JAMS

JOURNAL OF ASSOCIATED MEDICAL SCIENCES

Volume 53 Number 2 May-August 2020 E-ISSN: 2539-6056

Journal of Associated Medical Sciences

Aims and scope

The Journal of Associated Medical Sciences belongs to Faculty of Associated Medical Sciences (AMS), Chiang Mai University, Thailand. The journal specifically aims to provide the platform for medical technologists, physical therapists, occupational therapists, radiologic technologists, speech-language pathologists and other related professionals to distribute, share, discuss their research findings, inventions, and innovations in the areas of:

1. Medical Technology
2. Physical Therapy
3. Occupational Therapy
4. Radiologic Technology
5. Communication Disorders
6. Other related fields

Submitted manuscripts within the scope of the journal will be processed strictly following the double-blinded peer review process of the journal. Therefore, the final decision can be completed in 1-3 months average, depending on the number of rounds of revision.

Objectives

The Journal of Associated Medical Sciences aims to publish integrating research papers in areas of Medical Technology, Physical Therapy, Occupational Therapy, Radiologic Technology, and related under peer-reviewed via double-blinded process by at least two internal and external reviewers.

Types of manuscript

Manuscripts may be submitted in the form of review articles, original articles, short communications, as an approximate guide to length:

- **Review articles** must not exceed 20 journal pages (not more than 5,000 words), including 6 tables/figures, and references (maximum 75, recent and relevant).
- **Original articles** must not exceed 15 journal pages (not more than 3,500 words), including 6 tables/figures, and 40 reference (maximum 40, recent and relevant).
- **Short communications** including technical reports, notes, and letters to the editor must not exceed 5 journal pages (not more than 1,500 words), including 2 tables/figures, and references (maximum 10, recent and relevant).

Peer review process

By submitting a manuscripts to Journal of Associated Medical Sciences, the authors agree to subject it to the confidential double-blinded peer-review process. Editors and reviewers are informed that the manuscripts must be considered confidential. After a manuscripts is received, it is assigned by a specific Associate Editor. The Associate Editor prepares a list of expert reviewers, which may include some suggested by the Editor-in-Chief. Authors can indicate specific individuals whom they would like to have excluded as reviewers. Generally, requests to exclude certain potential reviewers will be honored except in fields with a limited number of experts. All potential reviewers are contacted individually to determine availability. Manuscripts files are sent to at least two expert reviewers. Reviewers are asked to complete the review of the manuscripts within 2 weeks and to return a short review form. Based on the reviewers' comments, the Associate Editor recommends a course of action and communicates the reviews and recommendations to the Editor-in-Chief for a final decision.

The Associate Editor considers the comments made by the reviewers and the recommendation of the Editor-in-Chief, selects those comments to be shared with the authors, makes a final decision concerning the manuscripts, and prepares the decision letter for signature by the Editor-in-Chief. If revisions of the manuscripts are suggested, the Associate Editor also recommends who should review the revised paper when resubmitted. Authors are informed of the decision by e-mail; appropriate comments from reviewers and editors are appended.

Publication frequency

Journal of Associated Medical Sciences publishes 3 issues a year

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

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

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

Journal E-ISSN:
2539-6056

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

Journal of Associated Medical Sciences

Vol. 53 No. 2 : May 2020

Content

Journal of Associated Medical Sciences 2020; 53 (2): 1-6

JAMS
Journal of Associated Medical Sciences

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

Journal of Associated Medical Sciences

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

Application program for wound healing measurement based on the region growing technique: A trial version

Phadngam S [*1] **Ruthiang J**[1] **Ina N**[2] **Ferraresi A**[3] **Isidoro C**[3]

[1] *Department of Radiologic Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand*
[2] *Department of Radiology, Faculty of Medicine, Prince of Songkla University, Hat Yai Province, Thailand*
[3] *Laboratory of Molecular Pathology, Department of Health Sciences, Università del Piemonte Orientale "A. Avogadro", Novara, Italy*

ARTICLE INFO

Article history:
Received 6 December 2019
Accepted as revised 14 April 2020
Available online 14 April 2020

Keywords:
Region growing, wound healing, migration

ABSTRACT

Background: The study of cell migration is one of the most interesting topics in cancer research. Wound healing assay is a basic 2D technique used to follow cell migration. The area of a wound's images are measured and calculated to demonstrate the percentage of moving cells. Normally, the measurements are estimated by direct scaling of observers, which unavoidably involves human error. Moreover, there is yet to be a gold standard technique for measurement. Recently, image processing tools have been provided to use and easily carry out measurement, such as ImageJ software and others.

Objectives: This study proposed to take the advantage of the region growing algorithm for wound healing measurement.

Materials and methods: Basic algorithm was used for segmentation of the low contrast area of the wound area from the background in each image. Moreover, contrast limited adaptive histogram equalisation and edge detection techniques were applied in order to improve the quality of images. Program codes were generated and conformed to be the finalised program, named the wound healing measurement program for the trial version.

Results: The program was compared to ImageJ, with the results showing that there were no statistically significant differences (p>0.05, n=3). However, time for all processes of this program was fast and not dependent on the shape of the wound. Intra observer and inter-observer reproducibility of this study found a correlation coefficient within groups close to 1. The average satisfaction of observers for using the program was 4.45.

Conclusion: Thus, it was concluded that this study could apply the region growing technique for the measurement of cell migration program. However, there were problems that occurred during the proceeding in this version. Therefore, we aim to resolve and improve the efficiency of this program in future work.

* **Corresponding author.**
Author's Address: *Department of Radiologic Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand*
** **E-mail address:** : suratchanee.phadngam@cmu.ac.th
doi: 10.14456/jams.2020.7
E-ISSN: 2539-6056

Introduction

Cell migration is one of main subjects that biology researchers have been studying, especially those cells associated with cancer. For in vitro 2D-study, the wound healing assay was performed by scratching the monolayer cell with 80-100% confluence. Then, the study followed the movement of the healing of wound and calculated in percentage of movement. Generally, measurement comprises the area of the wound each time by using a ruler or other measurement instrument.[1-3] Therefore, it is a manual technique and the values are approximate, with human error definitely possible. Moreover, the captured images in the study have appeared in low contrast properties by microscopy camera. Somehow, it was difficult to distinguish between cells and background, which caused more errors in their results. Avoiding errors will provide more accurate results. Ideally, it would be nice to have some kind of technique or program to measure an area easily and accurately, with acceptable error.[4] Nowadays, ImageJ software is used globally, whether for image adjustment or measurement.[5-6] The ROI function of ImageJ allows manual drawing along the wound area and gives a result in pixel units. However, some observers have been unable to get precise results due to the low contrast of images, as mentioned before. Moreover, in the case of low ability to use a hands-free function to draw an area of observes could be one problem for uncertainty in the area of the wound. Even though most images are low contrast, the wound area is apparently different in texture. The wound is a background of the image and almost uniform in intensity, while the cell layer part is individual but not uniform. To easily measure the wound area, each part must be separated from others. The one basic segmentation technique in image processing that could be applied for this purpose is region growing.[7-9] This algorithm can separate the uniform and communicated areas. Therefore, this study proposes to apply the basic region growing technique to measure the wound area in wound healing images. This study aims to develop a basic program for measuring the migration area in wound healing images to get precise, fast and convenient results. Moreover, we will develop a more complete version in the future.

Materials and methods

Materials

In this study, wound healing images were taken by a microscope, (Leica DMI6000B, Leica Microsystem AG) provided by the laboratory for Molecular Pathology, Department of Health Sciences, Università del Piemonte Orientale "A. Avogadro", Novara, Italy. All experiments were performed on a PC with HP Inter® Core™i3 CPU 3.07 GHz and 2.00 GB RAM using MATLAB version R2013a and ImageJ Software (freely available at http://imagej.nih.gov/ij/,). Thirty observers were students in the Faculty of Associated Medical Sciences, Chiang Mai University, during the period of study. They were 13 females and 17 males with an average age of 20.3 years.

Algorithm description

Region growing technique for image segmentation

Region growing is a region or pixel-based image segmentation. Its work involves the initial seed point, which is selected in the first step. The algorithm will then expand to the neighbouring pixels of its initial seed points and determine whether the pixel neighbours should be added to the region or ignored by using the threshold value. This approach to segmentation is used to find data clustering the same as general data clustering algorithms. A general discussion of the region growing algorithm includes:

1) For image R, let p denote the initial seed, after region growing, the region R1 generated from p ($p \in R1$) is connected.
2) Assume that R1 is unconnected, then there are at least two subsets $R1,1 \subset R1$, $R1,2 \subset R1$, and $R1,1 \cap R1,2 = \emptyset$ Setting the seed $p \in R1,1$, as $R1,1 \cap R1,2 = \emptyset$, the algorithm ends after finishing the search of the subset R1,1, we get the result $R1,2 \subset R1$. However, the result is paradoxical with the assumption. So, the proposition is correct, namely, R1 is connected.

We follow the algorithm to generate the basic software to measure the wound area image for users. The basic steps are described as below.

Step 1. Generate the matrix 1x5 as RG data collection to collect the data of only 5 areas in one image. Therefore, this software allows a user to select the seed point in 5 unconnected ROI. Each seed point is determined as the centre of each ROI as the queue Q.

Step 2. Check all the pixels around the Q, one by one, to determine whether they are similar to the seed. Let p denote the current seed, and q is one element of p's neighbouring pixel set, I(p) and I(q) denote the intensities of p and q, respectively. If $|I(p) - I(q)| < 1$, p and q belong to the same region. Otherwise, p and q belong to different regions. The segmentation result is denoted by RG after this region growing. The region that includes the initial seed is marked as "1", while other regions are marked as "0".

Step 3. Pop the first pixels and add to R1 set. Then check whether it is empty set. If not, take the first element from the current Q as the seed and repeat Step 2. Otherwise, push the elements that are not yet added to R1 to set R2. Find the total area of 1 which is connected and add as RG data to the RG data collection matrix. The final result of region growing is summarised from the RG data. Fig. 1 shows an example of region growing based image segmentation.

The wound healing measurement program developed and Algorithm framework

The code was generated and compiled by MATLAB program. Considering that wound healing images are often taken with low-contrast properties and weak edges (shown in Figure 2), it affects traditional region segmentation algorithms. Therefore, we added the improvement algorithms, the contrast-limited adaptive histogram equalisation[10, 11] and edge detection[12-14] to be a qualified image for the region growing algorithm. The entire procedure of the algorithm follows certain steps.

Step 1. Select image I and conduct the contrast-limited adaptive histogram equalisation on the image I to be the

higher contrast image.

Step 2. To avoid taking a long time for processing, select only the wound area with 10-20 % of cell layer by cropping to obtain Image I selected.

Step 3. Obtain the edge image of image I selected by the Sobel edge detection algorithm. Then dilate the edge to fill and connect all edges together using the dilated gradient mask technique.

Step 4. Enter the final image in Step 3 to the traditional region segmentation algorithms. The result will appear in a pop-up window in pixel units.

Results and discussion

The image results by the wound healing measurement program

This version of the wound healing measurement program enables true and grey scale imaging. We tested the proposed program on two set images with different format types. The results show the successful measuring in wound area for both types (data not shown). Somehow, the algorithm in the program is liable to produce isolated regions in some images epically, which are not clear or have artefacts in the area, as shown in Figure 3. However, this program provides the summation connected area according to the region growing technique. Therefore, the results depend on the appearance of an image. The segmentation results and area measurement shown in Figure 4 indicate that our propose program obtains satisfactory measurement results based on the region segmentation and improved image algorithms. Moreover, this program is useful for the maximum unconnected area in 5 areas, as shown in Figure 2C.

Image evaluation measure for the program results

In this section, we consider the performance and accuracy of the measurement algorithms, which are the important aspects of this program. We test the ability of the program for 3 different images with different manner of wound area, as shown in Figure 2, and compare the results using ImageJ software. The results are shown in Table 1. We observed that the results from both programs are similar and not statistically significant difference, (p>0.05, n=3). Also, both programs show the area measurement related to the area. In contrast, the time cost of both programs is different. These results indicate that Image J's proceeding time depends on the shape and complexity of the area. In the other hand, an uncertainty image, such as in Figure 6C, takes longer than others. However, our program shows the time cost depends on the size of the wound only. Moreover, 3 observers independently measured the 19 images for both programs two times. The results were analysed for intra observer and inter-observer reproducibility. The results are shown in Table 2. We found the correlation coefficient within groups to be close to 1 for both the intra observer and inter-observer reproducibility. These results indicated that this study program is reliable.

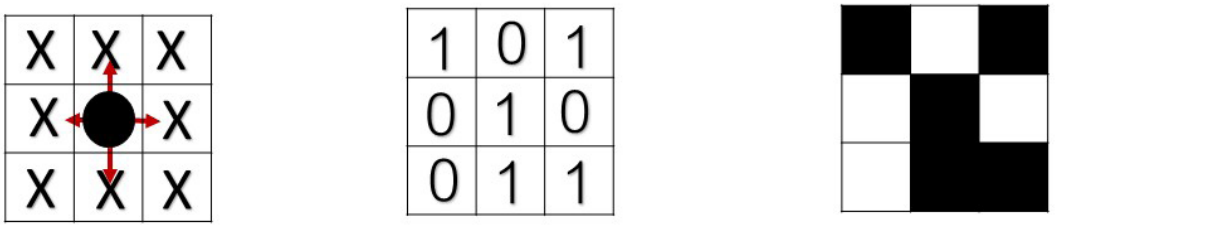

Figure 1. *The region growing algorithm.*

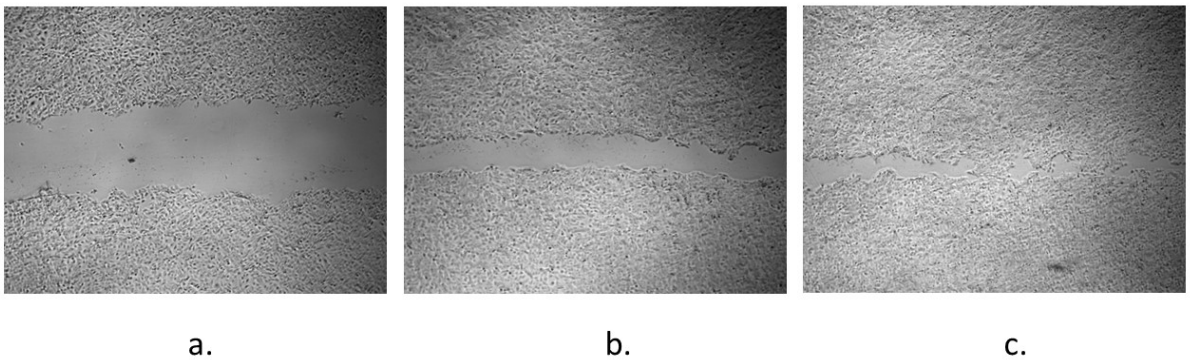

Figure 2. *Wound healing image with low contrast and weak edges.*

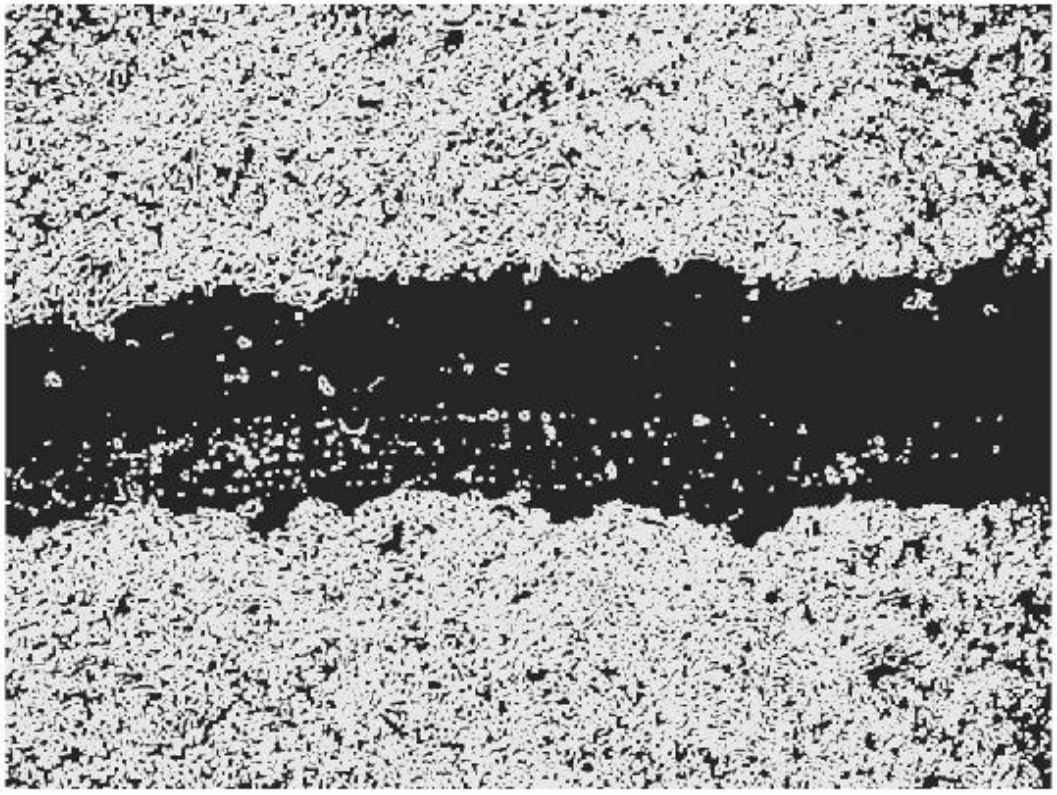

Figure 3. *Isolated point in the image.*

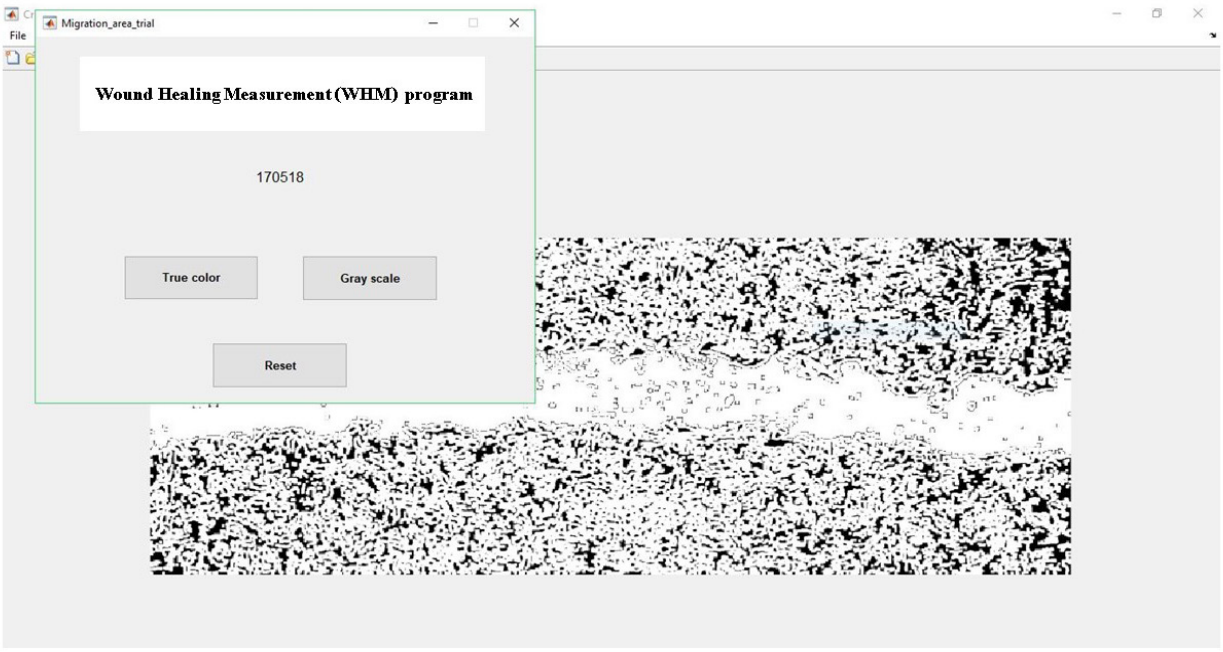

Figure 4. *The result of measurement area by program.*

Table 1 Results of measurement for both programs.

