang Mai province, Thailand. Twenty elderly with cognitive impairment were recruited through purposive sampling and assigned to the experimental group (n=10) and control (n=10). The experimental group received in a 36-sessions multimodal episodic memory training (MEMT) protocol including episodic memory training, multisensory stimulation, and mindfulness-based yoga training over 12 consecutive weeks. The attention and episodic memory scores were measured at baseline and after the intervention.

Results: Participants receiving the MEMT protocol intervention demonstrated statically significant improvement ($p<0.05$) on attention and memory as compared with the control group, which showed no statically significant differences at the post-intervention.

Conclusion: The findings of this study suggest that the 36 sessions within 12-week MEMT protocol intervention revealed the potential evidence in improving attention and memory in community-dwelling elderly with cognitive impairment.

Introduction

Aging populations are at a greatly increased risk of developing cognitive impairment and Alzheimer's disease. These conditions are associated with diminished cognitive function abilities that adversely affect them, their families, and society. Elderly with mild cognitive impairment referred to as objective cognitive complaints about age, in a person with essentially normal functional activities, who do not have dementia.[1] It affects 19% of people aged 65 and over.[2] Around 46% of people with mild cognitive impairment develop dementia within three years compared to 3% of the population of the same age.[3] Mild cognitive impairment often demonstrates deficits in certain cognitive domains, particularly in episodic memory, attention, and executive function.[4] Episodic memory impairment is a core feature of Alzheimer's disease and mild cognitive impairment. The

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doi: 10.14456/jams.2019.31
E-ISSN: 2539-6056

episodic memory impairment includes deficits in list recall of words, sentences or stories, and recognition memory for words, drawings or pictures. The issue of forgetting over time is of great importance. Indeed, it has been frequently shown that to-be-remembered information is rapidly lost, as measured by significantly impaired delayed free recall tasks; even when inducing deep semantic processing maximizes encoding.[5]

Medical service provision for elderly with mild cognitive impairment includes pharmacological treatment and non-pharmacological treatment. However, the standards of care did not recommend the approval to use any medications to reduce mild cognitive impairment symptoms.[5] Cognitive training is an alternative treatment used among the elderly with mild cognitive impairment to promote the decrement of the severity of mild cognitive impairment. Cognitive training program was effective in reducing the impairment in cognitive domains with memory, attention, and reasoning which affect the ability to perform activities in daily life and have better well-being.[6] A systematic review has reported on the effectiveness of cognitive interventions targeted at remediating memory processes; include training to stimulate visual and auditory attention, memory, abstract thinking, and constructional ability. The evidence-based of cognitive and episodic memory training in several studies have investigated non-pharmacological treatment effects of cognitive and episodic memory training intervention among individuals with mild cognitive impairment. This training revealed the increasing of cognitive score and functional performance[7] and uses of memory strategies have been found to enhance memory performance within an elderly population.[8] Effective strategies encourage deeper analysis and elaborative encoding of the material to be recalled, and a level of processing which enhances retrieval.[9] Another non-pharmacological treatment, multisensory stimulation (MSS) is a specifically designed room containing a variety of equipment to stimulate the senses of sight, hearing, touch, taste, and smell.[10] MSS provides a non-threatening environment through the sensory stimulation activities and an enriched environment that is beneficial to mediate relaxation and promote emotional benefits with enjoyment experience, with consequent enhancement of motivation to participate in occupations as well as improvement of attention span, memory and functional performance.[11] Additionally, the mindfulness-based yoga training is associated with physical yoga discipline includes asanas (postures), pranayama (breathing techniques), and dhyana (meditation).[12] Tools to withdraw the senses (pratyahara), concentrate the mind (dharana), and develop unwavering awareness (dhyana) manifest from dedicated yoga practice.[13] Yoga practice comprises not just stretching, but rather dynamic movements tied to the breath. Indeed, yoga is associated with multiple health benefits including increased physical stamina, balance, flexibility, and relaxation.[14] However, yoga also appears to offer potential psychological benefits through the inclusion of mindfulness training, involving the practice of meditation as well as the dynamic combination of proprioceptive and interoceptive awareness.[15] Regular practice of mindfulness skills results in both awareness and profound focus by drawing attention to the present moment without judgment. Previous evidence also supports the ability of mindfulness meditation to improve aspects of cognitive functioning, such as attention- and memory-related parameters.[16] Similar to aerobic exercise, even brief mindfulness meditation training has been found to be effective in improving cognition.[17]

Furthermore, previous studies demonstrated that the benefits of cognitive intervention combined with other promising non-pharmacological intervention like multi-facet cognitive program including mindfulness, yoga, physical activity, art therapy, music therapy resulted in cognitive performance improvement in mild Alzheimer's disease and mild cognitive impairment[17, 18] and sustained beneficial effect within 4-6 months after the end of the program.[19]

This study hypothesized that three combination multimodal interventions; episodic memory training, multisensory stimulation, and mindfulness-based yoga training would result in improvements in cognitive function. Therefore, the aim of the present study was to determine the benefits using a multimodal episodic memory training (MEMT) protocol intervention for enhancing cognitive and episodic memory performance in elderly with cognitive impairment.

