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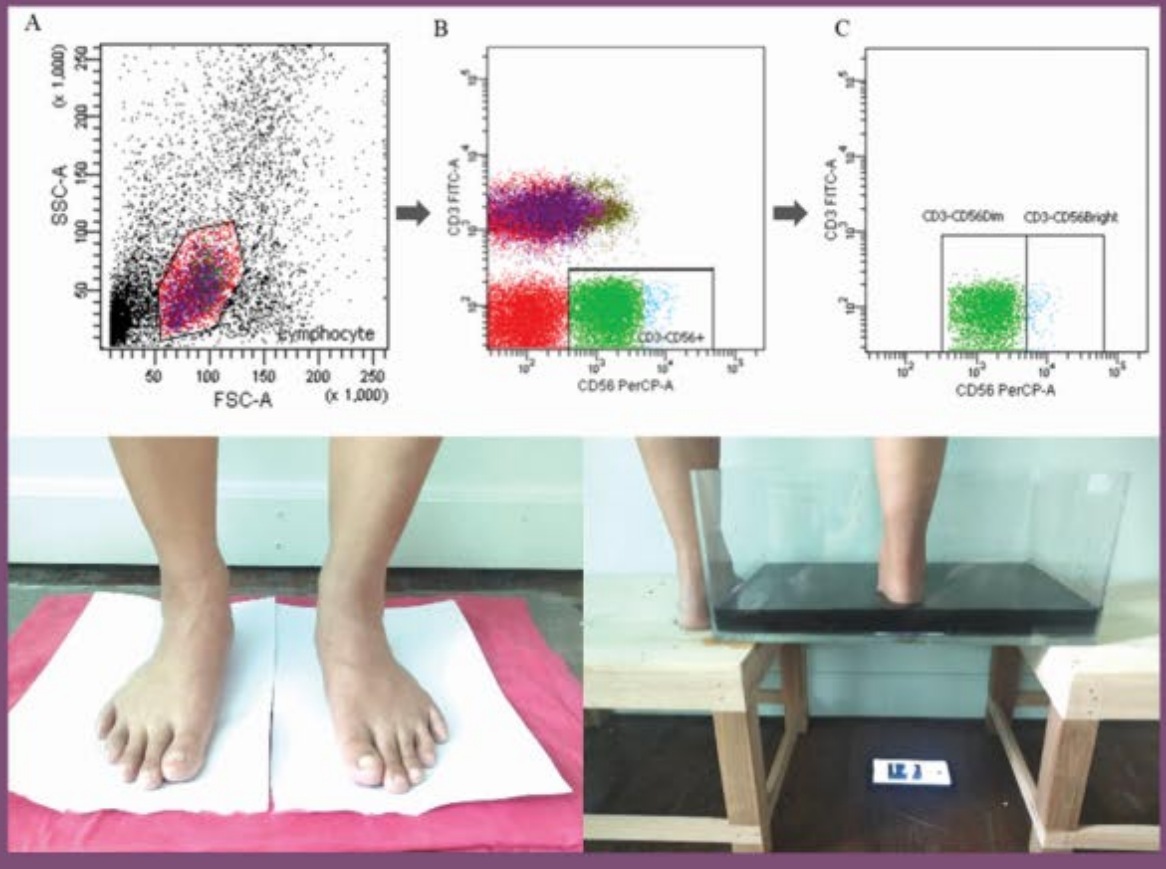

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ORIGINAL ARTICLE

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Effectiveness of URiSCAN 2 ACR strip test for albuminuria detection in screening of kidney disease

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KEYWORDS

Albumin/creatinine ratio (ACR);
Urine strip;
Screening test.

ABSTRACT

Albuminuria is a key marker for detection of kidney disease and an increased risk factor for cardiovascular diseases. Measurement of albumin/creatinine ratio (ACR) in urine is the best screening method to detect albuminuria. Nowadays, new version of URiSCAN 2 ACR strip test is developed to measure of albumin/creatinine ratio in urine and to report ACR value as semi-quantitative and quantitative results. Thus, we aimed to validate the effectiveness of the URiSCAN 2 ACR strip test by comparing with a quantitative automated analyzer, and to demonstrate the effectiveness of the strip test for screening chronic kidney disease in the community population. Measurements of ACR levels in 484 spot urine specimens of participants in CKDNET project were performed using the URiSCAN 2 Optima urine chemistry test system and using the SYNCHRON Lx20 PRO automated chemistry analyzer. The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of URiSCAN 2 ACR strip to detect ACR were 93.2%, 62.9%, 67.6%, 31.2% and 98.1%, respectively. URiSCAN 2 ACR strip showed 63.0% concordance rate with quantitative automated assay, 68.8% false-positive and only 1.9% false-negative results. In addition, the best cut-off of ACR value for detection of chronic kidney disease using the urine strip test was in the range from 29.50 to 33.00 mg/g with area under the curve of 0.7413, while the area under the curve of quantitative automated analyzer was 0.7515. Sensitivity, specificity and accuracy of the strip test for detection of chronic kidney disease were 70.5%, 61.8% and 63.8%, respectively. URiSCAN 2 ACR strip provided a high sensitivity, high negative predictive value and few false negative results for detection of albuminuria. Thus, this ACR strip test might be served as an optimal method to rule-out microalbuminuria. However, diagnostic performance of URiSCAN 2 ACR strip test at cut-off of ACR $\geq$30 mg/g is fair for screening CKD in community population.

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Introduction

Albuminuria is a marker of both active renal inflammation and renal injury. It is a strong risk factor associated with kidney disease, cardiovascular disease and a predictor of diabetic complications. Detection of albuminuria is a tool for early detection and monitoring kidney disease. Albuminuria usually appears before the decline of glomerular filtration rate (GFR). Thus, the screening test of albuminuria is a major role to prevent and to decline a development of chronic kidney disease (CKD)[1,2]. In healthy person, albumin excretion rate (AER) is less than 30 mg/24 hours or albumin/creatinine ratio (ACR) is less than 30 mg/g. Abnormal detection can be categorized when 30 to 300 mg/day of AER or 30-300 mg/g of ACR is called microalbuminuria, while >300 mg/day of AER and >300 mg/g of ACR is called macroalbuminuria[3].

Quantification of albumin in 24-hour urine collection (24-hr UAC) is the standard method to indicate albuminuria but it is an inconvenient, costly and has difficulty in sample collection causing poor patient compliance. There are several methods to detect the amount of protein in urine but the ACR is the best screening test for detection of albumin in spot urine. Previous studies showed that the results of spot ACR had high sensitivity (84.9%) and high specificity (95.8%) when compared with 24-hr UAC. In addition, they also showed positive correlation (R^2= 0.905: coefficient of determination) and not significantly statistic difference (*p*-value=0.724) for evaluation of microalbuminuria between the results from 24-hr UAC method and ACR method [4,5]. ACR is a convenient and alternatively rapid method with currently advocated by the major guidelines. The Kidney Disease Improving Global Outcomes (KDIGO) 2012 clinical practice guidelines determines that ACR is important for the diagnosis, prognosis and classification of CKD[3].

General technique to detect abnormal levels of proteinuria is urine dipstick. The usefulness of urine dipstick is not only rapid test but also a screening test in the community. This test is user-friendly, acceptably reliable and simple without additional equipment[6]. The importance of early detection of microalbuminuria is a marker of kidney damage such as glomerular disease. Additionally, early screening for microalbuminuria in diabetic patients with an initial stage of renal involvement has an advantage to prevent the progression of kidney complications.

URiSCAN 2 ACR strip test is a semi-quantitative urine dipstick as a screening tool for albumin and creatinine in urine. The URiSCAN Optima urine analyzer determines the ACR value with milligram of albumin per gram or mmol of creatinine. New version of the URiSCAN 2 ACR strip test reports both quantitative and semi-quantitative values of ACR. Thus, the objective of this study was to validate the effectiveness of the URiSCAN 2 ACR strip test by comparing with a quantitative automated analyzer, and to demonstrate the effectiveness of the strip test for screening chronic kidney disease in the community population.

Materials and methods

Participants and samples collection

This study was a cross-sectional community-based study investigating CKD events between June 2017 and August 2018 in Tambon Don Chang, Amphoe Meuang, and Tambon Khok Samran, Amphoe Ban Haet, Khon Kaen Province, Thailand. The study was a part of Chronic Kidney Disease Prevention in the Northeast of Thailand (CKDNET) project. The participants (more than 18 years old) were enrolled into this study. This study protocol (HE601035) was approved by the Ethics Committee of Khon Kaen University

The CKD subjects in this study presented with persistent kidney damage and/or loss of kidney function for more than 3 months according to the KDIGO 2012 guideline. Table 1 shows the baseline characteristics of all participants. The total of 484 random spot urine samples including 51 diabetes, 69 hypertension, 65 diabetes and hypertension and 299 non-diabetics and non-hypertension were collected. All samples were refrigerated in 2-4ºC during immediately transferred to the laboratory within 2 hours. The evaluation processes for all samples were performed at Community Laboratory, Faculty of Associated Medical Sciences, Khon Kaen University.

Table 1 Characteristic of the 484 participants in this study

Parameters	Total (n = 484)
Age (mean ± SD)	56.0 ± 12.5
CKD (%)	112 (23.1%)
Non-CKD (%)	372 (76.9%)
Diabetes Mellitus (%)	51 (10.5%)
Hypertension (%)	69 (14.3%)
Diabetes and hypertension (%)	65 (13.4%)
Non-diabetes and non-hypertension (%)	299 (61.8%)

Laboratory quantitative ACR method

SYNCHRON Lx20 PRO chemical analyzer (Beckman Coulter, Inc, USA) measured albumin level by turbid metric method (antigen-antibody complexes) and creatinine level by Jaffe rate method. The analytical range of albumin and creatinine in urine samples were 0.2-30.0 and 10-400 mg/dL, respectively. The quantitative ACR ratio (mg/g) was calculated by the results of albumin (mg/dL) and quantitative creatinine (g/L).

ACR urine strip test

URiSCAN 2 ACR strip test detected urinary albumin by dye binding method and detected creatinine by metal complex methods. URiSCAN Optima urine analyzer (YD Diagnostics Co., Ltd., Korea) read the color change on strips. The measurement of albumin concentration was categorized into four scales as negative, 1+, 2+, 3+ (0, 30, 80, and 150 mg/L) and five-scales of creatinine concentration as +/-, 1+, 2+, 3+, 4+ (10, 50, 100, 200, and 300 mg/dL). The results of ACR was also calculated and reported as a quantitative ACR were semi-quantitative. The quantitative results were a continuous value while semi-quantitative results were <30 mg/g, 30-300 mg/g and >300 mg/g referred to normoalbuminuria, microalbuminuria and macroalbuminuria, respectively.

Statistical analysis

Categories of ACR results in this study were positive and negative. The term "positive" was defined as samples with ACR results of ≥30 mg/g, whereas the definition of "negative" was samples with ACR results of <30 mg/g. The effectiveness of URiSCAN 2 ACR strip test for ACR detection was evaluated by sensitivity, specificity, accuracy, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (+LR) and negative likelihood ratio (-LR) compared with laboratory quantitative reference method. The diagnostic performance of strip test in CKD were analyzed by using receiver operating characteristic (ROC) curve, sensitivity, specificity, accuracy, positive predictive value, negative predictive value, positive and negative likelihood ratio. Statistical analyses were conducted by the SPSS for Windows version 19.0 (IBM Corp, Armonk, NY, USA).

Results

Comparison of the quantitative and semi-quantitative assay

Reference quantitative ACR assay of 484 random urine samples for normoalbuminuria (<30 mg/g), microalbuminuria (30-300 mg/g) and macroalbuminuria (>300 mg/g) were 84.7%, 12.6% and 2.7%, respectively while the semi-quantitative ACR detected by strip test were 54.3%, 37.6% and 8.1% (Table 2).

Table 2 Comparison of the ACR results between the quantitative assay and the semi-quantitative assay

URiSCAN 2 ACR strip (mg/g)	Quantitative ACR (mg/g)			
	<30	**30-300**	**>300**	**Total**
<30	258 (53.3%)	5 (1.0%)	0 (0%)	263 (54.3%)
30-300	146 (30.2%)	35 (7.2%)	1 (0.2%)	182 (37.6%)
>300	6 (1.2%)	21 (4.3%)	12 (2.5%)	39 (8.1%)
Total	410 (84.7%)	61 (12.6%)	13 (2.7%)	484 (100%)

The overall concordance rate of the ACR results between quantitative assay and semi-quantitative assay was 63.0%. The performance of URiSCAN 2 ACR strip test was 93.2% of sensitivity, 62.9% of specificity and 67.6% of accuracy. False positive and false negative rates of URiSCAN 2 ACR strip test for ACR detection were 68.8% and 1.9%, respectively. The value of their 95% confidence intervals for each parameter was also acceptable and presented (Table 3).

Table 3 The performance of the URiSCAN 2 ACR strip test for detection of ACR

	URiSCAN 2 ACR Strip
Sensitivity (%)	93.2 (84.9-97.8)
Specificity (%)	62.9 (58.1-67.6)
Accuracy (%)	67.6 (63.2-71.7)
Concordance rate (%)	63.0 (58.5-67.3)
False positive rate (%)	68.8 (62.2-74.7)
False negative rate (%)	1.9 (0.7-4.6)
Positive predictive value (%)	31.2 (28.3-34.3)
Negative predictive value (%)	98.1 (95.7-99.2)
Positive likelihood ratio	2.5 (2.2-2.9)
Negative likelihood ratio	0.1 (0.05-0.25)

Note: Values shown are percentage (95% confidence intervals).

Cut-off of ACR for indicating CKD

KDIGO 2012 guideline recommended the cut-off of ACR ≥30 mg/g for diagnosis and follow up of the complications of CKD. Based on the quantitative ACR reported by URiSCAN 2 ACR strip test, the efficiency for indicating CKD were analyzed using ROC analysis. The best cut-off of ACR value was 29.50 to 33.00 mg/g with AUC of 0.7413 (Figure 1). Sensitivity, specificity and accuracy of URiSCAN 2 ACR strip test for CKD detection were 70.5%, 61.8% and 63.8%, respectively. The diagnostic performance of URiSCAN 2 ACR strip test to indicate CKD was shown in Table 4.

Table 4 The diagnostic performance of the URiSCAN 2 ACR strip test for CKD detection

	URiSCAN 2 ACR Strip
Sensitivity (%)	70.5 (61.2-78.8)
Specificity (%)	61.8 (56.7-66.8)
Accuracy (%)	63.8 (59.4-68.1)
False positive rate (%)	64.3 (57.5-70.5)
False negative rate (%)	12.5 (8.9-17.3)
Positive predictive value (%)	35.8 (31.8-39.9)
Negative predictive value (%)	87.5 (83.8-90.4)
Positive likelihood ratio	1.9 (1.6-2.2)
Negative likelihood ratio	0.5 (0.4-0.6)

Note: Values shown are percentage (95% confidence intervals).

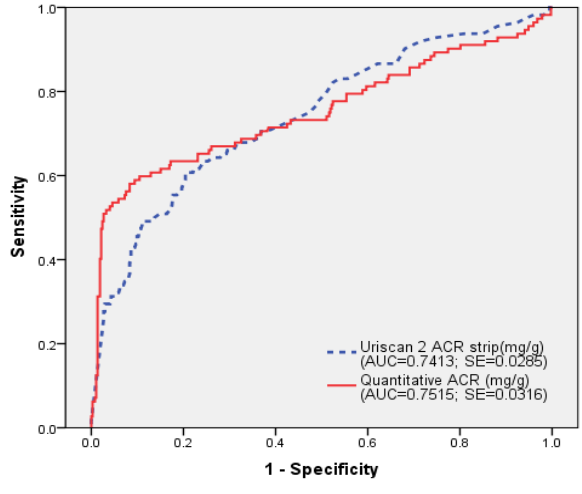

Figure 1 Receiver operating characteristic curves (ROC) for presenting the effectiveness of URiSCAN 2 ACR strip test to indicate CKD in the community population.

Abbreviations: AUC, area under the curve; SE, standard error.

Discussion

Detection of microalbuminuria is the earliest test for screening kidney disease and associated with the progression of CKD. It has a higher sensitivity and specificity than the total protein test[7,8]. KDIGOO 2012 guideline recommend that clinical laboratories should report ACR or protein/creatinine ratio (PCR) in spot urine samples rather than report the concentrations of albumin alone[3].

