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4. Radiologic Technology
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6. Other related fields

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Prevalence of high-risk human papillomavirus infection detected by real-time polymerase chain reaction in Lampang Cancer Hospital

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ABSTRACT

Background: Human papillomaviruses (HPV) infection can cause diseases such as warts, benign and tumors including cervical cancer. Latest, recent statistical results have revealed that cervical cancer is ranked the second highest of cancer diseases in Lampang Cancer Hospital (LPCH). Since 2013, HPV high risk (HR) test has been used to screen cervical cancer in combination with liquid-based cytology test in LPCH for early detection of cervix abnormalities.

Objectives: To report the prevalence of HR-HPV infection detected by real-time polymerase chain reaction (PCR) method in LPCH during 1st October 2014 to 30th September 2016.

Materials and methods: HR-HPV testing was done by real-time PCR technique using HPV-Risk assay kit. Cervical cell PAP smears were collected and prepared by liquid-based cytology system (PathTezt) and interpretation followed the Bethesda system.

Results: Results of the study detected 305 out of 4,789 women who participated in the screening program being positive with HR-HPV viruses related to cervical cancer. This represented 6% of positive cases. Of the 305 positive cases, 72 were infected with HPV 16 and 18 genotype which are the most common causes of cervical cancer.

Conclusion: Prevalence of HR-HPV infection detected by real time-PCR was no different from those detected by PCR and DNA hybridization technique. Advantages in using real time-PCR are primarily, time and cost savings which are of essential importance in laboratory operations. An added benefit in this new technique, is the greater degree of accuracy where the E7 region is probed instead of the L1 region where mis-detection is common.

Introduction

Cervical cancer is a serious health problem among Thai women as reported in "Cancer in Thailand vol.VIII". Incidence of cervical cancer in Thai women is second ranked with age-standardized rate (ASR) = 14.4.[1] In 2015, cervical cancer incidence in Lampang Cancer Hospital is also ranked the second among all types of cancer in female with 264 of new cases were diagnosed.[2] Cervical cancer screening has been applied for scaling down the incidence of cervical cancer.[3,4] Detection of precancerous stage is beneficial to patients because it reduces the time and cost of treatment at cancer stage.[5] Cervical cancer screening can be done by different methods: visual inspection with acetic acid (VIA), Papanicalaou test (PAP) smear, liquid-based Pap smear, and the latest one Human Papillomaviruses (HPV) high risk (HR) testing. Since persistent of HR-HPV is known as a cause of cervical cancer, using of HR-HPV testing for

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cervical cancer screening has started in many countries including Thailand.[6, 7, 8, 9] HR-HPV testing by polymerase chain reaction (PCR) and DNA hybridization technique (HPV-diassay kit) has been applied to screen cervical cancer in combination with liquid-based Pap smear in Lampang Cancer Hospital since 2013. After that, HPV-Risk assay kit using multiplex real-time Polymerase chain reaction technique was placed in October 2014. This new method provides four detection and typing result in single reaction: HPV16, HPV18, other HR-HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67 and 68) and internal control (human β-globin gene).[10] Hesselink *et.al.'s* study on clinical validation of HPV-Risk assay showed clinical sensitivity and specificity for CIN2+ at 97.1% and 94.3% respectively and concluded that HPV-Risk assay can be used for cervical cancer screening.[10] However, there is no study of the performance on this kit for the screening of cervical cancer in Thailand. Therefore, this study would like to reassure the results of the process to be reliable and less time and cost consuming.

In this paper, we report on the prevalence of HR-HPV infection detected by real-time PCR in Lampang Cancer Hospital. In addition, the purpose of this study is to reaffirm the claimed levels of efficiency of real-time PCR technique using HPV-Risk assay kit. We will also observe the user's friendliness of this technique and equipment.

Materials and methods

Study Population

Cervical cells in liquid based brush cytology stem (PathTezt, Biocytech Corp, Perak, Malaysia) obtained from 4,789 women who screened for cervical cancer at Lampang Cancer Hospital during October 2014 - September 2016 were used for this study.

DNA extraction

DNA extraction was done by Gene All® Exgene™ Clinic SV mini kit, Korea. Cell samples (1 mL) were centrifuged at 6,200g for 10 min and cell pellets were then mixed with proteinase K. Lysis buffer was added followed by pulse-vortex before reaction mix was incubated at 56 °C for 10 min. After that, absolute ethanol was added to precipitate DNA and mixtures were put onto silica membrane column. After centrifugation at 6,200g for 2 minutes, the membranes were washed twice with washing buffer. Finally, DNA was eluted by adding elution buffer and centrifuged at 9,600g for 2 minutes.

Human papilloma virus high risk type testing

HR-HPV testing was done by using HPV-Risk assay kit (Self-Screen, the Netherlands). PCR master mixture 15 µL and DNA sample 5 µL were mixed in low profile PCR tubes and they were put into a real-time PCR machine (Bio-Rad CFX96) for the thermal cycle run with 1 cycle of enzyme activation at 95 °C for 2 minutes, 40 cycles of denaturation at 95 °C for 5 seconds and also 40 cycles of amplification and data acquisition at 60 °C for 30 seconds. Data analysis and interpretation must concern 3 points as follow: 1) Ct values of positive control must be less than 35 2) There must be no signals above threshold for negative control 3) Ct value of HPV target >36 or no signal and Ct value of β-globin >33 in sample wells show invalid results, the sample must be repeated.

Table 1 Interpretation of real-time PCR results.

	Ct value HPV target (s)	Ct value β-globin	Interpretation
1	<36	Any	HPV-positive
2	>36 or no signal	<33	HPV-negative
3	>36 or no signal	>33	Invalid

Interpretation of data is shown in Table 1 as follow:

Cytology testing

Cervical cells were collected by using liquid-based cytology system (PathTezt, Biocytech Corp, Perak, Malaysia). Cervical smears were interpreted using the 2001 Bethesda system for cervical cytology reporting.

Statistical analysis

Descriptive statistic and Chi-Square analysis were used in this study.

Results

During government calendar year from October 2014 to September 2016, there were 4,789 women tended to screen for cervical cancer by high risk HPV testing combined with liquid-based Pap smear at Lampang Cancer Hospital. Age ranged from 23-79 years old and 2,230 out of 4,789 (46.57%) women were menopause. The prevalence of HR-HPV positive was 305 (6.36%) cases as shown in Figure 1. Comparative prevalence of HR-HPV test results using the old technique (PCR with DNA hybridization) shown in the year of October 2013 to September 2014, 2,435 women were tested for HR-HPV and 145 women out of this number or 6% were found to be positive[11] Comparatively, both the old testing method and the real-time PCR technique revealed similar percentage of prevalence. From this result, we can reasonably conclude that in term of prevalence, both methods of HR-HPV testing are found as reliable. Thirty-nine of these 305 cases were HPV 16, seventeen cases were HPV 18, two-hundred and thirty-three were other HR-HPV and sixteen were multiple infections which HPV 16 or 18 was included (Figure 2). The number of HR-HPV positive was shown in Table 2.

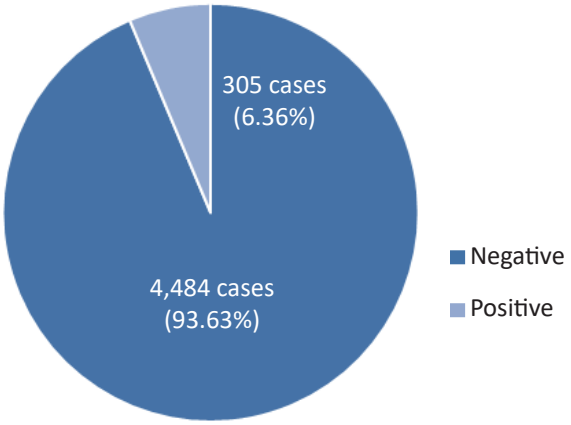

Figure 1 *Results of HPV-Risk assay (n=4,789) at Lampang Cancer Hospital during October 2014-September 2016.*

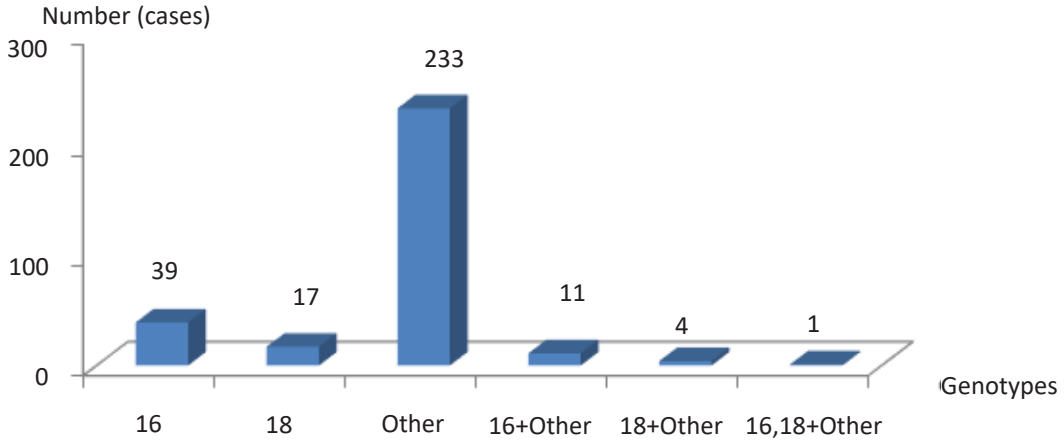

Figure 2 *Number of cases of HPV16, 18, other high risk HPV genotypes and multiple HPV genotypes in 305 HPV-Risk assay positive samples.*

Table 2 Diagnosis results of 305 HPV-Risk assay positive which were divided into normal, malignant, and non-malignant group and obtained from cytology and pathology reports.

Test group	Cytology result	HPV high risk type							Total cases
		16	18	other	16+other	18+other	16, 18, other	cases	
Narmal	Normal	10	6	57	1	-	-	74	74
Malignant	ASC-US	6	3	54	1	-	-	64	162
	ASC-H	3	-	14	1	-	-	18	
	LSIL	3	1	27	2	1	-	34	
	HSIL	7	1	24	5	2	-	39	
	Atypical glandular cell	-	2	2	-	-	-	4	
	SCC	2	-	1	-	-	-	3	
Non-malignant	Inflammation	3	-	15	-	-	-	18	69
	Atrophy	-	1	8	1	-	1	11	
	Atrophy with inflammation	-	-	3	-	-	-	3	
	Endocevicitis	1	-	-	-	-	-	1	
	Bacterial vaginosis	1	1	16	-	-	-	18	
	Candida infection	3	2	12	-	1	-	18	
	Total	**39**	**17**	**233**	**11**	**4**	**1**	**305**	**305**

Note: ASC-US, atypical squamous cells of underdetermined significance; ASC-H, atypical squamous cells, cannot rule out high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma

The relationship between HPV 16, 18, other HR-HPV, HR-HPV multiple infections and cervical abnormalities was analyzed by Chi-Square test. The results revealed that HPV 16, 18, other HR-HPV and HR-HPV multiple infections were not related to precancerous lesion as HSIL or more severe as cervical cancer (Fisher's Exact Test value >0.05) as shown in Table 2. In HSIL and greater cases, the other 13 HR-HPV types (HPV high risk types which are not type 16 or 18) was found the most followed by HPV type 16. However, in squamous cell carcinoma, 2 cases were positive for HPV16 and 1 case was positive for other types.

From October 2014 to September 2016, women who come for the HR-HPV testing were from age group of under 30 to age group of over 70 years old. An interesting yet critical aspect of this study showed the highest respondants group came from those in the 51-60 age group (1,931 cases) and followed by those in the 41-50 (1,471 cases) age group as shown in Table 3. Contrastingly, women from the age group of under 30 and over 70 years old appeared to be least concerned or interested in HR-HPV testing (Table 3). In the previous year of October 2013 to September 2014, women who came for HR-HPV testing were similar. Age profile also showed the same level of response to HR-HPV testing. Those in the age group of

51-60 were the highest respondants followed by those in the 41-50 age group.[11] The least number came from those two again in under 30 and over 70 years old.[11] Both different year time of HR-HPV testing, in term of age profile, showed that women from 41-60 years old being the most concerned about their health as again those in the younger age group of under 30 and in the advance age of over 70 years old being the least concerned in HR-HPV testing.

Table 3 Percentage of HPV-Risk assay positive in each age group.

Age (years)	HR-HPV positive (n/N, %)
<30	4/54 (7.40%)
31-40	83/653 (12.71%)
41-50	100/1,371 (7.29%)
51-60	94/1,837 (5.11%)
61-70	23/554 (4.15%)
>70	1/15 (6.66%)
Total	305/4,484

Note: n: number of HPV-Risk assay positive cases, N: total number of women in each age group

Discussion

HPV-Risk assay using primers which targets the E7 region of HR-HPV whereas the old method, HPV- diassay using GP5+/6+ primer sets which probes a conserved region within the L1 open reading frame of HPV.[12] It is already known that malignant lesions of cervix is related to the integration of HR-HPV DNA into the host genome and usually exist between the E1 and L1 regions.[13, 14] This integration may lead to mis-detection of L1 target region- based assay.[10] Therefore, using a new developed HPV-Risk assay will be a better choice to detect HR-HPV and the integrated HR-HPV samples are also included.

A comparative analysis was made between the old method by using PCR with DNA hybridization as against the real-time PCR for HR-HPV testing and positive conclusive findings are: in terms of prevalence, the finding is relatively similar. However, significant results in time saving, productivity of laboratory staffs and efficiency in administration out weight the PCR with DNA hybridization method of testing. The old method takes about 15 hours for the results to be obtained as against 4 hours or less for the real-time PCR one. Time saving leads to greater productivity of the lab plus a higher morale because of ease of use.

Result of the 2 years study has significant positive implications. Women in age group of under 30 and over 70 years old were the lowest respondents. We do not create and encourage these two groups to be more responsive to HR-HPV testing because the results are so revealing to the causes of cervical cancer and to which group would be more relevant for HR-HPV testing. HPV is in almost all human being. Women under age of 30 who may have HPV need not worry as they are usually transitional[15] and as our human immunities can get rid of them. Hence the correct age to be tested are for those age over 30 years old.[16] Moreover, women after the age of 65 are least sexually active and unlikely to contract HPV.[17] Therefore, these group of women who are least interested in HR-HPV testing is not a serious problem at all as the recommended screening age for HR-HPV testing is for 30-65 years old.

Conclusion

Prevalence of HR-HPV infection detected by real time-PCR was no different from those detected by PCR and DNA hybridization techniques (about 6% for both methods). Hybridization, on the other hand while it pales in terms of time efficiency and preference of use by laboratory technicians against real time PCR in the detection of HR-HPV because of its manual dependency on skilled and experienced laboratory personnel, it should be credited and recognized as the global first in the reliable detection of HR-HPV prior to the introduction of real time PCR technology. It is still an applicable and relevant tool in situations where institutions have no access to real time PCR equipment. HPV-Risk assay by real time PCR is different from DNA hybridization kit where the E7 region is probed instead of the L1 region where mis-detection is common. In this study, to determine the effectiveness and relevance of real time PCR and hybridization in the detection of HR-HPV, there is a limitation to its analytical results due to the time gap between the first pioneering use of hybridization, the eventual discovery, and application of the real time PCR technology. A finite conclusion on which method should eventually be the measuring parameter will require a further, wider, and deeper study and evaluation. However, as for now, both methods are still relevant and applicable.

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Characterization of G6PD genotypes in G6PD deficiency patients from Suratthani Hospital, Thailand

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ABSTRACT

Background: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is highly prevalent in Southeast Asia especially in the area of malaria endemic regions. Primaquine is used for the radical treatment of malaria. However, it causes hemolytic anemia in G6PD deficient patients.

Objectives: To characterize G6PD mutations in G6PD deficient patients around Surat Thani province which is one of the malaria-endemic areas in Thailand.

Materials and methods: One hundred and seventeen leftovered EDTA blood samples were received from primary hospital in Surat Thani Province. All the samples were evaluated for their hematological profiles using an automated hematological analyzer (Beckman Coulter LH 780 Analyzer) and genotyped for G6PD variants using the DiaPlexC G6PD Genotyping Kit.

Results: G6PD mutations were identified in 117 cases of G6PD deficient cases. G6PD Viangchan (51.1%) was identified in 43 Thais and 4 Myanmars, G6PD Mahidol (30.5%) in 16 Thais and 12 Myanmars, G6PD Canton (7.6%) in 4 Thais, 2 Myanmars and 1 Laos, G6PD Kaiping and G6PD Union (4.3% each) in 4 Thais each, and G6PD Mediterranean (2.2%) in 2 Thais. However, the G6PD mutations could not be identified in the remaining 14 samples. There was no evidence of significant differences between hematological parameters among different groups of G6PD variants.

Conclusion: Our study revealed various distribution of G6PD variants in the region, raising the awareness about the requirement for optimal dosage of primaquine in the treatment of malaria infection based on data on G6PD variants.

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathy which affects around ~400 million people worldwide, particularly in the malaria endemic area such as Asia, Africa, Mediterranean and the Middle East.[1] The G6PD plays a major role in the pentose phosphate pathway by catalyzing the production of nicotinamide adenine dinucleotide phosphate (NADPH). Cells require NADPH for maintaining the effective redox potential that helps against oxidative stress, the mechanism particularly important in red cells.[2] The *G6PD* gene is located on the X-chromosome (Xq28), consisting 13 exons and 12 introns. It encodes 515 amino acids of G6PD enzyme.[3] The mutations in *G6PD* gene usually result in a decrease of the G6PD enzyme in red cells which causes increased susceptibility to oxidative stress. Many drugs and chemicals trigger the oxidative stress in G6PD deficient person leading to hemolytic anemia, neonatal hyperbilirubinemia with kernicterus, chronic non-spherocytic hemolytic anemia (CNSHA), and

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spontaneous abortions.[4] The clinical severity depends on the level of enzyme deficiency (or type of G6PD variant), dose and duration of exposure to the oxidative agent, host factors such as age, level of hemoglobin and concurrent infection.[5,6]

Over 400 G6PD variants have been described based on biochemical properties, and approximately 186 mutations have been identified at the DNA level.[7,8] In Southeast Asia, several G6PD variants have been recognized. For instance, G6PD Surabaya (1291 G>A, Class II) is found in Indonesian Chinese, G6PD Mahidol (487 G>A, Class III) in Myanmar and Thai[9], G6PD Vanua Lava (383 T>C, Class II) in Amboinese, G6PD Viangchan (871 G>A, Class II) in Laos, and G6PD Coimbra (592 C>T, Class II) in Orang Asli.[10] Other mutations such as G6PD Gaohe, (95 A>G, Class III), G6PD Chatham (1003 G>A, Class II), G6PD Union (1360 C>T, Class II), G6PD Canton (1376 G>T, Class II), G6PD Chinese 4 (392 G>T, Class III), and G6PD Kaiping (1388 G>A, Class II) have also been reported. In Thailand, G6PD Viangchan variant is highly prevalent and predominates in the eastern part of the country while the Mahidol variant is obsessed in the western part of the country.[11-12] Other G6PD variants such as G6PD Kaiping, G6PD Union, G6PD Canton, G6PD Gaohe are found in Chinese, Indian and certain areas in Southeast Asia.

Since the early 1950s, primaquine has been the only drug to eliminate hypnozoidal for *P. vivax* and *P. ovale* and potent gametocytocide for *P. falciparum*.[13] However, it could cause dose-dependent, severe hemolysis for G6PD deficient person.[14] In recent years, Surat Thani Province is ranked one of top eight provinces in Thailand with high incidence of malaria.[15] The province is covered with rain forests, and rubber and palm oil plantations, making a good ground for malaria transmission.

In this work, we aimed to study the characteristics of glucose-6-phosphate dehydrogenase variants among G6PD patients around Surat Thani Province, Thailand.

Materials and methods

Sample collection

One hundred and seventeen leftovered EDTA blood samples were positive for routine G6PD fluorescent screening test done by Hematology Unit, Suratthani hospital, between March 2016 and November 2016. These samples were sent from the primary hospitals in Surat Thani Province which included Kanchanadit Hospital, Tha Rong Chang Hospital, Phanom Hospital, Khian Sa Hospital, Chaiya Hospital, Phunphin Hospital and Suratthani Hospital for routine G6PD screening test. All blood samples were evaluated for their hematological profiles using an automated hematological analyzer (Beckman Coulter LH 780 Analyzer). Other common characteristics such as age, gender, and nationality were also collected. The study protocol was approved by the ethical committee of Suratthani Hospital. (EC code 11/2560). For all the samples used in this study, written informed consent was obtained from the participants or from their parents.

Fluorescent spot test

Fluorescent spot test, a routine test for G6PD deficiency, was performed immediately after receiving the blood sample from the primary hospitals using the R&D G6PD kit (R&D Diagnosis, Greece) according to the manufacturer's recommendations (catalog number SQMMR500).[16] Briefly, 5 µL of EDTA blood was mixed with 100 µL of reagents (containing 1mmol/L of G6PD-6-phosphate, 0.75 mmol/L of NADP, 0.8 mmol/L of GSSG (oxidized glutathione), 0.2% of Saponin and 225 mmol/L, pH 7.8 of Tris (hydroxymethyl)-aminomethane). After 10 min of incubation at room temperature, 15 µL aliquot was spotted on filter paper and allowed to air dry. The spots were then visualized under UV light. The normal G6PD activity showed a fluorescence spot while no fluorescence signals G6PD deficiency.

Hematological data

Hematological data were collected from routine Hematology unit, comprising WBC count, RBC count, Hemoglobin (Hb) level, Hematocrit (Hct), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin concentration (MCHC). The Beckman Coulter LH 750 Hematology Analyzer (Beckman Coulter, Inc, Miami, FL, USA) was used to generate these hematological data.