Materials and methods

Participants

In the present study, twenty community-dwelling elderly were recruited from members at Nong-Kwai elderly school in suburban municipal district, Chiang Mai, Thailand. Inclusion criteria for participants were: 1) either male or female aged between 60-80 years old, 2) complained of memory decline, 3) absence of depressive symptom which determined by the TGDS[20] (Thai Geriatric Depression Scale), 4) had scored 19-23 points in MSET10[21] (Thai Mental State Examination), and 5) had graded with mildly impaired of cognitive function which measured by the KASCA[22] (Kendrick Assessment Scales of Cognitive Ageing). The exclusion criteria used in the study including; having previous diagnosis of either dementia, or Alzheimer's disease, or any cerebrovascular accident history. The study protocol was conformed to the ethical guideline of Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. Informed written consent was obtained from all participants prior to taking part in the study.

Multimodal episodic memory training protocol

Multimodal episodic memory training protocol (MEMT protocol) emphasized on attention and episodic memory functions and it was developed based on the knowledge and theoretical literature review of previous cognitive training programs,[18, 24, 32] multisensory stimulation[11] in person with cognitive impairment, and mindfulness based yoga training.[23, 32] MEMT protocol was integrated into group-based activities that consisted of the combination of the episodic memory training, multisensory stimulation and mindfulness based yoga training. Episodic memory training activities was included paper-based activities and mnemonic memory strategies training, the multisensory stimulation activities was provided a variety of sensory stimulation thoroughly

a specific environmental-setting or recreational and ludic activities, and the mindfulness based yoga training was based on the mindfulness Yin Yoga practice.[23] MEMT protocol contents are presented in Table 1.

Table 1 Detailed content of MEMT protocol component.

Component	Content
Episodic memory training Reality orientation	Group-based activities (paper-based, tabletop) • Inquire about the current date, place, weather, what happened today
Attention	• Stimuli through the techniques of attention in a group; i.e. clapping when a specific number or word was spoken out by the leader, naming the color of words
Autobiographical memory	• Recalling events of their personal lives, story-telling
Recall Memory	• Use mnemonics memory strategies, story-recall, shopping list recall, face-name association, remember the location on the map
Multisensory stimulation	Provided a variety of sensory stimulation thoroughly a specific multisensory room setting or recreational and ludic activities • Use relaxing sound and music • Use sound stimuli and body movement • Tactile stimuli of objects, hand massage • Use flavors odors
Mindfulness based yoga training	Based on the manual of mindfulness Yin Yoga practice[23] step by step training • Slow and hold position in each movement practice • Awareness of body movement and slow breathing exercise

Procedure

A matched pair was used with the matched by age, gender, and education as summarized in flow diagram (Figure 1). Twenty participants were allocated within pairs to either the control or the experiment group. Participants in the experimental group (n=10) received MEMT protocol intervention while those in control group (n=10) did not received the MEMT protocol intervention. The experimental group took part in a 36-session group-based MEMT protocol intervention. Each session of intervention lasting about 2 hours, with the 40-minute in combination of multisensory stimulation and mindfulness-based yoga training and followed the 80-minute episodic memory training activities, 3 days per week for 12 consecutive weeks.[24]

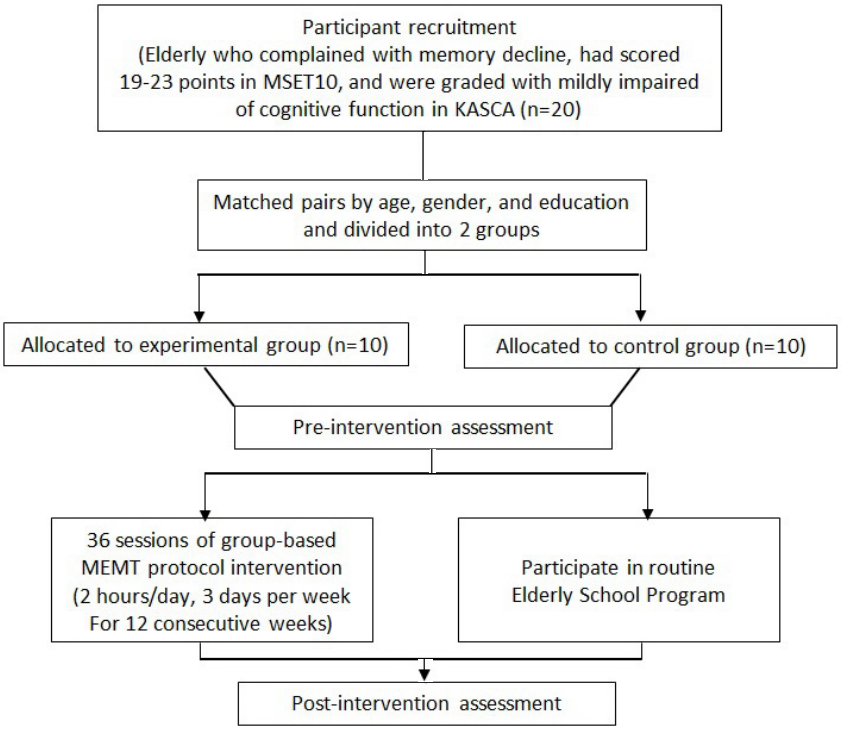

Figure 1. *Flow diagram of the study.*

Outcome measures

The outcome measurements of cognitive function are tests of specific domains of attention of everyday memory, episodic memory, and level of mindfulness are as follows.