Image	ImageJ program		This study program	
	Area (pixels)	Time(s)	Area (pixels)	Time(s)
A	552435±4211	47±6	554137±2746	68±2
B	192371±4008	38±8	189636±1524	17±2
C	81966±2154	41±18	72792±2895	12±2

Table 2 Intra-class correlation coefficient in this study.

Intra observer	ICC (intra-class correlation coefficient)
Observer 1	0.998
Observer 2	0.995
Observer 3	0.997
Inter observer	ICC (intra-class correlation coefficient)
All 3 observers	0.998

Satisfaction of users for the program

Finally, we provided the program to 30 users. All users had different performance using the image processing program and computer. The users were advised of the proceeding for the wound healing measurement (WHM) program. The program included the manual measurement by ruler and ImageJ program. Later, we asked all users to measure 3 times independently for each image in 3 different techniques, as mentioned. At the end, the satisfaction for this program was observed. Table 3 shows the results of satisfaction for this program. We were pleased that our program had a good average of satisfaction for users at 4.45. Moreover, we observed the SD data from 3 measurement techniques as shown in Table 4. The results showed that the highest SD (SD ≥±2.0) and lowest SD (SD ≤±1.0) were the manual techniques and this work, respectively. SD resulted can verify that our program has less human error, high accuracy and is similar to ImageJ.

Table 3 Satisfaction of 30 users for WHAM program.

Title	Mark
1. Time for proceeding	4.2
2. Steps of use	4.6
3. User interface	4.6
4. Reliability of the program compared to other technique	4.0
5. Reasonable wound healing image compared to other technique	4.8
6. Application for other images compared to other technique	4.5
Average	4.45

Table 4 Standard deviation of the percentage of migration from three techniques.

	Average Migration (%)					
	Manual technique		ImageJ		WHM	
	T1	T2	T1	T2	T1	T2
30 users	±2.04	±2.63	±1.12	±1.32	±0.72	±0.55

Conclusion

In this work, we proposed a beginning program based on the region growing algorithm for measuring the area in a wound healing assay. We named this program the wound healing measurement (WHM): the trial version program 2018. The program also introduced another algorithm to improve the image, such as the Contras limited adaptive histogram equalisation and Edge detection techniques for improving the quality of images. The program is able to measure in true scale and grey scale without any problem. Moreover, we designed an easier user interface for application. Briefly, the image needs to be loaded into the program from the data storage in a computer. The result is presented in a common window of the user interface in pixel units after selecting the area for 5 seed points. From our experience, we have not seen any wound image that had more than 5 unconnected areas, unless a region is small enough to ignore. That is the reason to set the seed point only 5 times. However, our program has a limitation of the maximum unconnected area, which has to be resolved in the next version. The experimental results show that our work is similar and not statistically significant difference to ImageJ, while the manual was quite different from both techniques. Moreover, the results strongly indicate that the WHM program can obtain reliable, satisfactory and reasonable measurement for wound images However, this program might depend on whether the image has high contrast and a high level of noise included in the image. Nevertheless, the program has a relatively high time cost depending on the size of the wound and the efficiency of the computer. Therefore, improving the efficiency of the program is the aim of future work.

Conflicts of interests

The authors reported no potential conflict of interest

Acknowledgements

The authors would like to thank Chiang Mai University, Department of Radiologic Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand, and Laboratory of Molecular Pathology, Department of Health Sciences, Università del Piemonte Orientale "A. Avogadro", Novara, Italy, for supporting this research.

References

[1] Morani F, Phadngam S, Follo C, Titone R, Aimaretti G, Galetto A, et al. PTEN regulates plasma membrane expression of glucose transporter 1 and glucose uptake in thyroid cancer cells. J Mol Endocrinol 2014; 53(2): 247-58.

[2] Morani F, Phadngam S, Follo C, Titone R, Thongrakard V, Galetto A, et al. PTEN deficiency and mutant p53 confer glucose-addiction to thyroid cancer cells: impact of glucose depletion on cell proliferation, cell survival, autophagy and cell migration. Genes & cancer 2014; 5(7-8): 226-39.

[3] Ferraresi A, Phadngam S, Morani F, Galetto A, Alabiso O, Chiorino G, et al. Resveratrol inhibits IL-6-induced ovarian cancer cell migration through epigenetic up-regulation of autophagy. Mol Carcinog 2017; 56(3): 1164-81.

[4] Mayrovitz HN, Soontupe LB. Wound areas by computerized planimetry of digital images: accuracy and reliability. Adv Skin Wound Care 2009; 22(5): 222-9.

[5] Nunes JPS, Dias AAM. ImageJ macros for the user-friendly analysis of soft-agar and wound-healing assays. Biotechniques 2017; 62(4): 175-9.

[6] Aragon-Sanchez J, Quintana-Marrero Y, Aragon-Hernandez C, Hernandez-Herero MJ. ImageJ: A Free, Easy, and Reliable Method to Measure Leg Ulcers Using Digital Pictures. Int J Low Extrem Wounds 2017; 16(4): 269-73.

[7] Yian LC, Xiaobo L. Adaptive image region-growing. IEEE Trans Image Process 1994; 3(6): 868-72.

[8] Zhang X, Xiongfei L, Feng Y. A medical image segmentation algorithm based on bi-directional region growing. Opt - Int J Light Electron Opt 2015; 126(20): 2398–404.

[9] Ozturk CN, Albayrak S. Automatic segmentation of cartilage in high-field magnetic resonance images of the knee joint with an improved voxel-classification-driven region-growing algorithm using vicinity-correlated subsampling. Comput Biol Med 2016; 72: 90–107.

[10] Yousefi S, Qin J, Zhi Z, Wang RK. Uniform enhancement of optical micro-angiography images using Rayleigh contrast-limited adaptive histogram equalization. Quant Imaging Med Surg 2013; 3(1): 5-17.

[11] Flores WG, Pereira WC. A contrast enhancement method for improving the segmentation of breast lesions on ultrasonography. Comput Biol Med 2017; 80: 14-23.

[12] Canny JF. A computational approach to edge detection. IEEE Trans Pattern Anal Machine Intell 1986; 8: 679-714.

[13] Davis LS. Survey of edge detection techniques. Comput Graph Image Process 1975; 4: 248–270.

[14] Qian RJ, Huang TS. Optimal edge detection in two-dimensional images. IEEE Trans Image Process 1996; 5: 1215–20.

Journal of Associated Medical Sciences 2020; 53 (2): 7-13

JAMS
Journal of Associated Medical Sciences

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

Journal of Associated Medical Sciences

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

Preliminary study on the combined effects of simple expiratory muscle strength device training with conventional exercise on submental muscles force and maximal expiratory pressure related to swallowing in patients with head and neck cancer

Erin Songdechagraiwud[1] Piyawat Trevittaya[1] Jirakrit Leelarungrayub[2*] Nuntigar Sonsuwan[3]

[1]Communication Disorders, Department of Occupational Therapy, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand
[2]Department of Physical Therapy, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand
[3]Department of Otolaryngology, Faculty of Medicine, Chiang Mai University, Chiang Mai Province, Thailand

ARTICLE INFO

Article history:
Received 16 March 2020
Accepted as revised 27 April 2020
Available online 27 April 2020

Keywords:
Head and neck cancer, maximal expiratory pressure, simple expiratory muscle strength trainer, surface electromyography.

ABSTRACT

Background: After chemo-radiotherapy, most patients with head and neck cancer (HNC) commonly show defect in swallowing and coughing function related to various weaknesses of oropharyngeal muscles. It is possible that strengthening those muscles via an expiratory force device could regain muscle strength.

Objectives: The aim of this preliminary study was to discover whether a combination of expiratory muscle strength training (EMST) together with a simple expiratory muscle strength device in conventional exercise could be performed for improving submental muscle force and expiratory muscle strength in patients with HNC during chemo-radiotherapy.

Materials and methods: The effects of combined EMST with a conventional exercise program on submental muscle force and maximal expiratory pressure (MEP) were evaluated when compare to a conventional exercise. Conventional exercise composed of neck, tongue, oral muscle, and supraglottic swallow exercise. Meanwhile, EMST was performed with a resistor at 50% and 70% of MEP. MEP and surface electromyography (sEMG) were tested at before and after a 6-week period of intervention. Satisfaction of using device was evaluated. Moreover, interviews on swallowing and pain sensation were also carried out.

Results: The results showed that sEMG amplitude decreased significantly in the conventional exercise group (n=5, aged 55.80±2.51 years). It was increased significantly in the combined EMST with conventional exercise group (n=5, aged 49.20±3.78 years). However, sEMG duration from submental contraction increased significantly in the conventional exercise group, but decreased significantly in the combined EMST with conventional exercise group. In addition, the MEP value decreased non-significantly in both groups after the 6-week period when compared to the baseline period. Finally, satisfaction of the training device from all of the five patients involved focused on comfort and easy-practical use without dyspnea. Moreover, the swallowing or food swallowing function was improved and less painful when compared to before the device was used.

Conclusion: This preliminary study showed that the combination of EMST with a simple expiratory muscle strength device, which was simple and easily developed, and a conventional exercise program, could improve the submental force among patients with HNC in the clinic, without adverse effects.

* **Corresponding author.**
Author's Address: *Department of Physical Therapy, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand*
** **E-mail address:** : donrawee.leela@cmu.ac.th
doi: 10.14456/jams.2020.8
E-ISSN: 2539-6056

Introduction

In 2020, the database of the World Health Organization (WHO) reported that the number of new patients with nasopharyngeal cancer (NPC) was expected to increase in the European Union (EU), United States (US) and Japan.[1] In addition, cancer is the most common cause of death in Thailand, with a higher mortality rate (48.4 to 95.2 per 100, 000) from 1998 to 2011. The age-standardized rate (ASR) of incidence for oral, nasopharyngeal and laryngeal cancers is 15.7 and 10.7 per 100,000 males and females, respectively.[2] General treatments for HNC can be performed with various interventions; for example, surgery and radiotherapy combined with chemotherapy, which depend on conditional variables such as primary site, clinical stage, and tumor removable, but most of the patients with HNC undertake clinical intervention by using chemo-radiotherapy.[3] The side-effects of radiotherapy have been reported as mucositis, hoarseness, erythema and desquamation3 and those of chemotherapy are nausea, vomiting and neutrophenia.[4] Thus, combination of both these interventions presents side-effects in the voice, swallowing, nutrition, and quality of life (QOL),[5] due to swallowing motility being dysfunctional from muscle weakness or damage to specific muscles such as the hypopharyngeal, laryngeal muscles or cervical esophageal function.[6,7] Of all clinical problems, swallowing dysfunction or dysphagia is the most common, and it diminishes QOL profoundly through aspirated pneumonia and malnutrition.[8]

At present, application of swallowing rehabilitation in patients with HNC is very necessary both before and after chemo-radiotherapy. Previous study reported that risk of dysphagia can be prevented,[9] by either direct therapy as modify food, promotion the swallowing and breathing coordination[10] or indirect therapy as increases on expiratory muscle strength by the expiratory muscle strength training (EMST).[11]

A systematic review showed that EMST improved airway safety during swallowing in dysphagia and increased the strength of expiratory muscles in all patient groups.[12] Previous studies reported that EMST enhanced the ability to swallow in patients with Parkinson's disease,[13] stroke[14] and multiple sclerosis[15] and also in healthy-elderly people.[16] They also showed that tongue strengthening exercise improved QOL and eating and speech efficiency in patients with oropharyngeal cancer, who were treated with radiotherapy with or without chemotherapy.[17] In addition, EMST increased maximal expiratory pressure (MEP) and swallowing safety in patients who were chronic radiation-associated aspirators.[18] Similar principle function of EMST device and inspiratory muscle trainer (IMT) is resistive pressure during inspiration or expiration. There are many commercial products available worldwide for IMT such as POWERbreath® (POWERbreathe International Ltd.) or the Threshold ® device (Respironcis Inc.), as well as the Inspiratory Muscle Trainer (Smiths Medical ASD, Inc., USA).[19] Although previous study has claimed that EMST can be performed with an inexpensive and a comfortable device for improving dyspnea and coughing, it is still expensive for individual patients and not used widely in Thailand. From all these devices, the IMT, which has six different color-coded resistors with a central hole of 2-7 mm diameter, is very interesting. Previous study adapted and modified plastic caps with various resistors of 2, 4 and 6 mm diameter in polyvinyl chloride (PVC) tubes for training inspiratory muscle strength in chronic obstructive pulmonary disease (COPD) patients.[20] Therefore, the aim of this preliminary study was to evaluate the effects of a modified device, *i.e*. an expiratory muscle strength trainer, among patients with HNC on swallowing force of submental muscles and duration of pharyngeal phase of swallowing.

Materials and methods

The protocol in this preliminary study was divided into two steps; 1) developing the expiratory muscle strength device before calculating the percentage of resistance, and 2) performing a pilot study on the effect of expiratory muscle strength device on submental muscles force and duration of pharyngeal swallowing phase in patients with HNC, while being treated with chemo-radiotherapy at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai University, Thailand. The protocol of this study was approved ethically by the Ethic Human Committee at the Faculty of Associated Medical Sciences, Chiang Mai University (AMSEC-61EX-025) and Faculty of Medicine, Chiang Mai University (NONE-2561-05329), Thailand. It was conducted in accordance with the Declaration of Helsinki, and all patients provided written informed consent.

Developing the device and calculating resistance percentage

The EMST device was designed by following a previous study.[20] It was produced by adapting a PVC water pipe (2 cm in diameter). Each EMST device comprised a main PVC tube 11 cm long and a plastic cap with various resistors having centrally placed holes of 1 mm diameter (Figure 1).

Figure 1. *Pilot expiratory muscle strength training (EMST) device.*

Percentage of resistors was performed in 10 sedentary and healthy subjects. All subjects had no hospital history of pulmonary conditions such as allergy, rib fracture and chest wall deformity, or scoliosis and pleural diseases for at least 3 months. They also should have been ex-smokers with normal blood pressure and body mass index of 18.50-22.90 $Kg.m^2$. Resistance of the resistors was determined with a MicroRPM device (MicroRPM, Medical Ltd, Kent, UK). Resistance percentage of each resistor was determined by calculating the MEP between with and without a resistor. MEP was evaluated under instruction of the standard European Respiratory Society (ERS) statement guideline.[21] All of the participants repeated three trial maneuver of

MEP by holding a disposable mouthpiece in their mouth in a state of maximum inhalation, and then exhaling maximally as strong and as fast as possible.[22] Percentage of resistance can be calculated by the following formula: $[(MEP_{non\text{-}resistor} - MEP_{resistor})/MEP_{non\text{-}resistor}]x100$. Finally, the resistors that presented 50% and 70% of MEP were selected for training.

Patient recruitments and Programs

Ten patients who treated with radiotherapy or chemo-radiotherapy were randomized into two groups following a randomized controlled trial (RCT) protocol (TCTR20180812002). Effectiveness of combined EMST on conventional exercise (n=5) was performed compared to a conventional exercise (n=5). Patient criteria were appropriate for the protocol of this study including good consciousness and no problem in lips and mouth closures. Patients with total laryngectomy, tracheostomy, oral cavity defect, underlying pulmonary diseases, dysphagia or hypertension were excluded. For good adherence to the conventional exercise and performance in EMST training device, log-book records were made daily and kept strictly by patients or close caregiver at home, and they were rechecked by the researcher who telephoned every two days.

Conventional exercise program

All ten patients with HNC were educated and well-trained on conventional exercise program. The program consisted of neck, tongue, oral muscle, and supraglottic swallow exercise under previous prescriptions.[22, 23] Patients were asked to perform each exercise five times per day for a six-week period.

The EMST program

All five patients in EMST program were educated and well-trained by the time it started. A total six-week period of training was divided into two steps: 1) the first 3 weeks of achieving 50% of MEP individually, and 2) the second 3 weeks of achieving 70% of MEP. The patients used EMST device at home, 5 days per week during the 6-week training period; performing 5 sets of 5 breaths through the device for a total of 25 breaths per day, with 2-3 minutes of rest between sets.[24,-26] Finally, satisfaction on use of device regarding sensation in swallowing and pain was found by individual interviews after completing the 6-week course.

Evaluation of outcomes

The main outcomes in this study were the maximal expiratory pressure (MEP) and surface electromyography (sEMG) on submental muscles before and after 6 weeks of complete interventions in both groups. MEP evaluation was performed according to the standardized protocol of the ERS guideline.[21] The protocol was repeated trials in the maneuver of exhaling maximally and as fast as possible from maximal inhalation.[22] The evaluated protocol of surface electromyography (sEMG) on submental muscles force was followed as detailed below.