This study demonstrated the performance of the URiSCAN 2 ACR strip test to detect ACR. Our results showed a good sensitivity (93.2%), adequate specificity (62.9%) and concordance rate (63.0%) between the URiSCAN 2 ACR strip and the quantitative assay, which was comparable to those reported in the previous studies[9,10]. Lim et al.[9] demonstrated sensitivity, specificity and concordance rate of the URiSCAN 2 ACR strip were 87.7%, 72.2% and 75.6%, respectively. Cho et al.[10] evaluated the performance of URiSCAN Super cassette ACR strips and found that the, the sensitivity, specificity and concordance rate of the ACR strip test were 86.3%, 93.8% and 81.9%, respectively.

Lower specificity and concordance rate between URiSCAN 2 ACR strip and the quantitative assay in this study is due to the different assay of the reference method and the variation for disease heterogeneities among these populations in the study area. The high sensitivity of URiSCAN 2 ACR strip and the disagreement between this strip test and quantitative method for ACR detection were caused by high false positive results of the ACR results (68.8%) in this study. The false positive cases of higher albumin levels with lower creatinine levels might result in false positive ACR results. The previous study has shown the same pattern with these results[9]. However, false positive results can occur because of highly concentrated urine, especially diabetic patients. Urine of diabetic patients have more glucose than the level that the renal tubule can absorb. It is caused by highly concentrated urine in diabetic patients[11]. The prevalence of diabetic patients was 10.5%, diabetic patients with hypertension was 13.4% whereas 35.3% of all diabetic cases had positive result with glucose dipstick strip test. Moreover, 1.2% of false negative cases showed low specific gravity and low creatinine levels in urine (data not shown), suggesting that the urine was diluted. False negative results have been reported with diluted urine in the cases where predominant protein is not the albumin[12,13].

Furthermore, microalbuminuria is a marker of the inflammatory process. It is often present in patients with hypertension as well. This study showed a high incidence of albuminuria (ACR $\geq$30 mg/g) found in hypertensive patients with or without diabetes compared with non-diabetic and non-hypertensive patients (55.4% vs 27.0%) by using the quantitative assay (data not shown). The other study reported the same results as our study. In a study of 140 hypertensive patients with age of 50.1±11.6 years, the average ACR results was significantly higher in hypertensive than normotensive patients (2.17±2.67 mg/mmol and 1.72±2.97 mg/mmol, respectively, P=0.012)[14]. The increased ACR in hypertensive patients was due to two main mechanism, an increased glomerular hydrostatic pressure and an increased permeability in the glomerular basement membrane. Increased blood pressure, increased peripheral resistance and augmented volume load from increased flow pulsation could change the renal hemodynamics and damage the renal microvascular. Hence, hyperfiltration of albumin exceeds the absorption ability of the proximal tubules. In addition, the destruction of the lysosomal degradation pathway and the leakage of albuminuria is caused by the elevated angiotensin II and transforming growth factor-β1 in patients with hypertension[15,16].

Moreover, the data demonstrated that the URiSCAN 2 Optima urine chemistry test system for ACR detection is reliable to rule out increased excretion of urinary albumin with high NPV (98.1%) and low negative likelihood ratios (negative LR 0.1) at ACR value of >30 mg/g in spot urine. The procedures guide for clinical evaluation showed the reliably accepted cutoff value for rule-out microalbuminuria with a negative likelihood ratio of less than 0.1[17], whereas the performance of the URiSCAN 2 ACR strip test in our study for

rule-in albuminuria is poor (positive LR 2.5). The positive likelihood ratios must exceed 3.0 and the considered reliable value for a test's ability to rule-in a condition was a value of greater than 10[15]. Thus, the URiSCAN test system can reliably rule out microalbuminuria. In another ACR strip performance test study, Guy M et al. evaluated the ability to rule in or rule out albuminuria by the CLINITEK microalbumin strip test (Siemens Healthcare Diagnostics Inc., Deerfield, US)[18]. Their data showed NPV above 90% and negative likelihood ratios less than 0.05. It was shown to be a reliable test for ruling out increased urinary albumin excretion at the same cutoff as in our study (AER>30 mg/24-hour).

In addition, this study accessed the effectiveness of urine ARC in distinguishing between CKD and non-CKD using the ROC curve. The best cutoff of ACR using URiSCAN 2 ACR strip for detection of CKD was in the range from 29.50 to 33.00 mg/g. The strip test had the same fair prediction (AUC 0.7413) as the quantitative automated assay (AUC 0.7515). Moreover, the URiSCAN 2 ACR strip test showed that PPV, NPV, positive LR and negative LR for diagnostic CKD were 35.8% 87.5%, 1.9 and 0.5%, respectively (Table 4). These results indicated that the effectiveness of the URiSCAN 2 ACR strip test as a screening test for ruling in CKD disease is poor; it is optimal for ruling out CKD disease. However, the previous studies have shown that the same cutoff of ACR (<3 mg/mmol or <30 mg/g) is used to determine albuminuria as with our study[19-21]. They showed the similarity of the high sensitivity and high NPV of the strip test for measuring the ACR. These results make this ACR strip test particularly useful for screening CKD in the general population, especially in the diabetic group.

There are several limitations for interpreting the results from this study. First, we have studied a few numbers of CKD samples when compared with non-CKD samples (approximately 1:4 of CKD vs. non-CKD) in this cohort. Imbalanced sample size may reduce the performance of the ACR strip test and increase the margin of error in this study. Secondly, the urine samples were collected from the wide-ranging parameter to enroll the population in the community into the study. The subjects were various groups instead of specific patients, which had a limitation in prediction of the test performance in specific diseases patients. Lastly, the measurement of ACR was only available from a single measurement. This will affect the imprecision of diagnostic performance of strip test in indicating CKD. KDIGO 2012 guideline recommends that the presence of kidney damage can be assumed by albuminuria. Findings evidence of albuminuria at least 2 occasions for more than 90 days should be classified as having CKD[3].

Conclusion

URiSCAN 2 ACR strip provides the high sensitivity and high negative predictive value but few false negative results to detect albuminuria. Therefore, this ACR strip test might be served as an optimal method for excluding microalbuminuria. However, diagnostic performance of URiSCAN 2 ACR strip test at cut-off of ACR $\geq$30 mg/g is fair for screening CKD in community population.

Take home messages

ACR strip test is the efficient method to rule-out microalbuminuria in random spot urine samples of the general population. It is still necessary to verify the effectiveness of the strip test for detection of microalbuminuria in chronic kidney disease patients in the community.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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ORIGINAL ARTICLE

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Alteration of natural killer cell subset proportion is associated with risk factors of coronary artery disease in aging

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KEYWORDS

Immunosenescence;
NK cell;
Coronary artery disease;
Chronic low-grade inflammation.

ABSTRACT

Currently, the world population is moving to aging society because of decreasing fertility rates and increasing survival in old age. In Thailand, the results of the National Statistics Office showed that the number and proportion of older people increase continuously. Age-related impairment of human immunity involves in many chronic inflammatory diseases such as coronary artery disease (CAD). Thus, age is one of the risk factors for CAD causing the chronic inflammation condition. Natural killer (NK) cell is one of the immune cells that change in aging. Thus, this study aims to investigate the alteration of NK cells and NK cell subsets in aging and their association with the risk factor of CAD. Blood samples from aging (≥ 60 years old) group and who had any risk factors for CAD, were collected. Additionally, blood samples of young group (age < 35 years old) without risk factors associated with CAD were included. Whole blood from these samples were stained with anti-CD3 and anti-CD56 labeled with FITC and PerCP, respectively to determine NK cell subsets by flow cytometry technique. The result showed that the proportion of NK cell subsets (CD56bright/CD56dim) was significantly decreased in aging (p-value < 0.001) and also tended to decrease in aging who had more than or equal to 2 CAD risk factors when compared with aging who had less than 2 CAD risk factors (p-value = 0.051). Thus, this result suggested that alteration of NK cell subsets in aging was associated with the risk factor of CAD and this might be the marker for CAD that needs to be investigated further.

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Introduction

Currently, fertility has decreased and is in contrast with life expectancy. This leads to global aging population growth and progresses to an aging society. In 2015, the proportion of the age population had risen 12%. It is expected to reach 22% in 2050 [1]. In Thailand, the results of the National Statistics Office revealed that the number and proportion of Thai older people increase rapidly and continuously from 6.8% in 1994 to 15.3% in 2014 [2]. Age-associated impairment of the immune system or in terms of immunosenescence leads to the aging immunity becoming more susceptibility to infections [3, 4] and being involved in many low-grade inflammatory diseases. Moreover, Age is one of the risk factors for coronary artery disease (CAD) causing deaths worldwide and especially in developed countries or more than 45% of males global death and 36% in females in 2011[5, 6]. Furthermore, metabolic-related diseases including type 2 diabetes mellitus (T2DM), dyslipidemia and hypertension were accepted to be risk factors of CAD[7-9]. Thus, the immune cells play an important role in these diseases of aging.

Innate and adaptive immunity function together in the immune response. The innate immunity provides the first-line defense against pathogen and drives and regulates the antigen-specific adaptive immunity. Mononuclear phagocytes, neutrophils, and Natural killer (NK) cells are important effector cells in innate immunity. NK cells are bone marrow-derived lymphocytes that characteristic as loss expression of CD3 and positive expression with CD56. Peripheral blood NK cells are present in up to 15% of total lymphocytes in peripheral blood. NK cells can be classified into two subpopulations by the density of CD56 expression [10], CD56dim NK cells and CD56bright NK cells. The major type of NK cell is CD56dim NK cell that is present in about 90% of peripheral blood NK cells involving in cytotoxic activity. These NK cells can produce perforin and granzyme to kill the target cell whereas CD56bright NK cells involve in cytokine production. In the elderly, the NK cells and their subsets were reported to be changed especially the CD56 bright NK cell being declined in advanced age [11]. In addition, the NK cell is also the important immune cell that involves in coronary artery disease.

Previous studies demonstrated that the reduction of the NK cell was associated with CAD patients and related to CAD condition. CD16/CD56 NK cells significantly decreased in CAD patients especially in unstable angina conditions [12, 13]. However, the involvement of the NK cell population changes in aging containing CAD risk factors has still not been reported. This study aimed to investigate the alteration of NK cell populations in the elderly and their association with the risk factors of CAD. We hypothesized that NK cell and their subpopulations would be decreased and these reductions would be involved in chronic inflammation.

Materials and methods

Study populations

Sixty-three aging individual blood samples were collected from Sum Song district, Khon Kaen province including subjects who had aged over or equal 60 years and contained any risk factor associated with CAD (hypertension, dyslipidemia, and diabetes mellitus). Additionally, twenty-seven samples of the young group without the 3 CAD risk factors were included. Subjects who were treated with anti-inflammatory drugs were excluded. All subjects had no diabetic complications, infections, immunological diseases, hematological diseases, malignancies, or inflammatory sickness. This study was approved by the Ethics Committee of Khon Kaen University (HE 622164)

Sample analysis

The blood samples were collected in a fasting state in the morning. WBCs were counted with an automated blood cell counter. Serum triglycerides, total cholesterol, and high-density lipoprotein cholesterol (HDL-C) were determined enzymatically on an automated analyzer system. Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula. The blood glucose samples were treated with a hemolytic reagent and glucose was determined enzymatically with glucose dehydrogenase. Blood pressure (BP) was measured to the nearest five

mm Hg with a mercury sphygmomanometer with the subjects in a supine position having rested for five minutes. All processes were operated at AMS Wellness Center, Faculty of Associated Medical Sciences, Khon Kaen University, Thailand.

Flow cytometry of cell surface expression

Cell surface staining was performed using 100 µl of fresh peripheral blood (within 24 hrs.) which was collected in heparin anticoagulant. The blood samples were stained with monoclonal antibodies (mAbs) conjugated with different fluorochromes as follows: anti-CD3-Fluorescein Isothiocyanate (FITC) (UCHT1, BD PharmingenTM, San Diego, CA, USA) and anti-CD56-Peridinin Chlorophyll Protein Complex (PerCP) (HCD56, BioLegend, San Diego, CA, USA) and then were incubated for 15 min at the room temperature in the dark. Red blood cells were lysed using the BD FACSTM lysing solution (BD Biosciences, San Jose, CA, USA) for 15 min before washing with 1X PBS. The samples were analyzed by flow cytometry using the BD FACSCantoTM II flow cytometer (BD Biosciences). The service was provided by Research Instrument Center, Khon Kaen University, Thailand. Isotype antibodies labeled with FITC and PerCP were also used to stain blood samples and used as the negative control for staining. The proportion of NK cell subset was calculated by using the percentage of CD56bright divided by CD56dim NK cell. The setting of flow cytometer was shown in Figure 1.

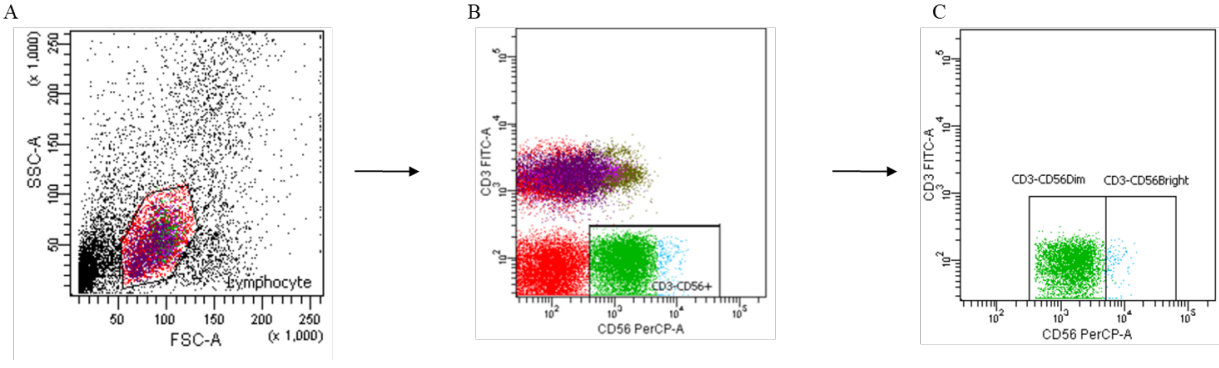

Figure 1 Analysis NK cell subsets by flow cytometer. Whole blood samples were stained with a monoclonal antibody specific to CD3 and CD56 labeled with FITC and PerCP, respectively, and then the stained samples were analyzed by FACSCanto II to determine NK cell subsets. Lymphocyte gate (A) was selected to analyze NK cell population (B) following NK cell subsets based upon the expression of CD56 (C).

Statistical analysis

All data were tested for normal distribution using Kolmogorov-Smirnov test for data set of more than or equal 50 data and Shapiro-Wilk test using for data set of less than 50. Data with normal distribution were explored for significance by Unpaired T-test, whereas non-parametric data were tested by Mann-Whitney U test using the software GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance at p-value < 0.05 was considered.

Results

Clinical characteristics of study subjects

The clinical characteristics of study subjects including total white blood cell count were presented in Table 1. The results showed that body mass index (BMI), systolic and diastolic blood pressure, triglyceride, blood urea nitrogen (BUN), uric acid, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum alkaline phosphatase (ALP), and uric acid were significantly increased. In contrast, high-density lipoprotein (HDL) was reduced in aging.

Table 1 Clinical characteristics of study subjects according to total white blood cell counts

Parameters	Young group Mean ± SD	Aging group Mean ± SD	Reference value*	P-value
Number of subjects	27	63		NA
Age (years)	22.9 ± 5.0	69.6 ± 5.4		<0.0001
BMI (kg/m^2)	21.4 ± 2.1	24.1 ± 4.4	18.5 – 24.99	0.0027
FBS (mg/dl)	84.4 ± 6.3	101.8 ± 45.5	70 – 110	0.0466
Total white blood cell count (cell/µl)	6325.0± 973.5	6722.1 ± 2082.2	4600 – 10600	0.5678
Blood pressure				
Systolic (mmHg)	114.7 ± 9.6	138.0 ± 20.5	100 – 140	<0.0001
Diastolic (mmHg)	70.9 ± 6.3	78.7 ± 12.1	60 – 90	0.0007
Lipid profiles				
Total cholesterol (mg/dl)	189.4 ± 19.8	192.8 ± 50.7	127 – 262	0.9181
Triglyceride (mg/dl)	67.3 ± 32.9	159.6 ± 91.1	10 – 200	<0.0001
HDL (mg/dl)	63.6 ± 17.9	38.7 ± 10.7	> 35	<0.0001
LDL (mg/dl)	113.1 ± 27.5	122.0 ± 41.2	10 – 150	0.5978
BUN	12.1 ± 2.8	14.7 ± 5.5	0.5 – 1.5	0.0208
Creatinine	0.9 ± 0.2	0.9 ± 0.3	0.67 - 1.17	0.5720
Uric acid	5.0 ± 1.2	6.0 ± 1.4	2.7 – 7.7	0.0022
AST	21.5 ± 5.2	31.0 ± 16.7	0 – 40	<0.0001
ALT	15.0 ± 5.8	22.9 ± 11.1	0 – 33	0.0007
ALP	54.6 ± 14.6	64.0 ± 14.3	30 – 120	0.0011

Note: * Reference value from Clinical Immunology and Chemistry Unit at Srinagarind Hospital, Khon Kaen, Thailand.