Detection of G6PD variants

DNA was extracted from 200 µL of individual blood sample using a NucleoSpin blood kit (Macherey-Nagel, Duren, Germany) and eluted in 100 µL of BE buffer. DNA was kept at -20°C until genotyping was performed. G6PD variants were detected using the DiaPlexC G6PD Genotyping Kit (Asian type; SolGent, ROK). The kit employed one-step multi-allelic specific PCR to detect 8 common mutations of G6PD frequently found in Asia. The 8 variants generated PCR products of different amplicons based on the G6PD mutation types: Vanua Lava (383 T>C, 154 bp), Mediterranean (563 C>T, 262 bp), Coimbra (592 C>T, 234 bp), Mahidol (487 G>A, 337 bp), Viangchan (871 G>A, 501 bp), Kaiping (1388 G>A, 557 bp), Canton (1376 G>T, 681 bp) and Union (1360 C>T, 803 bp). The PCR reactions were: initial denaturation at 95°C for 15 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s; and a final extension at 72°C for 5 min. 25 µL of each PCR mixture contained 5 µL of template DNA (25–50 ng or 100 ng), 12.5 µL of 2X multiplex PCR smart mix (G6PD Asian type), 2 µL of primer mixer (G6PD Asian type), 5.5 µL of nuclease free water. The PCR products were visualized using 3% agarose gel. The internal control of each PCR reaction was confirmed at band 1234 bp.

Data analysis

Data analysis was performed using the SPSS for Windows version 11.5 (SPSS Inc., Chicago, IL, USA). The descriptive statistics include the number and percentage of the distribution of the G6PD variants according to nationalities. One-way ANOVA or unpaired Student t-test was used to assess the mean difference of the hematological characteristics among G6PD variants. The statistical significance was considered at p-value of less than 0.05.

Results

In this study, we characterized the G6PD variants from the positive routine G6PD fluorescent screening test samples obtained from hospitals in the malaria risk area of Surat Thani Province. A total of 117 samples comprised 96 newborns (mean 5 days), 10 infants (mean 10 months), 4 children (mean 4.3 years) and 7 adults (mean 46 years). 93 subjects (79.5%) were males of Thai nationality (76 newborns, 8 infants, 3 children and 6 adults), 23 subjects (19.66%) (22 males and 1 female) were Myanmar (20 newborns, 2 infants and 1 adult), and 1 Laos (0.84%) male newborn. The most common variants detected were G6PD Viangchan (51.1%), which was observed in 43 Thais and 4 Myanmars (Table 1). The second most common was G6PD Mahidol (30.5%), found in 16 Thais and 12 Myanmars. G6PD Canton (7.6%) was detected in 4 Thais, 2 Myanmars and one Laos, while G6PD Kaiping (4.3%) and G6PD Union (4.3%) was found in 4 Thais each. Two other cases were G6PD Mediterranean (2.2%) and they belonged to Thais. The agarose gel detection of the G6PD variants was shown in Figure 1. The 14 samples could not be identified for G6PD variants and 11 other samples had no amplified PCR product due to the low concentration of genomic DNA.

To study the hematological effects of the different G6PD mutations, the hematological parameters were compared in each age groups. We categorized the subjects into four different age groups: newborns (NB, <30 days), infants (IF, 1-12 months), children (CH, 1-12 years) and adults (AD, >12 years). There was no significant difference between the parameters among different G6PD variants in each age category (Tables 2, 3). In our study, six patients had anemia with Hb level of less than 11 g/dL, and Hct levels less than 30.4%: (1) the eight months old female patient with Viangchan mutation, Hb level decreased to 2.8 g/dl, and Hct was 7.6%; (2) two infants males (1 month) with Viangchan mutation with Hb level of 7.8 and 9 g/dl, and Hct value was 22.8 and 27.3% respectively; (3) two adult males of 40 and 74 years old with Mahidol mutation, Hb values of 7.6 and 9.4 g/dl respectively, and Hct level of 12.8 and 26.7%; (4) one male child (2 years) with Union mutation, Hb value of 4.6 g/dl and Hct 12.8%.

Table 1 G6PD variants according to ethnic group in Surat Thani Province.

G6PD variants*	Thai [n (%)]	Myanmar [n (%)]	Laos [n (%)]	Total [n(%)]
Viangchan (871 G>A)	43 (46.8%)	4 (4.3%)	-	47 (51.1%)
Mahidol (487 G>A)	16 (17.4%)	12 (13.1%)	-	28 (30.5%)
Canton (1376 G>T)	4 (4.3%)	2 (2.2%)	1 (1.1%)	7 (7.6%)
Kaiping (1388 G>A)	4 (4.3%)	-	-	4 (4.3%)
Union (1360 C>T)	4 (4.3%)	-	-	4 (4.3%)
Mediterranean (563 C>T)	2 (2.2%)	-	-	2 (2.2%)
Vanua Lava (383 T>C)	Not found			
Coimbra (592 C>T)	Not found			
Total	73 (79.3%)	18 (19.6)	1 (1.1%)	92 (100%)

**Detected by DiaPlexC G6PD Genotyping Kit (Asian type) which could detect 8 G6PD variants.*

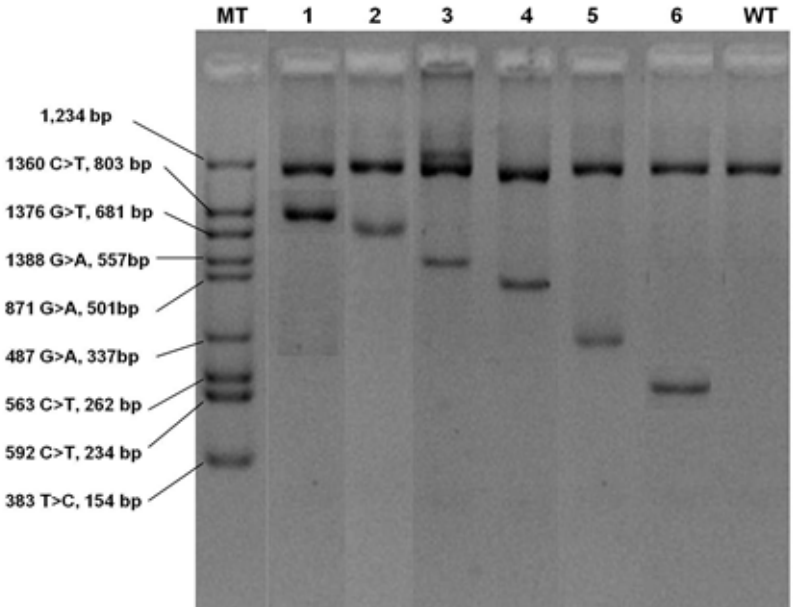

Figure 1. *Agarose gel detection of the G6PD variants using the G6PD genotyping kit (Asian type, DiaPlexC). MT: Mutant type control, 1: Union mutant, 2: Canton mutant, 3: Kaiping mutant, 4: Viangchan mutant, 5: Mahidol mutant, 6: Mediterranean mutant, WT: Wild type control.*

Table 2 Comparison of Rbc, Hb and Hct and G6PD genotypes with the respect in age group.

Parameter	Age	G6PD genotypes														*P* value
		Viangchan		Mahidol		Union		Canton		Kaiping		Mediterranean		Unknown		
		No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	
Rbc, 10^{12}/L	NB	37	4.5±0.7	24	4.7±0.7	3	4.9±0.5	7	4.4±0.7	14	4.4±0.5	1	4.04	11	4.6±0.7	0.555[a]
	IF	6	3.2±1.4	2	3.5±0.4	0	0	0	0	0	0	1	3.67	1	5.44	0.610[b]
	CH	2	4.3±0.2	0	0	1	3.5	0	0	0	0	0	0	0	0	-
	AD	2	4.1±0.1	2	2.0±0.8	0	0	0	0	0	0	0	0	2	2.5±1.8	0.299[a]
Hb, g/dL	NB	37	15.1±2.6	24	15.7±2.5	3	14.2±0.9	7	15.0±2.2	14	13.8±1.4	1	13.9	11	15.1±2.3	0.296[a]
	IF	6	10.2±4.7	2	10.8±0.4	0	0	0	0	0	0	1	16.5	1	12.3	0.629[b]
	CH	2	11.1±0.1	0	0	1	4.6	0	0	0	0	0	0	0	0	-
	AD	2	13.4±1.6	2	7±3.4	0	0	0	0	0	0	0	0	2	11.3±9.3	0.589[a]
Hct, %	NB	37	46.0±8.1	24	48.3±7.9	3	42.9±3.0	7	45.3±6.6	14	41.7±4.0	1	43	11	45.3±8.0	0.191[a]
	IF	6	30.8±15.0	2	32.8±1.8	0	0	0	0	0	0	1	48.3	1	40	0.628[b]
	CH	2	34.2±1.6	0	0	1	12.8	0	0	0	0	0	0	0	0	-
	AD	2	40.7±5.0	2	19.8±9.8	0	0	0	0	0	0	0	0	2	21.9±13.0	0.21[a]

Note: *Newborn (<30 days), IF: Infant (1-12 months), CH: Children (1-12 years), AD: Adult (>12 years).[a] p value according to one-way ANOVA. [b] p value according to unpaired t-test, Rbc: Red blood cell, Hb: Hemoglobin, Hct: Hematocrit*

Table 3 Comparison of MCV, MCH and MCHC and G6PD genotypes with the respect in age group.

Parameter	Age	G6PD genotypes														*P* value
		Viangchan		Mahidol		Union		Canton		Kaiping		Mediterranean		Unknown		
		No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	
Rbc, 10^{12}/L	NB	37	100.2±12.0	24	102.6±5.8	3	96.4±6.5	7	101.5±4.3	14	94.8±12.0	1	106.6	11	96.7±13.5	0.279[a]
	IF	6	92.4±15.2	2	93.9±4.2	0	0	0	0	0	0	1	104	1	70.8	0.930[b]
	CH	2	79.4±0.4	0	0	1	90.4	0	0	0	0	0	0	0	0	-
	AD	2	88.3±6.9	2	94.5±13.0	0	0	0	0	0	0	0	0	2	94.5±11.0	0.809[a]
Hb, g/dL	NB	37	34.6±7.2	24	34.9±8.3	3	38.2±2.7	7	33.4±1.8	14	31.4±4.1	1	34.3	11	31.1±3.9	0.283[a]
	IF	6	31.0±4.0	2	31±2.0	0	0	0	0	0	0	1	35.2	1	22.7	0.920[b]
	CH	2	26.8±1.2	0	0	1	24.5	0	0	0	0	0	0	0	0	-
	AD	2	28.7±2.6	2	33.3±4.2	0	0	0	0	0	0	0	0	2	33.8±5.6	0.510[a]
Hct, %	NB	37	33±1.2	24	32.5±0.8	3	34.5±3.2	7	32.7±0.7	14	33.1±0.3	1	32	11	32.6±1.2	0.069[a]
	IF	6	33.8±1.7	2	33±0.7	0	0	0	0	0	0	1	32	1	32	0.870[b]
	CH	2	33.6±1.6	0	0	1	22.8	0	0	0	0	0	0	0	0	-
	AD	2	32.6±0.3	2	35.3±0.4	0	0	0	0	0	0	0	0	2	55.7±26.6	0.381[a]

NB: Newborn (< 30 days), IF: Infant (1 – 12 months), CH: Children (1 – 12 years), AD: Adult (>12 years). [a] p-value according to one-way ANOVA. [b] p-value according to unpaired t-test (p<0.05). Rbc: Red blood cell, Hb: Hemoglobin, Hct: Hematocrit

Discussion

Mutations in G6PD gene were genotyped using DiaPlexC G6PD Genotyping Kit (Asian type). The method was rapid, highly sensitive and specific[17] and the kit could detect 8 variants: G6PD Vanua Lava, Union, Kaiping, Mahidol, Canton, Coimbra, Mediterranean and Viangchan. Five of these eight variants, G6PD Union, Kaiping, Canton, Coimbra and Mediterranean, can cause severe acute hemolytic anemia as only 1-10% of enzyme activity is present with these variants. Other G6PD mutations i.e. G6PD Viangchan, Canton and Mahidol have 10-60% of enzyme activity remains, causing less severe acute hemolytic anemia.[18]

The most frequent mutations in the present study was G6PD Viangchan which has been previously reported as the common variant in Thais from central part of Thailand, in Cambodian, Laotian who live along the border with Thailand[10-12,19] and in Malay.[20,21] However, G6PD Viangchan was not frequently found in Myanmar.[10] Instead, G6PD Mahidol (487, G>A) was the most common variant observed among Myanmars. This variant could also be found in the central part of Thailand[10], Indonesia, and Malaysia.[20] G6PD Mediterranean (563C>T), which is common in Mediterranean countries and Indo-Pakistan areas, was also detected in 2 Thais.

Surat Thani is a populous province with a significant number of migrant workers from other countries. As it is

one of the malaria endemic provinces in Thailand, the information about the G6PD variants would help in adjusting the dose of anti-malarial drugs. The dosage regimen of primaquine and duration of exposure to the drug for treatment of both *P. falciparum* and *P. vivax* malaria may need to be optimized based on G6PD variants.

Conclusion

Our study focused on the characterization of G6PD variants obtained from hospitals in the malaria risk area of Surat Thani Province. Six G6PD variants would be associated with clinical hemolysis during treatment with primaquine were found. Therefore, our results revealed various distribution of G6PD variants in the region, raising the awareness about the requirement for optimal dosage of primaquine in the treatment of malaria infection based on G6PD variants.

Limitations of the study

There were fourteen samples which could not be identified for G6PD variants due to the limitation of the DiaPlexC G6PD Genotyping Kit (Asian type). The kit is unable to detect a certain variants such as G6PD Quing Yuan (392 G>T), G6PD Songklanagarind 196 T>A, silent mutation (1311 C>T) and G6PD Gaohe (95, A>G). However, the results of this study still provided valuable information regarding diagnosis G6PD variants in southern regions of Thailand.

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In vitro evaluation of P-glycoprotein functions in human neuroblastoma cell lines

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ABSTRACT

Background: Neuroblastoma (NB) remains one of the most puzzling of paediatric cancers in which most patients develop progressive disease that is refractory to chemotherapy. Effective treatment is hampered by drug resistance related to the expression of multidrug-resistant proteins belonging to the ATP-binding cassette transporters family, especially P-glycoprotein (P-gp). Most previous studies focused on molecular evidence of P-gp expression, however, functional studies of P-gp efflux have not clearly demonstrated.

Objectives: The aim of this study was to determine whether human neuroblastoma cell lines (SH-SY5Y and SK-N-SH) express the functionally active P-gp efflux pump.

Materials and methods: Functional studies on P-gp–mediated pumping were performed using pirarubicin, a P-gp substrate, with verapamil, a multidrug resistance inhibitor, and analyzed by a spectrofluorometer. To confirm the gene expression, reverse transcription polymerase chain reaction (RT-PCR) was performed with specific primers for human multidrug resistance 1 (MDR1).

Results: MDR1 expression was observed in neuroblastoma cell line (SH-SY5Y) in the same degree of expression as in the sensitive K562 cell line, a negative P-gp model. Kinetic analysis showed that there was no difference in drug accumulation in the presence or absence of verapamil, indicating that no function of P-gp influenced the accumulation of pirarubicin (PIRA) in both human neuroblastoma cell lines. Combination treatment of verapamil and PIRA was also not found to increase the sensitivity of PIRA.

Conclusion: This study suggests that the existence of P-gp in neuroblastoma cell lines is not significant function.

Introduction

Neuroblastoma is a type of cancer in the sympathetic nervous system of infants and is characterized by highly heterogeneous clinical behaviour, ranging from spontaneous regression to rapid progression and, finally, patient death.[1-3] Strategy for treatment of NB remains a challenge because disease relapse and progression despite receiving intensive multimodal therapy are common in patients with high risk tumors.[2] Most patients achieve some response to aggressive chemotherapy. However, those in the poor-prognosis group fail the treatment and have early relapse. Subsequently, the tumors become refractory to a variety of chemotherapeutic agents.

One of the most significant impediments that hamper treatment of the cancer is resistance to the standard types of chemotherapy. This is a phenomenon in which tumor cells that have been exposed to a cytotoxic agent develop cross resistance to a whole range of drugs with unrelated function and structure.[4] This phenomenon is known as multidrug resistance (MDR). The development

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of drug resistance involves alteration of the signaling pathway,[5,6] induction of anti-apoptotic proteins,[7,8] alteration of tumor microenvironment,[9,10] and induction of drug metabolizing systems.[11] Clearly, the clinical obstacle associated with drug resistance is responsible for the reduced effect of chemotherapeutic drugs, mainly due to uptake from the targeted cells via the ABC transporter cassette and P-gp, which is a subfamily B member.[11,12] P-gp is a member of the ATP-binding cassette superfamily of active transporters and functions as an energy-dependent uptake pump that reduces intracellular concentration of cytotoxic compounds and their toxicity.[13,14] P-gp has a broad substrate specificity and can confer resistance to a wide range of different cytotoxic compounds.[15]

Most pre-clinical and clinical aim to overcome MDR in NB which is modulated by P-gp activity, however, success has been limited as regards efficiency in the outcome. To date, the focus has been primarily on molecular evidence of P-gp expression. However, evidence for functional expression has not been clearly elucidated. Therefore, it may be more appropriate to clearly understand the functionality of P-gp. Numerous methods have been adopted in order to elucidate the P-gp function. Up until now, feasibility of a method that deals with living cells without disturbing their drug equilibrium has not been investigated. Many proposed methods have been used in studies, such as calcein assay,[16,17] rhodamine 123 efflux assay,[16,18,19] and trans-epithelial transporter assay.[20] It was found that one step of these methods is based on incubation of the cells with drugs, centrifugation and washing, suspension in a buffer, and detection of the concentration of the drug within the cell or in the supernatant. However, these steps could not reflect exactly what happens with living cells; also, these steps might render the equilibrium among the drug concentration outside and inside the cell disturbed.[21] For this reason, to analyze the function of the multidrug transporter that mimics living cells, a method that uses the change in the fluorescent characteristics of the transported compound when it moves between intracellular and extracellular medium was employed.[21,22] This method allows measuring accurately the overall concentration of anthracycline accumulated inside the cell and the concentration that is intercalated between the base pairs in the nucleus in the steady state.[23,24]

In the present work, P-gp function in neuroblastoma cell lines, SK-N-SH and SH-SY5Y, was evaluated by using the kinetic uptake of anthracycline monitored by a spectrofluorometer. Pirarubicin (PIRA; a fluorescent P-gp substrate) was selected to examine the function of P-gp *in vitro* because its pKa is relatively low and it can penetrate the cells very rapidly.[21] Fluorescence behaviour of PIRA was made use of and the accumulation of PIRA was directly monitored in the presence of verapamil. By this technique, the sensitive and the resistant K562 cells were selected as the model and defined as the negative and the positive control, respectively, for the P-gp function study. Data obtained showed that the presence of endogenously expressed P-glycoprotein in neuroblastoma cells is not functional.

Materials and methods

Cell lines and cultures

Experiments were performed with human neuroblastoma SH-SY5Y and SK-N-SH. SH-SY5Y cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). SK-N-SH cells were a gift from Assistant Professor Dr. Chainarong Tocharus (Department of Anatomy, Faculty of Medicine, Chiang Mai University, Thailand). SH-SY5Y cells were maintained in minimum essential medium (Eagle's)/Ham's F12 medium with L-glutamine (Caisson, USA) and SK-N-SH cells in DMEM high glucose medium (Sigma-Aldrich, USA), all supplemented with 10% fetal bovine serum (Gibco®, Invitrogen, USA) and 1% penicillin/streptomycin (Caisson, USA), and kept in a humidified atmosphere of 5% CO_2 at 37 °C. For the experiment, both cell lines were initiated at a density of 20% confluence (T75 flask) in culture medium for 3 days to reach about 80-90% confluence. The K562 cell line is a human myelogenous leukemia derived from a 53-year-old female patient with chronic myeloid leukemia and the resistant phenotype line (K562/adr) were used as the control for studying the P-gp function.[25,26] These two lines were provided by Dr. Ruoping Tang (Hôpital de l'Hôtel Dieu, Pairs, France). Both cell lines were routinely cultured in RPMI-1640 medium (Caisson, USA) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin in a humidified atmosphere of 95%air and 5%CO_2. K562 and K562/adr cell initiated at a density of 10^5 cells/mL grew exponentially to about 10^6 cells/mL in 72 hrs. For the assays, the cells were initiated at 5×10^5 cells/mL to have the cells in the exponential growth phase and allowed to grow for 24 h until the experiments. Cell viability was assessed by trypan blue exclusion. The number of cells was determined by using a hemocytometer.

Drugs and chemicals

Pirarubicin (4-Q-tetrahydropyrayldoxorubicin) was purchased from APExBIO Technology (Houston, USA). Stock solutions were prepared in DMSO just before use. Concentrations were determined by diluting stock solutions in distilled water to approximately 1 mM and using ε_{480}=11500 $M^{-1}cm^{-1}$. Verapamil hydrochloride (Sigma, Singapore) was dissolved in absolute ethanol. Deionized double distilled water was used throughout the experiments for solutions and buffers. Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) was purchased from Sigma and dissolved in deionized double distilled water. The experiments were performed in Luckoff-Na^+ buffer solutions at pH 7.25 containing 20 mM HEPES, 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM D-glucose.

Real-time PCR

To evaluate the expression of MDR1 levels in the cell lines, total RNA was isolated from each of the cell lines using the PureLink® RNA Mini Kit (Thermo scientific, Invitrogen, USA) according to the manufacturer's instructions. UV absorbance of the spectrophotometrically isolated RNA was measured at 260 nm to determine its concentration and at 280 nm to check its purity from the 260/280 nm

absorbance ratio. cDNA was prepared from 5 µg of the extracted total RNA with MMLV reverse transcriptase using oligo(dT)18VN nucleotides and RiboSafe RNase inhibitor (Tetro cDNA Synthesis Kit, Bioline, London, UK). RT-PCR was performed using the primer sequences listed in Table 1. The amplification reaction components consisted of 5 µL of 10X PCR buffer, minus Mg (ThermoFisher Scientific, Invitrogen, USA), 1 µL of 10 mM dNTP mixture (ThermoFisher Scientific, Invitrogen, USA), 2.5 µL of specific-primer pair (10 µM), 0.1 µL of Taq DNA polymerase (ThermoFisher Scientific, Invitrogen, USA), and 1 µL of cDNA template (250 ng), and it was adjusted to a final volume of 50 µL with RNase-free water. The amplification reaction was performed under the following thermal cycling condition: 94 °C for 3 min, 36 cycles of 94 °C for 45 sec, 50 °C for 30 sec, 72 °C for 45 sec, and the final extension step at 72 °C for 10 min in a thermal cycler machine (TProfessional, Biometra, Göttingen, Germany). The PCR products were subjected to electrophoresis on 3% agarose gels and imaged using a ChemiDoc™Touch imaging system (Bio-Rad, Hercules, CA, USA); the band intensities were determined using the relative mRNA expression levels of the genes.