Digit Span forward-backward test

The test was used to assess attention, a series of lists of numbers present verbally to participants. Participants are asked to immediately repeat the numbers verbally either in forward or the backward numerical order.[25]

Thai Cognitive-Perception Test (Thai-CPT)

Thai-CPT test has a validity and reliability to assess cognitive and perceptual functions. The test consists of six subtests incuding visual perception, body scheme perception, praxis, memory, matching and categorization, and problem solving. This study emphasized everyday memory by using a memory subtest, which is divided into 5 topics; auditory Thai alphabet memory; visual Thai alphabet memory, auditory object names memory, visual object names memory, and object recognition.[26]

Episodic memory test

Episodic memory is examined using the Logical Memory-Delayed Recall and Logical Memory-Recognition subtests.[27] During the test, participants were instructed to listen carefully to two stories and remember the content of the stories. After a 30-minute delay, they would be asked to repeat each story as close to the original story as possible (Delayed Recall) and provide "Yes" or "No" answer in response to a series of 30 questions (Recognition).

Mindfulness Attention Awareness Scale (MAAS)

MAAS is a 15-item instrument measuring mindfulness.[28] Each item is scored from 1 (almost always) to 6 (almost never). Total score for MAAS is computed by calculating the mean across items. For MAAS, high scores indicate higher levels of mindfulness. It is a single factor measure that includes the following aspects of mindfulness: attention to and awareness of present-moment experiences in daily life.[30]

Data analysis

Chi-square test was used to analyze demographic comparison between control and experimental groups. Comparisons of the outcome measurement scores before and after the MEMT protocol intervention were carried out using the Mann-Whitney U Test. Significance was set at $p<0.05$. Statistical analyzes were carried out using SPSS (Statistical Package for the Social Sciences 22.0) (SPSS/IBM, Armonk, NY, USA).

Results

Demographic characteristics of participants in both groups including gender, age, and education level, screening scores from Thai Mental State Examination (MSET10), Kendrick Assessment Scales of Cognitive Aging (KASCA), and Thai Geriatric Depression Scale (TGDS) are presented in Table 2.

Table 2 Demographic characteristics of the participants (control group n=10, experimental group n=10).

Characteristics	Control group n (%)	Experimental group n (%)	Chi-square
Gender			
Male	3 (30.00)	4 (40.00)	
Female	7 (70.00)	6 (60.00)	
Education			0.78
Primary	2 (20.00)	2 (20.00)	
Secondary	7 (70.00)	8 (80.00)	
High school	1 (10.00)	0	
Age (years) (mean±SD)	67.30±3.94	67.00±4.59	0.64
Screening scores (mean±SD)			
MSET10	17.30±1.82	16.70±2.02	0.26
KASCA	8.10±0.99	7.80±0.91	0.79
TGDS	1.80±1.22	2.00±0.94	0.61

From Table 2, participant characteristics showed almost equal distribution of gender, age, and education level between control and experimental group. The average age of control and experimental group was 67.30±3.94 years and 67.00±4.59, respectively. Education level of control and experimental group were secondary level at 70% and 80%, respectively. Cognitive screening scores of MSET10 demonstrated the average scores for control group was 17.30±1.82 and 16.70±2.02 for experimental group. While KASCA assessment reported mildly cognitive function grade in both groups, the control group's average score was 8.10±0.99 and 7.80±0.91 for the experimental group. Both groups revealed no depressive symptoms with TGDS assessment, control group's average score was 1.80±1.22 and 2.00±0.94 for experimental group. On Mann-Whitney U test was used to test the differences of demographic characteristics of the participants before conduction of the experiment between control and experimental groups. The correlations were found on age, education level at 0.78

and 0.64. Correlation of screening scores from MSET10, KASCA, and TGDS were 0.26, 0.79, and 0.61, respectively. This demonstrated that before experiment, participant characteristics of control and experimental group were not statistically different.

Table 3 Mean rank and p value of the outcome measurement scores before and after experiment between control and experimental groups.

Component	Pre-intervention Mean rank		*p* value	Post-intervention Mean rank		*p* value
	Control group (n=10)	Experimental group (n=12)		Control group (n=10)	Experimental group (n=12)	
Attention						
Digit span forward	9.60	9.20	0.75	9.20	10.10	0.03*
Digit span backward	6.70	7.40	0.48	6.40	8.50	0.01*
Episodic memory						
Logical Memory-Delayed Recall (LM I)	12.40	11.60	0.52	12.10	14.80	0.01*
Logical Memory-Recognition (LM II)	17.80	16.70	0.78	18.10	22.10	0.01*
Everyday memory (Thai-CPT)						
Visual Memory	8.70	7.80	0.76	8.60	9.80	0.01*
Auditory Memory	8.80	9.00	0.85	8.80	9.70	0.01*
Object Recognition	8.80	8.30	0.75	8.30	9.30	0.01*
Mindfulness (MAAS)	3.40	3.20	0.93	3.25	5.10	0.01*

NB: **statistically significant difference in means.*

Table 3 reveals the statistical analysis of the outcome measurements score before and after experiment between control and experimental groups. At pre-intervention, scores of all components; attention, episodic memory, everyday memory, and mindfulness between control and the experimental group were not significantly different ($p>0.05$). This result indicated that two groups did not differ in attention, memory and mindfulness performance before intervention. After 36 sessions of multimodal episodic memory training intervention, the participants in control and experimental group showed significant differences ($p<0.05$) in their test performance on Digit Span forward ($p=0.03$), Digit span backward ($p=0.01$), Logical Memory-Delayed Recall ($p=0.01$), Logical Memory-Recognition ($p=0.01$), all everyday memory subtest of Thai-CPT ($p=0.01$), and Mindfulness Attention and Awareness Scale ($p=0.01$).