Evaluation of the surface electromyography (sEMG)

This study detected the effect of EMST on submental muscles that relate to swallowing function or dysphagia by surface electromyography (sEMG).[27] sEMG of swallowing has been confirmed as a non-invasive, simple, and reliable method for screening and initially evaluating dysphagia.[28] The 2-channel sEMG device (PowerLab, 4/35 Dual Bio Amp, AD Instruments, Australia), with Ag/AgCl electrodes (3M™ Red dot™ 2235, United State), was used for examinations. Parallel electrodes were placed on skin in the submental muscle groups (0.5 cm parallel to the left and right of the midline) and performed following previous description[29] in order to record changes in sEMG potential when having swallowed. sEMG signals from swallowing 3 times were amplified (1000 x) and filtered (width band: 500-1,000 Hz), before reporting the root of mean square values (RMSμV) of maximal amplitude and duration of pharyngeal swallowing (in seconds).

Statistical analysis

Data were represented with median (minimal and maximal data). Non-parametric Wilcoxon-Signed Rank Test was used within the groups for statistical analysis during the 6-week period. Mann-Whitney U test was used to compare the groups either before or after the training period. All statistical analyses were carried out using the Statistical Package for Social Software version 11.0 (SPSS Inc., Chicago, IL, USA) for Windows. The significant level was set at $p<0.05$

Results and discussion

The pilot study for the percentages of resistance on expiratory resistor in 10 healthy patients showed that 2 sewing needle holes and 1 mm diameter located centrally had 57.13±4.90 % and 72.54±2.30 % of resistance.

Results of the combined EMST with conventional exercise in 5 patients aged 49.20±3.78 (4 males and 1 female) and a conventional exercise in 5 patients aged 55.80±2.52 years (4 males and 1 female) are presented in Table 1. The age between groups was not statistically different. From various cancer types and stages under the standard cancer stage of the American Joint Committee on Cancer Guideline, [30] all patients were treated clinically with concurrent radiotherapy (CCRT) (9,996 gray weekly) and Cisplatin and carboplatin chemotherapy, using the intensity-modulated radiation therapy (IMRT) technique. The outcomes of both sEMG and MEP at before and after 6-week period individually are shown in Table 2.

The results of sEMG on submental muscle force showed the amplitude and duration during the swallowing test (Table 3). The median sEMG amplitude (0.0747 RMSμ) was increased significantly compared to those before training (0.0547 RMSμV) in a EMST combined with conventional exercise group. The significant decrease of sEMG amplitude after the 6-week period (0.0505 RMSμV) compared to baseline period (0.0605 RMSμV) was shown in the conventional exercise group (Figure 2.A). In addition, the median sEMG duration decreased statistically from before (1.17 second) to after 6-week period in the EMST and conventional exercise group (1.05 second) was contrast to a conventional exercise group (0.92 to 1.01 second) (Figure 2B).

Table 1 Characteristics, types & stage of cancer and medical treatments during 6-weeks period in both groups.

Gender	Age (years)	Cancer	TMN stage	Treatment	Radiotherapy		Chemotherapy	
					Technique	Dose	Type	Frequency
Conventional exercise group (n=5)								
male	47	NPC	T1N1M0	CCRT	IMRT	6996	Carboplatin	6
male	55	Unknown primary	T0M3M0	CCRT	IMRT	6996	Carboplatin	6
male	56	NPC	T4N2M1	CMT+CCRT	IMRT	6996	Cisplatin	6
male	62	Rt. Base of tongue	T2N0Mx	CCRT	IMRT	6996	Cisplatin	6
female	59	NPC	T3N3M0	CMT+CCRT	IMRT	6996	Cisplatin+ Carboplatin	6
Expiratory muscle strength training (EMST) with conventional exercise group (n=5)								
male	52	NPC	T2N2M0	CMT+CCRT	IMRT	6996	Cisplatin	6
female	46	NPC	T1N2M0	CCRT	IMRT	6996	Carboplatin	6
male	57	Glottis	T1aN2M0	CCRT	IMRT	6996	Cisplatin	6
male	55	Lt. Soft palate	T3N3bM0	CMT+CCRT	IMRT	6996	Cisplatin	6
male	36	Hypopharynx	T2N0Mx	CCRT	IMRT	6996	Carboplatin	6

Note: *NPC: Nasopharyngeal carcinoma, T: tumor, N: nodes, M: metastasis, CCRT: concurrent radiotherapy, CMT: chemotherapy, IMRT: intensity-modulated radiation therapy*

Table 2 sEMG and maximal expiratory pressure (MEP) in both groups before and after 6 weeks of intervention.

Gender	Age (years)	Amplitude (RMSμV)		Duration (seconds)		MEP (cmH_2O)	
		Before	After	Before	After	Before	After
Conventional exercise group (n=5)							
Male	47	0.069	0.064	0.75	1.00	125	114
Male	55	0.060	0.050	0.95	1.06	106	118
Male	56	0.058	0.040	0.92	1.02	114	79
Male	62	0.052	0.034	0.73	0.91	122	107
female	59	0.081	0.064	1.01	1.01	62	46
EMST with conventional exercise group (n=5)							
male	52	0.0611	0.0747	1.06	0.76	150	74
female	46	0.0505	0.0785	1.13	1.05	163	128
male	57	0.0547	0.0671	1.18	1.1	80	82
male	55	0.0459	0.0536	1.18	0.98	96	50
male	36	0.0696	0.084	1.17	1.12	166	127

Table 3 sEMG amplitude and duration of the swallowing maneuver test and MEP between conventional exercise and EMST with conventional exercise group.

Data / Group	Amplitude (RMSμV)		Duration (second)		MEP (cmH_2O)	
	Before	After	Before	After	Before	After
Conventional exercise group (n=5)	0.0605	0.0505*	0.92	1.01*	114	107
	(0.052-0.081)	(0.034-0.064)	(0.73-1.01)	(0.91-1.06)	(62-125)	(46-118)
EMST with conventional exercise group (n=5)	0.0547	0.0747*	1.170#	1.050*	150	82
	(0.045-0.069)	(0.053-0.084)	(1.05-1.17)	(0.76-1.12)	(80-166)	(50-128)

Note: *Data presents the median (min-max), * p<0.05 compared to pre period within the group by using the non-parametric Wilcoxon Signed Ranks Test, # p<0.05 compared between the groups pre period by using the non-parametric Mann-Whitney U test. EMST: Expiratory muscle strength training, RMS: root mean square.*

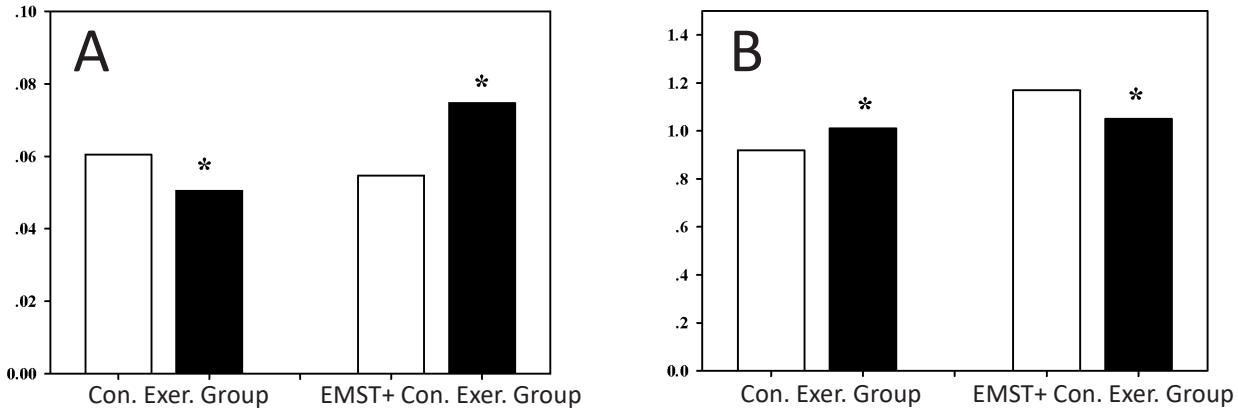

Figure 2. *Median of amplitude (RMSμV, A) and duration (seconds, B) of sEMG signal from the swallowing test before (white-color bar) and after the 6-week period (black bar) in the conventional exercise (Con. Exer. Group) and EMST with conventional exercise group (EMST+Con. Exer. Group) *p<0.05 when compared to pre period within the group by using the non-parametric Wilcoxon-Sign Rank Test.*

The results showed that median MEP value reduced non-significantly in the conventional exercise group from 114 at baseline to 107 cmH_2O after 6 weeks of chemo-radiotherapy. In addition, when EMST and conventional exercise were applied together during chemo-radiotherapy, the MEP still decreased from 150 at baseline to 82 cmH_2O after 6 weeks of training.

Furthermore, five patients who used EMST device for 6 weeks were satisfied. It was claimed to be comfortable and easy to use with no side effects such as dyspnea. Moreover, swallowing or food swallowing after 6 weeks of EMST was easier with less painful sensation when compared to before using the device.

Discussion

This preliminary study evaluated the influence of combined EMST with conventional exercise by developing a device based on previous study, in which its participants had inspiratory muscle strength training with stable COPD.[20] This was interesting because HNC patients had been suffered from the side the side effects of chemo-radiotherapy such as swallowing and coughing defects[5] from oropharyngeal swallowing motility dysfunction or weakness.[6-8]

To evaluate the effect of EMST with a simple device and very cheap, on submental muscles force or MEP among patients with HNC, EMST was designed with 50% and 70% of MEP for 6 weeks as in previous studies.[24-26] Moreover, the target pressure between 50% and 70% of MEP in this study also was consistent with a previous research. During the first week, the expiratory load was designed to 15% of MEP and up to 60% at the end of the first month of training.[31] Therefore, 50-70% of MEP is possibly suitable for training effect.

This preliminary study found increasing amplitude in the combination of EMST and conventional exercise group receiving chemo-radiotherapy for 6 weeks. This result was consistent with a previous study that found higher sEMG amplitude for swallowing in community-dwelling elderly people who performed EMST.16 Therefore, lower amplitude reflects the muscle weakness that presented in the conventional exercise group, whereas sEMG signal amplitude significantly increased in the combination of EMST and conventional exercise group. Therefore, EMST possibly increased the strength of submental muscles.

However, this preliminary study found a significant decrease of MEP in both groups, especially in the conventional exercise group who received chemo-radiotherapy for 6 weeks. The finding was consistent with previous evidence.[3, 6, 7] On the other hand, MEP decreased in the combination of EMST and conventional exercise group who could not maintain value of or increase MEP with EMST approach. This can be explained by a different protocol of training, in which the EMST was divided into two stages: starting with a lower load at 50% of MEP for 3 weeks before increasing to 70% of MEP for 3 weeks. However, a previous study was performed in 12 community-dwelling elderly people with 70% of MEP for 4 weeks.[16] Its results showed a significant increase in strength of the buccinators and orbicularis oris muscles. Interesting data of a previous study on EMST at 75% of MEP for 8 weeks in 23 patients with HNC presented significantly increased MEP indicating higher intensity of resistance and longer period applied in clinical training.[18] Therefore, the results of non-statistical differences of MEP in the combination of EMST and conventional exercise group were possibly from lower resistance of the resistor and shorter training period. In addition, a previous report suggested that impaired laryngopharyngeal function was presented dominantly from prolonged local post-radiotherapy and possibly reduced the expiratory force.[32] Therefore, the MEP values were decreased in both groups.

The result of sEMG duration in this study was in contrast to a previous report that documented a longer duration reflected on muscles weakness or dysphagia and swallowing when compared to healthy subjects.[27, 33] This study demonstraed the benefit of combined EMST with conventional exercise on submental muscles strength by significant higher amplitude and shorten duration of sEMG signal. Moreover, the result of swallowing sensation and pain showed an improvement after completing 6 weeks of

intervention. Thus, it can be suggested that combination of EMST and conventional exercise involves swallowing function by relating to submental muscles strength. The results of sEMG and pain possibly support the benefits of EMST by a previous theory proposing the mechanisms of EMST on peripheral nervous stimulation,[16] and central nerve of the respiratory control center.[34]

Although sEMG improved and possibly improved swallowing, but the swallowing function was not evaluated directly, as in a previous study with the Gugging Swallow Screening (GUSS).[35] Therefore, this study cannot confirm the benefit of EMST with conventional exercise for swallowing function or dysphagia. In future study, other specific exercise such as tongue strengthening or swallowing training program[17] should be added which may be more effective than an EMST program alone.

Limitation of study

The clinical recruitment of specifically diagnosed patients with primary HNC without any diseases or medical problems was very strictly under clinical trial design in this study. In addition, the long period of 6 weeks for data collecting caused some external confounding factors such as disease complication. The low sample size of patients also was a limitation in this study. Thus, the results in this preliminary study is need to be confirmed by future researches.

Conflicts of interests

The authors declare no competing interests in this study.

Acknowledgements

The authors thank the Faculty of Associated Medical Sciences, Chiang Mai University, Thailand for supporting a grant and the sEMG equipment in this study. Moreover, the authors also thanks Associate Professor Samatchai Chamnongkich for structuring the sEMG data, and all patients for their kind cooperation and practicing during this study.

References

[1] Carioli G, Negri E, Kawakita D, Garavello W, La Vecchia C, Malvezzi M. Global trends in nasopharyngeal cancer mortality since 1970 and predictions for 2020: focus on low-risk areas. Int J Cancer 2017; 140(10): 2256-64.

[2] Tangjaturonrasme N, Vatanasapt P, Bychkov A. Epidemiology of head and neck cancer in Thailand. Asia Pac J Clin Oncol 2018; 14(1): 16-22.

[3] Cook IJ. Oropharyngeal dysphagia. Gastroenterol Clin North Am 2009; 38(3): 411-31.

[4] Agarwal J, Dutta D, Palwe V, Gupta T, Laskar SG, Budrukkar A, et al. Prospective subjective evaluation of swallowing function and dietary pattern in head and neck cancers treated with concomitant chemo-radiation. J Cancer Res Ther 2010; 6(1): 15-21.

[5] Lazarus CL. Effects of chemoradiotherapy on voice and swallowing. Curr Opin Otolargyngol Head Neck Surg 2009; 17(3): 172-8.

[6] Stenson KM, MacCracken E, List M, Haraf DJ, Brockstein B, Weichselbaum R, et al. Swallowing function in patients with head and neck cancer prior to treatment. Arch Otolaryngol Head Neck Surg 2000; 126(3): 371-7.

[7] Eisbruch A, Schwartz M, Rasch C, Vineberg K, Damen E, Van As CJ, et al. Dysphagia and aspiration after chemoradiotherapy for head-and-neck cancer: which anatomic structures are affected and can they be spared by IMRT?. Int J Radiat Oncol Biol Phys 2004; 60(5): 1425-39.

[8] Pikus L, Levine MS, Yang YX, Rubesin SE, Katzka DA, Laufer I, et al. Videofluoroscopic studies of swallowing dysfunction and the relative risk of pneumonia. Am J Roentgenol 2003; 180(6): 1613-6.

[9] Ohba S, Yokoyama J, Kojima M, Fujimaki M, Anzai T, Komatsu H, et al. Significant preservation of swallowing function in chemoradiotherapy for advanced head and neck cancer by prophylactic swallowing exercise. Head Neck 2016; 38(4): 517-21.

[10] Saitoh E. Dysphagia rehabilitation. Rinsho Shinkejgaku 2008; 48(11): 875-9.

[11] Langmore SE, Pisegna JM. Efficacy of exercise to rehabilitation dysphagia: a critique of the literature. Int J Speech Lang Pathol 2015; 17(3): 222-9.

[12] Brooks M, McLaughlin E, Shields N. Expiratory muscle strength training improves swallowing and respiratory outcomes in people with dysphagia: a systematic review. Int J Speech Lang Pathol 2019; 21(1): 89-100.

[13] Byeon H. Effect of simultaneous application of postural techniques and expiratory muscle strength training on the enhancement of the swallowing function of patients with dysphagia caused by Parkinson's disease. J Phys Ther Sci 2016; 28(6): 1840-3.

[14] Hegland KW, Davenport PW, Brandimore AE, Singletary FF, Troche MS. Rehabilitation of swallowing and cough functions following stroke: an expiratory muscle strength training trial. Arch Phys Med Rehabil 2016; 97(8): 1345-51.

[15] Silverman EP, Miller S, Zhang Y, Hoffman-Ruddy B, Yeager J, Daly JJ. Effects of expiratory muscle strength training on maximal respiratory pressure and swallowing-related quality of life in individuals with multiple sclerosis. Mult Scler J Expe Transl Clin 2017; 3(2): 2055217317710829. doi: 10.1177/2055217317710829. eCollection Apr-Jun 2017.

[16] Park JS, Oh DH, Chang MY. Effect of expiratory muscle strength training on swallowing-related muscle strength in community-dwelling elderly individuals: a randomized controlled trial. Gerodontology 2017; 34(1): 121-8. doi: 10.1111/ger.12234. Epub 2016 May 16.

[17] Lazarus CL, Husaini H, Falciglia D, DeLacure M, Branski RC, Kraus D, et al. Effects of exercise on swallowing and tongue strength in patients with oral and oropharyngeal cancer treated with primary radiotherapy with or without chemotherapy. Int J Oral Maxillofac Surg 2014; 43(5): 523-30.

[18] Hutcheson KA, Barrow MP, Plowman EK, Lai SY, Fuller CD, Barringer DA, et al. Expiratory muscle strength training for radiation-associated aspiration after head and neck cancer: a case series. Laryngoscope 2018; 128(5): 1044-51.

[19] McConnell AK, Romer LM. Respiratory muscle training in healthy humans: resolving the controversy. Int J Sports Med 2004; 25(4): 284–93.

[20] Leelarungrayub J, Pinkaew D, Puntumetakul R, Klaphajone J. Effects of a simple prototype respiratory muscle trainer on respiratory muscle strength, quality of life and dyspnea, and oxidative stress in COPD patients: a preliminary study. Int J Chron Obstruct Pulmo Dis 2017; 12: 1415-25.

[21] Laveneziana P, Albuquerque A, Aliverti A, Babb T, Barreiro E, Dres M, et al. ERS statement on respiratory muscle testing at rest and during exercise. Eur Respir J 2019; 53(6): 1801214. doi: 10.1183/13993003.01214-2018.