Table 1 Primer Sequence in RT-PCR

Gene	Primer sequence	Amplicon (bp)
MDR1	5'-GAA ACC AAC TGT CAG TGT-3' (forward) 5'-AGC ATC ATG AGA GGA AGT-3' (reverse)	120
β-actin	5'-AAG GCC AAC CGC GAG AAG ATG A-3' (forward) 5'-TGG ATA GCA ACG TAC ATG GCT G-3' (reverse)	74

Cytotoxicity assay

Cells were seeded in 96-well plates (5×10^3 cells for K562 and K562/adr, 5×10^4 cells for SH-SY5Y and SK-N-SH,100 µL/well) and incubated with increasing concentrations of PIRA (0–5 µM) in the presence and absence of verapamil (at final concentrations of 0.25 µM, 2.5 µM, and 5.0 µM) for 3 days. After incubation, 20 µL of resazurin stock solution (0.02 mg/mL) was added to each well. The plates were further incubated for 4 h in a 5% CO_2 incubator. Cell viability was assessed by the ability of the remaining viable cells to reduce resazurin to resorufin. The fluorescence intensity of resorufin (570 nm excitation/590 nm emission) was measured with a spectrofluoroscopic microplate reader (PerkinElmer LS55 spectrofluorometer). The 50% inhibitory concentration (IC_{50}), defined as the drug concentration causing 50% reduction in cell viability, was determined by plotting the concentration of the drug in the x-axis and the percentage of the cell inhibition in the y-axis. The experiments were run in triplicate.

Determination of P-gp–mediated uptake of pirarubicin in presence or absence of verapamil

Kinetic uptake of PIRA by cells was followed by monitoring of the decrease in the fluorescence signal at 590 nm (λ_{ex} = 480 nm) by following the method previously described.[23,24,27] Using this method, it is possible to accurately quantify the kinetic parameters of the drug uptake by the cells without compromising cell viability. All experiments were conducted in 1 cm quartz cuvettes containing 2 mL of buffer at 37 °C using a circulating thermostat water bath. Briefly, cells (10^6 cells/mL, 2 mL per cuvette) were incubated with or without various concentrations of verapamil (0–10 µM) for 10 min in glucose containing Luckoff-Na^+ at pH 7.25, under incessant stirring. After the addition of 20 µL of 10^{-4} M pirarubicin, yielding a concentration C_T equal to 1 µM, the decrease in the fluorescence intensity F_0 at 590 nm was monitored as a function of time. In the steady state, the curve F=f(t) reached a plateau and the fluorescence intensity was equal to F_n. The drug-cells system was thus in a steady state and the overall concentration C_n of the drug intercalated between the base pairs in the nucleus was $C_n=C_T.(F_0-F_n)/F_0$. Once the steady state was reached, the cell membranes were permeabilized by the addition of 0.02% Triton X-100, yielding the equilibrium state which was characterized by a new value F_N of the fluorescence intensity. The overall concentration C_N of the drug intercalated between the base pairs in the nucleus was then $C_n=C_T.(F_0-F_N)/F_0$. P-gp function was assessed by the ratio value of C_n/C_N as a function of the verapamil concentration. In the presence of verapamil, the uptake of PIRA was found to increase, resulting in an increase in the overall concentration C_n. In case of complete inhibition of the P-gp function or no effect on the P-gp function, the overall concentration of the drug intercalated between the base pairs in the nucleus in the steady state is expected to be the same as that in the equilibrium state: thus, the ratio of C_n/C_N equals to 1.

Statistical analysis

All data are expressed as mean±standard deviation (SD). Statistical significance was determined using Student's t-test between the groups treated and the control. A probability (*p*) value less than 0.05 was considered statistically significant.

Results

Expression of MDR1/P-gp mRNA in cell lines

The objective was to investigate whether P-gp, encoded by the *mdr1* gene, is expressed in neuroblastoma cell lines. The determination of MDR1 mRNA was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). The PCR products of P-gp, 120 bp, and β-actin, 74 bp, are shown in Figure 2a. Expression of the MDR1 mRNA was determined relative to the β-actin used as the internal control. Data on the expression of MDR1 mRNA relative to β-actin are shown in Figure 2b. The MDR1 mRNA expression was identified in K562 cells as equal to 0.45±0.19. In contrast, their resistance cell line, K562/adr, showed higher expression with value equal to 0.74±0.17.

The expression of MDR1 mRNA in the neuroblastoma cells demonstrated the low-level expression in the SH-SY5Y cells, with value equal to 0.33±0.17, while no expression was observed in the SK-N-SH cells. Unexpectedly, there was found the presence of non-targeted PCR in the SK-N-SH cells in the PCR product range of 200-300 base pairs.

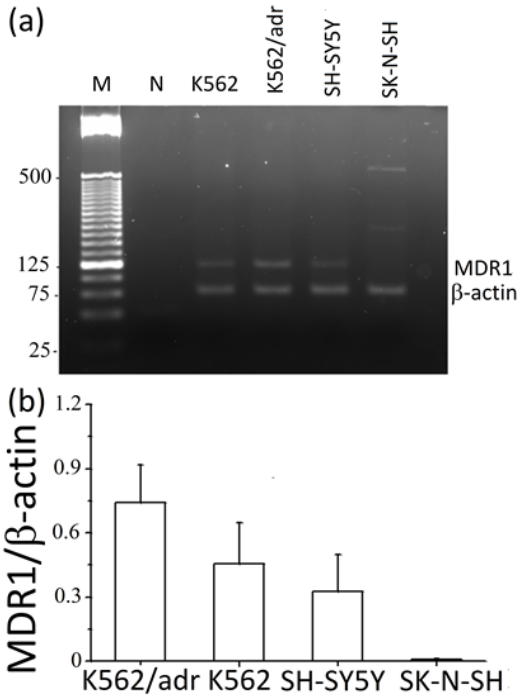

Figure 3 *RT-PCR analysis of MDR1 expression in K562, K562/adr, SH-SY5Y, and SK-N-SH cells. Total RNA from each cell line was subjected to RT-PCR analysis with primers specific for human MDR1. RT-PCR products were run on a 3% agarose gel to show the expression of the MDR1 genes. (a) The expected 120-bp product for MDR1 was present in the cell lines. The housekeeping gene human β-actin, 74-bp, was used as the internal control. (b) Relative mRNA expression of MDR1/P-gp normalized to human β-actin. Each value represents mean ± SD (n = 3).*

Uptake of pirarubicin by cells

Typical pattern of PIRA uptake by a P-gp overexpression model, K562/adr, is shown in Figure 1. After the addition of PIRA, the decrease in the fluorescence intensity at 590 nm was monitored as a function of time of incubation. Quenching of fluorescence intensity refers to the accumulation of PIRA within the nuclei of the cells. Once the steady state was reached, 0.02% Triton X-100 (v/v) was added, and the fluorescence intensity decreased to F_N. The overall nuclear concentration in the steady state (C_n) and the overall nuclear concentration in the equilibrium state (C_N) were calculated. As shown in Figure 1, C_n was lower than C_N, rendering the ratio of C_n/C_N less than 1. This observation provided evidence of a gradient in the concentration of PIRA, with a low free intracellular concentration against a high extracellular concentration. The gradient was generated by the plasma membrane protein transporter, P-gp. Therefore, the accumulation of PIRA in K562/adr was very low as a result of the P-gp function. In the negative control for the P-gp function, the sensitive K562 cell, the same experiment was performed (Figure 3a). It was observed that upon adding PIRA, the decrease in the fluorescence intensity at 590 nm was higher than that of the K562/adr cells. The fluorescence intensity did not change after the addition of 0.02% (v/v) Triton X-100; C_n was similar to C_N, rendering the ratio of C_n/C_N close to 1. These events indicated that no gradient in the concentration of PIRA was observed in the sensitive K562 cells. As for the drug uptake in both the neuroblastoma cell lines, SH-SY5Y and SK-N-SH, the behaviour of kinetic drug uptake was similar to that observed in the sensitive K562 cells. The ratios of C_n/C_N in all the cell lines tested are shown in Figure 3b.

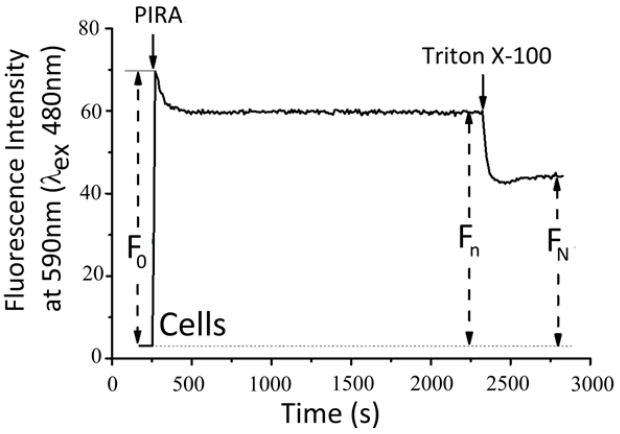

Figure 1 *Uptake of pirarubicin by K562/adr, the P-gp overexpression model, with fluorescence intensity at 590 nm (λ_{ex} =480 nm) was recorded as a function of time until the steady state. Cells, 2×10^6 cells, were suspended in a cuvette filled with 2 mL buffer of pH 7.25. At t=0, 20 µL of 100 µM PIRA was added to the cells, which yielded a C_T=1 µM PIRA solution. The fluorescence intensity was then F_0. Once the steady state was reached, the fluorescence was F_n, and the concentration of the drug intercalated between the base pairs in the nucleus was $C_n=C_T$. $(F_0 - F_n)/F_0$. The addition of 0.02% Triton X-100 yielded the equilibrium state. The overall concentration C_N of the drug intercalated between the base pairs in the nucleus was then $C_N=C_T$. $(F_0 - F_N)/F_0$.*

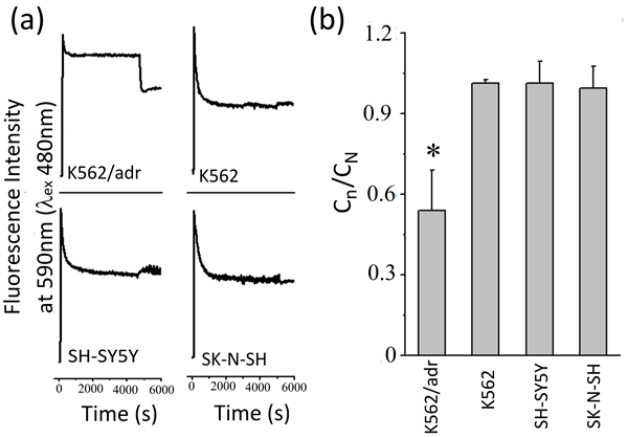

Figure 3 *The representative data of the uptake of pirarubicin (PIRA) by K562/adr, K562, SH-SY5Y, and SK-N-SH (a). The cells were analyzed in the experiment, as previously described. The ratio of C_n/C_N was determined to investigate the effect of the P-gp function on drug accumulation (b). The data are presented as mean±SD (n=3). *P<0.05 versus K562, SH-SY5Y, and SK-N-SH.*

Kinetic P-gp–mediated uptake of pirarubicin in the presence of verapamil

Verapamil, a calcium channel blocker, is a known P-gp inhibitor.[28,29] It was found that verapamil has the capability to reverse multidrug resistance.[30,31] Therefore, to evaluate the function of P-gp, the uptake of PIRA by cells was determined in the presence of verapamil. The typical experiment in the K562/adr cells, the positive control of the P-gp function, is shown in Figure 4a. In the experiment, when the cells were incubated in the presence of verapamil, the fluorescence signal decreased as a function of time of incubation and then plateaued after reaching the steady state. The addition of Triton X-100 did not yield any modification in the fluorescence signal, indicating that the P-gp function was completely blocked and there was no effect of the P-gp–mediated PIRA pump outside the cell. However, when the cells were incubated without verapamil, the accumulation of PIRA in the nucleus in the steady state was very low. The addition of Triton X-100 yielded a decrease in the fluorescence signal, indicating a gradient in the drug concentration which was generated by the presence of the pump. From the experiment, the overall concentration of the drug bound to the nucleus in the steady state, C_n, and the overall concentration of the drug bound to the nucleus in the equilibrium state, C_N, were determined. The ability of verapamil to inhibit P-gp-mediated uptake of PIRA, with the ratio C_n/C_N as the function of the verapamil concentration added, is demonstrated in Figure 4b. In K562/adr cells, the ratio of C_n/C_N increased continuously as the concentration of verapamil increased. When the same experiment was performed in K562, SH-SY5Y, and SK-N-SH cells, no modification in the fluorescence signal was observed either in the condition of the presence of verapamil or in the condition of the absence of verapamil. Thus, the concentration of the drug bound to the nucleus in the steady state, C_n, was not significantly altered by verapamil, rendering the ratio of C_n/C_N close to 1.0 (Figure 4b), which indicates that no function of P-gp was observed on the accumulation of PIRA in these cell lines.

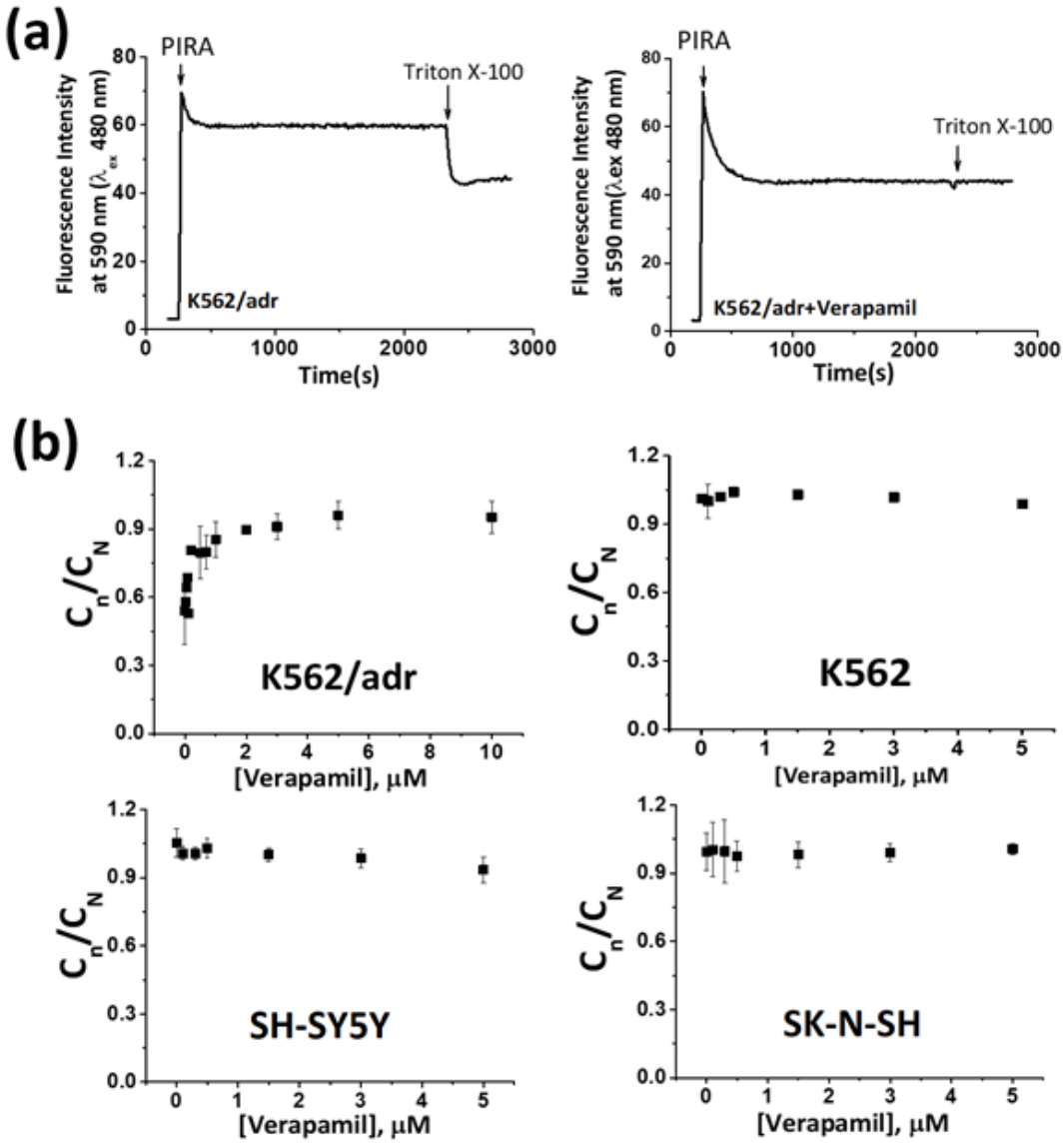

Figure 4 *The effect of verapamil on the drug accumulation in K562/adr, K562, SH-SY5Y, and SK-N-SH. In the model of the P-gp overexpressing cell line, K562/adr, the typical characteristics of P-gp–mediated uptake of PIRA were studied in controlled conditions and exposed to verapamil (a). The values of C_n/C_N are plotted as a function of the concentration of verapamil (b). Each value represents mean±SD (n=3).*

Effect of verapamil on cellular sensitivity to pirarubicin

To confirm the function of P-gp in long-term treatments, cotreatment assay of PIRA with various concentrations of verapamil was performed. The results of the cotreatment assays were expressed as IC_{50} values. As shown in Figure 5, addition of verapamil significantly increased the sensitivity of PIRA to K562/adr cells in a dose-dependent manner; however, no effect of verapamil was observed in K562, SH-SY5Y, or SK-N-SH. Interestingly, it was observed that 5 μM verapamil significantly decreased the IC_{50} value when compared with untreated control in SK-N-SH.

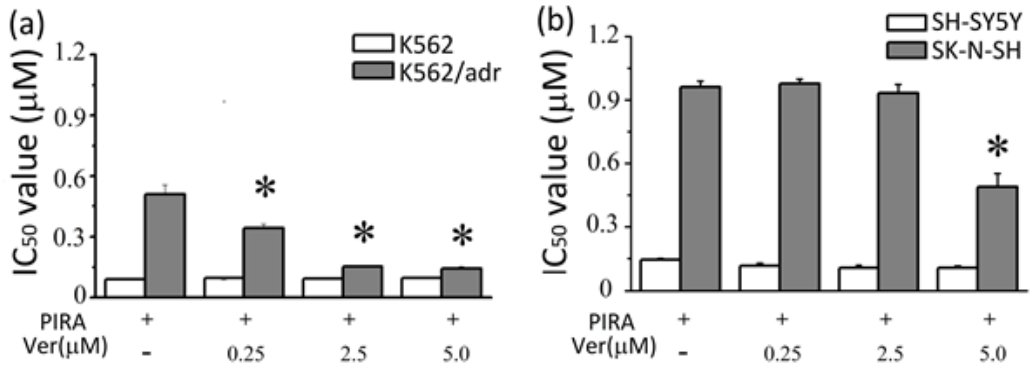

Figure 5 *The IC_{50} value after cotreatment of PIRA with verapamil in K562 and K562/adr cells (a), and SH-SY5Y and SK-N-SH (b). The cells were treated with different concentrations of PIRA in the presence of various concentrations of verapamil (0–5.0 μM) for 72 h and analyzed using the AlamarBlue® assay. The data are presented as mean±SD (n=3). *p<0.05 versus the control of each cell line.*

Discussion

Multidrug resistance phenomenon is a major hindrance to successful treatment of neuroblastoma patients. In tumor cells, the drug penetration across cell membranes can be hindered by increased presence of the ATP-binding cassette (ABC) which contains membrane proteins that translocate a wide variety of substrates across extra- and intracellular membranes.[32] As a result, intracellular drug concentration within the targets is reduced to a level below its therapeutic threshold. It should be noted that certain ABC transporters confer multidrug resistance to numerous drugs differing in chemical structure and mechanisms of action.[4] An important member of this family that has been extensively studied is P-gp.[33,34] Regarding the type of drug to be transported, conventional chemotherapeutics have been the obvious first consideration because those are the antitumor drugs in most widespread use. In this study, the sensitive and the resistant K562 cells were selected as the negative control and the positive control, respectively, for P-gp expression. The confirmation was by observing the increase in the IC_{50} value of anthracycline, PIRA, in the drug-resistant K562/adr cells compared with their corresponding sensitive cells, as shown in Table 2. Thus, the *in vitro* setup that was used can be considered to be a suitable model to investigate the function of P-gp.

Table 2 IC_{50} Values and ratio of C_n/C_N of Human Cancer Cell Lines after Exposure to pirarubicin (PIRA)

Cell line	IC_{50} (μM)	C_n/C_N
Erythroleukemic cell line		
K562/adr	0.51±0.05	0.54±0.15
K562	0.09±0.01	1.01±0.01
Neuroblastoma cell line		
SH-SY5Y	0.15±0.01	1.01±0.08
SK-N-SH	0.96±0.03	1.00±0.08

Note: *Each value is expressed as mean±SD (n=3). Means with different letters are significantly different at p<0.05.*

Abbreviations: *IC_{50}, 50% inhibitory concentration; C_n, overall concentration of PIRA bound to the nucleus in the steady state; and C_N, overall concentration of the drug bound to the nucleus in the equilibrium state.*

It is known that acquisition of drug resistance and/or changes in cellular drug accumulation may be related to changes in the molecular expression of the drug transporter.[35] Initial experiments with mRNA from neuroblastoma cell lines demonstrated that MDR1 mRNA expression was common as demonstrated by reverse transcription PCR. In this study's experiments, included as controls for MDR1 mRNA expression were the drug-sensitive parental K562 cell line and the drug-resistant sublines, K562/adr, which are 6-fold resistant to PIRA. Detectable levels of MDR1 mRNA were seen in SH-SY5Y, and not in SK-N-SH. The level of MDR1 mRNA expression in SH-SY5Y was close to the same level of expression in the sensitive K562 cells, indicating the presence of endogenously expressed P-glycoprotein. It was found that SH-SY5Y expressed both *mdr1* gene and *mrp1* gene.[36-38] A study by Bates et al. found that expression of *mdr1* gene in human neuroblastoma is related to the degree of differentiation modulated by retinoic acid.[36] SH-SY5Y cells are derived from a subclone of the parental neuroblastoma cell line SK-N-SH and can be differentiated into mature human neurons through a variety of different mechanisms including the use of retinoic acid.[39] Interestingly, apart from MDR1 mRNA expression, one study characterized the ABC transporter protein in SH-SY5Y and found that MRP1 mRNA in human SH-SY5Y cells had the highest expression levels of ABC transporters, while MDR1 mRNA was expressed at levels approximately 30% of those for MRP1 mRNA.[38] Michelle et al. showed that high levels of MRP1 mRNA expression are related to poor clinical outcome in a large prospective study of primary neuroblastoma;

MDR1 mRNA expression, nevertheless, was demonstrated to have no prognostic significance.[37] However, no evidence has been found for the expression of human BCRP mRNA in SH-SY5Y.[38] It seems that MRP1 protein plays a more prominent role in drug resistance than P-gp. However, no evidence has been found proving the prominent role of MDR protein in neuroblastoma cells. Further studies should be carried out to clearly demonstrate this phenomenon.