Discussion

The present study aimed to determine the effectiveness of multimodal episodic memory training protocol which was specifically designed for the community-dwelling elderly with cognitive impairment. The present findings are consistent with previous studies that demonstrated the effects of multimodal cognitive training improving the cognitive performance including attention and memory of individuals with amnestic mild cognitive impairment.[30]

In Table 3, after 36 sessions of multimodal episodic memory training intervention, participants in control and experimental group showed significant differences ($p<0.05$) in their attention, memory and mindfulness performance. The participants in experimental group demonstrated a significant improvement in attention and episodic memory and everyday memory scores. Attention score revealed the attention ability of Digit Span test,[26] and score of Mindfulness Attention Awareness Scale demonstrated attention and awareness of present-moment experiences in daily life.[29] MEMT protocol training was designed to take advantage of the combination of mindfulness yoga training and multisensory stimulation into the intervention will mediate the additional effect to improve attention. In particular, mindfulness yoga training has been shown to enhance key attention capacities, including orienting and alerting,[36] the increased moment-to-moment attention is associated with mindful task engagement.[37]

Furthermore, episodic memory scores of Logical Memory test and Thai-CPT test demonstrated the delayed recall and recognition memory.[24, 25] These findings contrary to previous studies that found the participants with mildly cognitive impairment did not improve on the Logical Memory-Delayed Recall test after 6-weeks cognitive training[18] and 8-weeks multifaceted rehabilitation program[31] and did not improve significantly in Thai-CPT memory score after 5-weeks cognitive training program in elderly with suspected dementia.[32] In the present study, improvement in episodic memory may be due to a MEMT protocol was specified on episodic memory training and being continuously performed with more frequency and time in the 36 sessions of 12-weeks lasting about 2 hours in each session. Prolong duration of MEMT protocol intervention enhancing the high availability of cue information[33] and repetitive training is an effective strategy in assisting the person with cognitive impairment to gain new information.[34] Also, a MEMT protocol was designed to take advantage of the combination of

mindfulness yoga training and multisensory stimulation into intervention will mediate the additional effect to improve attention and memory performance.[19, 35] Consistent with this, the multisensory stimulation enriched intrinsic motivation that willing to attend and participate in the activities.[11] Moreover, mindfulness yoga training has been shown to enhance associated with mindful task engagement may directly contribute to enhanced working memory[37] and in turn , the better episodic memory performance. [38]

Although the present study demonstrated the beneficial effect of multimodal episodic memory training protocol, a few limitations should be taken into account. Firstly, our limited sample size might introduce some error of inference, reduce power of the analysis and limit generalization. Secondly, it can't be assured whether the improvement in experimental group is sustainable afterward, long-term follow up is needed to confirm our findings to establish the long-term effects of MEMT protocol training on the attention and memory. Result from this study can be applied as a guideline for developing or applying multimodal episodic memory training protocol for elderly who live in the community or elderly school members. In conclusion, this study provides evidence of the effectiveness of non-pharmacological interventional approach, MEMT protocol intervention in community-dwelling elderly with mild cognitive impairment. Therefore, further study remains to be verified the sustained effects of this protocol in long-term effects.

Acknowledgements

This work was supported by the research fund for graduate studies, Faculty of Associated Medical Sciences, and Chiang Mai University research grant, Chiang Mai University, Thailand. The authors thank the elderly school members at Nong-Kwai municipal district, Chiang Mai province, Thailand for their willingness to participate in this study.

Conflicts of interests

The Authors declare to have no conflict of interest.

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Journal of Associated Medical Sciences 2019; 52 (3): 212-218

JAMS
Journal of Associated Medical Sciences

Thai-Journal Citation Index Centre (TCI) & ASEAN Citation Index (ACI)

Journal of Associated Medical Sciences

Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index

Relationships between clinical features of neck pain and upper limb disability and reaction and response times in individuals with chronic neck pain

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ARTICLE INFO

Article history:
Received 17 June 2019
Accepted as revised 16 August 2019
Available online 28 August 2019

Keywords:
Neck pain, reaction time, response time, disability

ABSTRACT

Background: Dysfunction of sensorimotor integration can influence the execution of the reaction time and motor task. Although evidence suggests the association of neck pain and sensorimotor dysfunction, relationship between clinical features of neck pain and reaction and response times is still unknown.

Objectives: To examine the relationship between clinical features of neck pain and upper limb disability, and reaction and response times in individuals with chronic neck pain.

Materials and methods: Fifty-six individuals with chronic neck pain aged between 18-59 years were recruited for the study. Clinical features included pain intensity using Visual Analogue Scale (VAS), pain duration, neck disability using Neck Disability Index-Thai version (NDI-TH), and upper limb disability using Disabilities of the Arm, Shoulder and Hand-Thai version (DASH-TH). Hand reaction and response times were assessed using hand-held electronic timer with a modified computer mouse and foot reaction and response times using a pedal switch. Pearson's correlation coefficient was used to analyze the relationships between variables.

Results: NDI-TH score was mildly correlated with hand and foot reaction and response times (r ranged from 0.29 and 0.32, $p<0.05$). DASH-TH score (8 items related to neck pain) was positively correlated with hand reaction and response times (r=0.26 and 0.34, respectively, $p<0.05$) but not with foot reaction and response times ($p>0.05$). There was no correlation between intensity and duration of neck pain and the hand and foot reaction and response times ($p>0.05$).