[22] Cnossen IC, van Uden-Kraan CF, Witte BI, Adlders, YJ, de Goede CJT, de Bree R, et al. Prophylactic exercises among head and neck cancer patients during after swallowing sparing intensity modulated radiation: adherence and exercise performance levels of a 12-week guided home-based program. Eur Arch Otorhinolaryngol 2017; 274(2): 1129-38.

[23] Hutcheson KA, Bhayani MK, Beadle BM, Gold KA, Shinn EH, Lai SY, et al. Eat and exercise during radiotherapy or chemoradiotherapy for pharyngeal cancers: use it or lose it. JAMA Otolaryngol Head Neck Surg 2013; 139(11): 1127-34.

[24] Baker S, Davenport P, Sapienza C. Examination of strength training and detraining effects in expiratory muscles. J Speech Lang Hear Res 2005; 48(6): 1325-33.

[25] Pitts T, Bolser D, Rosenbek J, Troche M, Okun MS, Sapienza C. Impact of expiratory muscle strength training on voluntary cough and swallow function in parkinson disease. Chest 2009; 135(5): 1301-8.

[26] Darling-White M, Huber JE. The impact of expiratory muscle strength training on speech breathing in individuals with parkinson's disease: a preliminary study. Am J Speech Lang Pathol 2017; 26(4): 1159-66.

[27] Tseng FF, Tseng SF, Huang YH, Liu CC, Chiang TH. Surface electromyography for diagnosing dysphagia in patients with cerebral palsy. World J Otorhinolaryngol 2013; 3(2): 35-41. https://dx.doi.org/10.5319/wjo.v3.i2.35.

[28] Vaiman M, Eviatar E. Surface electromyography as a screening method for evaluation of dysphagia and odynophagia. Head Face Med 2009; 5: 9. doi: 10.1186/1746-160X-5-9.

[29] Vaiman M. Standardization of surface electromyography utilized to evaluate patients with dysphagia. Head Face Med 2007; 3: 26. doi: 10.1186/1746-160X-3-26.

[30] Lydiatt WM, Patel SG, O'Sullivan B, Brandwein MS, Ridge JA, Migliacci JC, et al. Head and neck cancers-major changes in the American Joint Committee on cancer eighth edition cancer staging manual. CA Cancer J Clin 2017; 67(2): 122-37.

[31] Suzuki S, Sato M, Okubo T. Expiratory muscle training and sensation of respiratory effort during exercise in normal subjects. Thorax 1995; 50(4): 366-70.

[32] Hutcheson KA, Barrow MP, Warneke CL, Wang Y, Eapen G, Lai SY, et al. Cough strength and expiratory force in aspirating and nonaspirating postradiation head and neck cancer survivors. Laryngoscope 2018; 128(7): 1615-21.

[33] Rademaker AW, Pauloski BR, Logemann JA, Shanahan TK. Oropharyngeal swallow efficiency as a representative measure of swallowing function. J Speech Hear Res 1994; 34(2): 314-25.

[34] Wheeler KM, Chiara T, Sapienza CM, Surface electromyographic activity of the submental muscles during swallow and expiratory pressure threshold training tasks. Dysphagia 2007; 22(2): 108-16.

[35] Trapl M, Enderle P, Nowotny M, Teuschl Y, Matz K, Dachenhausen A, et al. Dysphagia bedside screening for acute-stroke patients: the gugging swallowing screen. Stroke 2007; 38(11): 2948-52.

Journal of Associated Medical Sciences 2020; 53 (2): 14-22

JAMS
Journal of Associated Medical Sciences

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

Journal of Associated Medical Sciences

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

Preservative methods for autologous peripheral blood stem cells collected from Thai patients with multiple myeloma

Banphot Shaengkhamnang[1] Pawaret Panyajai[1] Sawitree Chiampanichayakul[1,4] Singkome Tima[1,3,4] Rujirek Chaiwongsa[1,4] Tarika Thumvijit[2,4] Phennapha Klangsinsirikul[1] Adisak Tantiworawit[5*] Songyot Anuchapreeda[1,3,4*]

[1]Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand
[2]Department of Radiologic Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand
[3]Research Center of Pharmaceutical Nanotechnology, Chiang Mai University, Chiang Mai Province, Thailand
[4]Cancer Research Unit of Associated Medical Sciences (AMS-CRU), Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand
[5]Department of Internal Medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai Province, Thailand

ARTICLE INFO

Article history:
Received 6 January 2020
Accepted as revised 23 April 2020
Available online 28 April 2020

Keywords:
Multiple myeloma, autologous peripheral blood stem cell, blood preservation, bone marrow transplantation

ABSTRACT

Background: Bone marrow transplantation in multiple myeloma patients is one of the methods for multiple myeloma therapy. Blood stem cell preservation is very important for transplant therapy. Thus, preservative methods of peripheral blood stem cells (PBSCs) must be evaluated for successful transplantation.

Objectives: The aim of this study was to collect and preserve autologous peripheral PBSCs ($CD34^+/CD38^-$) from multiple myeloma patients at 4°C and deep-freezing.

Materials and methods: PBSCs were collected by leukapheresis before being cryopreserved and kept in liquid nitrogen. The number of $CD34^+/CD45^{dim}$ cells were investigated and subpopulation $CD34^+/CD38^-$ cells were evaluated by trypan blue exclusion method and 7-AAD by flow cytometry before and after cryopreservation.

Results: The result showed that $CD34^+/CD38^-$ cells constituted 45.08% of total $CD34^+$ cells and 0.56% of total nucleated cells (TNCs). After thawing, $CD34^+/CD38^-$ cell number did not show significant differences when compared to pre-storage. The CFU recovery after cryopreservation and storage at 4°C for 7 days were 93.53±5.83 and 63.77±12.40%, respectively. Storage at 4°C for 7 days showed significant decrease when compared to day 1. The remaining of total CFU after deep-freezing and storage at 4°C confirmed the tolerant and robust recovery of $CD34^+/CD38^-$ cells. The engraftments of deep-freezing cells were 100% successful within 11 days without graft failure.

Conclusion: In this present study, we assess the process of storage for high quality and recovery of PBSCs at 4°C within 3 days and cryopreservation for development of autologous hematopoietic stem cell (HSC) transplantation in multiple myeloma patients. Moreover, these conditions are important data guideline for HSC preservations and applications in the future.

* **Corresponding author.**
Author's Address: *Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand*
** **E-mail address:** : songyot.anuch@cmu.ac.th
doi: 10.14456/jams.2020.9
E-ISSN: 2539-6056

Introduction

Multiple myeloma (MM) is a malignancy of bone marrow, caused by defect or dysfunction of the B-cell in various origin of the body. The major characteristics of MM represent the hyperproliferation of plasma cells in bone marrow and found abnormalities of monoclonal protein or M-protein in serum and/or urine may result in end-organ dysfunction and patient died later. The incidence of the disease over the world approximately 4 cases in population of 100,000 persons and a total of 11,300 deaths per year.[1,2] In 2012, multiple myeloma, there are 386 cases in male, accounting for 1 per 100,000 population and 379 cases in female, accounting for 0.8 per 100,000 population and the incidence is between 0.5-2 cases of 100,000 people in Thailand.[2]

Autologous hematopoietic stem cell transplant (autologous-HSCT) has been developed for treatments of multiple myeloma,[3-5] by destroying the abnormal stem cells and replacing with normal patient's hematopoietic stem cells (HSCs). Currently, more advanced treatment regimens have been developed, especially hematopoietic stem cell transplant (HSCT) treatments. Autologous hematopoietic stem cell transplant (autologous-HSCT) and allogeneic hematopoietic stem cell transplant (allogeneic HSCT) are commonly used in routine treatment. Mobilization of autologous-HSCT is performed by chemotherapeutic treatments and/or combined with G-CSF to destroy the cancer or leukemic cells and stimulate HSCs releasing from bone marrow into the bloodstream as peripheral blood stem cells (PBSCs) and collected by leukapheresis. PBSCs were cryopreserved with dimethyl sulfoxide (DMSO) at concentration of 10% v/v is optimal and a common reagent for freezing solution[6] before storage in deep freezing while pretransplant and later thaws before reinfusion to patient for transplantation. PBSCs quality assessment depends on target $CD34^+$ cell concentration, viability, functional, and recovery. The International Society of Hematotherapy and Graft Engineering (ISHAGE) recommend to use HSCs at the concentration of 2-5x10^6 cells/kg body weight (BW) for the successful of HSCT,[7] $CD34^+$ cell enumeration wildly used single-platform ISHAGE protocol measured by flow cytometer to detect $CD34^+/CD45^+$ cells and viability with 7-aminoactinomycin D (7-AAD). The HSC or $CD34^+$ cell measurement by flow cytometry was required the ISHAGE[7] and ISCT & EBMT[8] guidelines for PBSCs, total nucleated cell (TNC) count, $CD34^+$ cell count, cell viability, and % recovery. The measurements of cell viability before and after storages affect $CD34^+$ cell viability.[9,10] Several studies have been found that, the cell viability, ability, and engraftment potential were decreased after cryopreserved PBSC thawing.[11-14] Moreover, it was reported that PBSCs can be stored at 4°C for more than 48 h without significantly decreased in $CD34^+$ cell number and viability.[15-17]

This study aims to assess the preservation methods of PBSCs before and after storage deep freezing cryopreservation and storage at 4°C. The investigation and quantification of $CD34^+/CD45^{dim}$ and subpopulation $CD34^+/CD38^-$ in PBSCs were measured by flow cytometry. Cell viability was performed by trypan blue exclusion method and flow cytometry (7-AAD). Assessed the potency of $CD34^+$ cells by CFU assay in a semi-solid medium to compare the number of total colony-forming unit (CFU) and colony type.[18-20] In this study, we assess the quality of PBSCs and the process of storage methods for the development of autologous-HSCT treatment in multiple myeloma.

Materials and methods

Patients

Seven collections of PBSCs by leukapheresis were obtained from 4 patients (1 male and 3 females) with multiple myeloma who were receiving autologous-HSCT (Table 1). Four multiple myeloma patients (less than 65 years old) who achieved at least very good partial response after treatments was the inclusion criteria. The stem cell mobilization was done with G-CSF alone (10 µg/kg/day) for 5 consecutive days before stem cell collection. All multiple myeloma patients were collected by random sampling during December 2017 to September 2018 at the Maharaj Nakorn Chiang Mai hospital, Chiang Mai province, Thailand. This study was approved by the Ethics Committee of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. The study code was NONE-2559-04138/Research ID: 4138, Date of Approval: September 23, 2016. The optimal stem cell dose was more than 2x10^6 cells/kg BW. The stem cell collection was done in 1-2 days to achieve optimal stem cell dose. PBSCs were assessed pre and post cryopreservation within 1-6 months after storage. Analysis of PBSCs including volume (mL), TNC count (x10^{10} cells), % recovery, $CD34^+$ cell count (x10^6 cells/kg BW), $CD34^+/CD38^-$ cell count (x10^6 cells/kg BW), and % cell viability. The potency of $CD34^+$ cells were measured by CFU assay. Number of each colony type was counted (x10^4 cells) and calculated into percentage (%). Cell number and viability of PBSCs were compared and analysed of each bag before and after storage in two conditions.

Table 1 Characteristics of autologous PBSCs (n=7) from 4 autologous-HSCT patients.

Parameter	Male	Female
Age* (year)	52.00 (29-60)	
Weight* (kg)	56.00 (41–80)	
Sex	1 (25%)	3 (75%)
Total PBSCs (7 bags)	1 (14.29%)	6 (85.71%)

**Data present as median (range).*

PBSCs collection, aliquot, storage at 4°C, deep freezing, and thawing

Autologous PBSCs (7 blood collection bags) from multiple myeloma patients (n=4) were collected by leukapheresis (COMTEC, Fresenius, Waltham, MA), target $CD34^+$ yield in PBSCs was ≥ 2.0x10^6 cells/kg BW of patient. PBSC bags were centrifuged at 2,000xg, 8 min at 22°C (Sorvall RC3, Thermo scientific, CA, USA) to concentrate TNC and reduce plasma approximately 20%. PBSC samples were immediately aliquoted from pre- and post-centrifugation of PBSCs into cryotubes (1 mL/tube) and measured for TNC and TNC recovery (%). Post-centrifuged PBSCs were aliquoted in two vials for pre-storage determination and CFU assay.

Remaining post-centrifuged PBSCs were diluted with cryopreservative solution with the ratio of 1:1 in cryobag. Cryopreservative solution was prepared by mixing DMSO and plasma with the final concentration of DMSO adjusted to 20% and then stored at 4°C for 15 min. The final concentration of DMSO after adding PBSCs was 10%. Cryopreserved PBSCs were aliquoted in two cryotubes (1 mL in each) for post-thawing determination and CFU assay. Then, cryopreserved PBSCs were stored into pre-frozen cassette at -20°C for 15 min and further deep freezing by controlled-rate freezer to -90°C. Finally, all samples were stored in a liquid nitrogen tank (-180°C). Post-thawing samples were thawed at the indicated time period at 37°C in water bath for 2-3 min. Samples were aliquoted into two tubes; the first tube was resuspended with EDTA at the sample to EDTA ratio of 500:50 for a viable TNC count by trypan blue exclusion method and flow cytometry analysis using 7-AAD. The second tube was resuspended with heparin at the sample to heparin ratio of 500:80 for the CFU assay. Moreover, 7 PBSC bags were aliquoted approximately 5 mL into sample bag and stored at 4°C for 7 days. Cold storage sample bags were aliquoted in two cryotubes. The first cryotube was measured for $CD34^+$ and $CD34^+/CD38^-$ cell counts by flow cytometry. TNC and cell viability were determined by trypan blue exclusion method and 7-AAD. The second cryotube was assayed for CFU at day 1, 3, 5, and 7.

TNC, $CD34^+/CD45^{dim}$, and $CD34^+/CD38^-$ cell counts

TNCs were counted using a cell counter (XE-5000, Sysmex, Kobe, Japan). HSC analysis were performed by using a modified ISHAGE protocol[7]. HSCs ($CD34^+/CD38^-$) were determined by stem cell enumeration kit (BD Bioscience, CA, USA). Briefly, samples were diluted to $5x10^5$ cells/100 µL with 0.5% BSA in PBS, pH 7.4. Sample (100 µL) was incubated with CD45-FITC/CD34-PE antibodies for $CD34^+$ cell or HSC identification and anti-CD38 antibody (CD-38-PECy7) for subpopulation $CD34^+/CD38^-$ identification in fluorescent bead tube (TruCount, BD Bioscience, CA, USA) followed by 20 min incubation at room temperature in the dark. After that, 2 mL red blood cell lysis buffer (ammonium chloride, BD Bioscience, USA) was added and followed by incubation for 10 min in the dark and analysis within 1 h. HSCs were enumerated using a single platform analysis on a Cytomics FC500 flow cytometer (Beckman Coulter, IN, USA) to identify target $CD34^+$ cell ($CD34^+/CD45^{dim}$, SS^{low}) and subpopulation of HSCs ($CD34^+/CD38^-$).

$CD34^+$ enumeration was completed by using known a fluorescent bead tube, collecting 75,000 of $CD45^+$ events in each sample. Calculation of $CD34^+$ or $CD34^+/CD38^-$ cells/µL = (number of $CD34^+$ or $CD34^+/CD38^-$ events/number of beads counted)x(total number of beads in each bead-coated tube) x(dilution factor/sample volume).

Cell viability test by trypan blue exclusion method

PBSCs were tested for their viability by trypan blue exclusion method. Samples were diluted with PBS, pH 7.4 with the ratio of 1:10 and then mixed with an equal volume of trypan blue vital stain (0.4%) at room temperature for 5 min. The dead cells were identified by a blue colour of cytoplasm (stained cells as dead cells cannot exclude trypan blue dye). Stained and unstained cells were counted under 10X light microscope (Olympus, USA) and calculated % viability = unstained cells/(unstained cells+stained cells)x100.

Cell viability test by flow cytometry

Samples were stained with 7-aminoactinomycin D (7-AAD) using stem cell enumeration kit (BD Bioscience, CA, USA) and analysed by FC500 flow cytometer (Beckman Coulter, IN, USA). Nucleus was stained with fluorescent dye 7-AAD, and used to detect the signal of viable cells by flow cytometry. This study measured the cell count and cell viability by diluting $5x10^5$ cells/100 µL with 0.5% BSA in PBS, pH 7.4. A BD-Trucount tube contained fluorescent bead that used to calculate the absolute cell count was used. Samples (100 µL) were incubated with 7-AAD (5 µL) in BD-Trucount tube for 20 min at room temperature in the dark. Next, 2 mL of lysing solution (1X ammonium chloride (NH_4Cl) solution, BD Bioscience, USA) was added into the tube, mixed by vortexing and incubated in the dark for 10 min. Samples were not fixed or washed before analysis. Finally, sample tubes were placed in ice bath and immediately analysed within 1 h by flow cytometer (Cytomics FC500, Beckman, USA). The results were analysed by Flowjo analysis software based on ISHAGE guidelines.

CFU assay

Colony-forming unit was used for the indirect assessment of viability as well as potency of PBSCs. The CFU assay represents the clonogenic potential of HSC differentiation to mature cells from viable progenitor cell lineages. Red blood cells in PBSCs were lysed by NH_4Cl solution with the ratio of 1:4 followed by suspended and washed 3 times with 2% FBS in IMDM (Invitrogen™, Carlsbad, CA, USA). A final cell suspension ($1-5x10^4$ cells) was resuspended in methylcellulose medium (HSC003, R&D system, MN, USA) supplemented with cytokine cocktails (50 ng/mL SCF, 10 ng/mL GM-CSF, 10 ng/mL IL3, 3 IU/mL EPO) and plated in 35 mm cell culture dish in triplicate following the manufacturer's instruction. The colony types from progenitors were identified and counted, including CFU-GEMM, CFU-GM, CFU-G, CFU-M, CFU-E, and BFU-E. Total colony count ($x10^4$ cells) and % CFU type were scored.

Statistical analysis

All results were expressed as mean±SD with significant difference level of $p<0.05$ and the differences were analysed by ANOVA and t-test.