As is known, mRNA levels cannot be used as surrogates for corresponding protein levels without verifying their function. All mRNAs are not equal with regard to function of proteins. Based on this notion, it was investigated whether the P-gp function takes place in the neuroblastoma cells where they are expressed. In this study, drug kinetic uptake using the spectrofluorometric method has been used to get a better understanding of the function of multidrug transporters that mimic living cells. This principle is used to investigate whether human neuroblastoma cells express the functionally active P-gp efflux pump. To demonstrate the functionality of P-gp, the impact of inhibiting P-gp–mediated PIRA efflux was investigated in the presence of verapamil. Verapamil is a specific first-generation MDR1 inhibitor. Its efficiency has been confirmed in certain types of cancer, both in preclinical studies and in clinical use.[28] In contrast to the findings of mRNA expression, kinetic data demonstrated that no gradient, generated by P-gp, of PIRA concentration was present in both the neuroblastoma cells. Thus, it can be suggested that accumulation of PIRA is not associated with the function of P-gp. Not only the inhibitory effect of verapamil on P-gp–mediated PIRA transport but also the effect of reversal of P-gp activity when combined with conventional chemotherapy, pirarubicin, for long-term treatment, was observed. Importantly, the apparent IC_{50} values for various concentrations of verapamil were not significantly different between SH-SY5Y and SK-N-SH, except at 5 µm verapamil, which is similar to what was observed in the sensitive K562 cells. At the same time, in the positive control of the P-gp model, verapamil enhanced the chemosensitivity of PIRA in the resistant K562 cell line in which the expression of MDR1 was highly detectable. In SK-N-SH, it was found that 5 µM verapamil enhanced the efficacy of PIRA which was not consistent with the kinetic study. However, it was found that the percentage of viable cells of SK-N-SH was reduced to 5% after treated with 5 µM verapamil (data not shown). Therefore, it was possible that this discrepancy results affect from the toxicity of verapamil on SK-N-SH. From both the kinetic and the cotreatment data, it can safely conclude that endogenous expression of P-gp in neuroblastoma cells is not functional and is associated with increased cytotoxic drug accumulation. The reason for this discrepancy was unclear. However, possible explanations for this effect have been proposed. It might be possible that the MDR phenotype is not fully developed at these lower levels of P-gp expression, as can be observed in the sensitive K562 cell. Other *in vitro* studies have also supported this notion that MDR proteins effect drug resistance more actively in higher levels of resistance.[40-42] Notion that different isoforms of P-gp may possess different specificities was proposed. This hypothesis is supported by Sieczkowski et al. who found that core-glycosylated P-gp, in contrast to the fully glycosylated 180-kDa species of P-gp, is inactive and accumulates in the endoplasmic reticulum.[43] Bates et al. showed that drug accumulation failed to show decrease in 3H-vinblastine accumulation in the SK-N-SH and the SH-SY5Y cell lines after treatment with retinoic acid over a time period in which a clear increase in P-gp was observed.[36] It can be inferred that increased levels of MDR1 mRNA expression are not necessarily associated with increased cytotoxic drug accumulation. This is in accordance with the finding of this study that verapamil showed no effect on the toxicity of PIRA in SH-SY5Y, where there is endogenous expression of P-gp.

Conclusion

This study has demonstrated no significant P-gp function. This result correlates with the mild expression of MDR1 mRNA in both human neuroblastoma cell lines.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Plan evaluation of intensity modulated radiation therapy and volumetric modulated arc therapy in bilateral breast irradiation with 3-isocenter technique

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ABSTRACT

Background: Bilateral breast irradiation needs a sophisticated treatment planning due to large treated volume and the concern regarding low dose to the volume of irradiated normal lungs and heart.

Objectives: Aim of this study was to compare the dosimetric parameter between intensity modulated radiation therapy (IMRT) and volumetric modulated arc therapy (VMAT) plan with 3-isocenter technique for bilateral breast irradiation.

Materials and methods: Retrospective cases from 5 bilateral breast cancer were reviewed. Eclipse treatment planning version 11.0.31 was used for IMRT and VMAT optimization. The prescribed dose was 50 Gy in 25 fractions. Three isocenters were used to setup position; left, middle and right of PTV volume in lateral direction changes while the longitudinal and vertical directions were fixed. PTV of $D_{95\%}$, conformity index ($V_{prescribed}/V_{PTV}$) and dose homogeneity ($D_{5\%}$-$D_{95\%}$), mean lung dose (MLD) and volume receiving 20 Gy (V_{20Gy}), heart volume received dose 25 Gy (V_{25Gy}), maximum dose of left anterior descending coronary artery (LAD), and the number of MUs per fraction were compared for both techniques.

Results: Mean $D_{95\%}$ of PTV was 48.7±0.2 Gy for IMRT and 48.7±0.8 Gy for VMAT. Mean CI for IMRT and VMAT techniques were 0.97±0.0 and 0.98±0.0, respectively. IMRT plans showed significantly better for homogeneity of dose distribution in PTV volume than VMAT with the values of 5.6±0.7 Gy for IMRT and 7.6±1.1 Gy for VMAT. MLD was not significantly different between the plans 16.2±0.6 Gy (IMRT) and 16.6±0.9 Gy (VMAT). However, V_{20Gy} of lung showed significant difference for IMRT (25.8±4.8%) and VMAT (31.6±2.4%). The volume of heart received dose 25 Gy was 8.3±3.3% (IMRT) and 12.2±5.0% (VMAT). Two tails student t-test exhibited no significant differences between IMRT and VMAT in almost all parameters. Ratio of MU_{IMRT} to MU_{VMAT} was 3.0 which was the crucial part of using VMAT plan for treatment.

Conclusion: The 3-isocenter technique of VMAT plan for bilateral breast irradiation shows comparable plan quality to IMRT with shorter treatment delivery time. It demonstrates feasible to apply in clinical used due to short treatment time and easy setup.

Introduction

Bilateral breast cancer is a rare clinical manifestation and treatment of bilateral breast is a real challenge. Bilateral breast irradiation needs a sophisticated treatment planning due to the large treated volume and the concern regarding low dose to the volume of irradiated normal lungs and heart. The 3D tangential fields seem to be an appropriate technique because of shorter treatment time but the gap junction between medial fields of both sides is a technical issue. IMRT technique is time-consuming in treatment delivery from high number of MUs. VMAT technique is

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investigated to be feasible for irradiation in many clinical cases including breast cancer.[1-6] There is an advantage over IMRT in terms of treatment time efficiency. However, the use of gantry rotation in VMAT has limited the efficacy of its planning for bilateral breast irradiation. Single isocenter is not capable for dose coverage in the treatment target. We, therefore, study the feasibility of using 3-isocenter technique for bilateral breast irradiation and compare DVH parameter between IMRT and VMAT plan with this technique.

Materials and methods

This study was the retrospective of 5 bilateral breast cancer cases. Patient's position was supine on breast board (CIVCO Medical Solution, Iowa, USA) with both hands overhead. Knee support (Civco Radiotherapy, Iowa, USA) was used for patients to feel more comfortable during simulation and treatment. The Computed Tomography (CT) data was acquired with Siemens Somatom Definition AS Open 64 slices (Siemens, Erlangan, Germany) in 2 mm slice thickness. PTV volume and the main organs at risk (OARs), lung, heart and left anterior descending coronary artery (LAD) were delineated following the study from Dijkema *et. al*.[7] by the same radiation oncologist. PTV volume was removed from skin surface for 5 mm using Boolean function. Three isocenters were used to setup position: left, middle and right of PTV volume while the longitudinal and vertical were similar. Eclipse treatment planning version 11.0.31 was used for IMRT and VMAT optimization by using Varian clinac iX (Varian Oncology systems, Palo Alto, CA, USA) 6 MV with 120 MLCs. All plans were optimized by the same medical physicist. Beam angles for IMRT were 220°, 235°, 320°, 45° and 60° for the right lateral isocenter, 0° for the middle isocenter and 310°, 325°, 45°, 60°, and 115° for the left lateral isocenter. For VMAT technique, the gantry angles of partial arcs were utilized from 220° to 50° (CW-CCW-CW) for right lateral isocenter, 140° to 310° (CCW-CW) for the middle isocenter and 310° to 140° (CCW-CW-CCW) for the left lateral isocenter. Collimator was rotated about 5° to 10° to minimize the tongue and groove effect.[8] Isocenter setup and beam angles for IMRT and VMAT plans are shown in Figure 1. The prescribed dose was 50 Gy in 25 fractions. Criteria for dose optimization were 95% of PTV volume receiving the prescribed dose. The maximum dose was lower than 107%. The mean lung dose (MLD) and volume receiving 20 Gy (V_{20Gy}) were lesser than 16 Gy and 22%, respectively. The dose in all plans were kept as low as possible to spare lung, heart and LAD including minimize mean and maximum dose. The PTV volume of $D_{95\%}$, Conformity index (volume of prescribed dose divided by the PTV volume: $V_{prescribed}/V_{PTV}$) and dose homogeneity ($D_{5\%}$-$D_{95\%}$), MLD and V_{20Gy} of lung, the heart volume received dose 25 Gy (V_{25Gy}), maximum dose of LAD and the number of MUs per fraction were compared for both techniques.

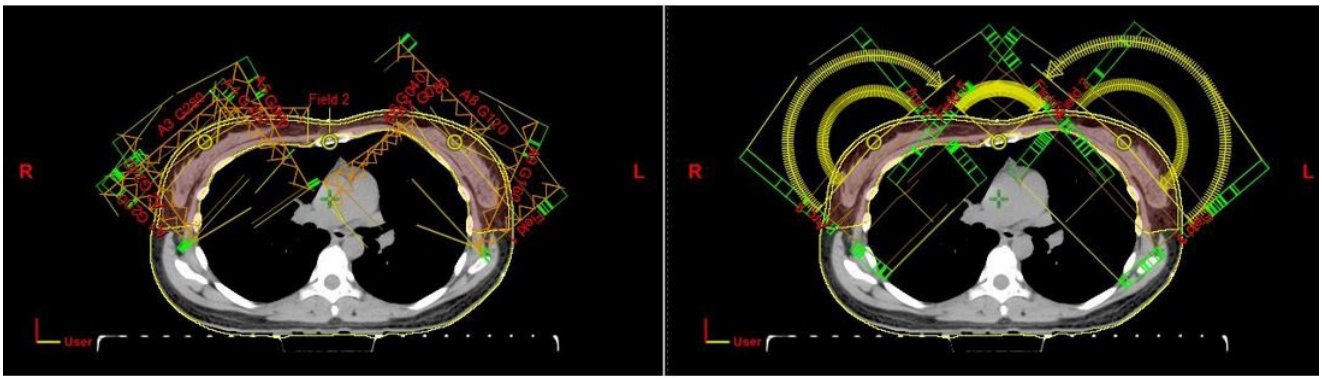

Figure 1 *Isocenter setup and beam angles for IMRT (left) and VMAT (right) planning.*

Results

Mean lung volume and PTV volume were 2624±93 cm^3 and 1459±440 cm^3, respectively. Mean dose of $D_{95\%}$ for PTV volume was 48.7±0.2 Gy for IMRT and 48.7±0.8 Gy for VMAT. Mean CI between IMRT and VMAT plans presented small differences with the value of 0.97±0.0 for IMRT and 0.98±0.0 for VMAT. IMRT plans showed significantly better dose homogeneity of dose distribution in PTV volume than VMAT plans was supported by p value. PTV dose homogeneity was 5.6±0.7 for IMRT and 7.6±1.1 for VMAT. Lung volume of V_{20Gy} showed higher dose than criteria (22 Gy), 25.8±4.8% for IMRT and 31.6±2.4% for VMAT, which was significantly difference. MLD was still within the criteria, 16.2±0.6 Gy (IMRT) and 16.6±0.9 Gy (VMAT). The volume of heart received dose 25 Gy was 8.3±3.3% (IMRT) and 12.2±5.0% (VMAT). Maximum dose of LAD was 37.3±3.0 and 37.0±6.4 for IMRT and VMAT, respectively. Ratio of MU_{IMRT} to MU_{VMAT} of 3.0 represented longer treatment time in IMRT. Summary of DVH based analysis is explored in Table 1. VMAT plan showed V_{20Gy} of lung higher significantly than the IMRT plan because the VMAT plan has more beam entry angles.[8] However, the mean lung dose was not significantly different in both plans and was within the criteria. This was consistent with Nicolini *et at*.[9] study where they found VMAT produced better sparing at the mid- to high- dose levels compared with IMRT. In addition, VMAT generates lower number of MU needed compared with IMRT plan. Another major factor to evaluate was the treatment time. The longer treatment time may induce intrafraction motion which lead to increased doses to OARs.[10] Overall, the VMAT showed more

effective plan than the IMRT plan in regards to the non-significant differences in two tails student t-test in almost parameters and lesser the treatment time. Isodose distribution as an example of both techniques is shown in Figure 2. DVH of PTV and OARs of the same case are shown in Figure 3.

Table 1 Summary of DVH based analysis of bilateral breast cancer.

Organs/parameters	Criteria	IMRT	VMAT	*p* value
PTV				
- $D_{95\%}$ (Gy)	At least 47.5 Gy	48.7±0.2	48.7±0.8	0.94
- CI	Close to 1	0.97±0.0	0.98±0.0	0.55
- Dose homogeneity	Close to 0	5.6±0.7	7.6±1.1	<0.05*
Lung				
- Mean (Gy)	22 Gy	16.2±0.6	16.6±0.9	0.54
- V_{20Gy} (%)	20%	25.8±4.8	31.6±2.4	<0.05*
Heart				
- V_{25Gy} (%)	25%	8.3±3.3	12.2±5.0	0.27
Max. LAD (Gy)	50 Gy	37.3±3.0	37.0±6.4	0.93
Total MUs		2621.05±390.3	865.7±81.4	<0.05*

**Significant relative difference tested by two-tailed student t-test*

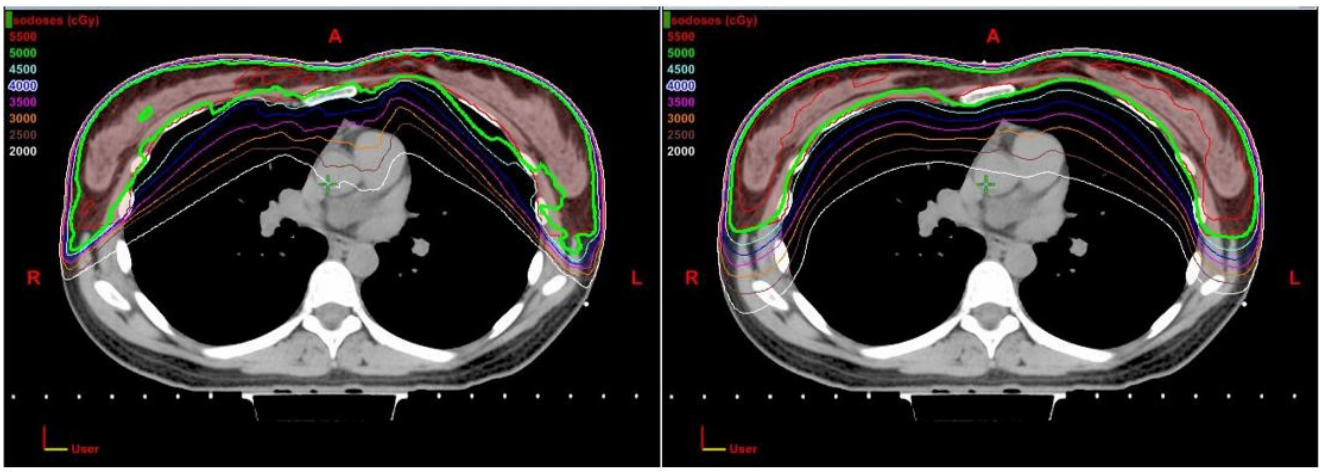

Figure 2. *Isodose distribution of the example case for IMRT (left) and VMAT (right) plan.*

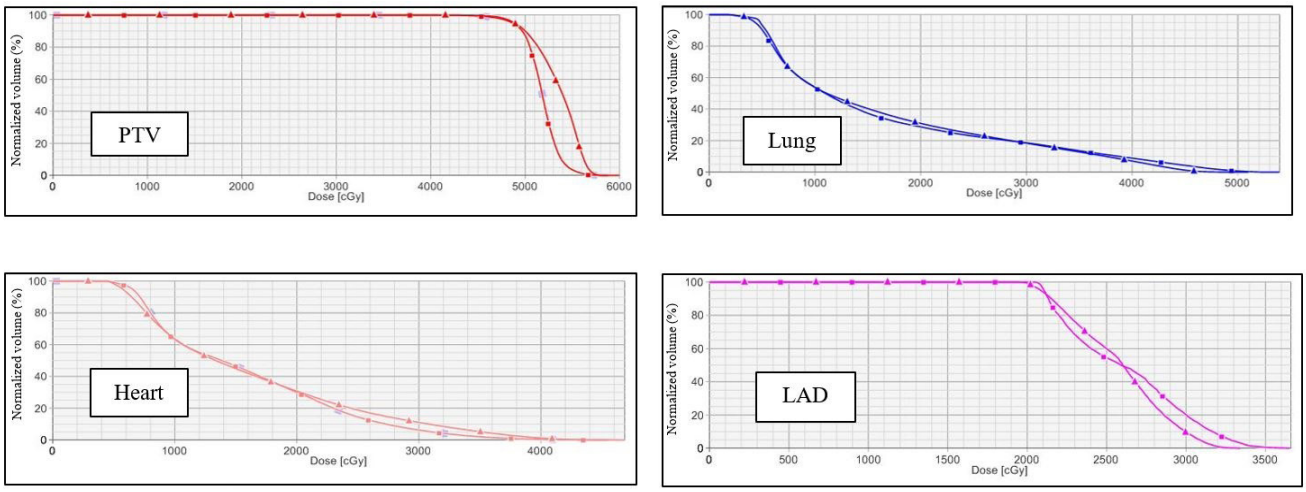

Figure 3. *Isodose distribution of the example case for IMRT (square) and VMAT (triangle) plan.*

Discussion

Our study discovered that IMRT and VMAT techniques for bilateral breast irradiation were comparable for DVHs analysis which which agreed well to the results from Nicolini *et al*.[8] However, the study by Nicolini had created the treatment plans with only single isocenter due to the supine position without breast board. In our study, patient was supine on the breast board with both hands overhead. If the plans were performed using a single isocenter, the isodose distribution to PTV volume would not be covered by the prescribed dose due to the limitation of the gantry rotation and the couch position. The other consideration taken into account was the treatment delivery of VMAT. Delivery time of VMAT plan was significantly shorter than IMRT technique.

Conclusion

Overall, the 3-isocenter technique of VMAT plan presented comparable plan quality to IMRT for bilateral breast irradiation although larger volume of lung receiving low dose was discovered in regards to more beam entry angles. However, the delivery time of VMAT was found shorter 3 times in comparison to IMRT. In addition, the setup technique was easy for technologist as it only shifted the couch position to lateral direction. Therefore, it is feasible to apply this technique in clinical situation.

Conflict of interest

We hereby state that there is no conflicts of interest.

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Large-scale preparation of purified monoclonal antibody from cell culture supernatant: A case study with a monoclonal antibody to ζ-globin chain

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ABSTRACT

Background: Nowadays, monoclonal antibodies have become important tools used in biomedical research, diagnosis and treatment of diseases. The mAbs are isolated from cell culture media, which usually contain different proteins in addition to antibodies. Obviously, antibody purification is becoming critical to guarantee its reliable application. Protein A and G resins are the most efficient and widely-used ligands in chromatographic methods. Nevertheless, some mAbs could not be purified by Protein A or G column.

Objectives: In order to explore the alternative method for purification of an IgG1 mAb, four different types of affinity chromatography were studied and compared in term of efficiency.

Materials and method: Chromatography using four commercial ligands, Protein A, Protein G, Protein L and engineered recombinant Protein A, was employed to purify the anti-zeta globin chain mAb clone PL3. The performance of the chromatography was compared in terms of yield, purity and biological activity of mAbs.

Results: The mAb PL3 could only be purified by using engineered recombinant Protein A column. The biological activity and purity of the purified mAbs were checked by ELISA and SDS-PAGE, respectively. It was found that the obtained purified mAbs were high purity and retained their biological activity.

Conclusion: In conclusion, engineered recombinant Protein A column is an alternative technique for large-scale purification of mAbs produced by unusual hybridoma clone that could not be purified by other columns.