White blood cell count influence by age

To investigate the influence of age on the changing of white blood cell subtype, subpopulation of white blood cells was compared between young and aging group which is presented in Figure 2. The total numbers of white blood cell count and any absolute count of white blood cell subtype did not differ significantly between groups. The result found that only the absolute eosinophil was statistically significant increased in the aging group (*p*-value=0.0022; Figure 2E).

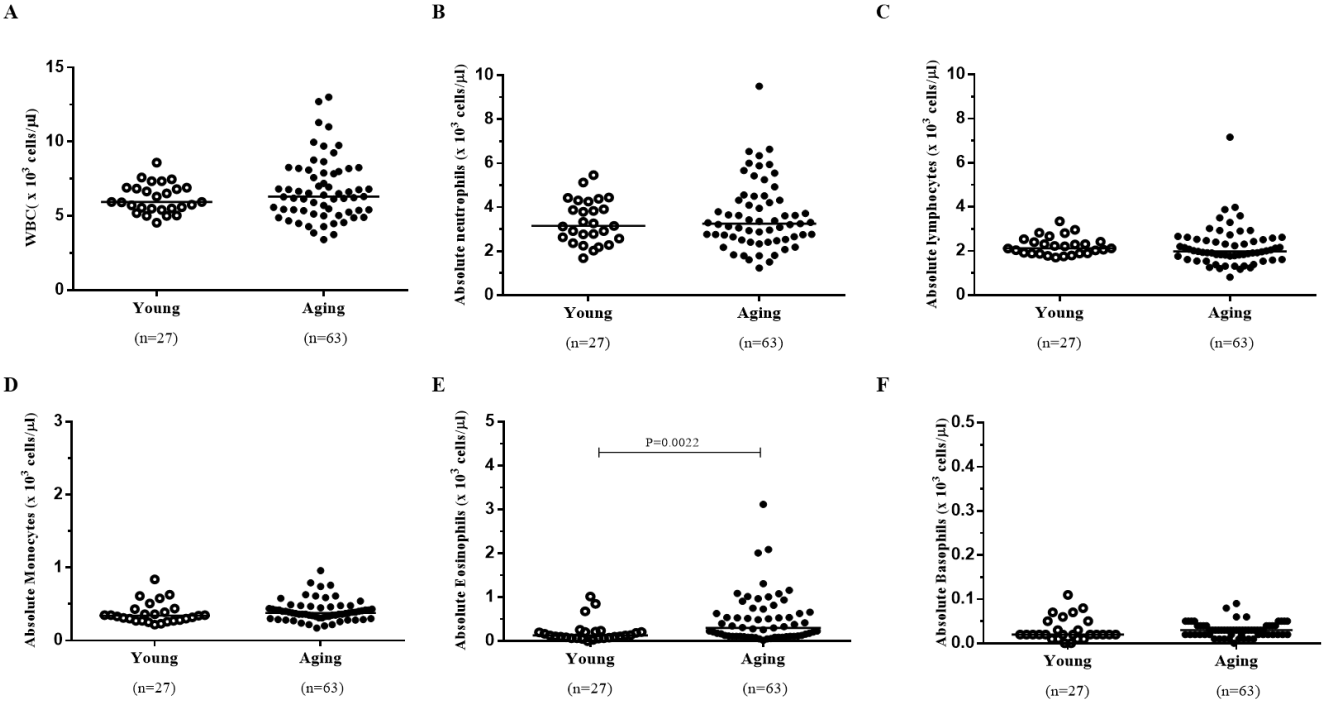

Figure 2 White blood cell count influence by age. Whole blood collected from young and aging individuals were measured for complete blood count at AMS-KKU Excellence Laboratory. Total white blood cell count (A) and the absolute number of each white blood cell types: Neutrophils (B), Lymphocytes (C), Monocytes (D), Eosinophils (E), Basophils (F) was compared between young and aging.

An effect of age on NK cell change

To determine the alteration of NK cells and NK cell subset, whole blood samples from the young and aging group were stained with anti-CD3 and anti-CD56 and detected by flow cytometer. The absolute total NK cell (CD3-CD56+ cell) did not differ significantly between the young and aging group (Figure 3A). The alteration of NK cell subsets found that CD56bright NK cells separated from aging individuals were significantly decreased (p-value<0.0001; Figure 3B.) This was in contrast) with the number of CD56dim NK cells that were slightly increased in the aging group (p-value=0.0446; Figure 3C). Moreover, the proportion of NK cell subsets was also declined in aging (p-value=0.0008; Figure 3D).

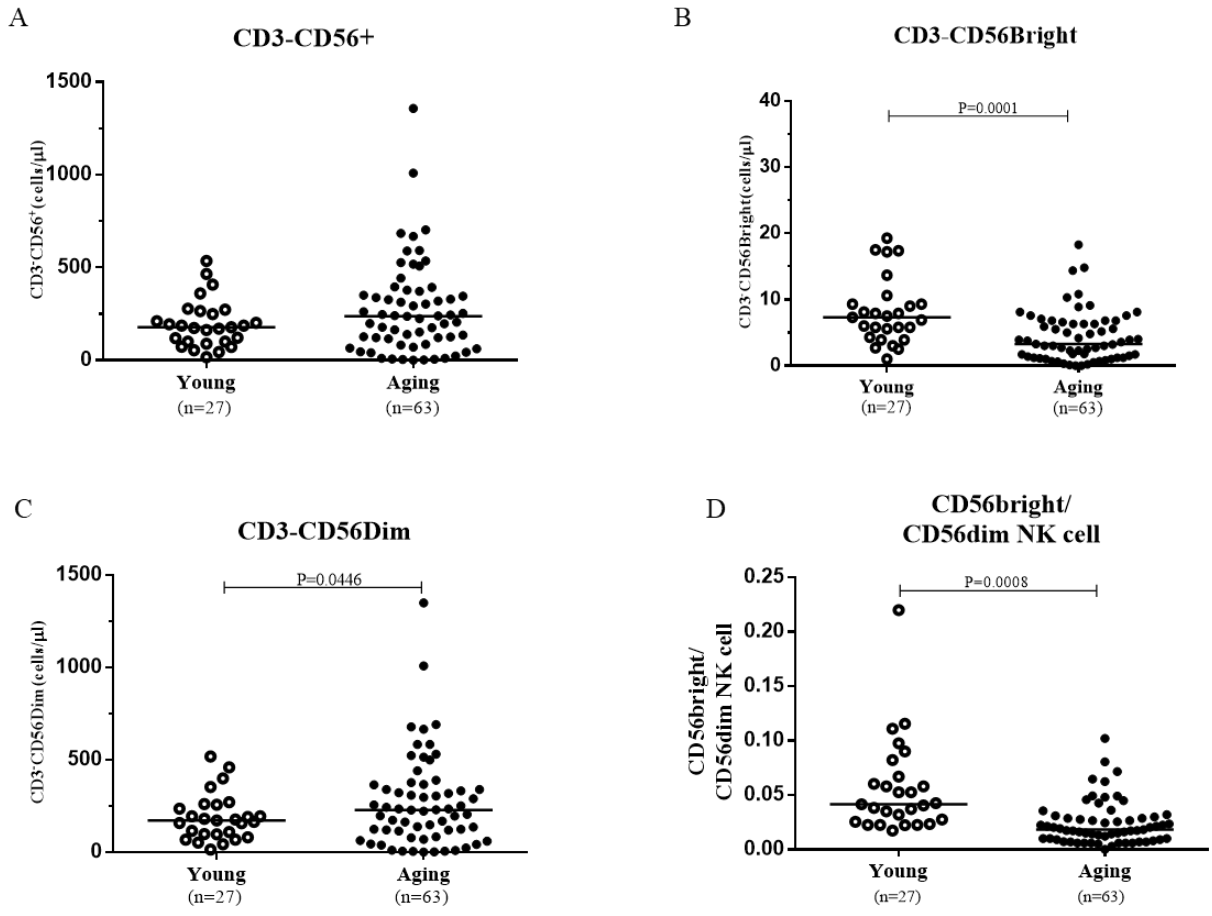

Figure 3 CD3-56+ NK cell and their subset distribution of young group compared with aging. Whole blood from the young and aging group was performed by multicolor staining with antibodies specific to CD and receptor markers and analyzed by FACS Canto II. The absolute number of each population: CD3-CD56+ cell (A), CD3-CD56Bright cell (B), CD3-CD56dim cell (C) was compared between young and aging group. Moreover, NK cell proportion was displayed by CD56bright cell/CD56dim cell ratio (D).

The association of NK cell subset proportion with CAD risk factors

Since hypertension, dyslipidemia, and diabetes mellitus were identified as common manifestation in aging individuals and defined as risk factors of CAD, we divided aging into two groups, aging with less than 2 CAD risk factors (n=30) and aging with more than or equal to 2 CAD risk factors (n=33) to investigate the alteration of NK cell subset influenced by CAD risk factor in aging. The results revealed that the number of overall NK cells and their subsets were not different between two aging groups (data not shown). The proportion of NK cell subsets was decreased in aging with more than or equal to 2 CAD risk factors. (*p*-value=0.0505; Figure 4)

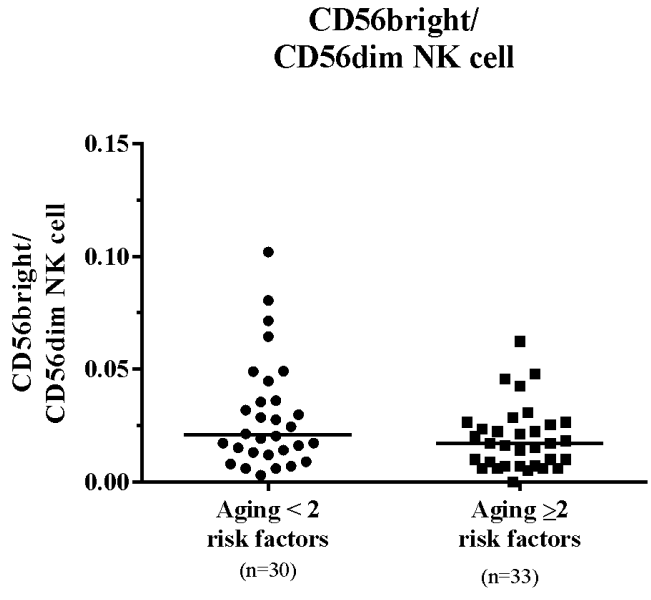

Figure 4 The proportion of NK cell subsets in aging with CAD risk factors. The samples were divided into two groups (aging containing less than 2 CAD risk factors and aging containing more than or equal to 2 CAD risk factors) and analyzed for the proportion of NK cell subsets.

Discussion

Currently, the proportion of aging is rising in several countries around the world. Age is associated with an impairment of the immune system, known as immunosenescence. Generally, aging individuals have the most common manifestation characterized by an elevation of peripheral blood components involving inflammation processes such as acute-phase protein and TNF-α, called chronic low-grade inflammation. These conditions can cause long period tissue damage and involve chronic age-related diseases including type 2 diabetes mellitus, dyslipidemia, and hypertension. Thus, impairment of the immune system in aging involves in many chronic inflammatory diseases such as coronary artery disease. Our study found that age did not influence the alteration of overall white blood cell count and their white blood cell subtypes except in eosinophils that were significantly high in the aging group. However, the previous study by Kubota K et al. suggested that the white blood cell count and platelet count tended to decrease with advancing age [14]. Besides, the previous studies were still a debate about the numbers of neutrophils change with aging. Most studies suggested that with healthy aging there are no changes in their number. Chatta GS et al. indicated that peripheral blood neutrophil and neutrophil precursors in the bone marrow are not lowered in healthy aging individuals[15]. However, the study for eosinophil was still not clear but an elevation of eosinophil in this study may be because the subjects were collected from the area of high prevalence of parasitic infestation[16].

NK cells are important immune cell composition of two subgroups based upon the expression level of CD56 molecule on the cell surface. Thus, there are two groups of NK cells, CD56bright and CD56dim NK cells. To investigate whether the NK cells and their subsets change is influenced by age, we compared the number of overall NK cells and their subsets in the young subject group and aging. Our results demonstrated that although overall NK cells were not changed in old age but both NK cell subsets were affected by age. This was in contrast with the study by Valiathan et al who indicated that the elderly population

presented the highest percentages of peripheral NK cells compared with infant and adulthood [17]. Furthermore, our results also showed that CD56bright NK cells collected from aging individuals were clearly decreased, whereas CD56dim NK cells were slightly high in old age. Interestingly, our study also found that the proportion of NK cell subsets (CD56bright/CD56dim) was reduced in aging. This result was similar to the previous studies showing that NK cells especially CD56bright NK cells were decreased in aging and the proportion of CD56bright/CD56dim NK cells were decreased[11]. Switching of CD56bright NK cells to CD56dim NK cells accumulation may be a marker of immunosenescence process. Moreover, CD94 molecule that involve in cytotoxicity was decreased in both CD56bright NK cells and CD56dim NK cells [18]. These results may cause impairment in NK cell cytotoxicity (NKCC). Thus, the expanding of circulating CD56dim NK cells that occurs in the elderly may be a compensatory mechanism for age-associated decrease in NKCC.

Coronary artery disease (CAD) is a single cause of death in approximately 12% of global death in 2004. Furthermore, age and age-related diseases including type 2 diabetes mellitus, dyslipidemia and hypertension are also risk factors for CAD. To investigate the association of NK cell subset and CAD risk factors, we divided 63 aging individuals into 2 groups based on CAD risk factors including type 2 diabetes mellitus, dyslipidemia, and hypertension, aging with less than 2 CAD risk factors and aging with more than or equal to 2 CAD risk factors. Our results found that overall NK cells and NK cell subsets did not differ in the two age groups. However, the proportion of NK cell (CD56bright/CD56dim) was decreased in aging with more than or equal to 2 CAD risk factors. This finding suggested that a high level of CD56bright/CD56dim ratio might be involved in CAD that needs to be investigated with the high number of sample size and prospective study is needed to determine the biological marker for CAD.

Conclusion

In conclusion, aging is a general phenomenon that has an impact on several immune cells. Our study demonstrates the advancing age is related to CD56dim accumulation and CD56bright depletion. These might be possibly pathogenic involving chronic low-grade inflammation and might be driven to coronary artery disease in advancing age. Thus, our study might suggest that the alteration of NK cell subset in the elderly with CAD risk factor might be a marker for CAD that has to be investigated in the future.

Take home messages

This study determined effects of CAD risk factors; T2DM, dyslipidemia, and hypertension, on NK cell subsets in aging. Our results demonstrated that age and CAD risk factors related to CD56dim alteration and the proportion of CD56bright/CD56dim was reduced. These finding suggested that NK subset alteration might involve CAD progress in aging.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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Effect of acute low-intensity exercise on polymorphonuclear cell function in type 2 diabetes patients with poor glycemic control

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KEYWORDS

Hyperglycemia;
Low physical activity;
Leukocytes;
Phagocytosis;
Oxidative burst.

ABSTRACT

Poorly controlled type 2 diabetes (T2D) patients, defined by glycated hemoglobin (HbA1c) ≥ 8.5%, show an impairment in polymorphonuclear (PMN) cell function leading to prone to infection. Exercise training can improve immune cell function, since it augments resistance to infections. However, there has been no study of PMN cell function following a single bout of low-intensity exercise in T2D patients with poor glycemic control though the exercise is practical for the patients. Therefore, we aimed to evaluate PMN phagocytosis and oxidative burst following a single bout of low-intensity exercise in T2D patients with poor glycemic control. In the experiment, the patients performed a cycling at low intensity for 20 min. Four milliliters of blood samples from the T2D patients (n=10) were collected at rest, immediatelyafter exercise (Post-Exs), and 24 hours after exercise (24h Post-Exs) to investigate PMN cell function. The percentage of PMN phagocytosis and oxidative burst were quantified by a two-color flow cytometric procedure. The phagocytosis was determined after incubation of fluorescein-isothiocyanate (FITC) labeled *Staphylococcus aureus (S.aureus)* with whole blood for 15, 30 and 60 min. Then, the activated cells were simultaneously added with dihydroethidium to determine oxidative burst. The results showed that phagocytosis and oxidative burst were not different immediately and 24 hours after the exercise compared to baseline. However, the function of PMN cell increased with the incubation time (15, 30 and 60 min; *p*-value <0.05) at rest and after the exercise. In conclusion, our findings revealed that a single bout of low-intensity exercise had no effect on PMN cell function in T2D patients with poor glycemic control.