Results

Effects of deep-freezing in cryopreservation and storage at 4^0C on $CD34^+$, $CD34^+/CD38^-$ cells, and TNC count by flow cytometry

$CD34^+$ cells were collected from multiple myeloma patients, acquiring an average number of $12.14±19.62x10^6$ cells/kg BW. $CD34^+$ cell population in the TNCs after leukapheresis was 3.03%. $CD34^+$ cell lost after deep-freezing was 3.29%. Pre-storage $CD34^+/CD38^-$ cells were $7.78±13.59x10^6$ cells/kg BW. Thus, $CD34^+/CD38^-$ cell numbers were 62.74% of total $CD34^+$ cells and 1.94% of TNCs. After thawing, $CD34^+/CD38^-$

cells (7.61±13.36x10^6 cells/kg BW) constituting 64.82% of total $CD34^+$ cells and 2.11% of TNCs were not significantly different (p>0.05) when compared to those of pre-storage. After 1-6 months (average 3.57±2.43 months) of cryopreservation, the number of $CD34^+$ and $CD34^+/CD38^-$ cells showed no significant difference when compared to pre-storage (p>0.05) data. However, TNCs showed significant differences after deep freezing (p<0.05) with total cell loss of 9.98% (Table 2).

$CD34^+$ cells after storage at 4°C for 3, 5, and 7 days decreased viable cell numbers by 15.6, 38.12, and 67.09%, respectively when compared to day 1. Moreover, $CD34^+/CD38^-$ cells decreased by 17.16, 30.08, and 58.20%, respectively when compared to day 1 (Table 3). However, there was no significant difference of both $CD34^+$ and $CD34^+/CD38^-$ cell number after storage at 4°C for 3, 5, and 7 days when compared to day 1.

Table 2 $CD34^+$ stem cells, $CD34^+/CD38^-$ cells, and TNC count from pre-storage (post-centrifugation) and post-thawing cryopreserved PBSCs.

Parameter	Pre-storage	Post-thawing	*p* value
$CD34^+$ cells (x10^6 cells/kg)	12.14±19.62	11.74±19.33	>0.05
$CD34^+$ cell loss (x10^6 cells/kg)	0.39±0.51		
$CD34^+/CD38^-$ cells (x10^6 cells/kg)	7.78±13.59	7.61±13.36	>0.05
$CD34^+/CD38^-$ cell loss (x10^6 cells/kg)	0.16±0.22		
TNC count (x10^8 cells/kg)	4.01±1.88	3.61±2.32*	<0.05
TNC count loss (x10^8 cells/kg)	0.62±0.67		
% TNC recovery	85.99±28.59		
Volume of PBSC bag (mL)	173.29±64.96	40.29±11.55*	<0.05

*All data are shown as mean±SD with significant difference *p<0.05 when compared to the pre-storage, n=7.*

Table 3 $CD34^+$ stem cells, $CD34^+/CD38^-$ cells, and TNC count of PBSCs storage at 4°C for 1-7 days.

Parameter	Day			
	1	3	5	7
$CD34^+$ cells (x10^6/kg)	12.14±19.62	11.19±18.05	9.15±14.62	5.53±8.72
$CD34^+$ cells (%)	100	85.40±10.89	61.88±23.39	32.91±18.54
$CD34^+/CD38^-$ cell (x10^6/ kg)	7.78±13.59	7.11±12.25	5.72±9.71	3.80±6.49
$CD34^+/CD38^-$ cell (%)	100	82.84±12.96	69.92±10.10	41.80±19.71
TNC count (x10^8/kg)	4.01±1.88	3.86±1.91	3.68±1.90	3.49±1.88

All data are shown as mean±SD, n=7.

Percentage of cell viability after deep-freezing in cryopreservation and storage at 4°C by trypan blue exclusion method and 7-AAD by flow cytometry

Cell viability after deep-freezing in liquid nitrogen were determined by trypan blue exclusion method and compared to those of 7-AAD by flow cytometer. Pre-storage and post-thawing cell viabilities were also determined and compared. The results showed that viable cells of pre-storage were 96.78±1.45 and 97.96±0.73% by trypan blue and 7-AAD, respectively (Table 4). After thawing, the viable cells were 64.80±6.19 and 70.43±13.48% when determined by trypan blue exclusion method and flow cytometry, respectively (Table 4). The percentage of cell viabilities were significantly decreased by 33.04 and 28.10%, respectively when compared to the pre-storage (p<0.05). Cell viability after storage at 4°C was determined by trypan blue exclusion method and compared to those of 7-AAD for 3, 5, and 7 days. The viable cells decreased by a time-dependent manner when determined by both methods. The percentages of cell viability at day 7 were significantly decreased by 17.71 and 14.26%, respectively by trypan blue exclusion method and flow cytometer, respectively when compared to day 1 (p<0.05). Moreover, the percentage of cell viability at day 5 was significantly decreased by 12.42% by trypan blue exclusion method when compared to day 1 (p<0.05) (Table 5).

Table 4 Percentage of cell viability after deep freezing and determining by trypan blue exclusion method and 7-AAD by flow cytometry.

Cell viability	Pre-storage	Post-thawing	*p* value
Trypan blue exclusion (%)	96.78±1.45	64.80±6.19*	<0.05
Flow cytometry (7-AAD) (%)	97.96±0.73	70.43±13.48*	<0.05

*All data are shown as mean±SD with significant difference,*p<0.05, when compared to the pre-storage, n=7.*

Table 5 Percentage of cell viability by trypan blue exclusion and flow cytometry (7-AAD) of PBSCs storage at 4 ^{O}C for 1-7 days (n=7).

Day	1	3	5	7
Trypan blue exclusion (%)	97.34±0.40	94.70±2.19	87.58±4.55*	79.63±7.44*
Flow cytometry (7-AAD) (%)	98.37±0.73	96.36±2.10	92.36±3.12	84.11±7.61*

*All data are shown as Mean±SD with significant p<0.05. *Result was significant difference when compared to day 1.*

Effects of deep-freezing cryopreserved and storage at 4°C on colony forming unit (CFU) of progenitor and committed cell growths

We next evaluated whether deep-freezing cryopreserved $CD34^+$ cells in liquid nitrogen had the ability to grow in the methylcellulose cultures with cytokine cocktails. $CD34^+$ cells gave rise to various types of myeloid colonies including CFU-granulocyte/erythrocyte/monocyte/megakaryocyte (CFU-GEMM), CFU-granulocyte/monocyte (CFU-GM), CFU-granulocyte (CFU-G), CFU-monocyte (CFU-M), burst-forming unit erythroid (BFU-E), and CFU-erythroid (CFU-E). BFU-E showed the highest colony counts in both pre-storage and post-thawing, followed by CFU-G and CFU-GM, respectively. Colony counts of post-thawing showed no significant difference when compared to the pre-storage as shown in Table 6.

After PBSCs were kept at 4°C for 3, 5, and 7 days. The CFUs were determined and identified all colonies. Total colony number after storage for 3, 5, and 7 days decreased by 3.90, 10.39, and 27.27%, respectively when compared to the day 1. CFU recovery (%) at day 5 was also significantly decreased that indicated in Table 7. However, CFU-GEMM, CFU-GM, CFU-G, CFU-M, BFU-E, CFU-E counts of storages in day 3, 5, and 7 were not significant difference when compared to the day 1 as shown in Table 7.

Table 6 Colony forming unit (CFU) counts of progenitor and committed cell growths before deep-freezing and after thawing from cryopreservation.

CFU	Pre-storage (%)	Post-thawing (%)	*p* value
CFU-GEMM	1.14±0.72	1.10±0.59	>0.05
CFU-GM	10.75±5.11	4.81±5.66	>0.05
CFU-G	26.48±15.89	26.59±25.40	>0.05
CFU-M	1.00±0.75	0.95±0.73	>0.05
CFU-E	0.48±0.38	0.57±0.69	>0.05
BFU-E	38.76±17.44	46.48±6.41	>0.05
Total (colonies/1.0x10^6 cells)	153.45±208.57	139.83±189.96	0.005
% CFU recovery	93.53±5.83		

All data are shown as mean±SD, n=7.

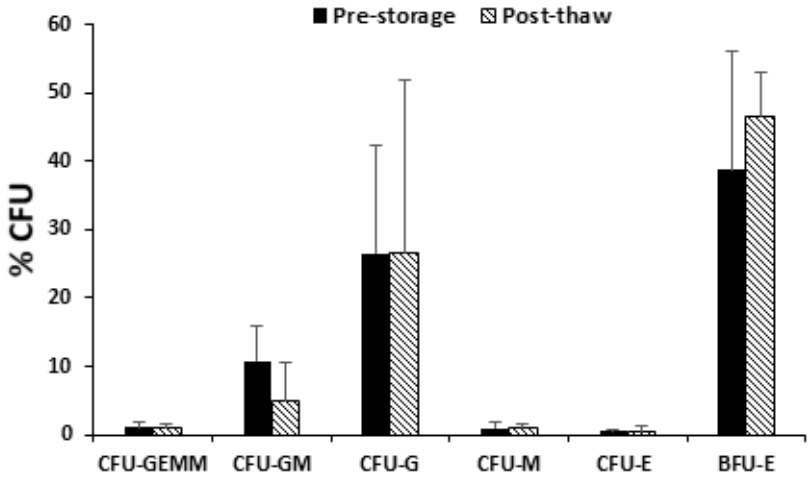

Figure 1. *Colony forming unit (CFU) counts of progenitor and committed cell growths before deep-freezing and after thawing from cryopreservation.*

Table 7 CFU counts and identification types of PBSCs storage at 4^{O}C for 7 days.

Colony forming unit	Day			
	1	3	5	7
CFU-GEMM (%)	1.23±0.99	1.24±0.52	1.15±0.44	0.76±0.69
CFU-GM (%)	9.27±4.74	10.95±3.13	9.57±6.93	8.69±7.17
CFU-G (%)	58.67±14.89	52.01±0.17	53.27±11.21	52.19.11±9.71
CFU-M (%)	0.76±0.73	0.75±0.72	1.00±0.76	0.52±0.50
CFU-E (%)	0.61±0.90	0.19±0.50	0.27±0.49	0.32±0.61
BFU-E (%)	44.27±7.80	40.44±7.11	41.07±4.29	44.09±6.26
Total (colonies/1x10^{6} cells)	1.54±2.07	1.48±2.00	138.15±192.32	112.43±161.59
%CFU recovery	100.00	93.93±3.14	83.39±7.24*	63.77±12.40*

*All data are shown as mean±SD with significant different *p<0.05 when compared to the pre-storage at day 1, n=7.*

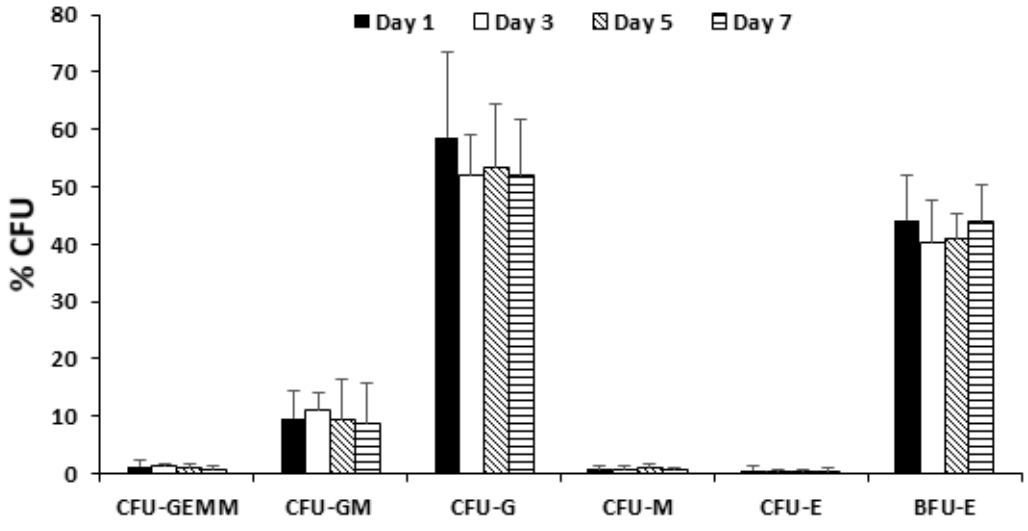

Figure 2. *CFU counts and identification types of PBSCs storage at 4^{O}C for 7 days.*

Evaluation of storage PBSCs by deep-freezing on clinical transplantation and engraftment in multiple myeloma patients

This study, storage PBSCs by deep freezing were transplanted to multiple myeloma patients. A day after stem cell collection, patients underwent autologous-HSCT with melphalan 200 mg/m^2 in 2 consecutive days. On the next day, non-cryopreserved stem cell was transfused. Thus, PBSCs were stored for 3 days after collection. To evaluate the engraftment, HSCs from 7 samples were transplanted to the multiple myeloma patients. All bags from leukapheresis procedures were infused autologously in each own patient (100%). The total CD34$^+$ cells were 12.14±19.62x10^6 cells/kg BW infused (CD34$^+$/CD38$^-$ cell number was ~11.74x10^6 cells/kg BW). After infusion, absolute neutrophil count (ANC) and platelets were engrafted within 11.25±0.96 and 11.50±1.00 days (Table 8). ANC was more than 0.5x10^9 cells/L and platelet counts were more than 20x10^9/L in 3 consecutive days without transfusion support. There were no graft failures in any cases.

Table 8 Clinical transplantation and engraftment in multiple myeloma patients after deep-freezing.

Leukapheresis procedures (Bag)	Total CD34$^+$ cells (x10^6/kg infused)	PBSC infused (Bag)	Engraftment	
			ANC (Day)	Platelet (Day)
1.75±0.96	12.14±19.62	2.00±1.15	11.25±0.96	11.50±1.00

All data are shown as mean±SD, n=7.

Discussion

Storage HSCs is very important for bone marrow transplantation. HSC preservation method are based on reasonableness of hospitals and doctors. Thus, both deep-freezing and storage at 4^{O}C have been used for bone marrow transplantation of multiple myeloma. Deep-freezing cryopreservation is the conventional method for hematopoietic stem cell (HSC) storage; it is a critical part of hematopoietic stem cell transplantation. Leukapheresis has been used to collect the peripheral blood stem cells (PBSCs) from multiple myeloma patients and transplant to those patients who have been recommended to replace their bone marrow with their own HSCs (autologous-HSCT). Cellular therapy products such as PBSCs may need to be cryopreserved until their use. There are two major methods of cell cryopreservation: deep-freezing and storage in liquid nitrogen at < -180^{O}C and freezing at -80^{O}C. Deep-freezing method is recommended for long term storage for years while freezing at -80^{O}C lacks evidence for its safety. However, freezing at -80^{O}C was reported to be used for storage in a region with limited resources.[21] The recovery of viable CD34$^+$ cell populations after deep-freezing was 91%. This result shows the same pattern of previous reports where the recovery of nucleated cells and viable CD34$^+$ cells for adult (n=51) stem cell collections were 92 and 91%, respectively.[22,23] CD34$^+$ cells were commonly reported on cell viability. However, CD34$^+$ is a common marker of both HSCs and progenitors in bone marrow. There is no report that the cell viability and engraftment of CD34$^+$/CD38$^-$ cells after deep-freezing and thawing. In this study, subpopulation of CD34$^+$/CD38$^-$ cells (HSCs) was focused and observed for total cell number of HSCs in PBSCs before and after deep-freezing cryopreservation by flow cytometry. Thus, this is a first report to specify the viability of CD34$^+$/CD38$^-$ cells after preservation. CD34$^+$/CD38$^-$ cell number after collection was 12.14±19.62x10^6 cells/kg BW, 3.03% of total CD34$^+$ cells, and 1.94% of TNCs. After thawing CD34$^+$/CD38$^-$ cell number did not show significant difference when compared to that of pre-storage (p>0.05). CD34$^+$/CD38$^-$ cell loss was 2.18%. Storage at 4^{O}C is a routine method used for multiple myeloma patients at the Maharaj Nakorn Chiang Mai hospital, Chiang Mai province, Thailand. CD34$^+$/CD38$^-$ cells after storage at 4^{O}C for 3, 5, and 7 days were decreased by a time-dependent manner. At day 7 showed significant decrease percentage of cell viabilities in both trypan blue exclusion method and 7-AAD assay. These results related to percentage of CFU recovery (Table 7). When CFU types were observed, there were no significant different between day 3, 5, and 7 as compared to day 1. The viability of collected PBSCs storage at 4^{O}C was significantly difference from day 1 (data from Table 5) therefore it might be suitable for transplant up to 3 days with cell viability of 94.70±2.19% by trypan blue exclusion method. If cell viability was determined by 7-AAD assay, the values would show the cell viability higher than that of trypan blue exclusion method at the same day. These two methods are popular for cell viability determination. Trypan blue exclusion method is the conventional method to show a loss of cell permeability that presents unsuitable cells for further application in patients. Cell viability of CD34$^+$ cells after 7-AAD assay was previously reported with values ranging from 58.50-99.48%.[24,25] However, the results after transplantations showed the good engraftment of HSCT. PBSCs were reported to store at 4^{O}C for more than 48 h without significantly decreased in CD34$^+$ cell number and viability.[15-17] Moreover, cell viability after deep freezing which (performed by trypan blue exclusion and flow cytometry) showed significant difference when compared to the pre-storage (p<0.05) by the values of 33.04 and 28.10%, respectively. This result suggested that other nucleated cells (not including the CD34$^+$ or CD34$^+$/CD38$^-$ cells) were dead after thawing. This phenomenon was observed in TNCs after thawing that were significantly decreased (p<0.05) by 9.98% when compared to the pre-storage. CD34$^+$/CD38$^-$ cells and CD34$^+$ (HSCs and progenitor cells) cells seem to be well adaptable compared to other nucleated cells.[26] CD34$^+$ cells tolerate up to 60 min exposure to 25% w/w (3.2 M) DMSO at +2^{O}C with no significant loss in clonogenic capacity.[6] Different cell types tolerated various ranges of solution osmolarities that effect by osmotic shocks, which

was evidenced by an increased necrosis in neutrophils and apoptosis in monocytes. Mature myeloid cells are more sensitive to osmotic stress than lymphocytes and $CD34^+$ cells. Moreover, $CD34^+/CD38^-$ cells appeared more resistant to cryoinjuries than their $CD34^+/CD38^-$ counterpart.[26,27] HSCs from 40 bone marrow and peripheral blood samples were previously reported to be successfully preserved for long-term cryostorage (5-14 years) using a standard in vitro method. Forty percent of harvests had $CD34^+$ cell counts of at least $0.7x10^6$/kg BW and 85% had CFU-GM counts of at least $1.0x10^5$/kg BW.[28] The potency of $CD34^+$ cells was assessed by CFU assay in a semi-solid medium to compare the number of CFUs and colony types.[19,20,29] The result completely showed that all CFU counts of deep freezing after thawing did not significantly differ from cell growth when compared to pre-storage (p>0.05). The percentage of CFU recovery before storage was 93.53±5.83. In this study, we assessed the quality and recovery of PBSCs as a function of storage process for the enhancement of autologous-HSCT treatment in multiple myeloma. The remaining of total CFU after deep-freezing and storage at 4^oC confirmed the tolerant and robust recovery of $CD34^+/CD38^-$ cells. The engraftments of 4^oC and deep-freezing cells were 100% successful within 11 days without graft failure. Absolute neutrophil count (ANC) should present more than $0.5x10^9$ cells/L[30] while platelet count should be more than $20x10^9$ cells/L in 3 consecutive days without transfusion support.[31]

Conclusion

In conclusion, deep freezing for 1-6 months and storage at 4^oC within 3 days are recommended methods for PBSC preservation. These methods provide cell viability and function for bone marrow transplantation with high successful probability in multiple myeloma patients.