Introduction

In 1975, Kohler and Milstein discovered hybridoma technology, which made possible the production of monoclonal antibodies (mAbs) possessing high affinity and specificity toward defined targets.[1-4] Nowadays, monoclonal antibodies have become obligatory tools used in biomedical analysis, purification, diagnosis and treatment of various diseases. For diagnosis, antibodies are the ideal biological recognition reagents; therefore, they are useful in a range of analytical platforms, e.g. immunohistochemistry, immunocytochemistry, enzyme linked immunosorbant assay (ELISA), flow cytometric analysis and immunosensors.[2, 5-7] The mAbs are usually isolated from cell culture media, which contain different proteins in addition to antibodies. Hence, the mAb purification becomes imperative for its reliable

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application and influences the reproducibility and stability of immunoassays and immunotherapy. Antibody purification can be achieved by several methods based on the specific physical and chemical properties of antibodies, such as size, solubility, charge, hydrophobicity and binding affinity.[5, 8-11] The techniques that are applied for antibody purification include precipitation, electrophoretic separation, filtration and liquid and affinity chromatography.[11] In general, affinity chromatography-based purification continues to be the most efficient and widely used.[5, 7-10, 12] However, the used ligands in chromatographic methods are species- and isotype-dependent and thus, adsorptions sometimes exhibit very low capacity.[5, 8-10, 12, 13]

From our previous study, mAb clone *PL3*, which is a mouse IgG1 anti-ζ-globin chain mAb, was generated by hybridoma technique.[14] The mAb clone *PL3* was used to develop the immunoassay for screening α-thalassemia 1 Southeast Asian (SEA)-type, which is the most common mutation of α-thalassemia 1 in the Thai population. Unfortunately, Protein G column, the common purification method for mouse mAb IgG isotypes, cannot be used to purify mAb clone *PL3*. Therefore, a suitable protein column ligand is needed to be investigated in order to produce a large amount of mAb clone *PL3*. We compared four commercial ligands (Protein A, Protein G, Protein L and modified Protein A) for the anti-ζ globin chain mAb clone *PL3* purification. The performance of the specific chromatography was compared in terms of yield, purity and biological activity of the mAb. Our study can be applied for purification of other unusual mAbs.

Materials and methods

Materials

Antibodies

Mouse anti-ζ globin chain mAb clone *PL3*[14]; mouse anti-γ4 globin chain mAb clone PB1[15]; mouse anti-γ4 globin chain mAb clone *Thal* N/B; mouse anti-Ag85B mAb clone *AM85B-8B*[16] were generated in our research center. The horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin Abs were obtained from Dako (Glostrup, Denmark).

Affinity chromatographic resins

Four commercial affinity chromatographic resins were used in this study (Table 1). The first resin was Protein A-coated agarose (Thermo Fisher Scientific, Waltham, MA, USA). Protein A is a cell wall component produced by *Staphylococcus aureus*. This ligand has the ability to bind specifically to the Fc region of immunoglobulin molecules, especially IgG. The second resin was recombinant Protein G immobilized on agarose (GE Healthcare Bio-Sciences, Uppsala, Sweden). The recombinant Protein G contains two IgG binding regions. The albumin binding region of native Protein G has been genetically deleted to avoid undesirable cross-reactions with albumin. The third resin was recombinant Protein L immobilized on agarose (GE Healthcare Bio-Sciences). This resin has strong affinity to the variable region of the antibody's kappa light chain. The fourth resin was MabSelect SuRe (GE Healthcare Bio-Sciences), an engineered recombinant Protein A, which is composed of an alkali-stabilized Protein A derivative immobilized on agarose. This ligand has enhanced alkali stability and a high binding capacity for IgG.

Table 1 Chromatographic conditions used for each affinity chromatography.

Chromatography name	Binding buffer	Elution buffer	Regeneration buffer
Protein A	20 mM sodium phosphate bufferf, 150 mM NaCl, pH 7.5	0.1 M glycine-HCl, pH 2.7	15 mM NaOH and 5 N NaOH
HiTrap Protein G	20 mM sodium phosphate buffer, pH 7.0	0.1 M glycine-HCl, pH 2.7	15 mM NaOH and 5 N NaOH
HiTrap Protein L	20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.0	0.1 M glycine-HCl, pH 2.7	15 mM NaOH and 5 N NaOH
Mabselect SuRe	20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.0	0.1 M sodium citrate, pH 3.0-3.6	15 mM NaOH and 5 N NaOH

Hybridoma cell culture

Hybridomas producing anti-ζ globin chain mAb clone *PL3*, isotype IgG1,[14] were cultured in completed IMDM (cIMDM), which is 10% fetal bovine serum (FBS)-IMDM supplemented with Gentamycin (4 mg/mL) and Fungizone (5 mg/mL), at 37°C in 5% CO_2 humidified incubator. To eliminate IgG contamination from supplement serum before antibody purification process, the *PL3* hybridoma cells were adapted for culturing in Hybridoma-Serum Free Media (SFM) (Thermo Fisher Scientific) by gradually increasing the ratio of Hybridoma-SFM to cIMDM from 0:100 to 25:75, 50:50, 75:25 until 100% Hybridoma-SFM.

Preparation of hybridoma culture supernatant

The *PL3* hybridoma cells ($1x10^6$ cells/mL) were cultured in 100% Hybridoma-SFM at 37°C in 5% CO_2 humidified incubator for 5 days. The culture supernatant was collected, centrifuged and filtered through a 0.45 µm filter to remove cells and cell debris.

Purification of mAbs

All affinity chromatography was performed at ambient temperature using an ÄKTAprime plus equipped with a

fraction collector (GE Healthcare Bio-Sciences). One mL packed beads in 1 cm internal diameter columns, were carried out. The buffer system was suggested by the individual medium manufacturers and described in Table 1.

Before antibody purification, all materials were equilibrated at room temperature. Air bubbles were removed from all the buffers using a sonicator. Both buffers and hybridoma culture supernatant were filtrated through a 0.45 µm filter. The column was equilibrated with 10 column volumes of binding buffer at 1 mL/min of flow rate. Culture supernatant was loaded by pumping into the column, followed by at least 10 column volumes of binding buffer to wash unbound protein. After the absorbance reached a steady baseline, the column was eluted with elution buffer, maintaining a flow rate of 1 mL/min. The fractions were collected in tubes that contained neutralizing buffer (1 M Tris-HCl, pH 9.0) to neutralize pH. The concentration of protein obtained in each fraction was measured using a Nanodrop (Thermo Fisher Scientific). After the purification process, the column was washed by adding 10 column volumes of regeneration buffer followed by binding buffer and filled up with 20% ethanol for new round purification.

Indirect ELISA

Indirect ELISA was performed as described before.[14] Hb Bart's hydrops fetalis hemolysates containing Hb Bart's and ζ globin chain (kindly provide by Professor Dr. Suthat Fucharoen) or HbF were coated on the ELISA plate. *PL3* hybridoma culture supernatant was added to the antigen-coated plate. The antigen-antibody complexes were monitored by adding horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins antibody (Dako) for 1 hr at 37°C. After that, 3,3',5,5' tetramethylbenzidine (TMB) substrate (Zymed, South San Francisco, CA, USA) was added and incubated for 15 mins at room temperature in the dark. The reaction was stopped with 1 N HCl. The colorimetric signal was measured at OD 450 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to determine the purity of the purified mAbs. Five micrograms of proteins were loaded in each lane and separated on 10% polyacrylamide gel by electrophoresis. The SDS-PAGE was performed as described elsewhere. The protein bands were visualized by PageBlue Protein Staining (Thermo Fisher Scientific).

Results

Determination of monoclonal antibodies against ζ-globin chains

Firstly, we determined the activity of mAb *PL3* after thawing and propagating of the *PL3* hybrodoma cells in cIMDM by indirect ELISA. As shown in Figure 1, the hybridoma culture supernatant showed positive reactivity with Hb Bart's hydrop fetalis hemolysates and did not show cross-reaction with HbF. The positive control, anti-γ-globin chain mAb clone *Thal* N/B, showed positive reactivity with both Hb Bart's lysate and HbF. In contrast, the isotype-matched control mAb, *AM85B-8B*, showed negative reactivity with all tested antigens. These results indicated that the *PL3* hybridoma cells could still produce and secrete the mAbs specifically reacting to ζ-globin chains

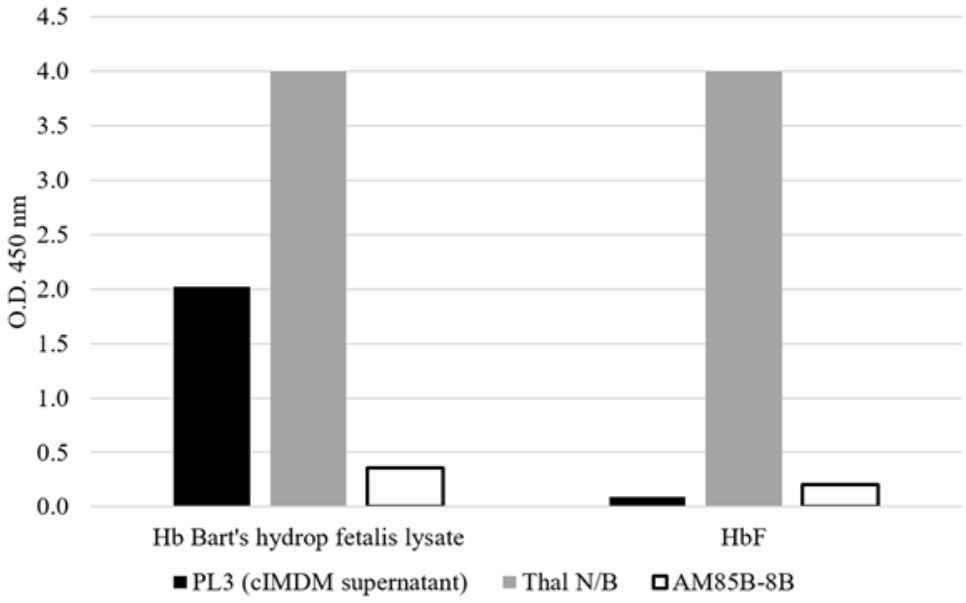

Figure 1. *Activity of mAb PL3 in hybridoma culture supernatant after culture in cIMDM. Indirect ELISA was performed using hemolysates of Hb Bart's hydrops fetalis (Bart's lysates) or purified HbF as antigen. Purified Thal N/B mAbs was used as positive control, whereas purified AM85B-8B mAb was used as isotope-matched control mAb. The antibody-antigen complexes were monitored by HRP-conjugated rabbit anti-mouse immunoglobulins.*

14 after long-term freezing.

For mAb purification, *PL3* hybridoma cells were adapted to culture in serum free media (SFM) to discard contaminated bovine immunoglobulins contained in cIMDM. We further checked the activity of mAbs produced by the *PL3* hybridoma cells cultured in SFM-IMDM. The *PL3* Hybridoma-SFM supernatant showed positivity with Hb Bart's hydrops fetalis hemolysates, as positive control mAb *Thal* N/B (Figure 2). No positive reactivity was detected in isotype-matched control mAb. This result suggested that mAb *PL3* was produced by hybridoma cells in serum free media condition. The culture of hybridoma in serum free media system could, therefore, be used to prepare culture supernatant for further experiments.

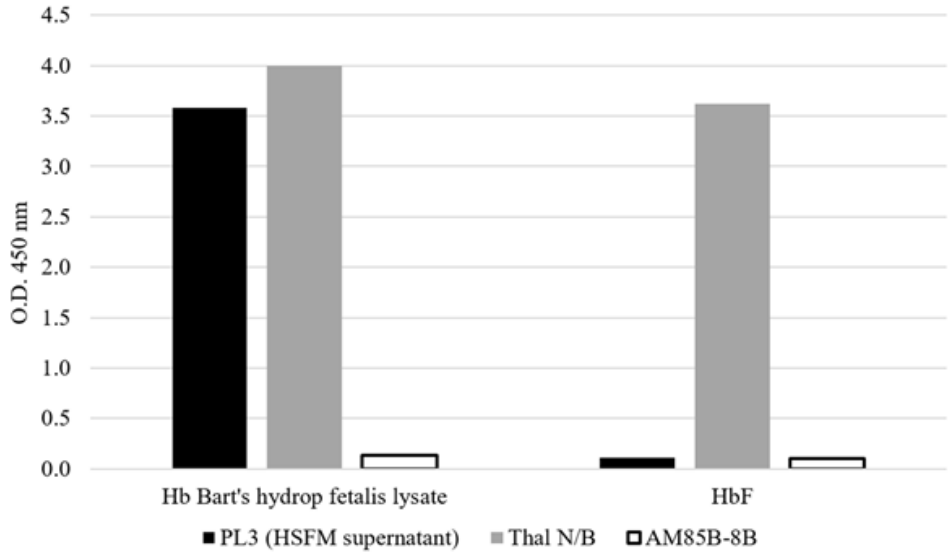

Figure 2. *Activity of mAb PL3 in hybridoma cell culture supernatant after culturing in hybridoma serum free media (HSFM) system. Bart's lysate or purified HbF was coated on plates. The culture supernatant and purified mAbs were added as indicated. HRP-conjugated rabbit anti-mouse immunoglobulins antibody was used to detect antigen-antibody complexes.*

Antibody purification

To purify mAb *PL3*, various types of affinity chromatography were employed. The yields of purified mAbs obtained from different affinity chromatographic columns were shown in Table 2. In fact, mAb *PL3* could not be purified using protein A, protein G and protein L columns. However, using the same columns, the irrelevant mAbs having the same isotype as mAb *PL3*, could be purified (Table 2). Surprisingly, mAb *PL3* could be purified only by the MabSelect SuRe column. These results suggested that different affinity chromatographic columns have a different ability in binding to immunoglobulins produced from various hybridoma clones. Using any affinity chromatographic column needs to be validated and optimized in order to purify different mAbs.

Table 2 Purification of mAb PL3 by using four different affinity chromatography.

Chromatography name	Protein binding capacity/mL	Purified PL3 mAb obtained (conc. per liter of culture supernatant)	Purified irrelevant mAb* obtained (conc. per liter of culture supernatant)
Protein A	12-19 mg human lgG	Not detected	0.773 mg (11 mg/L supernatant)
HiTrap Protein G	25 mg human lgG	Not detected	10 mg (100 mg/L supernatant)
HiTrap Protein L	25 mg human Fab	Not detected	5 mg (50 mg/L supernatant)
Mabselect SuRe	30 mg human lgG	2.064 mg (20 mg/L supernatant)	2.645 mg (37.8 mg/L supernatant)

*The irrelevant mAbs used in this study were IgG1 isotype. The anti-Ag85B mAb clone AM85B-9B were used for Protein A and anti-Hb Bart's mAb clone PB1 were used for Protein G, Protein L and MabSelect Sure.

Activity and purity of purified monoclonal antibodies *PL3*

The purified mAb *PL3* was further investigated for its activity and purity by indirect ELISA and SDS-PAGE, respectively. By indirect ELISA (Figure 3), the obtained mAb *PL3* purified using MabSelect SuRe column reacted specifically with Hb Bart's hydrop fetalis hemolysates and did not show cross-reaction with other hemoglobins. The positive control mAb *Thal* N/B showed positive reactivity with Hb Bart's hydrop fetalis hemolysate and HbF, whereas mAb *AM85B-8B* showed negative reactivity with all antigens.

The purity of the mAbs *PL3* eluted from the MabSelect SuRe column was examined by SDS-PAGE. The protein bands of purified mAb *PL3* under reducing condition appeared at approximately 55 and 25 kDa, corresponding to heavy chain and light chain, respectively (Figure 4). A major single band of approximately 180 kDa, corresponding to the intact IgG molecule, was observed in non-reducing condition (Figure 4). These results indicated that, using MabSelect SuRe column, purified mAb *PL3* could be obtained with high purity.

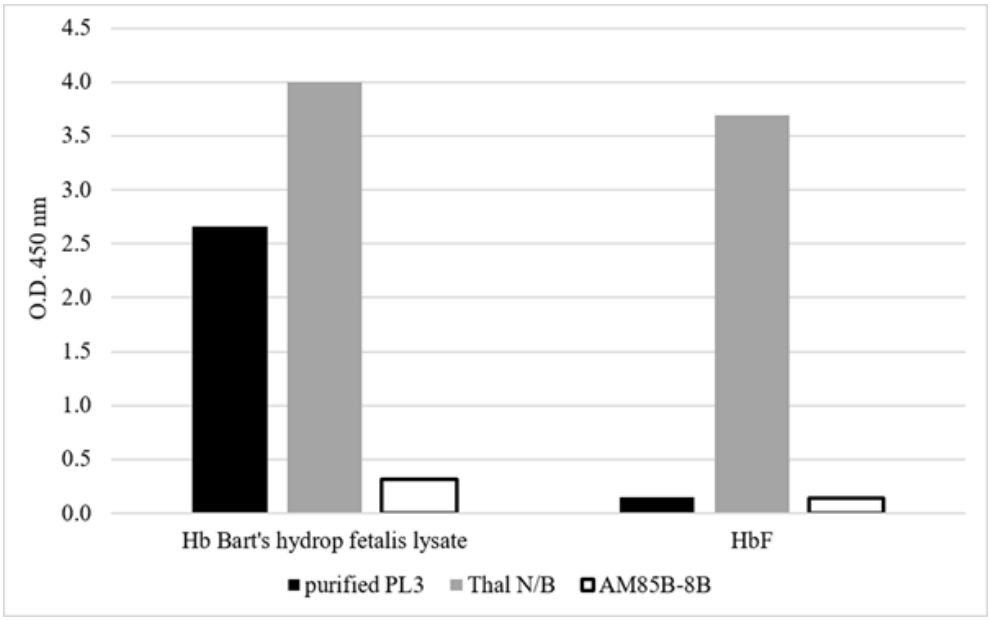

Figure 3. *Activity of purified PL3 mAb obtained by MabSelect SuRe column. Bart's lysate or purified HbF was used as the antigen of the poly-L-lysine pre-coated ELISA. Indicated purified mAbs were tested for their reactivity. The antigen-antibody complexes were detected using HRP-conjugated rabbit anti-mouse immunoglobulins antibody.*

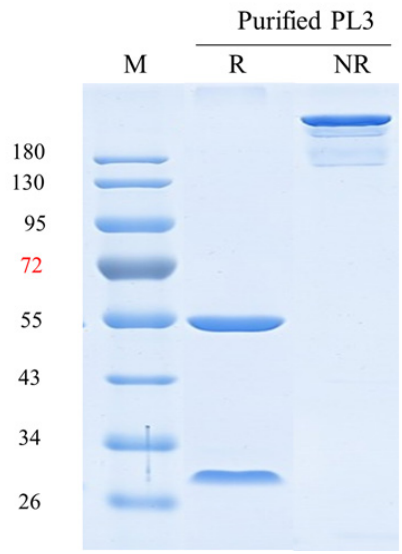

Figure 4. *SDS-PAGE analysis of the purified monoclonal antibody PL3. Five micrograms of proteins were loaded in each lane. The protein bands were visualized by PageBlue protein staining. Protein bands of purified mAb PL3 appeared at approximately 55 and 25 kDa in reducing condition. In non-reducing condition, the band showing molecular weight of PL3 appears at approximately 180 kDa.*

Discussion

In general, immunoglobulins or antibodies, are very important components of the vertebrate immune system.[17-21] Structurally, immunoglobulins are glycoproteins with a common Y-shaped building block comprised of two identical light chains (L) (~25 kDa) and two identical heavy chains (H) (~50 kDa), associated by disulphide covalent forces and by non-covalent interactions. Each chain is composed of constant (CL and CH) and variable (VL and VH) domains. These domains form into two antigen-binding fragments, Fab, and one constant region, Fc, involved in the effector function and biodistribution of the antibody. In addition to being the immune molecules in the body, antibodies can react to their specific antigen in vitro. This makes antibodies invaluable molecules for several applications. For the utilization of antibodies in several purposes, purified antibodies are required. Several methods for purification of antibodies have been developed; however, the most common and effective method is affinity purification. By affinity purification, antibodies of interest are captured in column by specific ligands and the unbound proteins are washed out. The retained antibodies in the column are eluted as purified antibodies.

Affinity purification of antibodies, actually, relies on the specific recognition between the antibody molecule and a complementary ligand. This can be achieved by two different approaches: 1) based on the specificity of antigen binding; and 2) targeting the constant part of the antibody.[5, 8, 9] Usually, the second option uses the biospecific ligand, a natural binding partner of antibody molecules, which is the most promising methodology for the large-scale production of purified mAbs.[5, 8-10] There are a number of naturally occurring immunoglobulin binding proteins that have been described.[10, 13] Protein A and Protein G for Fc portion of IgG[13, 22, 23] or Protein L for immunoglobulin light chains[10, 13] are the most common affinity ligands for the purification of antibodies. Protein A is a cell wall protein of Staphylococcus aureus, composed of five Ig-binding domains, which are independently capable of binding to the Fc part of IgG1, IgG2 and IgG4 with different affinities.[13, 22, 23] Protein A binds selectively to the Fc region of IgG (between the CH2 and CH3 region), but cannot form a complex with human IgG3 and binds to the Fab region of a subset of immunoglobulins with heavy chains belonging to the VH3 family.[13, 24-26] Protein G is a surface IgG-binding protein produced by Streptococci groups C and G and has a special affinity for the Fc region of IgG but also associates with the CH1 domain of the Fab portion through a β-zipper interaction.[13, 27] Protein L from Peptostreptococcus magnus recognizes κ1, κ3 and κ4, but not κ2 and λ light chains of antibodies and can be used to isolate antibody fragments that are not contained in the Fc domain.[10, 13, 28] The binding affinity of native biospecific ligands (Protein A, Protein G and Protein L) varies for the different IgG subclasses and species. Recently, recombinant and engineered forms of Protein A, G and L were developed in order to overcome the drawbacks associated with native proteins. The recombinant immunoglobulin ligands, currently, are procured by various commercial manufacturers and used as commercial products.[5, 8-10, 13]

Although various type of affinity column for purification of antibodies are available, in practice, some mAbs are difficult or cannot be purified. This becomes an obstacle for antibody utilization. In this study, we demonstrated an example for purification of an IgG1 mAb using four types of affinity chromatography. We revealed the use of four different biospecific ligands for purifying mouse IgG1, κ light chain mAb clone *PL3*. Protein L or Protein G beads were expected to be the best choice for mAb *PL3* purification, but it was not successful in this study. The reason might be that the mAb *PL3* consists of κ 2 light chain and imperfect CH1 domain resulting in mAb *PL3* unconfined. Surprisingly, the mAb *PL3* could only be purified by MabSelect SuRe (modified Protein A) column with high purity and it remained biologically activity. We hypothesized that mAb *PL3* contain complete CH2 and CH3 region, therefore it was captured by engineered recombinant Protein A-derived ligand of MabSelect SuRe. Moreover, Recombinant Protein A offers several potential advantages over the native Protein A. For recombinant Protein A construction, one of the Protein A domains has been engineered to favor a single-point oriented immobilization via thioether coupling, which results in enhanced binding capacity for IgG. Furthermore, amino acids that are particularly responsive to alkali on recombinant Protein A (Fab binding domain) were identified and replaced with more stable ones and the final construct was a tetramer of the engineered domain. Consequently, the Fab interaction is diminished, probably due to the fact that the engineered protein disturbed the interaction between the two molecules.[22, 29, 30] The different of IgG binding site and capacity between engineered recombinant Protein A and native Protein A revealed the different mAb *PL3* binding.