Conclusion: There was a mild correlation between neck and upper limb disability and slower hand reaction and response times. The neck disability was mildly correlated with slower foot reaction and response times

Introduction

Dysfunction in sensorimotor control has been demonstrated to be associated with neck pain. The sensorimotor dysfunction includes reduced cervical proprioceptive sense, visual disturbance, poor head-eye coordination and impaired balance.[1-3] Additionally, changes in the sensorimotor control often cause dizziness, unsteadiness and feeling of spinning in the head in persons with neck pain.[3] The dysfunctions and symptoms are proposed as a result of altered afferent information originating from the cervical spine, which mismatched with normal afferent signals from the vestibular and visual systems.[4]

There is evidence suggesting that sensorimotor integration involves in the execution of the reaction time and motor task.[5] Abnormality of sensorimotor integration can result in a delay in a reaction time and response time,[6]

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doi: 10.14456/jams.2019.32
E-ISSN: 2539-6056

which are important for everyday activities such as reaching and driving.[7] A recent study of Barr et al.[8] have demonstrated that patients with cervical dystonia had slower simple foot reaction time compared to healthy controls. The impaired step reaction time was also found to be moderately correlated with a higher fear of falling. Likewise, Sandlund et al.[9] showed that patients with traumatic neck pain and non-traumatic neck pain had deficits in upper limb coordination and position sense acuity. Huysmans et al.[10] also found that position sense acuity of the upper extremity and tracking performance were impaired in subjects with neck and upper extremity pain.

Several previous studies demonstrated that cervical musculoskeletal impairment was correlated to the magnitude of neck pain and disability[11-14] but not with pain duration.[12, 15, 16] For example, Falla et al.[12] demonstrated that the level of neck pain intensity was correlated with the function of the deep cervical flexor muscles. Kumbhare et al.[13] found that neck disability was correlated with cervical range of motion. Similarly, Chiu et al.[14] found relationships between physical impairments and neck pain. Alternatively, there was no correlation between duration of neck pain and cervical and axioscapular muscle impairment.[12, 15, 16] Patients with neck pain frequently have difficulties with functional use of their upper limb. A study demonstrated that patients with higher severity of neck pain had greater restrictions of the upper limb function.[17] Approximately 80% of neck pain patients reported that their neck pain was aggravated by upper limb activities. A significant moderate to high correlation was also found between the neck pain and disability and the upper limb disability.[17] While there is evidence for sensorimotor dysfunction in persons with neck pain and delayed reaction and response times are potentially modifiable risk factors associated with disability, the relationships between clinical features of neck pain and upper limb disability and reaction and response times in this population are yet to be addressed.

The purpose of this study was therefore to investigate the relationships between clinical features of pain (intensity, duration and self-perceived disability) and hand and foot reaction and response times in individuals with chronic neck pain.

Materials and methods

Participants

Fifty-six women and men with idiopathic neck pain aged between 18-59 years were recruited from physical therapy clinics, university, office and community in Chiang Mai. To be eligible for the study, participants had to have neck pain lasting for at least 3 months and have a score of ≥5/50 on the Neck Disability Index-Thai version (NDI-TH).[18] Participants were excluded if they had a previous history of trauma and surgery to the head, neck, upper and lower extremities, a previous history of neurologic disorders, musculoskeletal disorders that could affect reaction and response time, uncorrected visual problems, suspected vestibular pathology, and use of medications that could impact the reaction and response times. Participants were asked to refrain from consuming alcohol and caffeine 2 hours and sleep at least 6 hours prior to testing day.

The study was approved by the research ethics committee, Faculty of Associated Medical Sciences, Chiang Mai University (AMSEC-61EX-039). All participants received information about the study and signed written informed consent forms before the commencement of the study.

Questionnaires

Visual Analogue Scale (VAS)

Visual analog scale was used to measure intensity of neck pain.[19] VAS is a horizontal line with 0-10 cm in length. The scale is anchored on the left with the phrase "no pain", and on the right with the phrase "worst imaginable pain". The possible score ranges from 0 to 10.

Neck Disability Index-Thai version (NDI-TH)

NDI-TH was used to measure self-reported neck-pain related disability.[20] It contains 10 items including pain intensity, personal care, lifting, reading, headaches, concentration, work, driving, sleeping, and recreation. Score of each item ranges from 0 to 5. The maximum score is 50, with higher score indicating greater disability. Neck disability is defined as follows: 0-4 = no disability, 5-14 = mild, 15-24 = moderate, 25-34 = severe, and 35-50 = complete. NDI-TH was shown to be a reliable and valid tool for evaluating disability related to neck pain.[20]

Disabilities of the Arm, Shoulder and Hand-Thai version (DASH-TH) score

DASH-TH is a measurement of self-related upper extremity disabilities and symptoms.[21] It originally consists of a 30-item disability and symptom measure with a 5-point Likert scale (1=no difficulty to 5=unable). Eight items of DASH-TH were chosen from a study of Osborn and Jull [17] as they were mostly relevant to participants with neck pain. The eight items were 1) place an object on a shelf above your head, 2) do heavy household chore, 3) garden or do yard work, 4) carry a shopping bag or briefcase, 5) carry a heavy object, 6) change a light bulb overhead, 7) recreational activities in which you take some force or impact through your arm, shoulder, or hand, and 8) recreational activities in which you move your arm freely.[17] Response scores were summed and used for analysis. A possible total score of the DASH-TH ranges from 8 to 40.[17] DASH-TH was shown to have high content validity and internal consistency.[21]

Reaction and response time tests

Hand and foot reaction and response time tests were measured by a training examiner. Five practice trials were given to each participant for familiarization, followed by 10 experimental trials for each test.[22] The reaction and response times were recorded in seconds and an average value for each test was used for analysis.[22] The measurements used for hand and foot reaction and response times were as follow:

Hand reaction time and response time were measured using a hand-held electronic timer with a modified computer mouse. The light stimulus is located closed to response switches of the modified computer mouse.[22] A built-in timer was set with a delay of 1 to 5 seconds after pressing the start button. Participants sat on a chair with their feet flat on

floor and placed their index finger of the dominant hand over the right mouse button. For hand reaction time, participants were verbally instructed to press the button as fast as possible when red light came on. For hand response time, the modified computer mouse was positioned on a table with a distance of 40 centimeters from the starting home position.[23] Participants sat on a chair and rested their dominant hand on the starting point over the edge of the table. Participants were asked to press their index finger on the right mouse button as fast as possible when the red light came on. Following each trial, participants had to return dominant hand to the starting point. The trial was repeated if participant's hand was not return to the starting position.