Conflicts of interests

There are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgements

This work was supported by the research fund from the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand and the Thailand Research Fund (TRF) under grant number RSA6280034. We thank the Research Center of Pharmaceutical Nanotechnology, Chiang Mai University, Chiang Mai, Thailand, for instrumental support. We gratefully acknowledge Mr. Danial Griffin (Department of Pharmaceutical Chemistry, School of Pharmacy, The University of Kansas, Kansas, US) for his critical reading of the manuscript for English usage.

References

[1] Cancer incidence in five continents. In: Forman D, Bray F, Brewster DH, Gombe Mbalawa C, Kohler B, Piñeros M, et al., editors. Cancer incidence in five continents. X. Lyon, France: International Agency for Research on Cancer; 2013. p. 23-36.

[2] The International Agency for Research on Cancer. Cancer by organ site. In: Bernard W. Stewart, Wild CP, editors. World Cancer Report 2014. Lyon, France: The International Agency for Research on Cancer; 2014. p. 482-94.

[3] Attal M, Harousseau JL, Stoppa AM, Sotto JJ, Fuzibet JG, Rossi JF, et al. A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. N Engl J Med 1996; 335(2): 91-7.

[4] Blade J, Rosinol L, Sureda A, Ribera JM, Diaz-Mediavilla J, Garcia-Larana J, et al. High-dose therapy intensification compared with continued standard chemotherapy in multiple myeloma patients responding to the initial chemotherapy: long-term results from a prospective randomized trial from the Spanish cooperative group PETHEMA. Blood 2005; 106(12): 3755-9.

[5] Kumar L, Ghosh J, Ganessan P, Gupta A, Hariprasad R, Kochupillai V. High-dose chemotherapy with autologous stem cell transplantation for multiple myeloma: what predicts the outcome? Experience from a developing country. Bone Marrow Transplant 2009; 43(6): 481-9.

[6] Hunt CJ, Armitage SE, Pegg DE. Cryopreservation of umbilical cord blood: 2. Tolerance of $CD34^+$ cells to multimolar dimethyl sulphoxide and the effect of cooling rate on recovery after freezing and thawing. Cryobiology 2003; 46(1): 76-87.

[7] Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for $CD34^+$ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J Hematother 1996; 5(3): 213-26.

[8] Joint Accreditation Committee - ISCT & EBMT. International standards for cellular therapy product collection, processing and administration 2015. Available from: http://www.ebmt.org.

[9] Donmez A, Yilmaz F, Soyer N, Cagirgan S, Arik B, Tombuloglu M. The loss of $CD34^+$ cells in peripheral hematopoietic stem cell products cryopreserved by non-controlled rate freezing and stored at –80°C after overnight storage. Transfus Apher Sci 2014; 51(2): 188-92.

[10] Fisher V, Khuu H, David-Ocampo V, Byrne K, Pavletic S, Bishop M, et al. Analysis of the recovery of cryopreserved and thawed $CD34^+$ and $CD3^+$ cells collected for hematopoietic transplantation. Transfusion 2014; 54(4): 1088-92.

[11] Valeri CR, Pivacek LE. Effects of the temperature, the duration of frozen storage, and the freezing container on in vitro measurements in human peripheral blood mononuclear cells. Transfusion 1996; 36(4): 303-8.

[12] Liseth K, Abrahamsen JF, Bjorsvik S, Grottebo K, Bruserud O. The viability of cryopreserved PBPC depends on the DMSO concentration and the concentration of nucleated cells in the graft. Cytotherapy 2005; 7(4): 328-33.

[13] Castelhano MV, Reis-Alves SC, Vigorito AC, Rocha FF, Pereira-Cunha FG, De Souza CA, et al. Quantifying loss of $CD34^+$ cells collected by apheresis after processing for freezing and post-thaw. Transfus Apher Sci 2013; 48(2): 241-6.

[14] Berens C, Heine A, Muller J, Held SA, Mayer K, Brossart P, et al. Variable resistance to freezing and thawing of CD34-positive stem cells and lymphocyte subpopulations in leukapheresis products. Cytotherapy 2016; 18(10): 1325-31.

[15] Antonenas V, Garvin F, Webb M, Sartor M, Bradstock KF, Gottlieb D. Fresh PBSC harvests, but not BM, show temperature-related loss of CD34 viability during storage and transport. Cytotherapy 2006; 8(2): 158-65.

[16] Jansen J, Nolan PL, Reeves MI, Akard LP, Thompson JM, Dugan MJ, et al. Transportation of peripheral blood progenitor cell products: effects of time, temperature and cell concentration. Cytotherapy 2009; 11(1): 79-85.

[17] Kao GS, Kim HT, Daley H, Ritz J, Burger SR, Kelley L, et al. Validation of short-term handling and storage conditions for marrow and peripheral blood stem cell products. Transfusion 2011; 51(1): 137-47.

[18] Suzuki T, Muroi K, Tomizuka H, Amemiya Y, Miura Y. Characterization of enriched $CD34^+$ cells from healthy volunteers and those from patients treated with chemotherapy plus granulocyte colony-stimulating factor (G-CSF). Stem Cells 1995; 13(3): 273-80.

[19] Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 2000; 404(6774): 193-7.

[20] Morgenstern DA, Ahsan G, Brocklesby M, Ings S, Balsa C, Veys P, et al. Post-thaw viability of cryopreserved peripheral blood stem cells (PBSC) does not guarantee functional activity: important implications for quality assurance of stem cell transplant programmes. Br J Haematol 2016; 174(6): 942-51.

[21] Shima T, Iwasaki H, Yamauchi T, Kadowaki M, Kiyosuke M, Mochimaru T, et al. Preserved in vivo reconstitution ability of PBSCs cryopreserved for a decade at -80 °C. Bone Marrow Transplant 2015; 50(9): 1195-200.

[22] Sartor M, Antonenas V, Garvin F, Webb M, Bradstock K. Recovery of viable $CD34^+$ cells from cryopreserved hemopoietic progenitor cell products. Bone Marrow Transplant 2005; 36(3): 199-204.

[23] Makino S, Harada M, Akashi K, Taniguchi S, Shibuya T, Inaba S, et al. A simplified method for cryopreservation of peripheral blood stem cells at -80 degrees C without rate-controlled freezing. Bone Marrow Transplant 1991; 8(4): 239-44.

[24] Scerpa MC, Daniele N, Landi F, Caniglia M, Cometa AM, Ciammetti C, et al. Automated washing of human progenitor cells: evaluation of apoptosis and cell necrosis. Transfus Med 2011; 21(6): 402-7.

[25] Reich-Slotky R, Colovai AI, Semidei-Pomales M, Patel N, Cairo M, Jhang J, et al. Determining post-thaw $CD34^+$ cell dose of cryopreserved haematopoietic progenitor cells demonstrates high recovery and confirms their integrity. Vox Sang 2008; 94(4): 351-7.

[26] Pasha R, Elmoazzen H, Pineault N. Development and testing of a stepwise thaw and dilute protocol for cryopreserved umbilical cord blood units. Transfusion 2017; 57(7): 1744-54.

[27] Woods EJ, Perry BC, Hockema JJ, Larson L, Zhou D, Goebel WS. Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use. Cryobiology 2009; 59(2): 150-7.

[28] Spurr EE, Wiggins NE, Marsden KA, Lowenthal RM, Ragg SJ. Cryopreserved human haematopoietic stem cells retain engraftment potential after extended (5–14 years) cryostorage. Cryobiology 2002; 44(3): 210-7.

[29] Ikebuchi K. [Hemopoietic colony formation in semisolid and liquid culture system]. Rinsho Byori 1995; Suppl 99: 123-35.

[30] Wolff S. Second hematopoietic stem cell transplantation for the treatment of graft failure, graft rejection or relapse after allogeneic transplantation. Bone Marrow Transplant 2002; 29(7): 545-52.

[31] Teltschik HM, Heinzelmann F, Gruhn B, Feuchtinger T, Schlegel P, Schumm M, et al. Treatment of graft failure with TNI-based reconditioning and haploidentical stem cells in paediatric patients. Br J Haematol 2016; 175(1): 115-22.

Journal of Associated Medical Sciences 2020; 53 (2): 23-30

JAMS
Journal of Associated Medical Sciences

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

Journal of Associated Medical Sciences

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

Correlation between thoracic kyphosis and pulmonary function in elderly

Chanya Ueawattanasirikul Sirinun Boripuntakul Khanittha Wonglangka Decha Pinkaew*

Department of Physical Therapy, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand

ARTICLE INFO

Article history:
Received 16 January 2020
Accepted as revised 30 April 2020
Available online 30 April 2020

Keywords:
Thoracic kyphosis, pulmonary function, respiratory muscle strength, elderly

ABSTRACT

Background: Thoracic kyphosis is almost found in the elderly and reduces chest expansion which results in decreasing pulmonary function including lung function and respiratory muscle strength, then, it leads to dyspnea and exercises intolerance in the elderly.

Objectives: To evaluate thoracic kyphosis measured by flexicurve method in relation to pulmonary function in elderly.

Materials and methods: Sixty-six participants aged 65 years and older were recruited for the study. All participants received basic health screening and underwent thoracic kyphosis measurements using flexicurve method. All subjects went through the lung function tests by using spirometer and respiratory muscle strength measurements by using maximal voluntary respiratory pressures. Pearson's correlation coefficient was used to analyze the relationships between variables.

Results: Thoracic kyphosis was mild positive correlated with lung functions in Forced Vital Capacity (FVC), Forced Expiratory Volume in 1 second (FEV_1) and Vital Capacity (VC) parameter (r=0.265, 0.303 and 0.322, respectively, $p<0.05$) but not with FEV_1/FVC ratio (%) ($p>0.05$). There was no correlation between thoracic kyphosis and respiratory muscle strength (maximal inspiratory pressure (MIP) and maximal expiratory pressure (MEP)) ($p>0.05$).

Conclusion: Thoracic kyphosis measured by the flexicurve method had a mild positive correlation with the lung functions.

Introduction

Elderly population is taking place throughout the world.[1] The proportion of the elderly population in Thailand aged 65 years and older has been increasing with the rising estimation from 6.8% in 1994 and 16.7% of the population in 2017.[2] Therefore, rapidly change in the proportion of a population that is elderly result in healthcare professional awareness and preparation to deal with elderly changes in physical and mental functions, as for a good quality of life.[1] Physiological changes occur with elderly in all organ systems contribute to functional limitation that can predict mortality.[3] Changes in the respiratory system include internal and external structures such as thoracic wall, spine, and respiratory muscles lead to breathing control problem. Besides, musculoskeletal system changes in elderly for instance collapse wedging of vertebrae resultant in thoracic kyphosis with rib cage deteriorated.These changes in musculoskeletal system reflect on breathing control problem thus lead to respiratory infection[4] and increase vertebral and extremity fracture.[5, 6] Skloot et al.[7] suggested lung function testing is benefit for the elderly because it is a noninvasive technique used for severity assessment and prognosis in lung disease including restrictive, obstructive, or mixed abnormalities. Spirometry is the most popular in lung function testing because it is easy, quick, benefit, and high reliability.[8]

Shin et al.[9] reported that respiratory muscle strength including MIP and MEP variables related to SMI (skeletal

* **Corresponding author.**
Author's Address: *Department of Physical Therapy, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand*
** **E-mail address:** : decha303@gmail.com
doi: 10.14456/jams.2020.10
E-ISSN: 2539-6056

muscle mass index) in the elderly. This study expects that inspiratory and expiratory muscle strength may be related to thoracic kyphosis, which respiratory muscle strength are determined by using the maximal voluntary respiratory pressures. It is low cost, portable equipment, easy and rapid to perform, noninvasive as well. The maximal voluntary respiratory pressures has been proved to be a measurement determining the MIP and MEP.[10, 11]

Thoracic kyphosis affects 20-40% of elderly and can be considered as a kyphosis angle which is exaggerated anterior curvature of the thoracic spine more than 40 degrees.[5, 6, 12, 13] Furthermore, female tends to be more thoracic kyphosis than male, due to losses of estrogen in menopausal and postmenopausal period.[6] In addition, age is also an important factor that relate to thoracic kyphosis. The kyphosis angle varies in between 20 to 29 degrees in aged under 40 years,53 degrees in aged between 60-74 years and 66 degrees in aged above 75 years of age.[5] As a result, thoracic kyphosis can cause restrictive and obstructive ventilation. The biomechanics of kyphosis which increases the external moment, inducing trunk curvature and chest compression lead to limitations in rib cage expansion resulting in a restricted amount of oxygen taken in inspiration and expiration, additional leading to dyspnea and exercise intolerance; and consequently, causing a decrease in activity of daily living and an increase in mortality.[5, 6] Cobb angle measurement of spine from X-Ray images is a gold standard to measure thoracic kyphosis, however, it has disadvantages including cost, time-spent to interpret, and a requirement of specialist and invasive technique to measure.[14] In contrast, the non-invasive technique which is similar to gold standard (r=0.98)[15] is flexicurve method. It has a number of advantages including with low cost, easy to get, quickly to interpret, high validity and reliability (ICC>0.9).[16] Few studies have investigated the association between thoracic kyphosis and pulmonary function in elderly,[17-19] however, there are confounding factors which have an effect on research result such as history of smoking and gender. Lorbergs et al.[17] reported that elderly woman with thoracic kyphosis tended to decrease pulmonary function more than those with erect posture.It was found that FEV_1 decreased by 100 ml or 6.3 ml/years. However, results of research that has developed control confounding factor consist of unaware of history of smoking which is quit smoking at least 1 year might result in lung recovery fully[20] and thoracic kyphosis measurements used in previous study were invasive technique which is hard to measure in community.[17, 18] Moreover, at present there is no evidence measuring thoracic kyphosis and correlation with pulmonary function in elderly.

This study aimed to evaluate thoracic kyphosis measured by flexicurve method in relation to pulmonary function in elderly.

Materials and methods

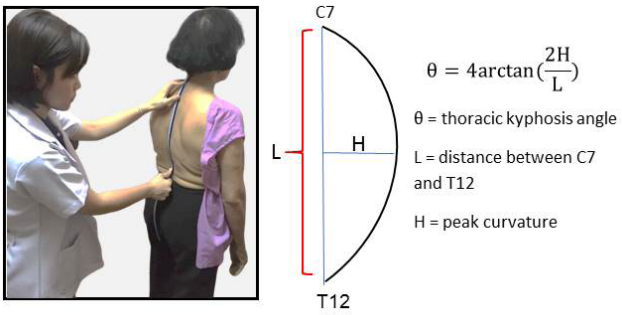

Figure 1. *Thoracic kyphosis measurement by using flexicurve method and calculated by using the equation of thoracic kyphosis angle.*

Participants

This study included 66 elderly, aged 65 years and older, living in Chiang Mai, Thailand. Flow chart of sources of study participants shows in Figure 2. All subjects underwent thoracic kyphosis measurements using flexicurve method and cognitive impairment measurements using the Montreal Cognitive Assessment-Thai version (MoCA-Thai). They were all non-smoker or quit smoking for 1 year and longer,[20] who walked with or without walking aids, understand command and consent to participate in the research, had thoracic kyphosis angle more than 40 degrees[5, 6, 12, 13] with no cognitive impairment (MoCA score≥23 points).[21] Subjects who had contraindications for spirometry measurements such as hemoptysis, [8, 22, 23] contraindications for respiratory muscle strength measurements such as aneurysm in thoracic, abdominal or brain,[24] had musculoskeletal complications involving thoracic kyphosis measurements such as leg length discrepancy (more than 3 cm)[25] or leg deformity which involve standing or pain on musculoskeletal system more than 5 points (visual analog scale 0-10) or severe scoliosis,[26] had neurological disorder which involve measurements such as cerebrovascular accident (CVA) or Parkinson's disease, had uncontrolled underlying disease such as hypertension, heart disease or asthma, had diagnosed spinal fracture by physician and take medication which involve measurements such as opioid or antidepressant[27] were excluded from the study. G-Power version 3.1 estimated a required sample size of 66 participants (effect size=0.32, α=0.05, and power=0.8).[18]

Thoracic Kyphosis

Thoracic kyphosis was measured using flexicurve method. The subject was instructed to be in standing position with comfortable and felt natural. A single trained reader used flexicurve ruler. The superior end of the ruler was placed at C7 and the inferior end of the ruler at T12. The ruler was then placed flat on 10x10 grid paper and then ruler position drawn from C7 to T12. The flexicurve was printed to the spine 3 times, being flattened between each measurement. The thoracic kyphosis angle was later calculated using the formula[12] displayed in Figure 1. The average of the 3 measurements of each subject was used for analysis.[28] If the calculated angle was greater than 40 degrees, the participant was considered to be thoracic kyphosis and included in this research. The intra-class

correlation coefficient (ICC) estimating as 0.984 (95% confidence interval: 0.939-0.996) for 10 participants, chosen at random and measured in duplicate.