From our study, we demonstrated that each mAb has its own properties even though they have the same isotype. In order to obtain a large scale of purified mAbs, researchers need to optimize the purification process and also the affinity column used. In this case study, mAb *PL3s* are not possible to purify using normal Protein A or Protein G column. Nevertheless, the mAb PL3 can only be purified by an engineered recombinant Protein A column. Essentially, various factors and conditions such as the isotype of mAbs, concentration of mAb, the flow rate, are still needed to be optimized for each mAb.

Conclusion

Antibody purification is one of the key success points for the development of immunodiagnostics and immunotherapeutics. To this end, three bacterial immunoglobulin binding proteins (Protein A, G and L) have been extensively used to purify an anti-ζ globin chain mAb clone *PL3*. We demonstrated here the success of only using the new engineering recombinant Protein A-derived ligand, but not the others. Accordingly, this modified Protein A column is probably an alternative technique to purifying the unusual hybridoma clone that could not be purified by other columns for a large-scale purpose.

Acknowledgements

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Conflict of interest

The authors declared that they have no competing interests.

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Use of biosensor as a tool for double antibody sandwich immunoassay establishment: Screening of appropriate matched monoclonal antibody pair

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ABSTRACT

Background: For biomarker detection, double antibody sandwich platform is the most sensitive and specific immunoassay. Development of double antibody sandwich platform requires the step of appropriate antibody pair selection. However, identification of matched antibody pairs is difficult, laborious and time-consuming.

Objectives: To illustrate the application of biosensor for selecting the suitable matched pairs of antibody for double antibody sandwich platform.

Materials and methods: Double antibody sandwich immunoassay for Ag85B protein detection was selected as a model study. Biolayer interferometry biosensor was used to search matched pairs of antibody for Ag85B protein detection. To prove the obtained information, the double antibody sandwich ELISAs were employed.

Results: Biosensor results revealed that the best-matched antibody pair might be mAb clone AM85B-8B as capture antibody and mAb clone AM85B-5B or AM85B-9B as detecting antibody. The results obtained from biosensor system and ELISA were similar.

Conclusion: Biosensor can be used as a tool for effectively screening and selecting the best pairs of antibodies for any immunoassays. Biosensor method is very useful for reducing processes and time for double antibody immunoassay development.

Introduction

Nowadays, immunoassays are widely used in several fields including clinical, pharmaceutical, environmental and food analysis.[1-5] Immunoassays are based on the principle of antigen-antibody reactions. Specificity and selectivity of the antibodies used lead to many applications in detection and quantification of biological molecules of interest. Immunoassay design for detection of any antigens can be classified into two broad categories: 1) label-immunoassay; 2) non-label immunoassay; which depend on the use of antibody or antigen labeling with radioisotopes, enzymes, fluorescence dyes and luminescent substances or non-labelling, respectively.[6]

For detection and measurement of antigen of interest, the most sensitive immunoassay design is double antibody sandwich immunoassays using two specific antibodies.[7] By this technique, the antigens to be measured are captured onto specific antibody-coated solid phase (capture antibody), samples are cleaned up and antigens are enriched. Subsequently, the captured antigens are allowed to react with a labeled secondary specific antibody

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(detection antibody). As a result, the antigen is 'sandwiched' between the two antibodies, the capture and detection antibodies. Amount of antigen measured is correlated with the amount of the labeled secondary antibody bound to the solid phase. Subsequently, antigen concentration is directly proportional to the intensity of the detecting signal.[6, 8]

Antibodies used in immunoassays for the detection of the molecule of interest can be either monoclonal or polyclonal. The efficacy of an immunoassay depends upon the properties of antibody such as specific recognition, affinity and avidity.[9] Importantly, the capture and detection antibodies must recognize two non-overlapping epitopes on the antigen, must not interfere with one another and must be able to bind to the antigen simultaneously.[8, 10] In immunoassay development for detection of molecules of interest, selection of appropriate antibodies is, therefore, one of the critical steps.[11]

In general, checkerboard assays using microplates are appropriate for the screening of appropriate antibody pairs.[11-13] However, identification of matched antibody pairs is difficult or cannot be determined in the case of the use of antibody pairs derived from one animal species.[11] Matched pair screening step requires a large amount of purified and labeled antibodies. These processes are laborious, time consuming and expensive.[11-13] Accordingly, new methods for effectively screening and selecting the best pairs of antibodies for any immunoassays are needed.[14] In this study, we introduced a new method for the aforementioned purpose. We demonstrated the use of biolayer interferometry biosensor can be applied for selecting the best matched pairs of antibody. The described strategy can be used to determine the antibodies that should be used as the capture antibody and which antibody should be used as the detection antibody. The described method is very useful for reducing processes and time for immunoassay development.

Materials and methods

Antibodies and reagents

The anti-Ag85B monoclonal antibody (mAb) clones AM85B-5B (IgG2b), AM85B-8B (IgG1) and AM85B-9B (IgG1) were produced in our laboratory.[15] The recombinant Ag85B protein linked with biotin carboxyl carrier protein (Ag85B-BCCP) was generated in our laboratory.[15] EZ-Link™ Sulfo-NHS-Biotin was obtained from Pierce (Rockford, IL, USA). The BLItz biolayer interferometry biosensor and the streptavidin sensor chips were purchased from FortéBio (Pall Life Sciences, Menlo Park, CA, USA).

Biotinylation of antibodies and generation of double antibody platform using biosensor system

All clones of antibodies were biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotin according to manufacturer instructions.[16] Each biotin labeled antibody was used as capture antibody and immobilized onto streptavidin sensor chips. The sensor chips were placed into sample diluent buffer (PBS containing 0.1% bovine serum albumin, 0.02% Tween-20, 0.05% NaN_3).

The biotinylated anti-Ag85B mAb sensors were immersed into Ag85-BCCP or CD147 BCCP (as control). The sensor tips were placed into sample diluent buffer. Then, anti-Ag85B mAb sensors were immersed into the non-biotinylated mAbs (detecting mAbs). The binding signal was measured using a BLItz biolayer interferometry biosensor and calculated as delta binding signal, which is obtained by subtracting the binding signal at the end point of the washing step from the end point of non-biotinylated detecting antibody adding step.

Detection of Ag85B-BCCP by ELISA

The capture antibody against Ag85B was coated on ELISA plate. The recombinant Ag85B-BCCP proteins were added and incubated at 37 °C for 1 hour. After washing, the biotinylated mAbs, which recognizes different epitopes from the capture mAbs to Ag85B-BCCP proteins, were added into plate and followed by HRP-conjugated streptavidin. The reactions were detected using a TMB substrate (Zymed) and optical density was measured at 450 nm.

Results

Methods for selecting an appropriate pair of antibodies using an immunoassay for detecting molecules of interest are always complicated. In this study, double antibody biosensor was designed (Figure 1A) for selecting the appropriate pair of mAbs for the development of any type of immunoassay. Capture antibody was labled with biotin and immobilized on a streptavidin biosensor, then antigen was added and bound with the immobilized capture mAbs on the sensor chip. Subsequently, the sensor was immersed into non-biotinylated specific antibody (detecting mAb). The binding signals at every steps were measured using a BLItz biolayer interferometry biosensor as displayed in sensogram (Figure 1B).

In this study, recombinant proteins Ag85B (*Mycobacterium tuberculosis* antigen), were used as study model. For Ag85B antigen detection system, three capture mAbs (anti-Ag85B mAbs clones AM85B-5B, AM85B-8B and AM85B-9B) were tested in order to obtain the best pair of mAbs in sandwich immunoassay which showed the highest detection signal when using different clones of detecting mAbs. Binding signal obtained from biotinylated anti-Ag85B capture mAb clone AM85B-8B showed the strongest binding signal when using either detecting mAb clone AM85B-5B or AM85B-9B (Figure 2). However, the best matched pair of mAbs that should be used in further development of sandwich immunoassay for detecting Ag85B proteins was using anti-Ag85B mAb clone AM85B-8B mAb as capture antibody and anti-Ag85B mAb clone AM85B-9B as detection antibody (Figure 2).

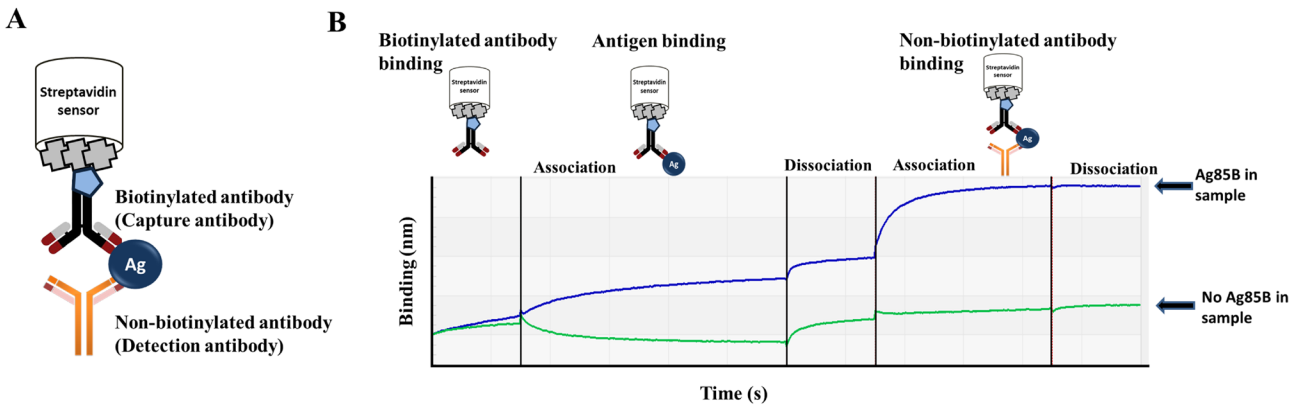

Figure 1. Schematic illustration of double antibody biosensor platform. *(A) The model of double antibody sandwich biosensor design. (B) The sensogram demonstrates the binding signal obtained from the BLItz biolayer interferometry biosensor at the indicated step.*

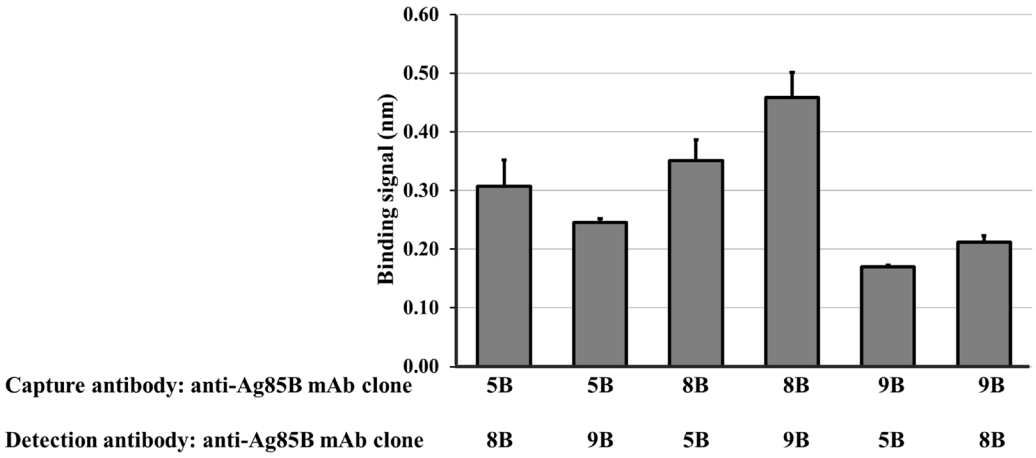

Figure 2. Detection of specific antigen using double antibody sandwich biosensor. *The capture antibodies as indicated were immobilized onto streptavidin sensor chip. The specific antigens were added and followed by the detection antibodies as indicated. The detection of Ag85B was plotted as delta binding signal. The bar graph shows mean±SD of three independent experiments.*

To confirm the finding of appropriate pair of mAbs by biosensor, we further performed the experiment by using double antibody sandwich ELISA which is the common immunoassay available as a commercial product. As shown in Figure 3, the use of anti-Ag85B mAb clone Ag85B-8B as capture antibody showed the highest absorbance signal, followed by clone 5B and clone 9B, respectively. This obtained data was similar to that using biosensor system. The result illustrated that, in agreement with the biosensor platform, anti-Ag85B mAb clone AM85B-8B as capture antibody and anti-Ag85B clone AM85B-9B as detection antibody was the appropriate mAb pair in using sandwich immunoassay for detection of Ag85B proteins.

Taken together, our study suggested that the biosensor system can be used as a simple and rapid screening method to obtain the pair of antibody for development of immunoassay.

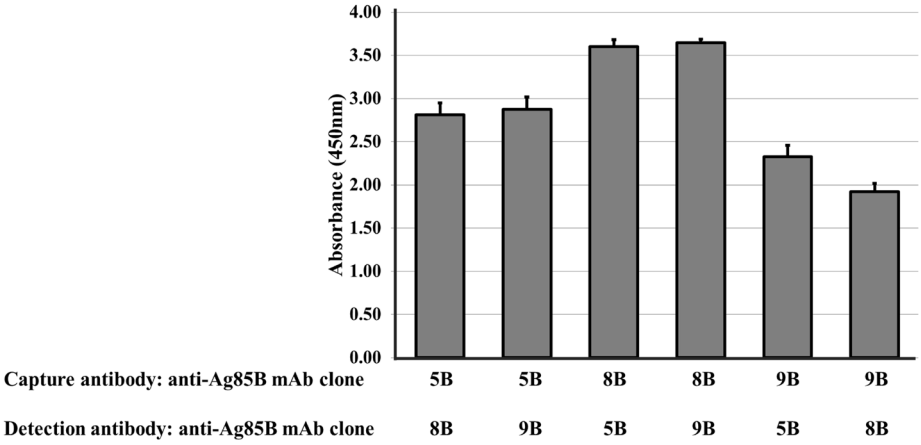

Figure 3. Detection of specific antigen using double antibody sandwich ELISA. *The capture antibodies as illustrated were coated onto ELISA plate. The specific antigens were added into each well. The detection antibodies, which bind to different epitope, were added and followed by conjugates and color substrate. The optical intensity for measurement of Ag85B is shown as mean ± SD of duplicate experiments.*

Discussion

Since an antibody binds specifically to its antigen, immunoassay becomes a popular strategy to detect the presence or absence of substances of interest such as biomarkers and drugs.[3, 4, 15, 17] Although the commercial immunoassays are available in different formats, ie. ELISA, immunochromatographic strip test, beads-based immunoassay[18, 19] all of the commercial immunoassay require the same steps during the set-upprocess including design of assay platform, selection of appropriate antibodies, optimization and validation.[8, 13, 17] Step of immunoassay development is time consuming; therefore, shortening time in each development process is a key point for the commercial development of diagnostic immunoassay.[14]

To develop immunoassay for antigen measurement, double antibody sandwich platform is usually designed according to the sensitivity and specificity.[2, 7, 12] Performance of sandwich immunoassay is affected by several factors especially characteristic of the antibody used in the assay. Selection of high affinity suitable pairs of antibodies is an important step of immunoassay development. Selection of the pair of antigen or antibody for use in assay is commonly based on ELISA which is laborious, requires a well-trained person and need a long incubation period.[11, 19] Moreover, purified specific antibodies are required for both capture and detection antibodies and pairs derived from one animal species are desired. The mAb purification step is also time-consuming, labor intensive and costly. As a consequence, there has been much interest in the development of a speedy, simple and cost-effective method for matched pairs monoclonal antibody identification that reduces the use of purified mAbs.[14]

Thus, in this study, the alternative approach for screening pairs of antibodies is introduced which is biolayer interferometry biosensor. This system is based on the principle of the optical interferometry called the interaction of light waves. Device detects the wavelength shift between before and after the biomolecule or antigen binds to its ligand or antibody. Then, the delta binding is real-time generated and plotted as binding signal which reflects the amount of detected antigen or antibody. Using this property, the binding characteristic of capture and detection antibodies with antigen in terms of overlapping epitope, interference and influence are revealed within 5 minutes. Therefore, it is fast and easy to discover the properly matched antibody pair. In addition, the kinetic rate of antibody-antigen binding can be obtained including association rate (Ka), dissociation rate (kd) and affinity constant (KD; Ka/Kd) by the biolayer interferometry biosensor.[20-22] By the biosensor system, binding signal of mAb and antigen could be determined in a real-time manner. Association rate of antigen binding signal can be visualized and the high association rate takes a shorter time to reach the highest stable binding signal whereas the low association rate appears over a longer time. Dissociation rate can be observed in the washing step after antigen binding, when the high affiniy antibody shows a stable binding signal. In contrast, the low affinity antibody illustrates a rapidly decreasing binding signal. Notably, the association and dissociation rate of antigen and antibody binding can be visualized and obtained from the information in the software. As a result, the suitable capture and detection antibodies are identified. In addition, the biosensor system requires a small volume and amount of antibody: only 4 ng of antibody is enough to use in the assay.[20-22]

In order to prove our concept, several clones of the monoclonal antibody against Ag85B and recombinant protein Ag85B were used as model of the study. Hence, labeling one of the antibodies with biotin or enzyme is required and then it is matched with a non-label antibody.[11, 12] In this study, antibodies were labeled with biotin which is a small biomolecule and does not interfere with the binding site of antibodies. Moreover, the reaction of biotin and streptavidin is a strong non-covalent reaction resulting in a tight binding between the biotinylated antibody and either streptavidin sensor chips or streptavidin-enzyme conjugate.[23]

The results obtained from biosensor system and

ELISA were comparable where the appropriate pair of antibody could be selected. Using biosensor system showed the benefit over ELISA method in several tasks. The incubation time could be reduced from at least 4 hours (ELISA) to 7 minutes (biosensor). In addition, the amount of antibody and antigen used in biosensor system required only 4 ul in the individual step, which is lower than the amount of antigen or antibody used in ELISA (50ul). Therefore, the scale of antigen or antibody production for the step of antibody screening could be minimized and translated to minimizing cost, time for antibody production and labeling. In addition, incubation time of biosensor system is less than ELISA. The early step in immunoassay development, screening pairs of antibodies, would be faster and could move forward the step of assay set up. Hence, the double antibody biosensor platform might be used as the screening system to select the appropriate pair of mAb for further immunoassay development.

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Conflict of interest

The authors declare that they have no competing interests.

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Effect of rehabilitation program on ability to perform activities of daily living and quality of life of physically disabled persons

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ABSTRACT

Background: The encouragement of a continuous rehabilitation program consisted of a manual, training and knowledge distribution about disabilities plus skills with appropriate techniques based on rehabilitation principles, follow-up and visiting the patients, and providing appropriate assistive devices can help the physically disabled persons return to activities of daily living and improve their quality of life.

Objectives: To investigate the results of rehabilitation program aimed at helping the physically disabled persons to be able to return to their activities of daily living and improve quality of life.

Materials and methods: This quasi-experimental research employed one group pretest – posttest design. Thirty physically disabled persons (19 males, 11 females) from Chiang Dao district, Chiang Mai were recruited by the inclusion and exclusion criteria. A Rehabilitation Program for Physically Disabled Persons with 10 weeks implementation program were developed from the patterns of care given to patients with cerebrovascular disease, from a literature review focusing on rehabilitation, knowledge and skill training for rehabilitation of physically disabled including follow-up and visiting the patients, and the appropriate assistive devices. It was intended to enhance the patient ability to perform their routine activities and to prevent complications. The data collection instruments consisted of activities of daily living assessment, physical complications questionnaire, anxiety and depression assessment, and quality of life questionnaire. Data were analyzed using descriptive statistics and McNemar test.

Results: After implementing the program, the physically disabled person had a significant increase in the level of ability in performing activities of daily living including mobility and overall activities of daily living (p<0.001 and p=0.003, respectively). The level of quality of life included social relationships, environment and overall quality of life were also increased significantly with p=0.048, p<0.001 and p<0.001, respectively. They also expressed some physical complications, and it was found that they had a significant increase in ankylosis and pain (p=0.031 and p<0.01, respectively). Additionally, the psychological complications including an anxiety and a depression from anxiety and depression assessment had significantly decreased after participated this rehabilitation program with p<0.001.

Conclusion: The rehabilitation program could be used as a guide to help rehabilitate the physically disabled persons in the community to continuously and efficiently improve their quality of life.

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Introduction

The World Health Organization (WHO) defined disabilities as a limitation in doing any activity, including being limited to engage in social functions. WHO reported that one billion people (about 15%) in the world were facing problems and the condition of disabled persons, which were 10% more than estimated in 1970. The increased number of disabled persons was due to the increase of chronic non-communicable diseases and the approach of an elderly society worldwide.[1] The reported of disabled persons in Thailand in September, 2016 revealed that 1,657,438 people were issued a disabled person card, which was 2.5% of the total population. Among these were the physically disabled person, 794,648 persons (48.47%), and were the highest in the group. Classified by age, they were in the range of 22-59 years of age and >60 years of age, and physically disabled persons came in the highest (47.54% and 53.65% respectively).[2] The causes of the disability were illness and some diseases (30.79%) mostly, such as hypertension, diabetes mellitus, coronary artery disease and epilepsy.[2] This argument corresponds to the research on disability preventive and surveillance system where it was found that the causes of physical disabilities came from such diseases as cerebrovascular disease, osteoarthritis, bone deformity, myo-paralysis, heart disease, diabetes mellitus, cancer, epilepsy and so on.[3] These diseases contributes to disability of various body systems causing difficulty of physical movement and dexterity, limiting people from performing daily living or routine activities, disability to help themselves or to work as well as loss of efficiency.[4] It can also result in physical complications physically such as ankylosis, pain and decubitus and mental complications, such as depression and anxiety affecting the patients' quality of life,[5] These can consequently, lead to the social and economic problems.