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Introduction

Type 2 diabetes (T2D) is a metabolic disorder defined by insulin resistance and pancreatic beta-cell dysfunction resulting from hyperglycemia[1]. The global prevalence of diabetes in 2019 was estimated to be 9.3% (463 million cases), rising to 10.9% (700 million) by 2045[2]. Recently, perception of diabetes has changed from a chronic metabolic disease to an immune-mediated disease[3]. It is well accepted that function of polymorphonuclear (PMN) cells such as neutrophil of T2D patients including phagocytosis and oxidative burst are impaired[4-6]. The defective innate immune system may underlie patients susceptibility to bacterial infections[5]. Elimination of pathogens by PMN cells follow a sequence of events including chemotaxis, phagocytosis, microbial killing and apoptosis[4,6].

It is well documented that exercise training at low intensity improves the immune system[7,8] such as stimulating the function of PMN cells[9]. The stimulation of PMN cell is particularly crucial, because it constitutes the host defense mechanisms against pathogens[10]. However, only a previous study in rats that investigated the effect of a single bout of low-intensity exercise and showed the increase in phagocytosis of PMN[11]. No human study investigated the effect of acute exercise at low intensity on immune function, although the low-intensity exercise is appropriate for the T2D patients. This information will encourage the diabetes patients who normally have impaired PMN function[12] and sedentary life style to start the exercise training. Unfortunately, mechanisms underlying the immune alteration in human induced by the low-intensity exercise are not reported. Up To our knowledge, a possible mechanism may be due to a decrease in blood glucose concentration induced by the exercise[13]. The purpose of this study was therefore to quantify the effect of a single bout of low-intensity exercise on the PMN cell function in T2D patients with poor glycemic control. We hypothesized that phagocytosis and oxidative burst in the patients are increased immediately after the low-intensity exercise.

Materials and methods

Subjects

Ten T2D patients (male=1, female=9) with poor glycemic control aged between 45-60 years participated in the study after providing written informed consent. To qualify for participation, they were medically diagnosed as having T2D for at least 12 months prior to enrollment and poor glycemic control defined as HbA1c levels ≥ 8.5% (≥ 69 mmol/mol). They had not participated in any regular exercise program for at least 6 months prior to commencement. This study was approved by the Human Ethics Committee of Khon Kaen University (HE 561129). Clinical trial registration number of this study is TCTR20180616002.

Recruitment occurred at Khon Kaen Hospital and Srinagarind Hospital, Khon Kaen Province, Thailand. At baseline, all patients were screened using a health screening questionnaire[14] and blood chemistry including lipid profiles, fasting plasma glucose (FPG), HbA1c levels, liver function and kidney function tests. Exclusion criteria were liver, kidney, or cardiovascular diseases; chronic infections (HIV, TB, Hepatitis); changed types and doses of anti-hypertensive, hypoglycemic, or lipid-lowering drugs prescription; blood pressure higher than 140/90 mmHg; abnormal resting electrocardiogram (EKG); neuromuscular disorders; and orthopedic problems. The characteristics of all patients are shown in Table 1. For optimization of the phagocytosis assay in our study, the heparinized blood samples (4 mL) were taken from non-diabetic volunteers (n=3). The volunteers were not diagnosed as T2D and they had not participated in any regular exercise program for at least six months prior to recruitment.

Research design and protocol

The experiment started with all patients performing a peak oxygen consumption ($\dot{V}o_{2,peak}$) test to determine the work load for the low-intensity exercise (30% $\dot{V}o_{2,peak}$, peak) session. Patients refrained from cigarette smoking, caffeine, alcohol consumption, and heavy exercise for 48 hours with 12 hours of overnight fasting before the experimental day. On the experimental day, one week after from the screening day, the patients

rested for 30 min and then they performed 20 min of cycling at a low intensity.

Blood sampling

Four milliliters of a peripheral venous blood sample from T2D patients were collected into a sodium heparin tube. The sampling was carried out at rest (Pre-Ex), immediately after exercise (Post-Ex), and 24 hours after exercise (24h Post-Ex). The blood samples were stored at 25 °C and measured within 6 hours after collection. For the varying conditions of fluorescein-isothiocyanate (FITC) staining and multiplicity of infection (MOI), samples of the non-diabetic control were obtained from three volunteers.

Peak oxygen consumption ($\dot{V}o_{2,peak}$) test

The peak oxygen consumption ($\dot{V}o_{2,peak}$) was tested with a graded maximal exercise test. Patients began with cycling at free workload for one minute (0 watt) and increasing to 20-30 watts depending on their fitness status[15]. Expired-gas samples, heart rate (HR), and electrocardiography were recorded throughout the test. Moreover, the fatigue and dyspnea symptoms were asked every three minutes and at the end of the test.

Bacterial growth and colony forming units (cfu) counting

Staphylococcus aureus strain ATCC 29223 were used for PMN phagocytosis (provided by Department of Microbiology, Faculty of Associated Medical Sciences, Khon Kaen University). The S.aureus were grown for 12-18 hours at 37 °C in Luria-Bertani (LB) broth and then inoculated into a new LB broth for growth at log phase (OD_{600nm} = 0.6-1). A loopful of the culture was subculture on LB agar for measurement of the colony-forming unit (cfu) which was used to estimate the number of viable bacteria. The bacterial cell concentration was adjusted to 1×10^9 cfu/mL, and stored frozen at -30 °C in a 60% glycerol for using throughout the study.

Labeling S.aureus with FITC fluorescence dye

A lyophilized fluorescein isothiocyanate (FITC) (Thermo Scientific, UK) for bacterial staining was dissolved in dry dimethyl sulfoxide (*DMSO*) at concentrations of 0.1, 0.5, 1 and 2 mg/mL. Then, 500 ul of the diluted dye to the pellet of *S.aureus*, incubated at 25 °C for 60 min in the dark. The varied conditions of FITC were subjected to flow cytometry to quantify mean fluorescence intensity (MFI) values. The highest MFI of FITC-stained *S.aureus* was selected for this study.

Dose of multiplicity of infection (MOI) for phagocytosis

Blood samples from three non-diabetic volunteers were used for optimization of the phagocytosis assay. The optimal condition of phagocytosis was firstly performed in suspension with FITC-labeled *S. aureus* and whole blood at different bacteria/PMN cell ratios (MOI). The bacterial cells were adjusted from MOI5, 10, 30, 50, 70, to 100, whereas the PMN cell were fixed at 3×10^6 cells/mL. After incubation PMN cells with different concentration of MOI for 15, 30, and 60 min at 37 °C, the condition giving the greatest percentage of phagocytosis was used to evaluate the PMN cell function of all T2D patients in our study. Data were analyzed by calculation the average of phagocytosis at 15, 30, and 60 min of incubation times. Finally, the phagocytosis of FITC-labeled *S. aureus* by PMN cell was evaluated in vitro using a flow cytometry.

Assessment of PMN phagocytosis and oxidative burst

The phagocytosis and oxidative burst of PMN cell obtained from T2D patients were measured using whole blood. For the experiment, the frozen *S. aureus* was fresh washed for two times with 1x PBS and stained in the dark with FITC at 25 °C, for 60 min in the dark. Then, the labeled bacteria were washed twice with 1x PBS to remove an excess FITC dye before use. In all heparinized blood samples of T2D patients, a complete blood count analyzer (Sysmex xs-800i, Germany) was used to estimate the number of PMN cell. The whole blood samples were adjusted to a final concentration of PMN cells at 3×10^6 cells/mL with 1x PBS. Phagocytosis was evaluated as follows: a 50 ul of FITC-labeled *S.aureus* was incubated at 37 °C for 15, 30, and 60 min with a 50 uL of the adjusted PMN cell that were collected at rest, immediately after exercise, and 24 hours after exercise. An oxidative burst of PMN obtained from T2D

patients was measured by production of reactive oxygen species (ROS). Following the phagocytosis assay, the reaction mixtures of PMN cells and FITC-labeled *S.aureus* were simultaneously performed to access oxidative burst by adding 3 ug/mL of hydroethidine (50 uL) (Santa Cruz Biotechnology, U.S.A.) at 37 °C for 5 min. The hydroethidine can passively diffuse into granules and was rapidly oxidized to red fluorescent ethidium bromide by the oxidative burst intermediates within the activated PMN cell. Then, the incubation mixtures were washed with 1 x PBS, and centrifuged at 3500 g for 10 min, discarding supernatant. The erythrocytes of the co-culture cells were lysed by adding 1 mL of red blood cell lysis buffer at 25 °C for 20 min. All activated PMN cell reactions were fixed by adding 200 uL of 4% formaldehyde before measurement. The green emission fluorescence of *S.aureus* ingested by PMN cell was quantified by the percentage of phagocytosis, while the red fluorescence of ethidium bromide was quantified by the percentage of oxidative burst. The red fluorescence indicating the oxidative burst was analyzed by the phycoerythrin (PE) channel in flow cytometry. Activation of PMN cell with 3 ug/mL of phorbol-myristate acetate (PMA) (Sigma-Aldrich incorporation, U.S.A.) at 37 °C for 15 min, which induced hydrogen peroxide production due to an oxidative burst was kept as a positive control, while PMN cell without activation were kept as a medium control.

Flow cytometry analysis

The mixture cells were acquired 10,000 events/reaction to quantify the PMN cell function using a BD FACSCanto™ II flow cytometer (BD Biosciences, U.S.A.). The service was provided by the Research Instrument Center (RIC), Khon Kaen University, Thailand. The PMN population was identified according to its specific forward- and side-scatter patterns. Data were presented as the percentage of phagocytosis and oxidative burst at 15, 30, and 60 min of incubation times at rest, immediately after exercise, and 24 hours after exercise. Flow cytometric data were analyzed with BD FACSDivas™ software (BD Biosciences, U.S.A)

Statistical analysis

Normal distribution of the data was tested using Shapiro-Wilk normality test. Data are expressed as means ± standard error (SE) as percentage of phagocytosis and oxidative burst using Prism software (version 5.0, GraphPad Software Inc., U.S.A.). Comparisons among conditions of FITC staining and dose of MOI were analyzed with the paired two-tailed Student's t-test. The effect of exercise on PMN cell function of T2D patients was determined at rest, immediately, and 24 hours after exercise using a repeated measure ANOVA with Bonferroni's post-hoc test (SPSS version 17.0 Inc., U.S.A. USA). Data were regarded as significant when *p-value* <0.05.

Using mean and SE from the study of Ortegaet al.[10], at least 10 patients were required to identify significant differences at the 5% level, and Cohen's d effect size was 0.23. The power was calculated based on the different means of the percentages of PMN cell phagocytosis between two dependent groups using the G Power (version3.1) software[16]. Apparently, the power (1-β) was 0.95 or 95%.

Results

Optimization of the FITC fluorescence dye for bacterial labeling

To determine an optimal concentration of FITC-labeled *S. aureus*, the data obtained with different FITC labeling after incubation of *S. aureus* with a solution of FITC from 0.1-2 mg/mL are shown in Figure 1. The results showed that a marked at 1 mg/mL of FITC labeling presented the highest value of MFI in comparison with lower concentrations of FITC. Moreover, after incubation of *S.aureus* with the FITC concentration of 2 mg/mL, it showed a stable MFI compared to 1 mg/mL of FITC (*p*-value >0.05). Therefore, we obtained a sufficient labeling of bacteria with 1 mg/mL of FITC as an optimal concentration used in this study.

Comparisons of PMN phagocytosis using different multiplicity of infection

To optimize a flow cytometric method for phagocytosis, we began by assessing a series of MOI to investigate the highest percentage of phagocytosis for using throughout this study (Figure 2). The highest percentage of PMN cell phagocytosis is presented when incubation at MOI30 compared to those with lower MOI. Moreover, at MOI30, the percentage of phagocytosis reached a plateau state. Moreover, Figure 2 demonstrates that the percentage of phagocytosis showed no significant difference between MOI30 and MOI50, 70, and 100 (*p*-value >0.05). Therefore, the MOI30 was chosen as the endpoint of the experiments.

Table 1 Characteristics of the patients with type 2 diabetes mellitus

Patients ID	Age (yr)	BMI (kg/m^2)	%HbA1c (mmol/mol)	FBG (mmol/L)
SN-08	60	24	8.8 (73)	7.4
SN-09	60	26	11.0 (97)	13.3
SN-12	60	25	9.9 (85)	5.7
SN-15	60	28	9.6 (81)	11.8
SN-16	51	28	11.9 (107)	7.8
SN-17	46	28	9.3 (78)	7.5
SN-18	59	28	9.5 (80)	8.3
SN-20	53	18	12.6 (114)	17.6
SN-21	54	23	8.8 (73)	13.8
SN-22	59	29	12.0 (108)	17.3
Mean*	56±2	26±1	10±1 (90±5)	11±1

Note: N=10 (male=1 or female=9), *Data are shown as mean ± SE. Abbreviations; BMI: body mass index; HbA1c: glycated hemoglobin A1c, FBG: fasting blood glucose.

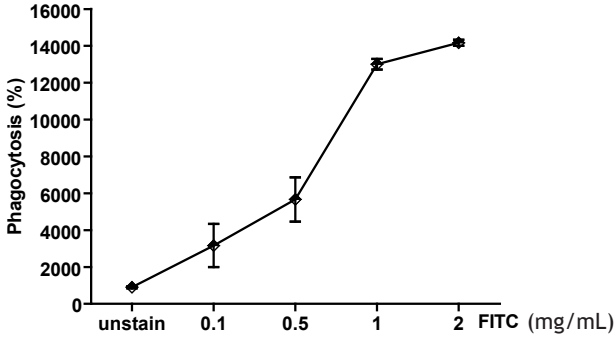

Figure 1 Variation of fluorescein-isothiocyanate (FITC) concentration for bacterial cell staining

Bacterial cells were labeled with varying concentrations of FITC from 0.1-2 mg/mL, for 60 min in the dark. The difference in the mean fluorescence intensity (MFI) of each concentration was plotted in mean ± SE. The highest MFI was observed after incubation of *S.aureus* with 1 mg/mL of FITC. Comparison between 1 and 2 mg/mL of FITC, the results showed no significant difference in the MFI values (*p*-value > 0.05).

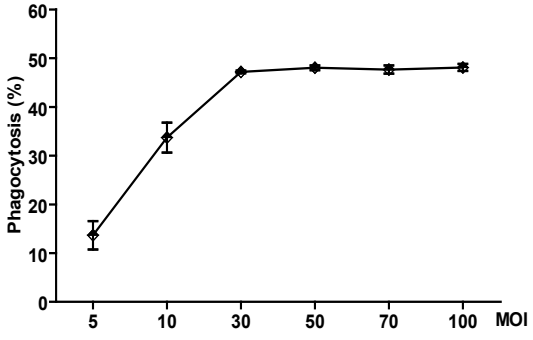

Figure 2 Effect of increasing dose of multiplicity of infection (MOI) on PMN phagocytosis

Phagocytosis of *S.aureus*-FITC by PMN cells (3 x 10^6 cells/mL) was incubated for 15, 30, and 60 min with a different MOI. Each MOI, the data were analyzed as the average of phagocytosis (%) after incubation of PMN cells with FITC-labeled *S.aureus* for 15, 30, and 60 min. The results presented the peak height of phagocytosis after the incubation at MOI30. We additionally analyzed the phagocytosis at MOI50, 70, and 100 compared to MOI30 and the results showed no significant increase in PMN phagocytosis (*p*-value > 0.05). The data were presented as the mean ± SE of the control blood samples (n=3).