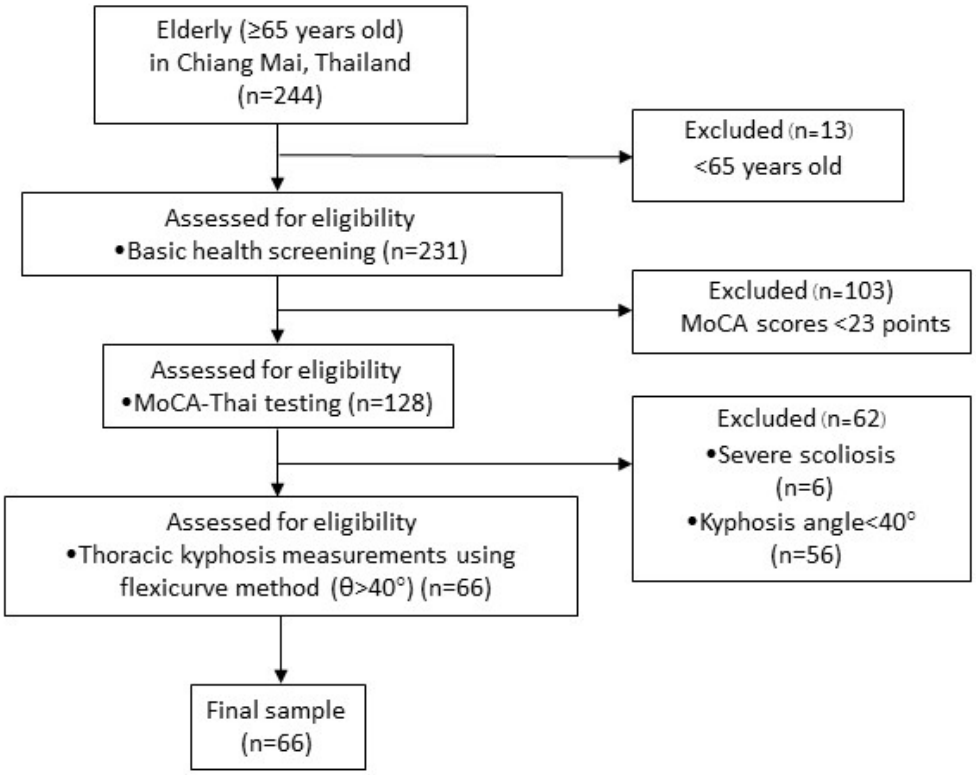

Figure 2. *A flow chart of the participants in the study.*

Lung function tests

Lung functions including forced vital capacity (FVC), forced expired volume in one second (FEV_1), FEV_1/FVC ratio and vital capacity (VC) were measured by CHESTGRAPH HI-105 spirometer (Chest MI, Inc, Tokyo, Japan) according to the American Thoracic Society (ATS) criteria.[29] Measurements were made using a closed-circuit spirometer with the subjects seated, feet resting on the floor, spine erect and wearing a nose clip. Subjects were asked to perform a maximum of five forced expiratory maneuvers for measuring FVC, FEV_1 and FEV_1/FVC ratio and were asked to perform a maximum of slow expiratory maneuvers to measuring VC to obtain three acceptable spirogram. Dejsomritrutai equations were used to determine the normal values based on age, sex, height and race.[8, 22, 23]

Respiratory muscle strength

Respiratory muscle strength was assessed by using the MicroRPM portable manometer's measurements of maximum inspiratory pressure (MIP) and maximum expiratory pressure (MEP)[30] (MicroRPM, Carefusion, Kent, and United Kingdom). Subjects were seated, feet resting on the floor, spine erect, wearing a nose clip and then, who were asked to perform a maximal expiratory effort after taking a maximal inspiration to measure a maximal expiratory pressure (MEP) and were instructed to ensure maximal inspiratory effort after exhaling a maximal expiration to measure maximal inspiratory pressure (MIP). Each participant was allowed to hold in 1.5 seconds per time, rest for about one minute and then repeat the maneuver 3 times. The highest value for MIP and MEP were chosen.[10, 11]

The thoracic kyphosis, lung function, and respiratory muscle strength measurements were examined by individual examiners. Besides, lung function and respiratory muscle strength measurements examiners were blinded to clinical status and kyphosis angle variables.

Statistical analysis

Descriptive analyses were presented as a mean±standard deviation, according to the results of the Kolmogorov-Smirnov test which was used to test the assumption of normality. The kyphosis angle, lung function and respiratory muscle strength data were normally distribution, thus all parameters were analyzed by using Pearson's correlation coefficient. The Correlation coefficient values were interpreted as follows: 0.00-0.10 negligible, 0.10-0.39 mild, 0.40-0.69 moderate, 0.70-0.89 strong and 0.90-1.00 very strong. The level of statistical significance was considered as $p<0.05$.[31]

Results

Participant's demographics

Demographic characteristic of participants is shown in Table 1. Among the 66 elderly participants, 15 (22.7%) of them were males and 51 (77.3%) were females. The mean age was 71.09±5.55 years. The mean BMI was 24.66±3.61 kg/m^2, and the mean kyphosis angle was 50.14±9.19 degrees. All participants had no history of smoking, did not work, and had underlying disease with medical supervision including hypertension, dyslipidemia, diabetes mellitus type 2, gout, and knee osteoarthritis. The percentage of participants in the three ranges of kyphosis angle were 57.58% for the 40-50 degrees, 33.33% for the 50-60 degrees, and 9.09% for the 60-100 degrees.

Table 1 Demographic characteristic of participants (n=66).

Variables	Min-Max	Mean±SD
Gender, (n%)		77.30
Male (n)		15 (22.7%)
Female (n)		51 (77.3%)
Age (years)	68-86	71.09±5.55
Height (cm)	143-172	153.83±5.60
Weight (kg)	39-75	57.62±7.87
BMI (kg/m^2)	17.3-35.7	24.66±3.61
Kyphosis angle (°)	40.15-93.04	50.14±9. 19
MoCA (score)	23-30	25.86±2.25

***Abbreviation**: BMI, body mass index.*

Correlations between the thoracic kyphosis angle and lung function

The correlation results are provided in Table 2 and Figure 3. There were mild positive correlations between the thoracic kyphosis angle and FVC, FEV_1 and VC (r=0.265, 0.303 and 0.322, respectively, p<0.05) (Figure 3A, 3B and 3D). There was no correlation between thoracic kyphosis angle and FEV_1/FVC ratio (%) (p>0.05).

Table 2 Correlations between the thoracic kyphosis angle and lung function (FVC, FEV_1, FEV_1/FVC ratio and VC).

Variables	Min-Max	Mean±SD	r	95% CI	*p* value
FVC (L)	0.43-3.28	1.72±0.58	0.265	0.009-0.434	0.032*
FEV_1 (L)	0.33-3.26	1.45±0.49	0.303	0.110-0.458	0.013*
FEV_1/FVC ratio (%)	64.56-100.00	85.08±9.03	0.100	-0.116-0.321	0.425
VC (L)	0.66-3.28	1.66±0.55	0.322	0.067-0.523	0.008*

*Data were analyzed using Pearson's correlation, FVC: Forced vital capacity, FEV_1: forced expiratory volume in one second, VC: Vital capacity, *p<0.05 showed significance.*

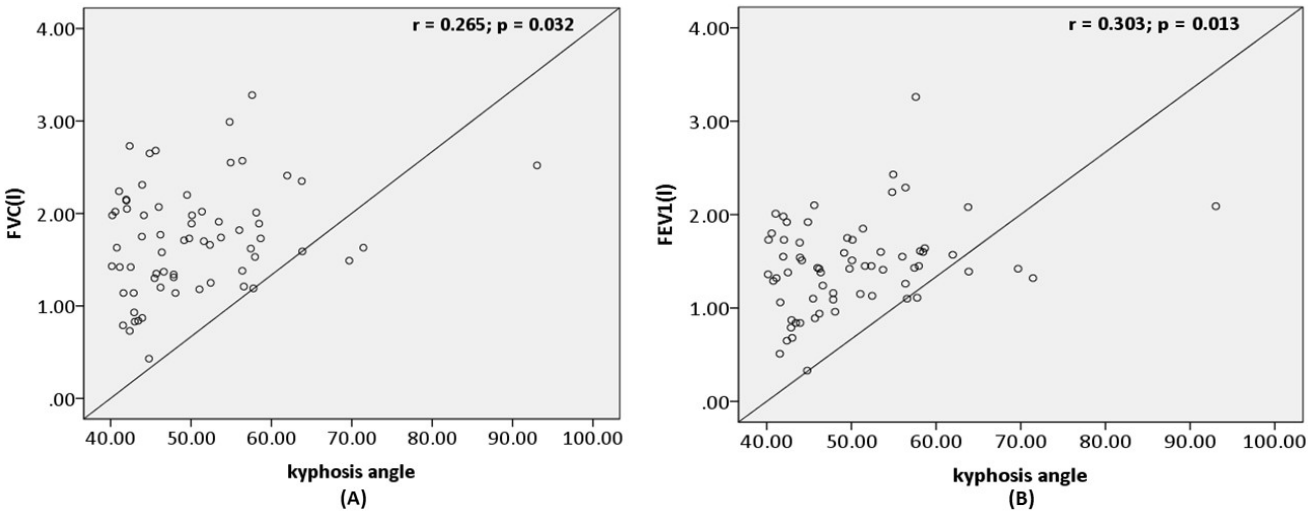

***Figure 3**. The correlations between the thoracic kyphosis angle and lung function. A) FVC B) FEV_1 C) FEV_1/FVC ratio D) VC.*

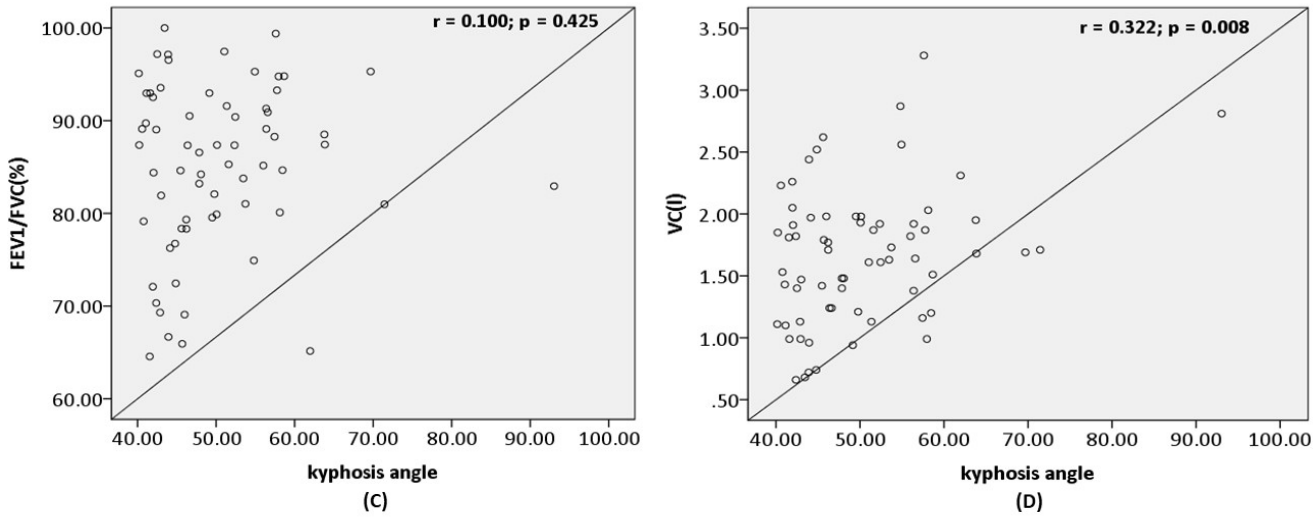

Figure 3. *The correlations between the thoracic kyphosis angle and lung function. A) FVC B) FEV_1 C) FEV_1/FVC ratio D) VC.*

Correlations between the thoracic kyphosis angle and respiratory muscle strength

There were no correlation between thoracic kyphosis angle and MIP and MEP (p>0.05). The correlation results are provided in Table 3 and Figure 4A and 4B.

Table 3 Correlations between the thoracic kyphosis angle and respiratory muscle strength (MIP and MEP).

Variables	Min-Max	Mean±SD	r	95% CI	*p* value
MIP (cmH_2O)	19-123	61.61±24.37	-0.081	-0.315-0.126	0.518
MEP (cmH_2O)	36-175	78.91±26.90	-0.167	-0.362-0.041	0.179

*Data were analyzed using Pearson's correlation, MIP: Maximal inspiratory pressure, MEP: maximal expiratory pressure. *p<0.05 showed significance.*

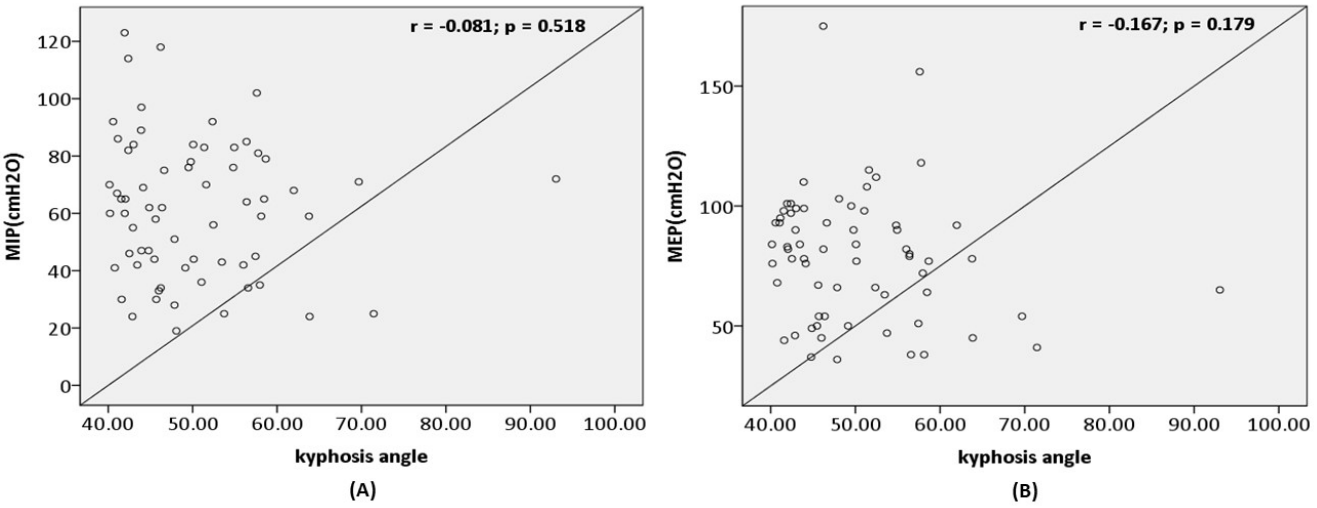

Figure 4. *The correlations between the thoracic kyphosis angle and respiratory muscle strength. A) MIP B) MEP.*

Discussion

The aim of the present study was to examine the correlation between thoracic kyphosis and pulmonary function in elderly. In the present study, we found that thoracic kyphosis angle was positive correlated significantly with the lung functions (FEV_1, FVC and VC) of elderly. However, thoracic kyphosis angle was not significantly correlated with FEV_1/FVC ratio and respiratory muscle strength (MIP and MEP).

In addition, the percentage of participants in the ventilatory defects was 34.85% for the normal, 59.09% for the restrictive, 1.52% for the obstructive, and 4.55% for the mixed lesion.[22] The thoracic kyphosis was mildly positive correlated with the lung functions in VC, FEV_1 and FVC parameters. Our results are consistent with the study of Mellin G et al.,[32] determining the relationship between thoracic spinal mobility and kyphosis and lung function. Thoracic kyphosis was measured by using an inclinometer and thoracic spinal mobility was measured by using a goniometer. They found that thoracic spinal mobility was significantly positive correlated with lung function (FEV_1 and VC parameters) (r=0.16, p<0.05) which may suggest that an increase in chest expansion, thereby an increase in lung volume. However, the results were in disagreement with the findings of previous studies regardless of thoracic kyphosis and pulmonary function.[17-19] Rahman et al.[18] found a significant mild correlation between the thoracic kyphosis measured by using electromagnetic motion tracking system and lung functions (FEV_1 and FVC) in elderly (r=0.232 and 0.317, respectively, p<0.05). A cross-sectional study of Bari et al.[19] reported that the worse kyphosis evaluated by using occiput to wall distance and standing stature adjusted for tibia length was associated with reduced FVC and FEV_1. Besides Lorbergs et al.[17], also found similar results in prospective studies and reported that elderly women with thoracic kyphosis measured by radiographic examination tended to have a decrease in pulmonary function (FEV_1) more than those with erect posture. Furthermore, thoracic kyphosis was found to be mildly positive correlated with vital capacity (r=0.322, p<0.05), which is inconsistent with the findings of a previous study[33] which reported that women with osteoporosis-related kyphosis had significantly lower VC (p<0.05). These differences in results between the studies may be related to the methodology used for the analysis of lung functions, the previous study[17, 19] used bell spirometer which low impact on restrictive ventilatory measurement and may result in overestimated. In addition, participants characteristics in the previous studied were different.[17-19] These included no variety in sources of study participants,[17, 19] low physical activity and activity daily living,[18] no control in respiratory disorder, history of vertebral fracture[19] and history of smoking[17, 19] which may cause of low lung functions. Hence, this result was inconsistent with other studies and resulted in thoracic kyphosis was positive correlation with lung functions. Accordingly, the factors which may impact on the positive correlation in thoracic kyphosis angle with lung functions (FEV_1, FVC and VC) in this study were healthy elderly (no respiratory disease and uncontrol in underlying disease), no diversity in the kyphosis angle because the number of participants considered for measuring the kyphosis angle almost in 40-50 degrees which is the low kyphosis angle result in mildly positive correlated. There was no correlation between thoracic kyphosis and FEV_1/FVC ratio, and also respiratory muscle strength (MIP and MEP). Suggestion participants in this study were healthy who not had a disease in the nervous system or neuromuscular which almost a problem leads to respiratory muscle weakness. This result is in accordance with a previous study,[18] which found that lung functions in FEV_1/FVC ratio parameter, MIP and MEP were not correlated with thoracic curvature. However, MIP and MEP significantly correlated with lumbar curvature, since the diaphragm is the main inspiration structure and it attaches mainly at the lumbar spine.[18] The researchers performed analysis into subgroups of gender (males and females) and kyphosis angles (40-50, 50-60, 60-100 degrees). It was found that both gender and kyphosis angles were not correlated with lung functions (p>0.05).