According to the expectation report by WHO, the situation of disability in the community was dramatically increasing. The data from Chiang Dao District, Chiang Mai were consistent with this report. According to the data of the medical rehabilitation service given to disabled patients at Chiang Dao Hospital in the fiscal year 2012-2016, as many as 713 patients with a disability certificate came for service and among them, the physically disabled persons formed the highest number of 480 cases or 67.32%. Although nowadays there is a wide range of rehabilitation services provided for the disabled, there are several problems occurred in the Chiang Dao district, Chiang Mai. One of the critical issues is that the patients are more likely to take a long time coming to receive the services because the long distance between home and the hospital caused by the geographic features. This obstruction also limits the medical personnels to deliver the services outside the hospital. Moreover, there are limitations on ethnic diversity, cultures and local living standard, and the number of medical personnel. These obstructions primarily cause the disabled lack of the opportunity to receive an appropriate and continual rehabilitation. If the disabled experiences this kind of difficulty incessantly, at the end, their quality of life will be downgrades. This incidence will more or less negatively affect the community, and overall economic system.

Currently, rehabilitation guidelines for patients suffering from cerebrovascular disease suggested that the health care for rehabilitation should be focusing on holistic health care. The primary objective is to gain more the recovery of cerebrovascular disease patients, After a disability occurred the efficacy of the rehabilitation depends on several factors like the therapist team, the duration of treatment and most importantly the patient's cooperation as well as their family members or caregivers.[6,7] Previous studies have examined the components of rehabilitation services for physically disabled persons. It was commonly found that the physically disabled persons need to receive medical rehabilitation services in order to adjust their living environment suiting for their lifestyle and society. Moreover, they have to get access the services and rehabilitation information regarding the disabled from the government, caregiver or community like home visiting by medical personnels for instance.[8] A model of caring for the physically disabled persons have been widely provided since it is an important part of promoting proper rehabilitation for the physically disabled persons. It was claimed that the pattern of caring for physically disabled persons could improve their quality of life. And it must be completely composed of personal, family, community and social factors. The results from the rehabilitation services which were included by the components mentioned above indicates that the physically disabled persons could get better in their activities performing ability of daily living. As a result, this consequence could also lead the physically disabled persons to have a better quality of life. It prevents the complications or reduces the rate of complications after the disability.[9,10] However, it can be stated that there has been very little development of rehabilitation programs for this group of the disabled persons and it is a component that is not covered by the rehabilitation as mentioned above. Example is a model of a rehabilitation program for the physically disabled persons developed from principles and techniques of specialized rehabilitation. By training to educate and practice about rehabilitation for the physically disabled, the rehabilitation program consist of physical therapy exercise (to strengthening muscles and flexibility to prevent limitation of joint motion, contraction of muscles and ligaments, as well as techniques to reduce muscle spasms), bed mobility and gross motor function training, balance training, ambulation with assistive devices training, providing appropriate assistive devices, and follow-up visits to the disabled persons (Home Health Care). In this research, the results of the rehabilitation program for physically disabled persons were investigated as the main interest. This program was conducted by applying and developing guidelines for a rehabilitation program for this group of patients and the patterns used for the rehabilitation from the previous studies. Also, it was included the complement of training and knowledge distribution about disabilities plus skills training on rehabilitation of physically disabled persons with appropriate techniques based on rehabilitation principles, as well as follow-up and visiting the patients. The appropriate assistive devices and a rehabilitation manual for primary care of this group of

patients and lifestyle were provided in order to ensure that they will be receiving continuum care and rehabilitation as much as possible. The community was also encouraged to take part in the process to enable the physically disabled patients to conduct their daily life to the full potential and competence in their community and society.

Objectives

To investigate the results of rehabilitation program aimed at helping the physically disabled persons to be able to return to their activities of daily living and to improve the quality of life.

Materials and methods

A quasi-experimental research (one group pre-test-posttest design) was utilized in order to investigate the outcomes of the rehabilitation program for physically disabled persons in their ability to conduct their daily living activities and their quality of life. The populations were 480 physically disabled patients at Chiang Dao District, Chiang Mai Province. The sample size was calculated by Power Analysis with the power =0.08, Alpha =0.05 and effects =0.50 before checking with the Burn and Grove Table.[11] The result was 24 samples. With the 20 percent dropout rate considered, sample of 30 persons were included. The recruitment criteria were as followed 1) the physically disabled person must be 20 years of age or older and hold a disability certificate, 2) having been a chronic patient due to cerebrovascular disease before the disability occurred and having had the symptoms for not longer than 3 years, 3) having a Modified Barthel Index (Thai version) less than 75, 4) willing to participate in the research and residing in the area during the research period, 5) having some relatives to look after him/her. Patients were excluded if 1) they had unable to communicate in the Thai language and not able to communicate verbally, 2) having double-disability of other kinds of diseases, 3) withdrawing from the research during the process.

Research tools can be classified into two parts. First, the rehabilitation program for physically disabled persons which was developed from 1) the patterns of care given to patients with cerebrovascular disease and 2) from a literature review focusing on rehabilitation for this group and knowledge and skill training for rehabilitation of physically disabled persons by physical therapists and occupational therapists. It was intended to enhance the patients' ability to perform their routine activities and to prevent complications. This came in the forms of a manual for rehabilitation of the physically disabled persons, training on caring of the physically disabled persons. Furthermore, there was a determination on the content validity of the tools by five experts including a physical therapist, a doctor, a nurse, an instructor of physical therapy and a nursing instructor. Results of determination on the content validity of the tools were reported as follows.

Item-Objective Congruence (IOC) came out at 0.95. Second, the data collection form consisting of an ability evaluation form or Modified Barthel Index.[12] A reliability test showed Cronbach's alpha Coefficient at 0.91. The WHOQOL – BREF – THAI[13] had reliability corresponding to Cronbach's alpha Coefficient at 0.85. The questionnaires assessing anxiety and depression or the Hospital Anxiety and Depression Scale (HADS)[14] had reliability on Cronbach's alpha Coefficient at 0.80 whereas those of the physical complications after disability took place based on Cronbach's alpha Coefficient was 0.86.

This research was approved by the ethical committee of the Interdisciplinary Program in Public Health Science, the Graduate School, Chiang Mai University, on June 8, 2016 (No. 015/2017). Moreover, the process of operation was informed about the research objectives and protecting the rights of the sample group and the caregivers. After that, proceeding with data collecting before providing the rehabilitation program to the physically disabled persons using questionnaires for general information, and the evaluation form for the ability to perform daily living activities as well as questionnaires about the physical and psychological complications, including questionnaires about their quality of life. In the first week holding training session to provide knowledge and skills practice on rehabilitation for physically disables persons consisting of content: knowledge, understanding about disabilities (a 60 minutes lecture), taking care of a disabled person in a daily life basis, routine practice and emotional management (a 60 minutes lecture, 45 minutes of practice) and basic rehabilitation for physically disabled persons (a 90 minutes lecture, 75 minutes of practice). Then actual practice on rehabilitation for a physically disabled person, including follow-up and home visits, giving assistance related to equipment by a physical therapist, and occupational therapist and a caregiver. Home visiting took place in the 3rd, 5th, 7th and 9th weeks (4 times, 30-60 minutes each) and telephone follow-up in the 2nd, 4th, 6th and 8th weeks (4 times). Finally, evaluation after 10 weeks of the activities using the evaluation form on the ability to perform daily activities and a questionnaire on physical and mental complication as well as one on the quality of life. In this research, training was provided knowledge and skills practice on rehabilitation for physically disables persons. Including data collecting, telephone follow-up and home visits mainly operated by the researcher. (Figure 1)

Data Analysis

The SPSS version 17 (serial no. 5068035) was purposely used for the data analysis. The demographics data of sample were analyzed by descriptive statistics showing numerous parameters including frequency, percentage, mean and standard deviation. The level of competency in performing daily activities and quality of life were compared between before and after implementing the rehabilitation program by using the McNemar test p-value ≤0.05 were considered as statistical significance.

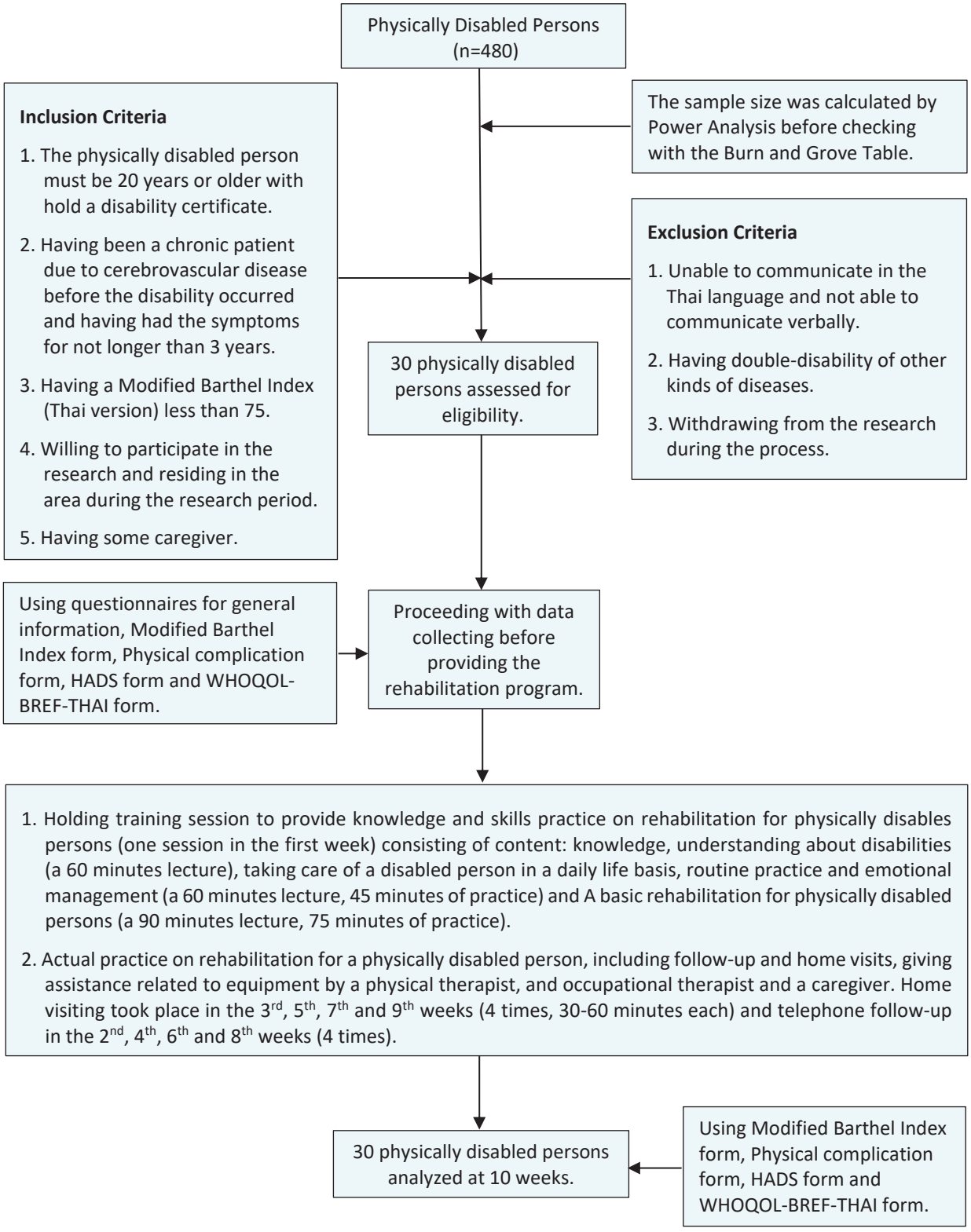

Figure 1. *Operating procedures*

Results

From the general data it was found that most of the physically disabled patients were male (63.3%) between 60-69 years old (43.3%). Their average age was 65.9±9.03 years old. The oldest physically disabled patient was 87 and the youngest was 49 years old respectively. The most two types of diseases in health conditions they generally had were hypertension (43.3%), followed by hypertension plus dyslipidemia (26.7%). The causes of disabilities had ischemic stroke (73.3%) and intracranial hemorrhage (26.7%). The duration of the disability were mostly 26-36 months (43.8%), with an average 19.23±12.19 months. Most of the patients were right to health access by universal coverage scheme (disability) (90.0%) (Table1).

Table 1 Personal data of physically disabled persons. (n=30)

Personal data	n	%
Gender		
Male	19	63.3
Female	11	36.7
Age (years)		
40–49	1	3.3
50–59	7	23.3
60–69	13	43.3
70–79	7	23.3
≥80	2	6.8
Mean±SD = 65.93±9.03 years, Min=49 years, Max=87 years		
Marital status		
Married	18	60
Widowed	8	26.7
Single	3	10
Divorced	1	3.3
Religion		
Buddhism	28	93.3
Christian	2	6.7
Educational Background		
Not education	3	10
Primary	22	73.3
Secondary/Vocational/Technical	5	16.7
Cause of Disabilities		
Ischemic stroke	22	73.3
Intracranial hemorrhage	8	26.7
Health Conditions		
Hypertension	13	43.3
Hypertension and dyslipidemia	8	26.7
Diabetes mellitus, hypertension and etc. (i.g. COPD, nephropathy)	5	16.7
Hypertension and etc. (i.g. Osteoarthritis Knee, Heart disease)	2	6.7
Dyslipidemia	1	3.3
Diabetes mellitus and hypertension	1	3.3
Periods of Disabilities (month)		
0-6	4	13.3
7-12	8	26.7
13-24	5	16.7
25-36	13	43.8
Mean±SD = 19.23±12.19 month, Min=3 month, Max=36 month		

Table 1 Personal data of physically disabled persons. (n=30) (continuens)

Personal data	n	%
Right to Health Access		
Universal Coverage Scheme (disability)	27	90
Government or State Enterprise Officer Scheme	2	6.7
Social Security Scheme	1	3.3
Income of Family per month (Baht)		
<5,000	12	40
5,000-10,000	13	43.3
10,001-15,000	3	10
>15,000	2	6.7
Mean±SD = 7,750±7,573.49 Baht, Min=1,500 Baht, Max=40,000 Baht		

Regarding the ability to perform daily routine after receiving rehabilitation, the ability to take care of themselves, mobility ability, excretory control and daily living performance of the sample group were improved. The sections that had statistical significance were Mobility ability and overall daily activity performance (p<0.001, p=0.003 respectively). It was also found that the sections on self-care and excretory control were better but with no statistical significance (p=0.090, p=0.120 respectively) (Table 2).

Regarding the quality of life, it was found that after the rehabilitation program, the physically disabled persons generally had a better quality of life in terms of physical, psychological, social relations and environment. Their social relations, environment and overall quality of life showed a statistical significance improvement (p=0.048, p<0.001 and p<0.001 respectively) while the physical and psychological improvement showed no statistical significance improvement (p=0.847, p=0.635 respectively) (Table 3). As for follow-up on their quality of life assessment, there was also a follow-up on complications emerging after the disability occurred, which could affect their quality of life. However, there were no incidents of decubitus in the sample group either before or after undergoing the rehabilitation. Nevertheless, after the rehabilitation, some incidence of ankylosis and pain decreased significantly (p=0.031, p=0.008 respectively) while shoulder joint subluxation and edema decreased but with no statistical significance. The anxiety and depression were significantly better as well (p<0.001) (Table 4).

Table 2 Comparison of competency levels in performing daily routine before and after the rehabilitation program. (n=30)

ADL	Self Care		Mobility Ability		Excretory Control		Overall	
	Before n (%)	After n (%)	Before n (%)	After n (%)	Before n (%)	After n (%)	Before n (%)	After n (%)
Very low	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Low	5 (16.7)	0 (0)	7 (23.3)	0 (0)	0 (0)	0 (0)	5 (16.7)	0 (0)
Moderate	7 (23.3)	1 (3.3)	7 (23.3)	4 (13.3)	11 (36.7)	2 (6.6)	17 (56.7)	5 (16.7)
High	13 (43.3)	10 (33.3)	16 (53.4)	15 (50.0)	3 (10.0)	5 (16.7)	8 (26.6)	20 (66.6)
Normal	5 (16.7)	19 (63.4)	0 (0)	11 (36.7)	16 (53.3)	23 (76.7)	0 (0)	5 (16.7)
***p* value**	0.090		0.001*		0.120		0.003*	

** p<0.05*

Table 3 Comparison of the ration of their quality of life before and after the rehabilitation program. (n=30)

QOL	Physical domain		Psychological domain		Social relationships		Environment		Overall	
	Before n (%)	After n (%)	Before n (%)	After n (%)	Before n (%)	After n (%)	Before n (%)	After n (%)	Before n (%)	After n (%)
Poor	5 (16.7)	0 (0)	2 (6.7)	0 (0)	5 (16.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Moderate	25 (83.3)	23 (76.7)	27 (90.0)	14 (46.7)	24 (80.0)	24 (80.0)	26 (86.7)	8 (26.7)	30 (100.0)	10 (33.3)
Good	0 (0)	7 (23.3)	1 (3.3)	16 (53.3)	1 (3.3)	6 (20.0)	4 (13.3)	22 (73.3)	0 (0)	20 (66.7)
***p* value**	0.847		0.635		0.048*		<0.001*		<0.001*	

** p<0.05*

Table 4 Comparison of physical and psychological complications before and after rehabilitation program. (n=30)

Complications	Before n (%)	After n (%)	*p* value
Physical			
Decubitus	0 (0)	0 (0)	1
Ankylosis	14 (46.7)	8 (26.7)	0.031*
Pain	17 (56.7)	9 (30.0)	0.008*
Shoulder subluxation	6 (20.0)	6 (20.0)	1
Edema	3 (10.0)	1 (3.3)	0.5
Psychological			
Anxiety	26 (86.7)	2 (6.7)	<0.001*
Depression	16 (53.3)	3 (10.0)	<0.001*

** p<0.05*

Discussion

This study found that the sample group's levels of ability to perform daily routine before and after the rehabilitation program had been changed positively, and the levels of ability to perform daily activities was assessed by the modified Barthel index based on score range defined in the evaluation form. They were better in taking care of themselves, their mobility ability and excretory control as well as general routine performance. This corresponded to the study by Photong et al[9] on the rehabilitation of the disabled in the community, which comprised of three components such as management, process definition, and define results. The study indicated that the effectiveness of the integrated rehabilitation model for persons with physical disability in the community, showing a statistical significance $p<0.05$. The rehabilitation program consisted of giving knowledge and skills practice on rehabilitation to the group of patients and providing simple equipment, adjusting their habitat and the environment, giving them a handbook for rehabilitation along with constant visits and follow-up. This also agreed with the study by Petchroung et al.[15] indicating that providing continual care for patients with cerebrovascular disease in a community could be efficient with policy and resource support from the community. Studies showed that collaboration of medical personnels and communities in rehabilitation and follow-up for people with disabilities in the community made the rehabilitation continue resulting in the patient being able to perform daily living activities better.[4,16]

The quality of life was measured by the WHOQOL - BREF - THAI questionnaire. The sample group was better overall regarding physical, psychological, social relations and environment. The follow-up tests focused on complications after being disabled which can affect the patient quality of life. The physical complications after disability was assessed by the physical complication questionnaire. It was not found the evidence of decubitus among the samples both before and after the rehabilitation program. Also, the patients in the sample group have been able to help themselves at a low to moderate level they were in little risk of decubitus. As for ankylosis and pain, the sample group suffered less with a statistical significance ($p=0.031$, $p=0.008$ respectively) because the rehabilitation program used in this study had improved their relevant knowledge and skills as well as evaluated the complications along with effective rehabilitation activities, plus home and telephone visiting throughout the study period, so the sample group received constant care and rehabilitation to prevent

complications, Regular physical rehabilitation and exercise helped reduce pains and boost all the systems in the body to function more efficiently.[17] In addition to, the rehabilitation program in this study were physical therapy exercise for rehabilitation physically disabled persons such as passive stretching exercise for prevent ankylosis and muscle shortening, strengthening exercise, bed mobility training, gross motor function training, balance training, transfer and ambulation training all of these had resulted in prevention and reduction of ankylosis. Suitable types of exercise were able to strengthen the muscles and reduce injury or complications.[18] This study also provided knowledge to promote environmental adaptation to suit the condition of the disabled patients making them able to help themselves or do activities better; thus, it reduced complications related to ankylosis and pain. This corresponded to the study by Photong et al[9] which discovered that complications reduced after undergoing integrated rehabilitation for persons with physical disabilities where complications in the muscular and skeletal system as well as in the alimentary system decreased and were much different than before the rehabilitation. In the case of shoulder joint subluxation among the sample group, it was found to be insignificantly different in terms of statistics because the subluxation could have been caused by several factors such as myopathia, paratonia around the shoulder joint, shoulder joint subluxation, improper exercising or improper posture. There are several methods of rehabilitation and treatment of shoulder joint subluxation such as exercising the muscle around the shoulder joint, adjusting the body posture, electrical stimulation, using some kinds of support and tape; each method has its own benefit and limitation. For a good result, one could choose more than one method and they must be those that suit the patients the most.[19] Rehabilitation program in this study was not designed specifically for shoulder exercise. As a result, the samples with shoulder joint subluxation were no significantly. It was also found that the sample group's edema improved. The causes of edema in the sample group could be due to several factors, personal diseases or a combination of diseases, medicine side effects or irregularity in the blood circulation system, chronic inflammation, problems in the blood vessels, phoroplasts and so on.[20] In addition to regular rehabilitation programs, people with mobility impairments will be able to promote and improve physical activity, helping to improve blood circulation and reduce swelling. The samples might need some other medical treatments suitable for their diseases to reduce or limit the edema more efficiently.