Effect of exercise on PMN cell function

After determining the optimal condition for PMN cell function, we then evaluated the effect of low-intensity exercise on phagocytosis and oxidative burst. According to incubation of PMN cell with labeled *S.aureus* for 15, 30, and 60 at MOI30, the results showed a significant time-dependent increase in PMN phagocytosis and oxidative burst (*p*-value <0.05) (Figure 3). The incubation time at 60 min presented a higher percentage of phagocytosis and oxidative burst when compared with 30 and 15 min, respectively. Moreover, the PMN cell function significantly increased in 30 min compared to 15 min. To quantify the effect of exercise-induced PMN cell function, the percentages of phagocytosis and oxidative burst of T2D patients were investigated Post-Exs and 24 hours Post-Exs compared to rest. We found no significant exercise-dependent increase in PMN phagocytosis and oxidative burst (Figure 3A, 3B, respectively). The data showed no significant changes in comparison between rest and Post-Exs, rest and 24 hours Post-Exs, and Post-Exs and 24 hours Post-Exs, respectively (*p*-value >0.05). The pairwise layouts of all T2D patients which were additionally analyzed to investigate the PMN cell function are shown in Figure 3C, 3D, respectively.

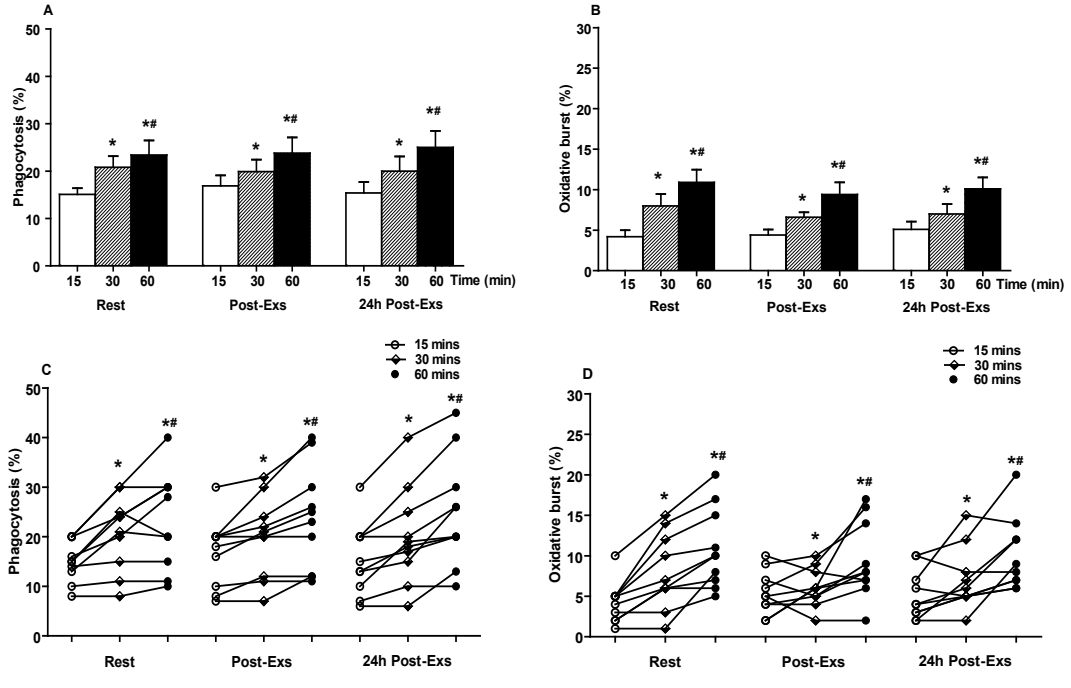

Figure 3 Effect of exercise on polymorphonuclear (PMN) phagocytosis and oxidative burst

A trend of PMN phagocytosis and oxidative after incubation of PMN cells with FITC-labeled *S.aureus* at 15 (white bar), 30 (striped bar), and 60 min (black bar)are shown as mean ± SE (n=10), in the bar graph (A, B). We observed time-dependent increases in phagocytosis (A) and oxidative burst (B). Additionally, the pairwise layouts of individual T2D patient were plotted (C, D) to determine the PMN cell function after incubation at 15 min (white circle), 30 min (square), and 60 min (black circle). *Significant difference from 15 min (*p*-value<0.05); #Significant difference compared between 30 min and 60 min (*p*-value <0.05). To investigate the effect of exercise-induced PMN cell function, the percentages of phagocytosis and oxidative burst were compared among rest, immediately-(Post-Exs), and 24 hours-after exercise (24 hours Post-Exs). There were no significant increases (*p*-value >0.05) in the PMN cell function at any time of exercise. The repeated measures ANOVA and Bonferroni were used to compare the data.

Correlations of BMI, HbA1c, FBG, and* PMN *cell function

According to the characteristic data in Table 1, we additionally analyzed the correlations between BMI-HbA1c, BMI-FBG, HbA1c-FBG (data not shown). We did not find significant correlations in any variables (*p*-value >0.05). The correlations of PMN cell function with BMI, HbA1c, and FBG were herein evaluated. The data presented that there were no significant correlations between PMN cell function and other variables (*p*-value > 0.05, data not shown).

Discussion

In this article, we tested the hypothesis that an acute low-intensity exercise induced PMN cell function of T2D patients with poor glycemic control. The findings did not support our hypothesis because we did not find the effect of a single bout of 20-min low-intensity exercise on PMN phagocytosis and oxidative burst in the patients.

We detected phagocytosis and oxidative burst of PMN cell by combination of FITC-labeled *S.aureus* and HE-labeled ROS production respectively, using flow cytometry procedure[17-19]. We have evaluated the PMN cell function from whole blood which then the PMN cell were diluted and fixed the final concentration at 3×106 cell/mL for all samples before testing. The fixed concentration of PMN cells is important for the reliability of the procedure[17-19]. The mixture cells acquired 10,000 events/reaction to quantify the PMN cell function using a flow cytometry. In this study, the quality control of the instrument was performed using Cytometer Setup & Tracking Beads (BD Biosciences, no. 641319, San Jose, CA, USA) which was daily calibrated to provide the accuracy and precision of this study. The laser source of the flow cytometry was also warm-up before star-up to allow reliable stabilization of the system. Therefore, this method was reliable, practicable and powerful to quantify PMN cell function.

Patients with T2D are known as being in an immunocompromised state that leads to increased susceptibility to infections[20]. It has been established that PMN cells play a vital role in the first stage for defense against microbial infection. The mechanisms of PMN cell that are important in microbial killing are migration to the site of infection, phagocytosis, and killing microorganisms[21]. Activated PMN clearance is completed following apoptosis from phagocytosis by macrophages leading to inflammation[22]. As mentioned from the previous data, PMN cells from poor glycemic control (HbA1c ≥ 8.5% or ≥ 69 mmol/mol) patients with diabetes had impaired phagocytosis and oxidative burst, reduced the migration response to IL-8 and the inability to delay apoptosis[4]. A study mentioned that T2D patients with poor glycemic control is powerfully associated with serious infections and should be more prone to infection compared with non-diabetic control, and good glycemic control[23]. The treatment goal of T2D is to maintain the blood glucose level to delay its complications[23]. In addition to taking medications, a good nutritional plan and optimal exercise program are generally recommended for T2D patients[24].

A randomized controlled trial showed that exercise training significantly reduced upper respiratory tract infections (URTI)[25]. The improved phagocytosis of PMN may reduce URTI after the training. However, the study

examined the effect of exercise training at moderate-intensity. Besides, some previous studies reported that acute exercise at moderate and high intensity activated PMN cells function[26,27]. The prolonged exercise at moderate intensity could stimulate the signaling pathway of NADPH-oxidase and myeloperroxidase causing migration and ROS production of PMN cells[26]. The other study in intense exercise found that activation of PMN cells increased the antimicrobial peptides (AMPs) concentration[27].Only a previous publication that investigated the effect of a single bout of low-intensity exercise in rats showed the increase in phagocytosis of PMN cell[11]. Unfortunately, no human study investigated the effect of acute exercise at low intensity on immune function. We suggest that exercise may have effect on PMN function via the blood glucose concentration. Briefly, exercise increases glucose uptake resulting in decreasing blood glucose concentration. Jafar et al (2016) suggested that high blood glucose concentrations inhibit neutrophil migration and function, decrease phagocytosis capacity, and impair immunoglobulin-mediated opsonization of bacteria[13]. It is comparable to our study since we did not find any changes in blood glucose concentration following the exercise. This may result in unaltered PMN cell function after the exercise. Unfortunately, we did not measure the effects of acute low-intensity exercise on PMN phagocytosis and oxidative burst in non-diabetic individuals and good glycemic control patients. It may be worth further investigating the exercise-induced PMN cell function in both groups. The research results may provide useful knowledge to prevent or reduce infection risk of the people.

Furthermore, the people with age older than 65 years were shown to have impaired PMN cell function[28,29]. Accordingly, different results of the effect of low-intensity exercise on the PMN cell function in the older people may be shown. Importantly, it is worth examining the PMN cell function following the exercise in the patients older than 65 years.

A limitation of this study seems to be the short duration of the exercise. A recommended duration of exercise for patients with T2D is at least 30 min[30]. Thus, a further research exploring the acute or training effect of low-intensity exercise for 30-60 min on PMN cell function is needed.

Conclusion

In conclusion, we show that a single bout of 20-min low-intensity exercise did not have any effects on PMN cell function in T2D patients with poor glycemic control. We suggest that a longer duration of the exercise bout may increase the PMN cell function in these patients.

Take home messages

- Polymorphonuclear (PMN) phagocytosis and oxidative burst are impaired in T2D patients with poor glycemic control.
- A longer duration of the exercise bout may increase the PMN cell function in these patients.
- The PMN cell functions did not change in response to a single bout of low-intensity exercise in the patients.

Conflicts of interest

All authors declare no conflicts of interest.

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ORIGINAL ARTICLE

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Impact of *G-6-PD*, *SLCO1B1* and *UGT1A1* variants on severity of neonatal hyperbilirubinemia in Northeastern Thailand

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KEYWORDS

G-6-PD; *SLCO1B1*; *UGT1A1*; Northeastern Thailand; Neonatal hyperbilirubinemia.

ABSTRACT

Neonatal hyperbilirubinemia is a common complication in Thailand. The polymorphisms of *SLCO1B1* (encoding solute carrier organic anion transporter 1B1) and *UGT1A1* (uridine diphosphate glucuronosyltransferase 1A1) as well as *G-6-PD* mutations associated with glucose-6-phosphate dehydrogenase deficiency have been reported as genetic risk factors for this condition. This study investigated the association between these genetic variations with severity of neonatal hyperbilirubinemia in northeastern Thai newborns. Neonates (n = 204) with hyperbilirubinemia were analyzed for common *G-6-PD* mutations and polymorphisms of *SLCO1B1* c.388G>A, *SLCO1B1* c.521T>C and *UGT1A1* g.-3279T>G using restriction fragment length polymorphism-PCR assay. *G-6-PD* mutations are significant genetic risk factors for severe neonatal hyperbilirubinemia indicated by significantly higher peak total serum bilirubin (coefficient = 0.93, 95% CI: 0.22-1.64, *p-value* = 0.011), longer duration of phototherapy (coefficient = 14.45, 95% CI: 6.92-21.99, *p-value* = 0.0001), early (≤48 hours) onset of hyperbilirubinemia (OR = 2.29, 95% CI: 1.22-4.31, *p-value* = 0.010) and more hospital readmission (OR = 4.13, 95% CI: 1.09-15.67, *p*-value = 0.037). *SLCO1B1* c.388G>A, *SLCO1B1* c.521T>C and *UGT1A1* g.-3279T>G polymorphisms were present in northeastern Thai neonates with allele frequencies similar to those of other Asian populations, but they were not associated with severity of neonatal hyperbilirubinemia. These findings indicate that if genetic factors impacting on neonatal hyperbilirubinemia are to be more fully understood, a larger cohort study of these genetic variations and other pertinent genes involved in neonatal bilirubin will be needed.

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Introduction

Hyperbilirubinemia (jaundice) is a common clinical manifestation of newborns during the first week of life, associated with a variety of maternal and neonatal factors, such as blood group (ABO or Rh) incompatibility, prematurity, birth trauma, infection, Asian ethnicity, breastfeeding, maternal diabetes, and previous sibling with hyperbilirubinemia[1]. However, in the majority of cases, the underlying cause of hyperbilirubinemia remains unidentified, but gene variants have been increasingly recognized as risk factors of neonatal hyperbilirubinemia.

Bilirubin is produced mainly from degradation of hemoglobin in senescent erythrocytes. The free (unconjugated) form of bilirubin is water-insoluble and is bound to albumin in the plasma. It is taken up by hepatocytes through predominantly organic anion transporter polypeptide 1B1 (OATP1B1) [encoded by *solute carrier organic anion transporter family member 1B1* (*SLCO1B1*)] and then conjugated with glucuronic acid by uridine 5'diphospho (UDP)glucuronosyltransferases (UGT) [encoded by *UDP-glucuronosyltransferase 1A1* (*UGT1A1*)], before being secreted into bile[2]. Therefore, variations of genes involved in bilirubin metabolism as well as erythrocyte life span, such as *G-6-PD*, are important candidate genes associated with neonatal hyperbilirubinemia. Moreover, two genome-wide association studies (GWAS) for serum bilirubin metabolism identified three loci of significance, namely, *UGT1A1*, *SLCO1B1* and *G-6-PD*[3, 4].

G-6-PD is the rate-limiting enzyme of the pentose phosphate pathway, which provides reduced nicotinamide adenine dinucleotide phosphate (NADPH) essential for all cells including erythrocytes. Limited production of NADPH increases susceptibility of erythrocytes to oxidative stress, which may shorten their life span and lead to low-grade hemolysis causing an increase in bilirubin production[5]. G-6-PD deficiency is considered as risk factor of neonatal hyperbilirubinemia in the American Academy of Pediatrics clinical practice guideline for management of hyperbilirubinemia in newborns[6]. G-6-PD deficiency is highly prevalent in malaria-endemic regions including Africa and Southeast Asia[7]. In Thailand, prevalence of G-6-PD deficiency among neonates with hyperbilirubinemia is as high as 22.1% in males and 10.1% in females[8].

Of the genes involved in bilirubin metabolism, *UGT1A1* has been widely studied as it is the key enzyme for bilirubin conjugation. Three major polymorphisms, namely insertion of TA (TA_7) in promoter TATA box, c.211G>A and g.-3279T>G, have been identified in different populations. Meta-analysis showed TA_7 and c.211G>A mutations significantly increase the risk of neonatal hyperbilirubinemia in both Caucasian and Asian populations[9]. However, more recent meta-analyses have reported different conclusions: Mehrad-Majd *et al*. confirmed c.211G>A polymorphism significantly increases the risk of neonatal hyperbilirubinemia in Asian population, but results in Caucasian population require further well-designed epidemiological investigation[10]. Li and Zhang demonstrated TA_7 polymorphism may not be associated with risk of neonatal hyperbilirubinemia[11]. In 2002, Sugatani *el al*. identified another polymorphic mutation, g.-3279T>G, in the *UGT1A1* promotor, located in phenobarbital responsive enhancer module (gtPBREM) and reduces transcriptional activity of *UGT1A1* promotor by 60%[12]. Several other studies demonstrated g.-3279T>G polymorphism is a genetic risk factor for neonatal hyperbilirubinemia[13-15].

Variations in *SLCO1B1* may predispose individuals to hyperbilirubinemia due to limitation in hepatic bilirubin uptake[16]. A recent meta-analysis demonstrated c.388G>A polymorphism is a risk factor for developing neonatal hyperbilirubinemia in Chinese neonates, but not in white, Thai, Brazilian, or Malaysians populations, and c.521T>C variant provides protection for neonatal hyperbilirubinemia in Chinese neonates, but not in white, Thai, Brazilian, or Malaysian populations[17].

Neonatal hyperbilirubinemia occurs more frequently and with greater severity among Asians, especially Southeast and Far East Asians, including Thais[18]. In Thailand, studies on genetic risk factors of neonatal hyperbilirubinemia are limited. Prachukthum *et al*. reported G-6-PD deficiency and *UGT1A1* c.211G>A, but not *SLCO1B1*

c.388G>A, polymorphisms are associated with neonatal hyperbilirubinemia[19]. In addition, in northeast Thailand *UGT1A1* c.211G>A and TA_7 promotor mutations are associated with higher peak total serum bilirubin (TSB) in G-6-PD deficient neonates with hyperbilirubinemia[20]. However, prevalence of *SLCO1B1* c.388G>A, *SLCO1B1* c.521T>C and *UGT1A1* g.-3279T>G polymorphisms have not been investigated in northeastern Thailand. Here, prevalence of these polymorphisms and impact of these gene variants as well as *G-6-PD* variants on severity of neonatal hyperbilirubinemia in northeastern Thailand were investigated.