The limitation of the present study was that the participants recruited in the study were women more than men (77.30% and 22.70%, respectively). In addition, this study used medium effect size (effect size=0.32) which lead to small sample size (n=66) and had effect on data distribution, in which there was no diversity in the kyphosis angles. Hence, this study has several strengths. The examiners and evaluations were blinded to clinical status and kyphosis angle variables. Besides, it was difficulty to select participants and noticed that 66 participants from 244 participants who passed in screening of inclusion criteria. Almost elderly has memory and cognition problem to assess the spirometry and maximal voluntary respiratory pressures, therefore, this study was used MoCA-Thai testing to assesse memory and cognition which is easy to collect data and good reliability tool. Additionally, these findings had represented diversity of participants including community-dwelling older adults living in rural areas, older adults living in residential institutions and citizen club at a suburban area. However, this study did not assess parameters on chest expansion, thoracic spinal mobility, flexibility, and physical activity which may impact on lung functions. Further study is needed to focus on these parameters. Especially, physical activity may be measured by using questionnaires such as the Community Healthy Activities Model Program for Seniors (CHAMPS), Physical Activity Scale for the Elderly (PASE) or Modified Baecke Physical Activity questionnaire.

Conclusion

We found that thoracic kyphosis was mildly positive correlation with lung functions in elderly, but FEV_1/FVC ratio parameter, MIP and MEP were not correlated with thoracic kyphosis. Thoracic kyphosis measurements using the flexicurve method may provide a trend to predict lung function associated with thoracic kyphosis. Further study is warranty.

Conflicts of interests

There were no conflicts of interest.

Acknowledgements

This study was granted by the Faculty of Associated Medical Sciences, the Graduate School, and TA/RA scholarship, Chiang Mai University, Chiang Mai Province, Thailand. We thank to all participants who participated in this study. The authors also thank to Miss Wannaporn Mapiwong, Miss Thanutcha Chaiyai, and Miss Wichaya Noibangyang for collecting the part of the data.

Ethical approval

Approved number AMSEC-62EX-041.

References

[1] Sangthong J. Aging Society (Complete Aged) :The elderly condition of good quality. Rusamilae 2017; 38(1): 6-28.

[2] National statistical office Thailand. Statistics of elderly [internet]. 2017 [cited 3 Mar 2019]. Available from: http://www.nso.go.th/sites/2014/Pages/Press_Release/2561/N10-04-61-1.aspx.

[3] Bean JF, Kiely DK, Leveille SG, Herman S, Huynh C, Fielding R. The 6-minute walk test in mobility-limited elders: what is being measured? J Gerontol. 2002; 57(11): 751-6.

[4] Robnett GH and Chop WC. Gerontology for the health care professional. 3rd, editor. Burlington: Jones&Barlett learning; 2015.

[5] Katzman WB, Wanek L, Shepherd JA, Sellmeyer AD. Age-related hyperkyphosis: its causes, consequences, and management. J Orthop Sports Phys Ther. 2010; 40(6): 352-60.

[6] Ailon T, Shaffrey CI, Lenke LG, Harrop JS, Smith JS. Progressive spinal kyphosis in the aging population. J Neurol Surg. 2015; 77: 164-72.

[7] Skloot GS. The effects of aging on lung structure and function. Clin Geriatr Med. 2017; 33(4): 447-57.

[8] Patronage. Guideline for spirometric evaluation. Bangkok: Parbpim; 2002.

[9] Shin HI, Kim DK, Seo KM, Kang SH, Lee SY, Son S. Relation between respiratory muscle strength and skeletal muscle mass and hand grip strength in the healthy elderly. Ann Rehabil Med. 2017; 41(4): 686-92.

[10] Troosters T, Gosselink R, Decrame M. Respiratory muscle assessment. Eur Respir J 2005; 31: 57-71.

[11] Caruso P, Albuquerque AL, Santana PV, Cardenas LZ, Ferreira JG, Prina E. Diagnostic methods to assess inspiratory and expiratory muscle strength. J Bras Pneumol. 2015; 41(2): 110-23.

[12] Wongsa S, Amatachaya S. Kyphosis assessments. J Med Tech Phy Ther. 2014; 26(2): 105-16.

[13] Augustus A et al. Clinical biomechanics of the spine. 2nd ed. Philadelphia: Lippincott Williams & Wilkins; 1990.

[14] Kado DM, Huang MH, Nguyen CB, Barrett-Connor E, Greendale GA. Hyperkyphotic posture and risk of injurious falls in older persons: the Rancho Bernardo Study. J Gerontol A Biol Sci Med Sci. 2007; 62: 652-7.

[15] Teixeira FA, Carvalho GA. Reliability and validity of thoracic kyphosis measurements using flexicurve method. Rev Bras Fisioter. 2007; 113: 199-204.

[16] Amatachaya P, Wongsa S, Sooknuan T, Thaweewannakij T, Laophosri M, Manimanakorn N. Validity and reliability of a thoracic kyphotic assessment tool measuring distance of the seventh cervical vertebra from the wall. Hong Kong Physiother J. 2016; 35: 30-6.

[17] Lorbergs AL, O'Connor GT, Zhou Y, Travison TG, Kiel DP, Cupples A et al. Severity of kyphosis and decline in lung function: The Framingham Study. J Gerontol A Biol Sci Med Sci. 2017; 72(5): 689-94.

[18] Rahman NN, Singh DK, Lee R. Correlation between thoracolumbar curvatures and respiratory function in older adults. Clin Interv Aging. 2017; 12: 523-9.

[19] Bari MD, Chiarlone M, Matteuzzi D, Zacchei S, Pozzi C, Bellia V et al. Thoracic kyphosis and ventilatory dysfunction in unselected older persons: an epidemiological study in Dicomano, Italy. J Am Geriatr Soc. 2004; 52: 909-15.

[20] Ramesh PM, Saravanan M, Divya. Effects of smoking cessation on pulmonary function and quality of life. Int J Adv Med. 2018; 5(5): 1177-80.

[21] Carson N, Leach L, Murphy KJ. A re-examination of Montreal Cognitive Assessment (MoCA) cutoff scores. Int J Geriatr Psychiatry. 2018;33(2): 379- 88.

[22] Summacheeva. Guideline for standardization and interpretation of pulmonary function test by spirometry in occupational health setting Chonburi: Summacheeva 2018.

[23] Kacmarek RM, Stoller JK, Heuer AJ. Egan's fundamentals of respiratory care. 11th ed. Missouri: Elsevier; 2013.

[24] Carefusion. MicroRPM simple tests for respiratory muscle strength Germany: Carefusion; 2011 [updated 2011].

[25] Eliks M, Tomaszewska WO, Lisiński P, Koczewski P. Does structural leg-length discrepancy affect postural control? Preliminary study. BMC Musculoskelet Disord. 2017; 18: 346.

[26] Tsiligiannis T, Grivas T. Pulmonary function in children with idiopathic scoliosis. Scoliosis Spinal Disord. 2012; 7: 7.

[27] Clarke CL, Witham MD. The effects of medication on activity and rehabilitation of older people – opportunities and risks. Rehabilitation Process and Outcome. 2017; 6: 1-7.

[28] Hart LD, Rose JS. Reliability of a noninvasive method for measuring the lumbar curve. J Orthop Sports Phys Ther. 1986; 2: 180-6.

[29] Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A et al. Sandardisation of spirometry. Eur Respir J. 2005; 26: 319-38.

[30] Dimitriadis Z, Kapreli E, Konstantinidou I, Oldham J, Strimpakos N. Test/retest reliability of maximum mouth pressure measurements with the MicroRPM in healthy volunteers. Respir. 2011; 56(6).

[31] Mukaka MM. A guide to appropriate use of correlation coefficient in medical research. Malawi Med J 2012; 24: 69-71.

[32] Mellin G, Harjula R. Lung function in relation to thoracic spinal mobility and kyphosis. Scand J Rehabil Med. 1987; 19(2): 89-92.

[33] Culham EG, Jimenez HA, King CE. Thoracic kyphosis, rib mobility, and lung volumes in normal women and women with osteoporosis. Spine J. 1994; 19: 1250-5.

Instructions for Authors

Instructions for Authors

Original article/thesis can be submitted through the on-line system via website https://www.tci-thaijo.org/index.php/bulletinAMS/

General Principles

Journal of Associated Medical Sciences is a scientific journal of the Faculty of Associated Medical Sciences, Chiang Mai University. The articles submitted to the journal that are relevant to any of all aspects of Medical Technology, Physical Therapy, Occupational Therapy, Radiologic Technology, Communication Disorders, and other aspects related to the health sciences are welcome. Before publication, the articles will go through a system of assessment and acceptance by at least three experts who are specialized in the relevant discipline. All manuscripts submitted to Journal of Associated Medical Sciences should not have been previously published or under consideration for publication elsewhere. All publications are protected by the Journal of Associated Medical Sciences' copyright.

Manuscript categories

1. **Review articles** must not exceed 20 journal pages (not more than 5,000 words), including 6 tables/figures, and references (maximum 75, recent and relevant).
2. **Original articles** must not exceed 15 journal pages (not more than 3,500 words), including 6 tables/figures, and 40 reference (maximum 40, recent and relevant).
3. **Short communications** including technical reports, notes, and letter to editor must not exceed 5 journal pages (not more than 1,500 words), including 2 tables/figures, and references (maximum 10, recent and relevant).

Manuscript files

To submit your manuscript, you will need the following files:

1. A Title page file with the names of all authors and corresponding authors*
2. Main document file with abstract, keywords, main text and references
3. Figure files
4. Table files
5. Any extra files such as Supplemental files or Author Biographical notes

Manuscript Format

1. **Language**: English, Caribri 10 for text and 7 for all symbols. PLEASE be informed that the Journal only accept the submission of English manuscript.
2. **Format**: One-side printing, double spacing. Use standard program and fonts and, add page and line number for all pages.
3. **A Title page**: Include article title, names of all authors and co-authors, name of the corresponding author and acknowledgements. Prepare according to following contents;
   - *Title of the article*: Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulate where possible.
   - *Author names and affiliation*: Where the family name may be ambiguous (e.g. a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with superscript number immediately after author's name and in front of appropriate address. Provide the full postal address of each affiliation, including the province, country and, if available, the e-mail address of each author.
   - *Corresponding author*: Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication, ensure that telephone and fax numbers (with postal area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.
   - *Acknowledgements*: Acknowledgements will be collated in a separate section at the end of the article before the references in the stage of copyediting. Please, therefore, include them on the title page, List here those individuals who provided help during the research (e.g. providing language help, writing assistance or proof reading the article, etc.)
4. **Main article structure**: The manuscripts should be arranged in the following headings: Title, Abstract, Introduction, Materials and Methods, Results, Discussion and Conclusion, and Reference. Prepare according to following contents;
   - *Abstract*: Not exceeding 400 words, abstract must be structured with below headings in separated paragraph:
     - Background,
     - Objectives,
     - Materials and methods,
     - Results,
     - Conclusion, and
     - Keywords (3-5 keywords should be included)
   - *Introduction*: State the objectives of work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.
   - *Materials and Methods*: Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference, only relevant modifications should be described. Ensure that each table, graph, or figure is referred in the text. According to the policy of ethical approval, authors must state the ethical approval code and conduct informed consent for human subject research (If any) and for animal research, authors must include a statement or text describing the experimental procedures that affirms all appropriate measures (if any) in this section.
   - *Results*: Results should be clear and concise. Present the new results of the study such as tables and figures mentioned in the main body of the article and numbered in the order in which they appear in the text or discussion.
   - *Discussion*: This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion

section is often appropriate. Avoid extensive citations and discussion of published literature.
- *Conclusion*: The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a "Discussion" or "Results and Discussion".
- *Conflict of interes*t: All authors must declare any financial and personal relationship with other people or organization that could inappropriately influence (bias) their work. If there is no interest to declare, then please state this: "The authors declare no conflict of interest".
- *Ethic approval*: Ethic clearance for research involving human and animal subjects.
- *References*: Vancouver's style.

5. **Artwork Requirements**
   - Each table, graph and figure should be self-explanatory and should present new information rather than duplicating what is in the text. Prepare one page per each and submit separately as supplementary file(s).
   - Save the figures as high resolution JPEC or TIFF files.

Note: Permission to reprint table(s) and/or figure(s) from other sources must be obtained from the original publishers and authors and submitted with the typescript.

Ensuring a blind peer review

To ensure the integrity of the double-blinded peer-review for submission to this journal, every effort should be made to prevent the identities of the authors and reviewers from being known to each other. The authors of the document have deleted their names from the main text, with "Author" and year used in the references and footnotes, instead of the authors' name, article title, etc. After the journal was accepted, the name of authors and affiliation and the name of the corresponding author must be included into the document and re-submitted in the copyediting stage.

Proof correction

The Proofs of final paper approved for publication are to be returned by email to the researcher before publication.

Page charge

No page charge.

References Format

1. References using the Vancouver referencing style (see example below).
2. In-text citation: Indicate references by number(s) in the order of appearance in the text with superscript format. Reference numbers are to be placed immediately after the punctuation (with no spacing). The actual authors can be referred to, but the reference number(s) must always be given. When multiple references are cited at a given place in the text, use a hyphen to join the first and last numbers that are inclusive. Use commas (with no spacing) to separate non-inclusive numbers in a multiple citation e.g. (2-5,7,10). Do not use a hyphen if there are no citation numbers in between inclusive statement e.g. (1-2). Use instead (1,2).
3. References list: number the references (numbers in square brackets) in the list must be in the order in which they are mentioned in the text. In case of references source from non-English language, translate the title to English and retain "in Thai" in the parentheses.
4. Please note that if references are not cited in order the manuscript may be returned for amendment before it is passed on to the Editor for review.

Examples of References list

Multiple Authors: List up to the first 6 authors/editors, and use "et al." for any additional authors.

Journal Articles (print): In case of reference source contains DOI, retain doi: at the end of reference. Vancouver Style does not use the full journal name, only the commonly-used abbreviation: "Physical Therapy" is cited as "Phys Ther". As an option, if a journal carries continuous pagination throughout a volume (as many medical journals do) the month and/or issue number may be omitted. Allow one space after semi-colon and colon and end each reference with full stop after page number.

- Pachori P, Gothalwal R, Gandhi P. Emergence of antibiotic resistance Pseudomonas aeruginosa in intensive care unit; a critical review. Genes Dis. 2019; 6(2): 109-19. doi: 10.1016/j.gendis.2019.04.001.
- Hung Kn G, Fong KN. Effects of telerehabilitation in occupational therapy practice: A systematic review. Hong Kong J Occup Ther. 2019; 32(1): 3-21. doi: 10.1177/1569186119849119.
- Wijesooriya K, Liyanage NK, Kaluarachchi M, Sawkey D. Part II: Verification of the TrueBeam head shielding model in Varian VirtuaLinac via out-of-field doses. Med Phys. 2019; 46(2): 877-884. doi: 10.1002/mp.13263.
- Velayati F, Ayatollahi H, Hemmat M. A systematic review of the effectiveness of telerehabilitation interventions for therapeutic purposes in the elderly. Methods Inf Med. 2020; 59(2-03): 104-109. doi: 10.1055/s-0040-1713398.
- Junmee C, Siriwachirachai P, Chompoonimit A, Chanavirut R, Thaweewannakij T, Nualnetr N. Health status of patients with stroke in Ubolratana District, Khon Kaen Province: International Classification of Functioning, Disability and Health-based assessments. Thai J Phys Ther. 2021; 43(1): 45-63 (in Thai).

Book / Chapter in an Edited Book References

PLEASE be informed that references of books and chapter in edited book should not be include in the research article, but others manuscript categories.

- Grove SK, Cipher DJ. Statistics for Nursing Research: A Workbook for Evidence-Based Practice. 3rd Ed. St. Louis, Missouri: Elsevier; 2019.
- Perrin DH. The evaluation process in rehabilitation. In: Prentice WE, editor. Rehabilitation techniques in sports medicine. 2nd Ed. St Louis, Mo: Mosby Year Book; 1994: 253–276.

E-book

- Dehkharghani S, editor. Stroke [Internet]. Brisbane (AU): Exon Publications; 2021 [cited 2021 Jul 31]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK572004/ doi: 10.36255/exonpublications.stroke.2021.
- Tran K, Mierzwinski-Urban M. Serial X-Ray Radiography for the Diagnosis of Osteomyelitis: A Review of Diagnostic Accuracy, Clinical Utility, Cost-Effectiveness, and Guidelines [Internet]. Ottawa (ON): Canadian Agency for Drugs and Technologies in Health; 2020 [cited 2021 Jul 31]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK562943/

Dissertation/Thesis

- Borkowski MM. Infant sleep and feeding: a telephone survey of Hispanic Americans [Dissertation]. Mount Pleasant (MI): Central Michigan University; 2002.
- On-Takrai J. Production of monoclonal antibody specific to recombinant gp41 of HIV-1 subtype E [Term paper]. Faculty of Associated Medical Sciences: Chiang Mai University; 2001 [in Thai].

Conference Proceedings

- Lake M, Isherwood J, Clansey. Determining initial knee joint loading during a single limb drop landing: reducing soft tissue errors. Proceedings of 34th International Conference of Biomechanics in Sport; 2016 Jul 18-22; Tsukuba, Japan, 2016. Available from: https://ojs.ub.uni-konstanz.de/cpa/article/view/7126.
- Ellis MD, Carmona C, Drogos J, Traxel S, Dewald JP. Progressive abduction loading therapy targeting flexion synergy to regain reaching function in chronic stroke: preliminary results from an RCT. Proceedings of the 38th Annual International Conference of the IEEE Engineering in Medicine and Biology Society; 2016: 5837-40. doi: 10.1109/EMBC.2016.7592055.

Government Organization Document

- Australian Government, Department of Health. Physical activity and exercise guidelines for all Australian. 2021 [updated 2021 May 7; cited 15 Jul 2021]. Available from: https://www.health.gov.au/health-topics/physical-activity-and-exercise/physical-activity-and-exercise-guidelines-for-all-australians.
- Department of Health. Situation survey on policy and implementation of physical activity promotion in schools for first year 2005. (in Thai). Nonthaburi: Ministry of Public Health; 2005.
- Department of Local Administration, Ministry of Interior Affairs. Standard of Sports Promotion. (in Thai). Bangkok. 2015:7–9.
- World Health Organization. WHO guidelines on physical activity and sedentary behaviour. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0 IGO.

Journal History

Established in 1968

- 1968-2016 As the Bulletin of Chiang Mai Associated Medical Sciences
  - Vol1, No1 - Vol.49, No3
- 2017, the Journal of Associated Medical Sciences
  - Vol.50, No1 and forward.

Journal Sponsorship Publisher

Faculty of Associated Medical Sciences, Chiang Mai University

Sponsors

Faculty of Associated Medical Sciences, Chiang Mai University

Sources of support

Faculty of Associated Medical Sciences, Chiang Mai University