As psychological complications was assessed by the Hospital Anxiety and Depression Scale (HADS) questionnaire, it was found that anxiety part and depression part were reduced significantly (p<0.001). As the rehabilitation program provided some training on relaxation and stress management and required a continual home and telephone visiting, the result of the spiritual dimension cause to hear the acceptance of the patient/family and community, clarification on suspicion from the medical personnel, encouragement, comfort, participation, freedom of expression, palliative self-esteem.[21] This situation makes the disable patient feel warm, in good spirits. In other words, they do not feel lonely. They become less depressed, more confident to meet people, and be able to better engage in the community.[15] So, disabled patients have reduced anxiety and depression score than before receiving a rehabilitation program. This went well with the study results of the personal information of the sample group for this study which was selected from patients with stroke or cerebrovascular disease for not longer than 3 years or an average duration of 19.23 months of disability (SD=12.19), which was the recovery stage of the patients. The period began from the first three months after having cerebrovascular disease when the symptoms remained stable, and the patients were able to receive rehabilitation and needed close care to reduce the severity of the disability and were likely to have a better quality of life. This corresponded with the studies by Raksaken et al[22], Clarke[23] and Muus[24] which found that the level of quality of life of the patients with cerebrovascular disease was related to the duration of the disease. The first 3-6 months could be of a low quality of life and from three months to five years the quality of life would be at a moderate level. Although cerebrovascular disease patient could be recovery by a recovery stage or an illness period, several studies reported that the cerebrovascular disease patients will be showed the disability outcome especially among patient didn't co-operated with the rehabilitation program.[4,25,26] However, the rehabilitation program for physically disabled persons, from our study could encourage the patient to do the rehabilitation program, empower family caregiver to care, and motivate the patient to continuous rehabilitation. Therefore, this program can develop the competency of rehabilitation program for physically disabled persons and could improve the quality of life in the near future. Since the rehabilitation program in this study involved giving knowledge about rehabilitation for persons with physical disability to improve the disability and promote proper environmental arrangement as well as follow-up by home and telephone visits along with individual rehabilitation with involvement of family/caregiver and the village public health volunteers in the community. This covered physical, psychological, social and environmental rehabilitation; along with proper equipment and support for individual patients, it could result in holistic rehabilitation, ensuring better performance of daily activities and a better quality of life in every dimension.[9,10,27] The follow-up and visiting the sample group showed that most of them need rehabilitation service and continual care, which means constant home and telephone visits became the key to success to increase their capability to cope more with daily activities and to prevent physical and psychological complications for a better quality of life. Since each physically disabled person had different types of daily life, housing and environment an individual visit helped us to be better informed about their problems and to be able to provide support and advice accordingly and properly in all aspects. This close and continual care boosted their morale and provided opportunities for counseling which improved their performance of routine activities and their quality of life as well as decreased complications that could

happen after the disability.[10,16]

Conclusion

The study results proved that after undergoing the rehabilitation program designed especially for the physically disabled persons, persons with physical disabilities could perform better in their daily routine and the quality of life. The physical and psychological complications as well as ankylosis and pain is prevented and reduced. It is recommended that this rehabilitation program be a guideline for rehabilitation of such groups of patients in a community to ensure continual quality rehabilitation and a better quality of life.

Recommendations

1. Evaluation and follow-up test on the rehabilitation program for persons with physical disabilities should be done in a long term.

2. Further study should be conducted with regard to exercising or the rehabilitation program to prevent complications and problems about shoulder joint dislocation among persons with physical disabilities.

3. There should be a promotion of community participation and network to provide care for rehabilitation for physically disabled persons in the community to stress continual rehabilitation suitable to the context of lifestyle of the patients and help them to receive a good quality of care.

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Immediate and short-term effects of thoracic manipulation with neck muscles stretching compared with neck muscles stretching on alteration of pain and range of motion in mechanical neck pain

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ABSTRACT

Background: Mechanical neck pain is generally found among manual workers. Manual therapy is one of treatment for patients with neck pain. Alternatively, thoracic spine thrust manipulation may efficiently treat mechanical neck pain. Therefore, treatment using biomechanical connection between cervical and thoracic spine can increase joint mobility for improvement of neck disorders.

Objectives: To investigate the immediate effects of post-test (48-72 hours and short-term 2 weeks follow up) of thoracic manipulation in combination with stretching exercise on mechanical neck pain, pressure pain threshold, Neck Disability Index (NDI), forward head posture, and cervical range of motion.

Materials and methods: Forty patients with neck pain were recruited in the study. Participants were divided into four groups: (1) thoracic manipulation; (2) thoracic manipulation with stretching exercise; (3) stretching exercise; and (4) control.

Results: There was a statistically significant difference between outcomes of the groups. Thoracic manipulation with stretching exercise group had better results than that of manipulation, stretching group, and control group on visual analog pain scale. Statistically significant difference was found when its effect was to the stretching and control group on NDI and pressure pain threshold. Thoracic manipulation with stretching exercise group had a statistically significant difference when compared to stretching, control group on forward head posture, and cervical range of motion in extension direction.

Conclusion: Thoracic manipulation may be an alternative treatment in mechanical neck pain in producing more benefits when is used in combination with stretching exercise to decrease neck pain, increase cervical range of motion, and tends to neutralize head posture decreasing neck disability.

Introduction

Neck pain is prevalent in thirteen percent of the population at any given time, and is experienced by fifty percent of the population over their lifetimes.[1] Mechanical neck pain is generally found among the general and workforce population. Risk factors for this disorder include poor posture and occupational activities.

Manual therapy like cervical manipulation and exercise is an alternative therapy for neck pain.[2] The advantages of cervical spine manipulation must be weighed against the risks, such as cervical spine fracture.[3] Treatments that incorporate an understanding of the biomechanical connection between the cervical and thoracic spine can improve joint mobility, which alleviates pain and improves range of motion in patients with neck disorders.[4] For this reason, it has

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been recommended that the incorporation of thoracic spine manipulation interventions may reduce inherent risks associated with manual therapy directed at the cervical spine while producing the same effects.[5]

Neck pain prevalence in the general population has been reported to be twenty-three percent in women and fifteen percent in men.[6] A study on both thrust and non-thrust manipulation treatment directed at the cervical spine shows pain reduction, an improvement of cervical range of motion, and function in patients with neck pain.[7] When procedures are compared, the study has reported similar clinical outcomes.[8] However, cervical manipulation directed at the cervical spine must be reconsidered since it has potential disadvantage including vertebrobasilar artery injury.[9] Because of the kinematic link between the cervical and thoracic spine, improvement of thoracic spine may alleviate neck pain and improve joint mobility of the cervical spine.[4] Measurement of forward head posture by three-point markers and a comprehensive test with subjective and objectives has not been established from thoracic manipulation in neck pain. Therefore, thoracic manipulation may be an efficient method for treatment of mechanical neck pain.

The objectives of this study were to investigate the immediate and short-term (2 weeks) effects of thoracic manipulation, thoracic manipulation in combination with stretching exercise, and stretching exercise alone on mechanical neck pain, pressure pain threshold, Neck Disability Index (NDI), forward head posture, and cervical range of motion. This study has been approved by the ethical committee (69/2557), Burapha University, Thailand.

Materials and methods

Forty participants (9 males, 31 females) with mechanical neck pain, with an average age of 21 years old, were enrolled in this study.

Inclusion criteria were patients who were included in the study had mechanical neck pain for more than three months and limited range of motion of neck in all directions, the sixth or seventh thoracic spine hypomobility, and forward head posture. The participants were assessed for thoracic vertebral mobility and neck posture, confirmed by two five-year experienced orthopedic physical therapists. This study was conducted at the Faculty of Allied Health Sciences, Burapha University.

Exclusion Criteria were patients with cervical or thoracic spine subluxation or instability by assessment of passive physiological intervertebral movement and passive accessory intervertebral movement, central or peripheral nervous system involvement, signs consistent with nerve root compression and a diagnosis of cervical spinal stenosis, history of a whiplash injury within six weeks, osteoporosis, bone tuberculosis, tumors, fibromyalgia, spinal malignancy, and having cervicothoracic surgery or serious accidents within the previous four weeks and having received cervical or thoracic spine manipulation within the previous two weeks.

Participants were divided into four groups by simple randomization and received research protocols, ethics and consent information:

1. **Thoracic Manipulation Group.** Participants were asked to assume the prone position with their hands beside the trunk. Thoracic manipulation was then performed on the seventh thoracic spine by a single manipulation (central posteroanterior direction). If the cavitation (popping sound) was not produced during the first thoracic manipulation, participant would rest for one minute, after which there was second attempt at manipulation. Thoracic manipulation attempts were limited to two times.[10]
2. **Thoracic Manipulation with Stretching Exercise Group.** A combination of thoracic manipulation (same procedure as thoracic manipulation group) along with stretching exercise for neck (suboccipital muscles, neck extensor muscles, and scalene muscle), and shoulder (upper trapezius muscle, pectoralis muscle) were performed. Stretching procedure was an active stretching exercise performed by patients on both sides, for 20-30 seconds per set, with five sets, was performed two times daily during a two-week home program in the morning and the evening, for about 10-15 minutes per session. Stretching exercise was provided and assessed by the same physical therapist that performed the thoracic manipulation.
3. **Stretching Exercise Group.** Participants received only neck and shoulder stretching exercise, and performed active stretching exercise for two-week home program as applied in group 2.
4. **Control Group.** Participants received 15 minutes of health education about correct neck and trunk posture, ergonomics, and lifting by the second physical therapist who has more than five years of orthopedic clinical experience.

Outcome Measurements

Primary and secondary outcome parameters were measured four times by the third physical therapist. The measurements occurred before, immediately, post-test (48-72 hours), and short-term after treatment.

<u>Primary Outcome Measurements</u>

Visual Analog Scale for Pain (VAS-P 100 mm): Participants were asked to use a pencil marked in a 100 mm straight line. The therapist evaluated the distance from zero point. A higher score signifies more pain. Pain scales were evaluated on time periods.

<u>Secondary Outcome Measurements</u>

1. Neck Disability Index: (NDI)[11] was evaluated using questionnaires.
2. Pressure Pain Threshold (PPT) using a PPT Device; Algometer (The Commander Algometer; JTECH MEDICAL INDUSTRIES, INC., USA) was evaluated using a perpendicular press on the suboccipital muscle.
3. Forward Head Angle, using a three-point marker software, with high reliability (ICC=0.99)[12] was evaluated using a real time image of the lateral view, reporting the angle degree using a marker that was placed on the mastoid bone, the spinous process of

the seventh cervical spine, and the acromion process.

4. The study incorporated the Cervical Range of Motion (neck extension ROM), using a double inclinometer (Baseline Bubble Inclinometer, Baseline® measurement instrument, USA) by placing the first one on the top of head and the second one on the spinous process of the seventh cervical spine. Then, extension occurred until the second one's score moved. Finally, the score for the first one on the top of the head was read. Average score from repeating this process three times were determined.

Sample Size Determination

Simple randomizing by random lots was applied. It was a single blind randomized control trial. Sample size determinations, (the accuracy for this study is considered to be eighty percent) were conducted in reference to Cleland et al[14] by means of thrust and non-thrust logic plus a 20 percent drop out. There were ten persons per group and this study compared four different treatment groups. Therefore, the four groups consisted of forty persons. Calculation was derived from the primary outcome measurements by mean different of visual Analog Scale for Pain.

Data Analysis

Parametric data mean±SD (VAS-P, PPT, forward head angle, neck extension ROM) and non-parametric data of NDI were compared between groups in the following time periods: pretests followed by immediate tests; pretests followed by post-tests within 48-72 hours; and pretests followed by post-tests performed two weeks later (such as comparing group 1 and group 2 in immediate post-tests), not calculated within group on time periods. Independent paired t-Test (ratio scale with Parametric data; NDI used non-parametric test) was used by matching comparisons between time periods between groups at statistical significance of $p<0.05$.

Results

Forty participants (9 males and 31 females) with an average age of 21 years old enrolled in this study. The average body mass index was within normal range. All participants were right-handed. Details of age, gender, weight, height, and body mass index are shown in Table 1. Study parameters making comparisons between groups and time periods are shown in Table 2.

Table 1 Baseline characteristic of participants.

Group	Manipulation	Stretching	Manipulation and stretching	Control
Age mean±SD	21.3±0.8	21±0.7	21.6±2	21
Gender	Male 2 Female 8	Female 10	Male 3 Female 7	Male 4 Female 6
Weight (Kg.) mean±SD	59.5±5.4	58.9±9.1	54.8±14.1	63.7±12
Height (Cm.) mean±SD	165.6±7.2	161.2±3.7	161.6±6.1	163.5±5.4
Body Mass Index (Kg./m^2) mean±SD	21.8±3.8	22.6±5.1	20.6±4	24.7±5.2
Handed	All right handed	All right handed	All right handed	All right handed

Table 2 Study parameters compared between groups and time periods.

Group	Compared with	Time periods	Pain	NDI	PPT	FHP	Neck extension ROM
Manipulation with stretching group	Manipulation group	Post test immediately	0.606	0.054	0.635	0.118	0.002*
		Post test 48-72 Hour	0.012*	0.701	0.029*	0.634	0.137
		Post test 2 weeks	0.002*	0.219	0.373	0.981	0.001*
	Stretching group	Post test immediately	0.011*	0.003*	0.436	0.475	0.002*
		Post test 48-72 Hour	0.047*	0.489	0.342	0.195	0.137
		Post test 2 weeks	0.029*	0.21	0.034*	0.001*	0.001*
	Control group	Post test immediately	0.001*	0.001*	0.034*	0.004*	0.002*
		Post test 48-72 Hour	0.027*	0.014*	0.002*	0.129	0.137
		Post test 2 weeks	0.008*	0.001*	0.02*	0.103	0.001*

Table 2 Study parameters compared between groups and time periods. (continues)

Group	Compared with	Time periods	Pain	NDI	PPT	FHP	Neck extension ROM
Manipulation group	Stretching group	Post test immediately	0.21	0.309	0.211	0.041*	0.002*
		Post test 48-72 Hour	0.467	0.423	0.007*	0.093	0.137
		Post test 2 weeks	0.239	0.879	0.376	0.001*	0.001*
	Control group	Post test immediately	0.015*	0.002*	0.004*	0.004*	0.002*
		Post test 48-72 Hour	0.63	0.013*	0.001*	0.059	0.137
		Post test 2 weeks	0.404	0.018*	0.003*	0.123	0.001*
Stretching group	Control group	Post test immediately	0.001*	0.002*	0.015*	0.001*	0.002*
		Post test 48-72 Hour	0.783	0.089	0.007*	0.687	0.137
		Post test 2 weeks	0.622	0.07	0.001*	0.001*	0.001*

*NDI: neck disability index, PPT: pressure pain threshold, FHP: forward head posture, ROM: range of motion; * p<0.05*

Visual Analog Scale for Pain

Manipulation with the stretching group had decreased pain statistically significant when compared with the manipulation group in the 48-72 hour post-test (p=0.012) and the two-week post-test (p=0.002). There were statistical differences compared with stretching group in the immediate post-test (p=0.011), 48-72 hour post-test (p=0.047) and the two-week post-test (p=0.029) as well as statistical differences compared with the control group in the immediate post-test (p=0.001), the 48-72 hour post-test (p=0.027) and the two-week post-test (p=0.008).

Manipulation group had no statistical difference in pain from stretching group on three time post-tests. However, there was a statistical difference in pain compared with control group in the immediate post-test (p=0.015). There was also statistical difference between stretching group and control group in the immediate post-test (p=0.001).

Neck Disability Index (NDI)

Manipulation with stretching group had no statistical difference compared with manipulation group in all post-test time periods. Statistical differences was found when compared with stretching group in the immediate post-test (p=0.003) (a high score signifies high disability). There was also found the statistical differences when compared to control group in all three post-test time periods.

There was no statistical difference between the manipulation group and the stretching group in all post-test time periods. There was a statistical difference between stretching group and control group in immediate post-test (p=0.002).

For NDI parameter, a high score signifies high disability. Manipulation with stretching group had no statistical difference in NDI scores compared with the manipulation group in all post-test time periods. On the other hand, NDI scores were statistical different when compared with the stretching group, in the immediate post-test (p=0.003). There was also found the statistical differences when compared to the control group in all three post-test time periods.

The manipulation group showed no statistical difference in NDI scores compared to the stretching group, in all post-test time periods. There was a statistical difference between stretching group and control group in immediate post-test (p=0.002).

Pressure Pain Threshold

Manipulation with stretching group had statistical differences when compared to manipulation group in the 48-72 hour post-test (p=0.029) (increased pain threshold), the stretching group in the two-week post-test (p=0.034), the control group in the immediate post-test (p=0.034), the 48-72 hour post-test (p=0.002), and the two-week post-test (p=0.02).

Manipulation group had statistical differences compared to stretching group and control group in the 48-72 hour post-test (p=0.007). There were also statistical differences between the stretching group and the control group in the immediate post-test (p=0.015), the 48-72 hour post-test (p=0.007) and the two-week post-test (p=0.001)

Forward Head Posture[12]

Manipulation with stretching group had no statistical difference compared with manipulation group in all post-test time periods. Statistical difference was found when compared to stretching group in the two-week post-test was (p=0.001, and control group in the immediate post-test was (p=0.004). A decreased forward head posture angle signified good posture.

Manipulation group had statistical difference compared to stretching group in immediate post-test (p=0.041) and two weeks post-test (p=0.001). Statistical difference was also found when compared to control group in the immediate post-test (p=0.004).

Stretching group had statistical difference compared with control group in the two-week post-test (p=0.001).

Neck Extension Range of Motion

Manipulation with stretching group had statistical differences compared to manipulation group in the immediate post-test (p=0.002) and the two-week post-test (p=0.001). Statistical differences were found when compared to stretching group in the immediate post-test (p=0.002), and in the two-week post-test (p=0.001); control group in the immediate post-test (p=0.002), and in the two-week post-test (p=0.001).

Manipulation group had statistical differences compared to stretching group in the immediate post-test (p=0.002) and in the two-week post-test (p=0.001). Statistical differences were found when compared with the control group in the immediate post-test (p=0.002), and in the two-week post-test (p=0.001).

There was statistical difference between stretching group and control group in immediate posttest (p=0.002) and the two-week post-test (p=0.001).

Discussion

Manual therapy can improve all parameters but different in specific mechanisms by applying mechanical stress to different structures, such as thoracic manipulation, specifically thoracic zygapophyseal joint and stretching specifically to the neck muscles.[2] Manipulation with stretching group had statistically significant improved in the visual analog scale for pain between groups compared to manipulation, stretching, and control group in all time periods. The exception was found in manipulation group in immediate effect. It meant that manipulation has the same benefit in decreasing pain by increasing pain threshold in suboccipital muscle. Since it was found that improvement of thoracic spine mobility by manipulation also increases cervical spine mobility when compared to control group in all post-test time periods. Therefore, decreasing forward head posture will decrease load on suboccipital muscles in holding head all the time in order to correct head posture and leads to neck pain. New advantage of this study was real time forward head posture by three-point marker.[12] The results suggest that thoracic spine manipulation has immediate analgesic effects in patients with mechanical neck pain through use alone[11] and pain decreases after thoracic manipulation in mechanical neck pain, as measured by the numeric pain rating scale.[13]

The pressure pain threshold improved in all but not control group. More benefits were found in stretching group. The reasons may arise from the direct manual stretch and measurement at the neck muscle. Interestingly, thoracic manipulation group has benefited from decreased neck muscle pain.

NDI had a statistically significant improved in immediate effects when compared to manipulation and stretching group. Moreover, stretching group has shown a statistically significant improvement in all time periods when compared to control. Manipulation played a significant role in decreasing neck disability, according to Cleland *et al*[14] contrary to Cleland *et al*[11] according to which neck disability has no statistically significant improvement after thoracic manipulation. Conversely, Lau *et al*[13] reported that thoracic manipulation improves neck disability using the Northwick Park Neck Disability Questionnaire.[13] Interestingly, stretching group has benefited in the 48-72 post-test and the two-week post-test to decrease neck disability. However, there was no statistical significance when compared to manipulation and stretching group. When compared to previous studies, this study had the advantage of using participants who, on average, were younger and had less pain and lower neck disability scores. NDI parameters was improved after manipulation with stretching or manipulation. This result may closely correlate to neck function and thoracic mobility.[15] Improvements on the thoracic spine increase flexibility of neck muscles.

There was improvement on forward head posture after thoracic manipulation and stretching as found in manipulation with stretching group treatment. Participants could erect the neck to a neutral position better than manipulation group. The result was according to Lau *et al*[13] that reported thoracic manipulation increased the craniovertebral angle (CV angle).[13,16] The authors suggested that there are close and link chain of thoracic spine and cervical spine. Increasing the mobility of the thoracic spine, as when the spine returns to the neutral position, results in improved cervical spine mobility. An increase in thoracic spine mobility causes increased vertebral spine mobility in case of thoracic spine decreases movement or stiffness. The effect is to decrease the curve of thoracic area which tends to increase activity and load on the neck and occipital muscle. Forward head posture leads to a slouch posture and causes head to point down. This effects suboccipital muscles to increase the load by pulling the head to the correct position. These mechanisms lead to neck pain and explain how thoracic manipulation can treat neck pain. Suboccipital muscle is an important part of neck extensor, which responsible for high load and continuous contraction. Alterations of neck extensor muscle action in neck pain study has reported that superficial cervical extensors regularly present increased activation in patients with neck pain[17] and delayed offset (relaxation).

An increased in neck extension range of motion leads to improvement of forward head posture. Stiffness of thoracic spine also affects the cervical spine. Therefore, thoracic manipulation promotes cervical movement. Moreover, neck muscle stretching showed more advantage by maintaining better neck movement in the two-week post-test. Quality of life may increase. However, it was not measured in this study. It is likely to be measurable using a health-related quality of life status questionnaire (SF36 Questionnaire) which has found benefits of thoracic manipulation with patients who have chronic neck pain.[14]

The theory that biomechanical link between thoracic spine and cervical spine may lead to abnormal connection and distribution of forces and mobility in the cervical spine has lately been investigated.[19,20] Thoracic spine manipulation may cause a restoration of more normal spine biomechanics. This may decrease mechanical stress and increase the distribution of joint forces from thoracic to cervical spine. Limitations are short-term follow-up and

a confounding factor needed to be controlled for in this study was the relative youth of the participants. Future research in this area should be focused in the long-term effects of thoracic spine manipulation in patients with mechanical neck pain on patient satisfaction and costs. Questionnaires might also be used.

Conclusion

Thoracic manipulation may be combined with standard physical therapy treatment in mechanical neck pain, and may provide more benefit when used in combination with stretching exercise to decrease neck pain, increase cervical range of motion, and improve head posture, leading to decreased neck disability.

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