Materials and methods

Study subjects

Based on previous studies on prevalence of genetic polymorphisms in Southeast Asia,[13, 19, 21] sample size was statistically calculated at 204. Leftover EDTA blood samples of near full term and full term neonates (gestational age 35 - 42 weeks) with hyperbilirubinemia were collected from the Diagnostic Microscopy Unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand from October 2016 to March 2018. Neonatal hyperbilirubinemia is defined according to the American Academy of Pediatrics Guidelines 2004[6]. Demographic and clinical data of subjects acquired from medical records by a pediatrician were gender, gestation age, birth weight, delivery method, type of feeding (exclusive breast-feeding, formula or combined), percent weight loss, and peak TSB. Neonates with known risk factors of hyperbilirubinemia, viz. gestational age <35 weeks, birth weight <2000 g, cephalhematoma, ABO incompatibility, maternal diabetes, and infection were excluded. The study protocol was approved by the Institutional Review Board of Khon Kaen University (HE591531).

Molecular analysis of gene variants

Genomic DNA was isolated from whole blood using DNAzol reagent kit (Invitrogen, Carlsbad, USA); concentration and purity ($A_{260\ nm}/A_{280\ nm}$ = 1.8 - 2.0) were determined employing an Eppendorf BioSpectrometer (Eppendorf, Hamburg, Germany) and adjusted to 10 ng/µL. PCR-restriction fragment length polymorphism (RFLP) assay was carried out to detect common *G-6-PD* mutations and *SLCO1B1* c.388G>A, *SLCO1B1* c.521T>C and *UGT1A1* g.-3279T>G polymorphisms. The seven common *G-6-PD* variants present in northeastern Thai population, namely, *G-6-PD* Canton (c.1376G>T, Arg459Leu), Chinese-4 (c.392G>T, Gly131Val), Chinese-5 (c.1024C>T, Leu342Phe), Kaiping (c.1388G>A, Arg463His), Mahidol (c.487G>A, Gly163Ser), Union (c.1360C>T, Asp454Cys), and Viangchan (c.871G>A, Val291Met), were detected as previously described[8, 22]. Identification of *SLCO1B1* c.388G>A, *SLCO1B1* c.521T>C and *UGT1A1* g.-3279T>G polymorphisms was performed in a 50-µL reaction mixture containing 10x ASPCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 30 mM $MgCl_2$, and 0.01% gelatin), 1.0 U *Taq* DNA polymerase (New England Biolabs, Ipswich, USA), 20 ng of each primer,[19, 23, 24] 10 mM dNTPs and 50 ng of DNA template. Thermocycling (conducted in TProfessional thermocycler; Biometra, Gottingen, Germany) conditions were as follows: 94°C for 5 minutes; 35 cycles of 94°C for 1 minute, 60°C (for *UGT1A1* g.-3279T>G) or 62°C (for *SLCO1B1* c.388G>A and *SLCO1B1* c.521T>C) for 1 minute and 72°C for 1 minute; and a final step of 72°C for 10 minutes. Then, a 5-µL aliquot of PCR solution was incubated with 5 U restriction enzymes (New England Biolabs, Ipswich, USA), *Dra* I for *UGT1A1* g.-3279T>G, *Taq*α I for *SLCO1B1* c.388G>A and *Hha* I for *SLCO1B1* c.521T>C, in a 20-µL reaction solution at 37°C for *Dra* I and *Hha* I or 65°C for *Taq*α I for 2 hours. A 7-µL aliquot from each reaction solution was subjected to 3% agarose gel-electrophoresis, stained with ethidium bromide and visualized under UV illumination. Two samples of wild type, homozygote and heterozygote of each gene polymorphism were randomly selected to be confirmed by DNA sequencing (First BASE Laboratories, Selangor, Malaysia).

Statistical analysis

Statistical analysis was performed using STATA software version 10.1 (StataCorp LLC, College Station, USA). Genotype frequency of each genetic variant was calculated as percent total samples and allele frequency as percent total

alleles. Association between genetic variations and continuous variables including peak TSB and duration of phototherapy were evaluated by multiple linear regression analysis. Multiple logistic regression analysis was applied to test association between genetic variations and categorical variables (onset of hyperbilirubinemia ≤48 hours, requirement of phototherapy and hospital readmission). Significance of association was demonstrated either by coefficient value or odds ratio (OR) with 95% confidence interval (CI). Statistical significance is accepted at *p*-value <0.05.

Results

Demographic and clinical characteristics

Demographic and clinical characteristics of the 204 neonates are presented in Table 1. There were twice as many male (64.7%) as female (35.3%) neonates, with 45.6% delivered by Cesarean-section, and with average gestational age and birth weight within normal limits[6]. However, a minority of neonates (14.2%) suffered >10% weight loss. Almost all infants (98.5%) were exclusively breastfed. Average ± SD peak TSB was 14.8 ± 2.6 mg/dL, indicating hyperbilirubinemia[16] and the majority of neonates (83.8%) required phototherapy.

Frequency of gene variants

Genotype and allele frequencies of gene variations are presented in Table 2. *UGT1A1* g.-3279T>G polymorphism was the most common (G allele frequency 40.4%) in the studied population, followed by *SLCO1B1* c.388G>A polymorphism (A allele frequency 22.3%), then *SLCO1B1* c.521T>C polymorphism (C allele frequency 10.3%). The observed allele frequencies of *UGT1A1* g.-3279T>G, *SLCO1B* c.388G>A and *SLCO1B1* c.521T>C polymorphisms were in Hardy-Weinberg equilibrium (*p*-value 0.51, 0.59 and 0.91, respectively). *G-6-PD* variants were detected in 50.5% of neonates, with 37.2% hemizygotes, 11.8% heterozygotes and 1.5% homozygotes.

Gene variants and severity of neonatal hyperbilirubinemia

Multiple linear regression analysis indicated *G-6-PD* variant is a significant genetic predictor of higher peak TSB (coefficient = 0.93, 95% CI: 0.22-1.64, *p-value* = 0.011) and longer duration of phototherapy (coefficient = 14.45, 95% CI: 6.92-21.99, *p-value* = 0.0001) while *UGT1A1* g.-3279T>G, and *SLCO1B1* c.388G>A and c.521T>C polymorphisms are not significant genetic predictors for these parameters (Table 3).

Multiple logistic regression analysis also confirmed *G-6-PD* variant is a significant genetic risk factor for early onset (≤48 hours) of hyperbilirubinemia (OR = 2.29, 95% CI: 1.22-4.31, *p-value* = 0.010) or hospital readmission due to hyperbilirubinemia (OR = 4.13, 95% CI: 1.09-15.67, *p-value* = 0.037) but not of requirement for phototherapy. *UGT1A1* g.-3279T>G, *SLCO1B1* c.388G>A and c.521T>C polymorphisms are not significant genetic risk factors for any of these conditions (Table 4).

Table 1 Demographic and clinical characteristics of neonates with hyperbilirubinemia in northeastern Thailand (n = 204)

Characteristics	Result
Peak TSB (mg/dl)	14.8 ± 2.6
Gestational age (week)	38.0 ± 1.4
Birth weight (g)	3,083.6 ± 431.7
Sex	
- Male	132 (64.7)
- Female	72 (35.3)
Delivery mode	
- Normal	102 (50)
- Cesarean section	93 (45.6)
- Vacuum extraction	6 (2.9)
- Forceps extraction	1 (0.5)
- No record	2 (1)
Type of feeding	
- Exclusive breast feeding	201 (98.5)
- Combined breast and formula feeding	1 (0.5)
- No record	2 (1)
Body weight loss	
- ≤10%	168 (82.4)
- >10%	29 (14.2)
- No record	7 (3.4)
Phototherapy	
- Yes	171 (83.8)
- No	33 (16.2)

Note: Data are presented as mean ± standard deviation or n (%).

Discussion

The study confirms and expands our previous report on the relationship between G-6-PD deficiency, *UGT1A1*(TA_6/TA_7) and *UGT1A1* c.211G>A with neonatal hyperbilirubinemia in northeastern Thai newborns. We have determined the frequency of *SLCO1B1* c.388G>A and c.521T>C, and *UGT1A1* g.-3279T>G polymorphisms in the northeasternThai newborns with hyperbilirubinemia. Furthermore, we have examined the association of *G-6-PD* variant and these polymorphisms with severity of neonatal hyperbilirubinemia.

G-6-PD deficiency is well documented as a risk factor for severe neonatal hyperbilirubinemia[25]. The present study confirmed *G-6-PD* variant is associated with more severe hyperbilirubinemia (higher peak TSB than average), longer duration of phototherapy, early onset (≤48 hours) of hyperbilirubinemia, and more hospital readmission. These findings were consistent with previous studies of neonates in northeastern Thailand[20] and with those reported from African American male newborns with G-6-PD deficiency[26] and newborns in Taiwan with G-6-PD deficiency[27]. In the present study, neonates with G-6-PD variants did not reach a statistically significant risk factor for requirement of phototherapy. This might be due to the majority (83.8%) of neonates with hyperbilirubinemia in our setting received phototherapy. Although hemolysis is a major etiologic factor in G-6-PD deficiency-related neonatal hyperbilirubinemia,[28] severity of neonatal hyperbilirubinemia is the result of complex interactions among various mutant genes, especially those involved in bilirubin metabolism, and environmental factors[25].

To the best of our knowledge, the present study is the first to report the presence of *UGT1A1* g.-3279T>G polymorphism in Thai neonates. The allele frequency of *UGT1A1* g.-3279G in newborns with hyperbilirubinemia (40.4% or 0.404) was similar to those reported in Egyptians (0.49),[15] Indians (0.55-0.58)[14, 29] and Malays (0.49)[13]. Although *UGT1A1* g.-3279G allele frequency is significantly higher in neonates with hyperbilirubinemia than that of control neonates, there is no statistically significant association with severity of this condition.

Table 2 Genotype and allele frequencies of *UGT1A1*, *SLCO1B 1*, and *G-6-PD* variants

Gene variant	Number	Genotype frequency (%)	Allele frequency (%)	P_{HWE}
***UGT1A1* g.-3279 T>G**				
T/T	75	36.8		
T/G	93	45.6		
G/G	36	17.6		
T allele	243		59.6	0.51
G allele	165		40.4	
***SLCO1B1* c.388 G>A**				
G/G	125	61.3		
G/A	67	32.8		
A/A	12	5.9		
G allele	317		77.7	0.59
A allele	91		22.3	
***SLCO1B1* c.521 T>C**				
T/T	164	80.4		
T/C	38	18.6		
C/C	2	1.0		
T allele	366		89.7	0.91
C allele	42		10.3	
***G-6-PD* variant**				
Hemizygote	76	37.2	NA	NA
Heterozygote	24	11.8	NA	NA
Homozygote	3	1.5		
Total	103	50.5		

Note: NA, not applicable due to G-6-PD deficiency is an X-linked disorder; P_{HWE}, p-value of Chi-Square test for Hardy-Weinberg equilibrium.

Yusoff *et al*. reported among Malay infants with hyperbilirubinemia there is no significant difference in mean peak TSB or early onset of hyperbilirubinemia among those carrying *UGT1A1* g.-3279T>G of various genotypes[13]. There is a high frequency (50%) of *UGT1A1* g.-3279T>G polymorphism among Indonesian neonates with hyperbilirubinemia but this polymorphism is not associated with severity of hyperbilirubinemia[30]. On the other hand, among Egyptian newborns presenting with jaundice, *UGT1A1* g.-3279T>G polymorphism is significantly associated with higher mean peak TSB, higher bilirubin/albumin ratio, and longer duration of hospital stay[15].

Table 3 Association between genetic variations and continuous variables analyzed by multiple linear regression analysis

Genetic variation	Peak TSB (mg/dL)	Duration of phototherapy (hours)
G-6-PD variant	0.93 (95% CI: 0.22-1.64)	14.45 (95% CI: 6.92-21.99)
UGT1A1 g.-3279T>G	-0.36 (95% CI: -1.10-0.38)	-2.61 (95% CI: -10.48-5.27)
*SLCO1B1*c.388G>A	0.66 (95% CI: -0.52-1.83)	2.45 (95% CI: -9.79-14.69)
SLCO1B1 c.521T>C	-0.25 (95% CI: -1.39-0.90)	-0.28 (95% CI: -12.01-11.45)

Note: Data are presented as coefficient value (95% CI) and statistically significant results (*p*-value < 0.05**)** are indicated in bold text**.**

Table 4 Association between genetic variations and categorical variables analyzed by multiple logistic regression analysis

Genetic variation	Onset of Hyperbilirubinemia ≤48 hr.	Requirement of phototherapy	Hospital readmission
G-6-PD variant	**2.29 (95% CI: 1.22-4.31)**	2.17 (95% CI: 0.92-5.13)	**4.13 (95% CI: 1.09-15.67)**
UGT1A1 g.-3279T>G	0.86 (95% CI: 0.45-1.65)	1.62 (95% CI: 0.71-3.68)	0.39 (95% CI: 0.12-1.22)
SLCO1B1 c.388G>A	1.18 (95% CI: 0.43-3.22)	0.41 (95% CI: 0.08-2.00)	0.15 (95% CI: 0.02-0.85)
SLCO1B1 c.521T>C	0.98 (95% CI: 0.37-2.62)	1.49 (95% CI: 0.30-7.32)	1.96 (95% CI: 0.53-7.25)

Note: Data are presented as odd ratio (95% CI) and statistically significant results (*p*-value < 0.05**)** are indicated in bold text**.**

These controversial results of association between *UGT1A1* g.-3279T>G variant and severity of hyperbilirubinemia can be partly explained by the differences in genetic background and environment among different populations[31].

Genetic variations in *SLCO1B1* have been investigated in different populations. *SLCO1B1* c.388G allele is highly prevalent in Asian populations with allele frequency of 60-90%, but is less frequent in Caucasians, with allele frequency of 30-45%[32]. On the other hand, *SLCO1B1* c.521C allele is less prevalent, being present in Asian populations with allele frequency of 10-15%, in Caucasians with allele frequency of 15-20%, and in African-Americans with allele frequency of 1-2%[32]. The c.521C allele is associated with markedly reduced transport activity, while transport function of c.388G allele shows normal, decreased or increased uptake activity toward various substrates[33]. The roles of *SLCO1B1* c.388G>A and c.521T>C polymorphisms in neonatal hyperbilirubinemia are controversial[31]. This is the first report of *SLCO1B1* c.388G>A and c.521T>C polymorphisms in neonates of northeastern Thailand. Their allele frequencies (c.338G 77.7% and c.521C 10.3%) were in the same range of Asian populations[32]. Statistical analysis showed both *SLCO1B1* c.388G>A and *SLCO1B1* c.521T>C polymorphisms were not genetic risk factors for severe neonatal hyperbilirubinemia, consistent with the previous meta-analysis study[17] and more recent studies conducted in Chinese,[34] Indonesian[35] and Taiwanese[36] neonates. However, an earlier report from India indicated association of both c.388G and c.521C alleles with neonatal hyperbilirubinemia[29]. In addition, Liu *et al.* showed in China *SLCO1B1* c.388A, but not c.521C, allele is associated with neonatal hyperbilirubinemia[24]. Therefore, the role of *SLCO1B1* c.388G>A and c.521T>C polymorphisms on severity of neonatal hyperbilirubinemia remains controversial among different populations or ethnic groups.

The etiology of neonatal hyperbilirubinemia consists of complex multifactorial factors, both genetic and environmental,[31] and focusing on a single factor (genetic or environmental) might be fruitful. Expression of multiple bilirubin metabolism gene variants can contribute to increase risk for severe neonatal hyperbilirubinemia[37].

Conclusion

In summary, the study is the first report of *UGT1A1* g.-3279T>G, *SLCO1B1* c.388G>A and *SLCO1B1* c.521T>C polymorphisms in neonates with hyperbilirubinemia in northeast Thailand; however, their carriage is not significantly associated with severity of this condition. Inheritance of G-6-PD variants is independent risk factor for severe neonatal hyperbilirubinemia. A large cohort study will be necessary to understand more fully the interactions among bilirubin metabolism gene variants, other variants of pertinent gene and environmental factors impacting neonatal hyperbilirubinemia.

Take home messages

- The first report of *UGT1A1* g.-3279T>G, *SLCO1B1* c.388G>A and *SLCO1B1* c.521T>C polymorphisms in northeast Thailand.
- *UGT1A1* and *SLCO1B1* polymorphisms are not associated with severe neonatal hyperbilirubinemia.
- *G-6-PD* variants are independent risk factors for severe neonatal hyperbilirubinemia.