Foot reaction time and response time were measured with a foot pedal switch as described in Lord et al.'s study.[22]

For foot reaction time, participants sat on a chair with dominant foot flat on a foot pedal switch. Participants were asked to press the foot pedal switch as fast as possible when the red light came on. For foot response time, participants stood in a comfortable position behind the starting line. Foot pedal switch was positioned on the floor in front of the participants at a distance of 40% of subject's lower limb length (LLL).[24] LLL was measured vertically from the femoral trochanter center to the ground.[24] Participants were asked to use dominant foot stepping on the foot pedal switch as fast as possible when the light came on and back to the starting position.

Procedure

Participants were screened according to the inclusion and exclusion criteria. All eligible participants were asked to sign a consent form and complete the questionnaires (general questionnaire, VAS, NDI-TH and DASH-TH). The experiment was performed in random order (hand reaction time, hand response time, foot reaction time and foot response time). The experiment took place in a quiet room and a 2-minute rest interval was given between each test.

Statistical analysis

Sample size for the study was calculated according to Roscoe's recommendation (n>50).[25] Kolmogorov Smirnov test was used to test the assumption of normality. Pearson's correlation coefficient was used to analyze relationships between the clinical features of neck pain and the reaction and response times (hand and foot). Correlation coefficient values were interpreted as follows: 0.00-0.10 negligible, 0.10-0.39 mild, 0.40-0.69 moderate, 0.70-0.89 strong and 0.90-1.0 very strong. A statistical significance level was at 0.05.

Results

Participant demographics

Demographics and characteristics of the participants are shown in Table 1. Based on NDI-TH score, 44 participants had mild disability, 11 had moderate disability and one had severe disability.

Table 1 Demographic data and clinical characteristics of the participants (n=56).

Demographic data	
Gender (% Female)	89.29
Age (years)	33.77±10.65
Height (cm)	160.20±5.74
Weight (kg)	58.69±12.00
Dominant foot (% right)	82.14
Dominant hand (% right)	100
Characteristic of pain	
Neck pain intensity (VAS, 0–10 cm)	4.55±1.53
Neck pain duration (months)	23.75±16.87
NDI-TH (0-50)	12.05±4.84
DASH-TH (8-40)	15.54±4.97

VAS: *Visual Analogue Scale, Data are expressed as mean±standard deviation, otherwise as indicated, NDI-TH: Neck Disability Index-Thai version, DASH-TH: Disabilities of the Arm, Shoulder and Hand-Thai version*

Correlations between the clinical features of neck pain and reaction and response times

There were mild correlations between NDI-TH score with hand and foot reaction and response times (r ranged from 0.29 and 0.32, $p<0.05$) (Figure 1A, 1B). DASH-TH score was mildly correlated with hand reaction time and hand response time (r=0.26 and 0.34, respectively, $p<0.05$) (Figure 2A) but not with the foot reaction and response times ($p>0.05$) (Figure 2B). There was no correlation between intensity and duration of neck pain and hand and foot reaction and response times ($p>0.05$). The correlation results are provided in Table 2 and 3.

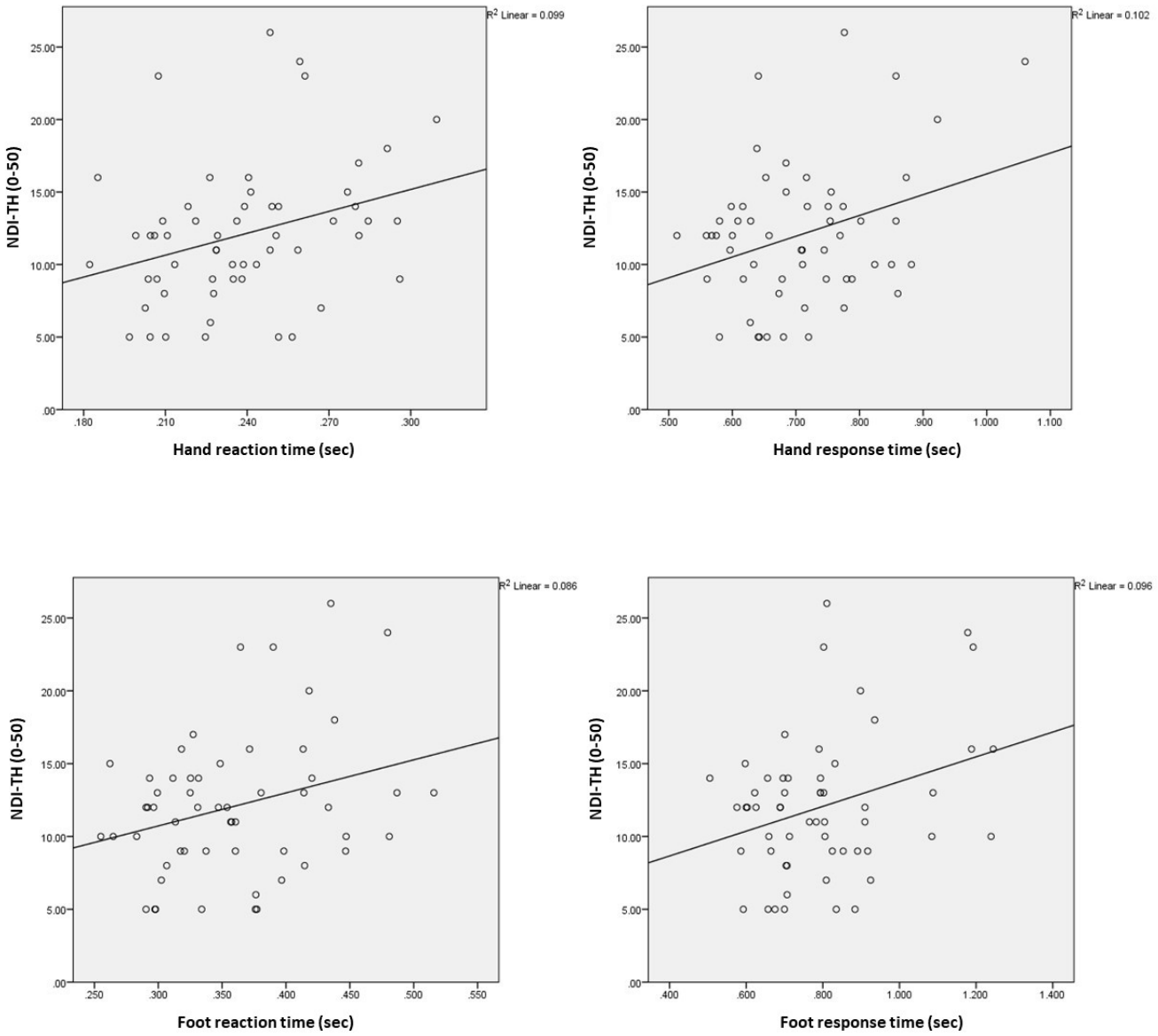

Figure 1. *The correlations between NDI-TH and reaction and response times A) hand B) foot*

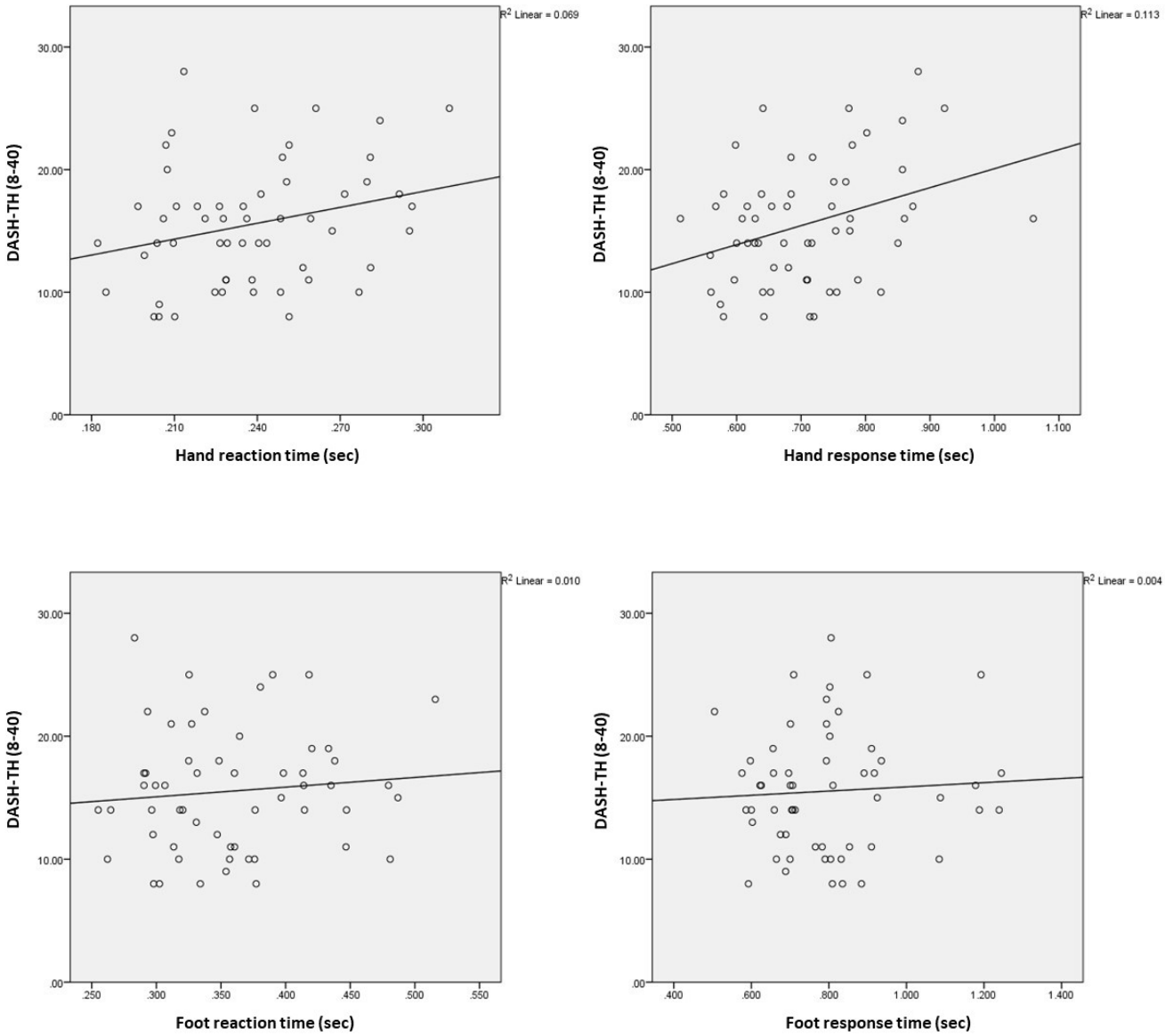

Figure 2: *The correlations between DASH-TH and reaction and response times A) hand B) foot*

Table 2 Correlations between the pain and disability and hand reaction and response times.