Conflicts of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

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Effects of oral vitamin C treatment on metabolism at rest and in response to an acute exercise in patients with poorly controlled type 2 diabetes mellitus

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KEYWORDS

Ascorbic acid; Hyperglycemia; Insulin resistance; Lipid oxidation; Physical activity.

ABSTRACT

Metabolic disturbances at rest and in responses to exercise are the hallmarks of type 2 diabetes mellitus (T2DM). Although vitamin C was shown to improve resting glycemia and lipid profile in patients with T2DM, a study investigating the effects of vitamin C treatment on the metabolic responses at rest and to low-intensity exercise in poorly controlled T2DM patients has not been conducted yet. This study aimed to investigate the effects of oral vitamin C treatment on metabolism at rest and in response to an acute exercise in patients. Twenty T2DM patients were randomly participated in the following two six-week arms with a six-week washout period: either daily placebo or 1000 mg vitamin C. On the first and last day of each session, they performed 20-min low-intensity cycling. Five-min expired gas was collected before starting and finishing the exercise. Immediately before and after the exercise, venous blood samples were collected. Vitamin C decreased resting cholesterol concentration compared with placebo treatment (p-value < 0.05) without any effect after the exercise. Post-vitamin C treatment, fat oxidation rate was higher during exercise than at baseline (p-value < 0.05). Resting total cholesterol/high-density lipoprotein-cholesterol and glucose concentrations in plasma at rest and after exercise were lower at post- compared with pre-vitamin C treatment (p-value <0.05). Additionally, pre- and post-treatment, rates of oxygen consumption and carbohydrate oxidation, and energy expenditure were higher during exercise than at baseline in both treatment arms (p-value <0.05). The findings suggest that daily treatment with 1000 mg of vitamin C for six weeks reduced the resting cholesterol concentration in patients with poorly glycemic controlled T2DM. However, this effect was not observed immediately after exercise. Nonetheless, post-vitamin C treatment, fat utilization during exercise was higher than baseline. Besides, low-intensity exercise increased oxygen consumption rate, carbohydrate oxidation rate, and energy expenditure, pre- and post- both treatment arms.

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Introduction

Worldwide, type 2 diabetes mellitus (T2DM) causes high morbidity and mortality rates and has an estimated healthcare cost as high as 1.3 trillion US dollars, which is expected to increase from 2015 to 2030[1]. T2DM has been known to impair metabolism at rest and in response to exercise including insulin resistance[2,3], dyslipidemia[4], skeletal muscle mitochondrial dysfunction[5] substrate utilization[6,7], and oxygen consumption [2,5,8].

Vitamin C (ascorbic acid), has been shown to play a significant role in improving metabolic responses in patients with T2DM[2]. Vitamin C is a cofactor of enzyme that stimulates carnitine synthesis[9,10]. This results in an improved mitochondrial function and oxygen consumption, leading to increased fat oxidation[5]. Importantly, the improved fat oxidation has been shown to improve insulin sensitivity[11]. The improved insulin sensitivity is confirmed by a study conducted by Dakehale et al. (2011) who found an improved glycemic control with T2DM patients taking oral 500 mg vitamin C twice a day for 12 weeks[12]. Afkhami-Ardekani and Shojaoddiny-Ardekani (2007) confirmed the hypoglycemic effect of the same dose of oral vitamin C for 6 weeks in patients with T2DM[13]. Furthermore, vitamin C was demonstrated to improve lipid profile including cholesterol[14,15] (by facilitating the conversion of cholesterol into bile acids) and low-density lipoprotein[13]. Importantly, previous studies examining the effects of oral vitamin C on low-intensity exercise in patients with poorly controlled T2DM, defined as HbA1c>8.5% for longer than 1 year[16], have not been conducted yet. The level of glycemic control may yield different changes in metabolic responses to the exercise. However, we have insufficient information regarding the effects of vitamin C on metabolic responses at rest and in response to low-intensity exercise in the patients.

Therefore, we aimed to primarily investigate whether oral 1000 mg vitamin C can improve fat oxidation rate and subsequently improved metabolic responses at rest and in response to low-intensity exercise in patients with poorly controlled T2DM. We hypothesized that vitamin C treatment would improve the metabolic responses at both conditions in patients with T2DM.

Materials and methods

Subjects

Of the 95 T2DM patients from Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, 24 were considered eligible to participate in the study. They were informed of their role in this study both verbally and in writing before signing a consent form. The entire protocol and consent form were approved by the Human Ethics Committee of Khon Kaen University (HE561129) in accordance with the 1964 Declaration of Helsinki. The subjects were screened according to their fasting blood chemistry and completed health questionnaires and underwent physical examinations before participating in the study. Patients with the following characteristics were included in the study: patients aged 45–60 years, patients diagnosed with T2DM at least 12 months prior to the study, patients with glycated hemoglobin A1c (HbA1c) level ≥ 8.5%, patients on oral hypoglycemic drug treatment, patients with normal lipid profile or dyslipidemia with or without lipid-lowering drugs, patients with systolic blood pressure (BP) ≤ 140 or diastolic BP ≤ 90 mmHg, patients on antihypertensive drug treatment at a similar dose throughout the study to maintain a BP of 140/90 mmHg, patients who had sedentary lifestyle, patients who did not participate in any regular exercise program for at least 6 months before the study, and patients residing in Khon Kaen Province, Thailand. Subjects were excluded from the study if their treatment plan changed (Table 1).

Twenty-four subjects (considering a 20% dropout rate) were recruited in this study according to the statistical calculations, where the mean was compared to the hypothesized value based on Johnston (2006)[16] using the WinPepi program [17]. Johnston (2006)[16] reported that vitamin C can increase fat oxidation rates from 0.48 g/min to 0.68 g/min; these data were significant with the following conditions: *p*-value<0.05, α = 0.05, β = 0.2 and power = 0.80. We measured the substrate oxidation rate using oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) from the expired gas, similar to what was used by Johnston[16].

Procedures

This study was conducted at the Nutrition and Exercise Sciences Laboratory, Faculty of Medicine, Khon Kaen University. This study was a prospective, double-blind, placebo-controlled crossover study. The capsules were provided and coded by Blackmores Co. Ltd, Australia, The 1000 mg of vitamin C capsule consisted of vitamin C 1000 mg (ascorbic acid 400 mg, sodium ascorbate 350 mg and calcium ascorbate 400 mg). In addition, 1000 mg of placebo consisted of calcium hydrogen phosphate anhydrous, carnauba wax, cellulose microcrystalline, citric acid anhydrous, Daucus Carota root dry powder, starch – tapioca, talc – purified. Both the researchers and patients were blinded on the code until the study was completed. On the testing day, the subjects were asked to sleep overnight for at least 8 hours, refrain from taking in alcohol and caffeine and from performing strenuous exercise the day before the study, and arrive fasted at the laboratory at 6:30 am. On the first visit, the subjects' peak $\dot{V}O_2$ ($\dot{V}O_{2,peak}$) was measured. One week later, all subjects were randomly divided into two arms receiving 6 weeks of daily treatment of either placebo (PLA) or vitamin C with a 6-week washout period between the treatments. The supplements were administered immediately following breakfast. On the first and last day of each treatment arm, all subjects performed cycling exercise at 30% $\dot{V}O_{2,peak}$ (16.5±2 watts) for 20 min. Immediately before and after the exercise, 16-mL blood samples were obtained from the subjects' antecubital vein. Besides, before starting and finishing the exercise, 5-minute expired gas were collected and analyzed using a gas analyzer (mixing chamber system) (PowerLab 8/30 ADInstruments, Australia) to determine the $\dot{V}O_2$ and $\dot{V}CO_2$ (g/min). The $\dot{V}O_2$ and $\dot{V}CO_2$ were used to calculate the respiratory exchange ratio (RER) and carbohydrate and fat oxidation rates using the Péronnet and Massicotte equation, without considering the protein oxidation rate[18]. Venous blood samples were obtained immediately before and after exercise to determine the plasma ascorbate, glucose, insulin, and lipid concentrations.

Randomization and blinding

The allocation sequence was generated by computer-generated random numbers and kept in sequentially numbered and sealed envelopes. Predictability of a random sequence was reduced by keeping in a separate document that was unavailable to those who enrolled participants or assigned interventions.

Peak oxygen consumption ($\dot{V}O_{2,peak}$) test

The $\dot{V}O_{2,peak}$ was determined by an incremental exercise test. All subjects began with a 2-min free workload cycling (0 watt). They subsequently continued the cycling with a workload of 30–50 watts depending on their fitness status. Workloads were increased by 20–30 watts every 3 min. The test continued until the subjects presented the maximum symptoms of dyspnea (9–10) and fatigue (18–20), determined by the rating of perceived dyspnea and rating of perceived exertion scales; were unable to maintain a cycling speed of at least 60 rpm; had an increased heart rate (HR) (calculated using the following formula: HR_{max} [220 – age]); had steady or decreasing VO2; and had RER >1.15. The expired gas samples, oxygen saturation, and electrocardiogram (ECG)

were recorded throughout the test, and the dyspnea and fatigue symptoms were evaluated every 3 min and at the end of the test. During the test, room temperature was 25±1°C, and humidity was 57±7%.

Measurements

Anthropometry and body composition

Body mass and height were measured using a stadiometer (Detecto, USA); subsequently, the body mass index (BMI) was calculated by kilograms per meter squared. Waist circumference was measured at the midpoint between the lower rib margin and the iliac crest, and hip circumference was measured at the widest point. The total body composition, lean body mass, and fat mass were measured by dual-energy X-ray absorptiometry (Lunar Prodigy whole body scanner, GE Medical Systems, USA). All scans were performed while the subjects were lying supine and wearing light indoor clothing.

Physiological measurements

Heart rate and BP were measured while the subjects were assuming a sitting position using an automatic sphygmomanometer (UA-767 Plus, UK) with the cuff wrapped around the upper arm.

Substrate utilization

From breath-by-breath expired gas ($\dot{V}O_2$, $\dot{V}CO_2$) measurements, total fat and carbohydrate oxidation rates were calculated using the Péronnet and Massicotte equation[18].

Blood chemistry

A total of 16-mL blood samples were obtained from the subjects' antecubital vein. Subsequently, blood samples were immediately separated into four tubes: an ethylenediaminetetraacetic acid tube (12 mL) to measure lipid concentrations, a sodium fluoride tube (1 mL) to measure glucose concentrations, a blood clotting tube (1 mL) to measure insulin concentrations, and a heparinized blood tube (2 mL) to measure plasma vitamin C concentrations. The heparinized blood tube was wrapped in aluminum foil and placed in an ice bath before being centrifuged. All of the tubes except the sodium fluoride tube were then centrifuged at 3000 g for 10 min at 4°C. Aliquots of plasma were frozen immediately and stored at -20°C. Blood glucose concentrations were measured by the glucose oxidase method immediately after blood collection (YSI 2300 STAT Plus™, USA, which was auto-calibrated in every five-sample test). Plasma insulin concentrations were measured with a radioimmunoassay kit (MP Biomedical, GmbH, Germany) at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. Fasting insulin and glucose concentrations were used to calculate insulin resistance (homeostatic model assessment for insulin resistance, HOMA-IR) scores and determine ß-cell function (homeostatic model assessment of ß-cell function, HOMA-ß). Cholesterol, high-density lipoprotein cholesterol (HDL-C), and triglyceride concentrations were analyzed using the cholesterol oxidase-peroxidase method, the homogeneous HDL-C plus method, and the glycerol phosphate oxidase- phenol 4-aminoantipyrine peroxidase (GPO-PAP) method, respectively (Reflotron Plus, USA, which was calibrated daily). Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated using the Friedewald formula. All measurements were performed in duplicate.

Physical activity and dietary assessment

Subjects were asked to maintain their daily physical activity and dietary habits during the study period. They were asked to record their physical activity and food intake for 3 days per week: two weekdays and one weekend day. The records were used to analyze energy expenditure (EE) following the compendium of physical activities. Energy intake was analyzed using the INMUCAL program (INMUCAL software, Mahidol University, Thailand).

Treatment compliance

We counted the number of tablets remaining at each visit, and checked the subjects' medical records to examine treatment compliance, and phoned to follow up the subjects' treatment adherence.

Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences statistics software package for Windows version 20. The differences within and between the supplement groups were

tested by analysis of variance with repeated measures. When a significant difference was observed, a post hoc analysis using the Bonferroni adjustment was performed. All differences were considered significant at p-value < 0.05. All data were expressed as the means ± SD, except when stated elsewhere.

Results

Of the 24 patients (20 females, 4 males) in the study, 20 (83% of the total patients, 16 females and 4 males) were fully compliant with the study. Three patients withdrew from the study due to a change in their treatment plans; they began receiving daily insulin injections during the washout period. Another patient had acute knee pain; thus, she could not perform the cycling exercise Figure 1. Patients' baseline anthropometrics are shown in Table 1. All subjects maintained their medication and treatment plan during 2 treatment arms. Several subjects took hypoglycemic, antihypertensive, and lipid-lowering agents, but none of them required insulin injections (Table 2). The intensity of the exercise was confirmed to be low by percent of $\dot{V}O_2$ (approximately 30% $\dot{V}O_{2,\,peak}$) and workload (27.8±9.5 %Wmax) was not different between the treatment arms (Table 1).

Table 1 Baseline anthropometric and physiological characteristics of T2DM patients

	Baseline	
	Placebo	Vitamin C
Age (yr)	53±7	53±7
M/F (n)	4/16	4/16
Body mass (kg)	61.2±2	61.1±10
Height (m)	1.6±0.1	1.6±0.1
BMI (kg/m^2)	23.9±1	23.9±2
Waist circumference (cm)	88.7±2	89.3±8
Hip circumference (cm)	98.3±6	98.2±3
W/H ratio	0.9±0.2	0.9±0.1
Body fat (%)	34.6±6	34.6±6
Fat mass (kg)	21.5±5	21.5±5
Lean body mass (kg)	21.5±5	21.5±5
Resting HR (/min)	79±1.43	81±11
SBP (mmHg)	121±2.52	121±9
DBP (mmHg)	79±1.79	81±11
$\dot{V}O_{2,\,peak}$ (mL/kg/min)	20.1±6	20.1±6
% $\dot{V}O_{2,\,peak}$	34.1±1.2	33.1±8.3
Workload (%Wmax)	27.8±9.5	27.8±9.5
Time since diagnosis (yr)	7.9±4.7	7.9±4.7
FBG (mg/dL)	194±3	227±5
HbA1c (mmol/mol)	87.7±2.8	85.9±3
HbA1c (%)	10±0.3	10±0.3
ALT (mg/dL)	32.3±28	32.3±28
Cr (mg/dL)	0.7±0.2	0.7±0.2

Note: Values are means ± SD, n = 20 (16 females, 4 males). M, males; F, females; BMI, Body mass index; W/H, Waist:Hip circumference ratio. SBP; Systolic blood pressure, DBP; Diastolic blood pressure, $\dot{V}O_{2,\,peak}$; Peak oxygen consumption, HbA1c; The glycated haemoglobin A1c, FBG; Fasting, ALT; Alanine aminotransferase, Cr, Creatinine.