Clinical features	Reaction time (sec)	*p* value	Response time (sec)	*p* value
Neck pain intensity (VAS, 0-10 cm)	0.19	0.16	-0.05	0.72
Neck pain duration (months)	0.03	0.81	-0.07	0.61
NDI-TH (0-50)	0.32	0.02	0.32	0.02
DASH-TH (8-40)	0.26	0.05	0.34	0.01

Data were analyzed using Pearson's correlation, VAS: Visual Analogue Scale, NDI-TH: Neck Disability Index-Thai version, DASH-TH: The Disabilities of the Arm, Shoulder and Hand-Thai version

Table 3 Correlations between the pain and disability and foot reaction and response times.

Clinical features	Reaction time (sec)	*p value*	Response time (sec)	*p value*
Neck pain intensity (VAS, 0-10 cm)	0.09	0.53	0.04	0.76
Neck pain duration (months)	0.08	0.54	0.00	1.00
NDI-TH (0-50)	0.29	0.03	0.31	0.02
DASH-TH (8-40)	0.10	0.47	0.06	0.66

Data were analyzed using Pearson's correlation, VAS: Visual Analogue Scale, NDI-TH: Neck Disability Index-Thai version, DASH-TH: Disabilities of the Arm, Shoulder and Hand-Thai version

Discussion

Results of this study demonstrated that NDI-TH was mildly correlated with the hand reaction and response times. This suggests that patients with higher levels of neck disability are likely to have slower hand reaction and response times. The results also demonstrated that higher level of disability related to upper extremity (DASH-TH scores) was mildly correlated with slower hand reaction and response times in patients with neck pain. Nevertheless, the correlations observed were relatively mild. The relationships between neck and upper extremity disabilities and hand reaction and response times are expected and considerably relevant as both NDI-TH and DASH-TH involved limitation of activities of daily living caused by neck pain.[20, 21] However, it is important to note that NDI-TH measures disability related to specific activity like personal care, reading, lifting and driving whereas DASH-TH is more focused on disability of arm, hand, and shoulder. Eight activity items on DASH-TH were chosen in accordance with a study of Osborn and Jull [17] which found that such eight activity items scored positive by 50% of participants and there was a moderate to high correlation between the neck disability index and the disabilities of the arm, shoulder and hand scores. In addition, the result from this study revealed mild relationships between NDI-TH score and the foot reaction and response times, suggesting that patients with neck pain with greater disability took a long time to respond to a stimulus. NDI-TH is a functional status questionnaire concerning neck pain and limitation of activities of daily living due to neck pain. The association between NDI-TH and foot reaction and response times was likely to be found, although its association was relatively mild in the study. This result is in accordance with a previous study which showed significant negative correlations between stepping reaction time and cervical range of motion and mobility in patients with cervical dystonia.[8] Not surprisingly, DASH-TH was not correlated with foot reaction and response times, like the results of hand reaction and response times as DASH-TH only focused on the upper extremity impairments. It has been well documented that deep cervical muscles, which contain higher density of muscle spindles play an important role for cervical proprioception and are associated with the sensorimotor dysfunction in patients with neck pain[26, 27] A mismatched cervical afferent input can also lead to sensorimotor symptoms and impairment.[4] Thus, the overall slower hand and foot reaction and response times may be indicative of impaired sensorimotor integration caused by abnormal cervical afferent input.

There was no correlation between pain intensity and duration of pain and hand and foot reaction and response times. These results may suggest that a higher level and a longer duration of neck pain did not influence time taken to respond to a stimulus. On other words, delayed reaction and response times occurred with no relation to the length of history of symptoms. Our results are consistent with previous studies which found no correlation between the duration of neck pain and disability [12,15,16] but are in contrast to most studies which demonstrated a correlation of pain intensity and physical impairment.[12-14] Discrepancy of the results may be due to variations of participants' characteristics and pain severity. Further research is needed to confirm the findings of the relationships between pain intensity and reaction and response times in persons with neck pain.

There are some limitations to this study. Most participants recruited into the study were women (89.29%). The average intensity of neck pain was mild to moderate and the average neck disability was only mild. These potentially limit the generalizability of the study's results. Further research is needed to confirm the study findings. Additionally, further investigation of reaction time and response time between individuals with neck pain compared with healthy control is also warranted.

Conclusion

The study showed that the higher level of neck and upper limb disability was mildly correlated with slower hand reaction and response times. There was also a mild correlation between a higher neck disability and foot reaction and response times. Pain intensity and duration were not found to be associated with reaction and response times. The overall results may suggest the higher the disability, the slower reaction and response times in individuals with neck pain.

Acknowledgements

This study was granted by the Faculty of Associated Medical Sciences, the Graduate School, and TA/RA scholarship, Chiang Mai University, Chiang Mai Province, Thailand.

Conflicts of interests

The authors declare no conflict of interest.

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Established in 1968

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