Table 2 Number of the T2DM patients taking the medicines

	Placebo	**Vitamin C**
Diabetes Mellitus type 2		
Metformin (500 mg)	16	16
Glibenclamide(5 mg)	6	6
Glipizide (5 mg)	7	7
Hypertension		
Enalapril (2.5 mg)	3	3
Propranolol (10 mg)	4	4
Aspirin (300 mg)	1	1
Amlodipine (20 mg)	1	1
Hydrochlorothiazide (50 mg)	1	1
Dyslipidaemia		
Simvastatin (20 mg)	4	4
Atorvastatin (20 mg)	2	2

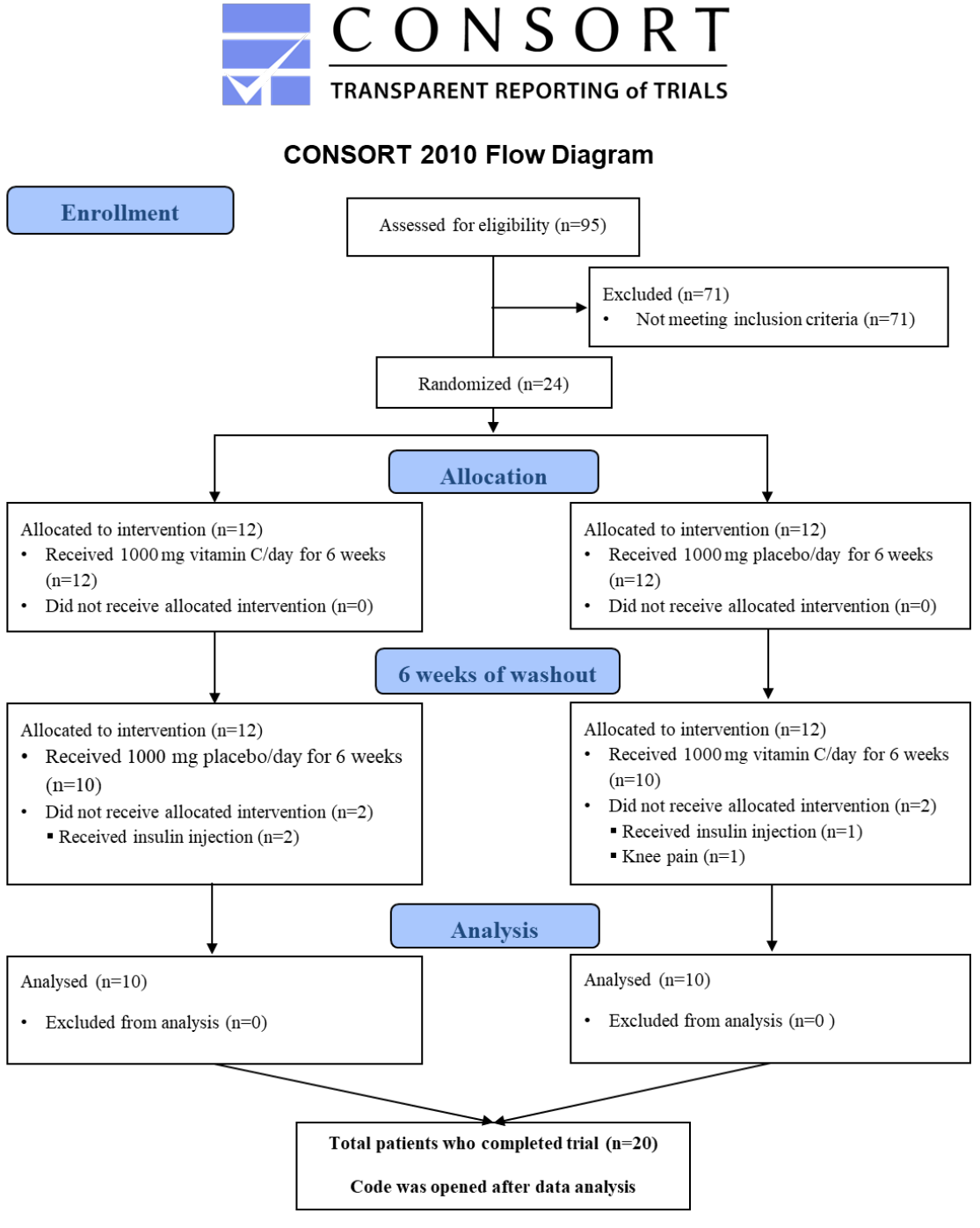

Figure 1 Consort flow chart of patient enrollment

Daily energy intake and energy expenditure

There were no significant differences in the amount or composition of dietary and energy intake and energy expenditure (EE) the week before starting and finishing the treatment between the vitamin C and PLA arms (Supplementary Table 1).

Effect of vitamin C on substrate utilization

All patients had more carbohydrate than fat oxidation rate both at rest and during exercise in both treatment arms.

At rest

Pre-treatment, the fat oxidation rate (Figure 2A) and contribution to total energy expenditure (TEE) Figure 3. were significantly lower than the carbohydrate oxidation rate Figure 2B, with similar values for PLA (oxidation rate, fat [0.02±0.01 g/min, 0.21 ± 0.1 mg/kg/min] vs carbohydrate [0.20±0.1 g/min, 3.36±2.4 mg/kg/min]; contribution to TEE, carbohydrate [87.5 ± 5.1%] vs fat [12.5 ± 5.1%]) and vitamin C treatment (fat [fat 0.02±0.01 g/min, 0.30 ± 0.1 mg/kg/min] vs carbohydrate [0.22±0.1 g/min, 3.56 ± 2.4 mg/kg/min]; fat [16.4 ± 4.9%] vs carbohydrate [83.6 ± 4.9%]) (all were *p*-value<0.05) Figure 2A and 2B, and Figure 3. Post-treatment, the oxidation rates and contributions of carbohydrate and fat had not changed from their pre-treatment values.

During exercise

Interestingly, post-treatment, vitamin C caused a significantly higher fat oxidation rate Figure 2A (0.05±0.01 g/min, 0.59 ± 0.1 mg/kg/min) and contribution to TEE (13.4 ± 5.5%) (*p*-value<0.05) during exercise than at baseline, but these did not change after PLA treatment Figure 3. The fat oxidation rate and contribution to TEE were still lower than the carbohydrate oxidation rate and contribution to TEE (fat [0.05±0.01 g/min, 0.6 ± 0.1 mg/kg/min] vs carbohydrate [0.64±0.3 g/min, 11.2 ± 7.8 mg/kg/min] and fat [13.4 ± 5.5%] vs carbohydrate [86.6 ± 5.5%]) (all were *p*-value<0.05) Figure 2 and Figure 3.

Pre-treatment, during a single bout of low-intensity exercise, the carbohydrate oxidation rate Figure 2B. and contribution to TEE were significantly higher compared to their rates at rest for PLA (0.64±0.3 g/min, 11.3 ± 6.9 mg/kg/min, 93.8±3%) and vitamin C treatments (0.65±0.3 g/min, 10.7 ± 6.1 mg/kg/min, 87.6 ± 4.3%) (all were *p*-value<0.05) Figure 2B and Figure 3. Similarly, post-treatment, carbohydrate oxidation rate and contribution to TEE were significantly higher compared to their rates at rest for PLA (during exercise, 0.56±0.2 g/min, 9.9 ± 6.6 mg/kg/min, 93.8±3%) and vitamin C (during exercise, 0.64±0.3 g/min, 11.2 ± 7.8 mg/kg/min, 87.6 ± 4.3 %) treatments (all were *p*-value < 0.05) Figure 2B and Figure 3. However, there were no significant differences in the carbohydrate oxidation rate and contribution to TEE during exercise between the treatment arms.

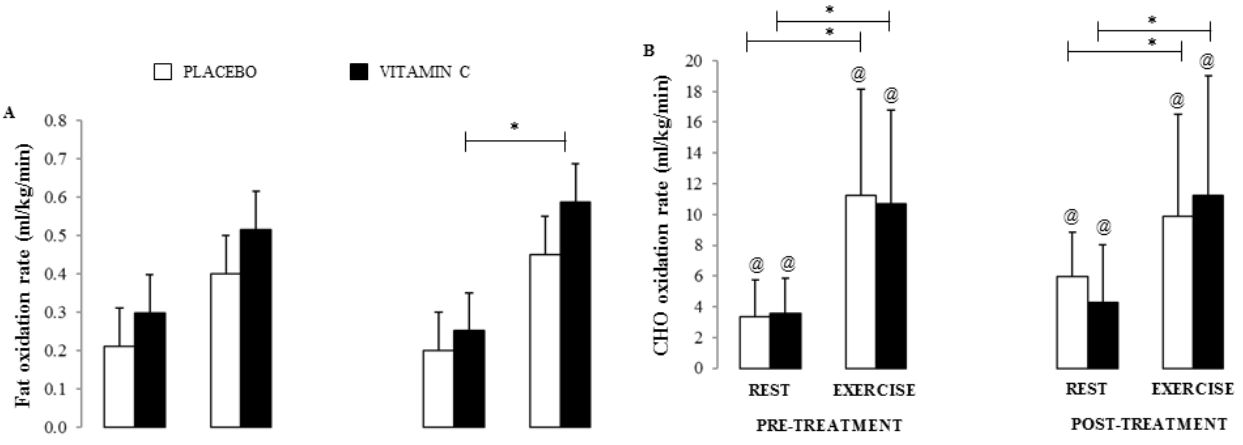

Figure 2 Substrate oxidation rate at rest and during low-intensity exercise pre- and post-treatment. (A) Fat oxidation rate (ml/kg/min). (B) Carbohydrate oxidation rate (ml/kg/min). Data are expressed as mean ± SD. (n=20; 16 females, 4 males). *Significantly different from rest (baseline) in the same treatment arm (p-value < 0.05), @Significantly different from fat oxidation rate in the same treatment arm (p-value<0.05).

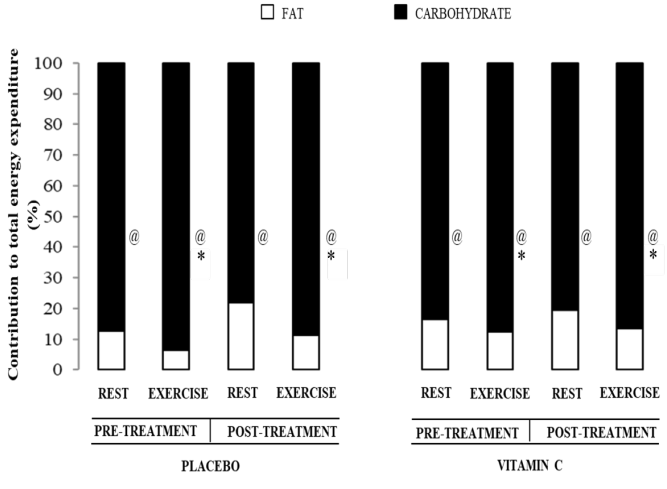

Figure 3 Contribution of substrate to total energy expenditure (%) at rest and during low-intensity exercise, pre- and post-treatment. Data are expressed as mean (n=20; 16 females, 4 males). *Significantly different from rest (baseline) in the same treatment arm (p-value<0.05), @Significantly different from fat oxidation rate in the same treatment arm (p-value<0.05).

Effect of vitamin C on circulating substrate and insulin concentrations

At rest

Cholesterol concentrations were significantly lower in post-treatment concentration compared to the PLA arm (-12.6%, p-value<0.05) (Table 3).

Post-vitamin C treatment, the TC/HDL ratio significantly lower (-14%, p-value<0.05) compared to the pre-vitamin C treatment concentration with no change in PLA treatment (Table 3).

On the contrary, post-vitamin C treatment, blood glucose concentrations significantly decreased at rest (-17.5%, *p*-value<0.05), whereas the blood glucose concentrations did not change after receiving PLA treatment (Table 3). However, after 6 weeks of vitamin C treatment, there were no changes in other plasma lipid concentrations, insulin resistance, or beta cell function at rest in either arm (Table 3).

After exercise

In both pre- and post-vitamin C treatments, fasting blood glucose concentrations after exercise were significantly lower compared to at baseline (-5.8%, pre-treatment; -10.5%, post-treatment; *p*-value<0.05), whereas the blood glucose concentrations did not change after receiving PLA treatment (Table 3). However, post-treatment, there were no changes in plasma lipid concentrations, insulin concentrations, or beta cell function immediately after exercise in either group (Table 3).

Table 3 Blood chemistry parameters at rest and during low-intensity exercise before and after treatment of T2DM patients

	Placebo				Vitamin C			
	Pre		Post		Pre		Post	
	Rest	Exercise	Rest	Exercise	Rest	Exercise	Rest	Exercise
Blood glucose (mg/dL)	193.8±3	184.2±3	190.5±4	179.9±3	226.6±5	213.3±4*	186.9±2$^{\$}$	167.1±2$^{* \$}$
Cholesterol (mg/dL)	197.8±2.3	201.3±2.2	205.4±1.8	206.3±2.0	207.1±2.3	205.2.2±2	181.0±8.3$^{\$ \#}$	203.4±1.6
TG (mg/dL)	183.0±5.4	177.8±5.0	169.5±4.2	162.5±3.6	162±2.8	163±2.9	177.43±3.8	172.11±3.7
HDL (mg/dL)	44.5±0.4	45.8±0.4	44.6±0.5	45.3±0.4	43.7±0.47	45.4±0.47	46.6±0.5	46.7±0.6
LDL (mg/dL)	128.1±2.2	131.3±2.1	136.3±1.8	140.4±2.0	136.2.1±4	133.9±2.3	127.2±1.2	130.1±1.4
LDL/HDL ratio	2.9±0.2	2.9±0.2	3.2±0.2	3.2±0.3	3.2±0.3	3.1±0.3	3.0±0.3	3.0±0.3
TC/HDL ratio	4.5±0.3	4.5±0.3	4.8±0.3	4.7±0.3	4.9±0.3	4.7±0.3	4.2±0.4$^{\$}$	4.7±0.3
Serum insulin (μIU/mL)	13.8±0.2	18.3±0.8	17.1±0.4	17.2±0.4	14.1±0.3	14.6±0.4	15.8±0.3	14.0±0.2
HOMA-IR	6.59±0.87	-	8.16±1.35	-	8.05±1.2	-	7.34±1.01	-
HOMA-β	47.34±7.8	-	63.84±10.4	-	42.5±6.6	-	48.14±4.9	-
EE (kcal/min)	0.85±0.5	2.88±1**	0.97±0.6	2.54±0.8**	0.99±0.6	2.82±1.5**	1.04±0.8	2.75±1.6**
VO2 (L/min)	0.16±0.1	0.50±0.1**	0.19±0.1	0.47±0.1**	0.16±0.1	0.44±0.1**	0.18±0.1	0.47±0.1**

Note: Values are mean ± SE, n = 20 (16 females, 4 males). Pre-, pre-treatment; Post-, post- treatment; TG, Triglycerides; HDL, high density lipoprotein; LDL, Low density lipoprotein HOMA-IR, homeostatic model assessment for insulin resistance; HOMA-β, homeostatic model assessment of β-cell function; EE, energy expenditure; VO_2, oxygen consumption. ***** Significantly different from rest in the same treatment arm (*p*-value<0.05), ****** Significantly different from rest (baseline) in the same treatment arm (*p*-value<0.001), $^{\$}$ Significantly different from before treatment in the same treatment arm (*p*-value<0.05), $^{\#}$ Significantly different from PLA treatment arm in the same condition (*p*-value<0.05).

Effect of vitamin C on $\dot{V}O_2$ and energy expenditure

At rest

There were no significant differences in the values of $\dot{V}O_2$ and EE at rest pre- and post-treatment in both arms (Table 3).

During exercise

At pre- and post-treatment in both PLA and vitamin C arms, $\dot{V}O_2$ and EE during low-intensity exercise were significantly higher than at rest (pre-treatment [PLA, 150%; vitamin C, 100%], post-treatment [PLA, 150%; vitamin C, 150%], all *p*-value 0.001) without any differences between arms (Table 3).

Discussion

To the best of our knowledge, this study is the first showing that the oral vitamin C treatment at 1000 mg/day for 6 weeks decreased plasma TC at rest in patients with poorly controlled T2DM. Moreover, we found that post-vitamin C treatment showed increased fat oxidation rate during low-intensity exercise, decreased resting plasma TC/HDL-C, and decreased plasma glucose concentration at rest and immediately after the exercise. Furthermore, pre- and post-treatment, rates of oxygen consumption and carbohydrate oxidation, and EE were increased from baseline for both treatment arms. Nonetheless, any significant effects of vitamin C treatment on other metabolic variables at both conditions were not observed.

We primarily hypothesized that oral 1000-mg vitamin C for 6 weeks can improve fat oxidation rate and subsequently improved other metabolic parameters at rest and during a single bout of low-intensity exercise in patients with T2DM. However, the hypocholesterolemic effect at rest is the only outcome that supports our hypothesis. This effect was supported by the previous studies in patients with T2DM using 1000–2000 mg of vitamin C[13,19,20], but other studies using 50–500 mg of vitamin C[13,21] did not reveal a similar effect. The high dose in the former may contribute to the hypocholesterolemic effect. However, Afkhami-Ardekani and Shojaoddiny-Ardekani did not observe the hypocholesterolemic effect following the patients' oral intake of 1000 mg of vitamin C for 6 weeks, which is possibly due to the difference in the research design used. Afkhami-Ardekani and Shojaoddiny-Ardekani used a double-blind, placebo-controlled, noncrossover design, which may have been influenced by the confounding covariates and has less statistical efficiency than the crossover design. This non-crossover design may weaken the result.

Regarding the important lipid indicators, that is, TC and TC/HDL-C ratio, which are positively associated with cardiovascular disease [22,23], the 12.6% reduction in TC concentration after vitamin C treatment shown in this study is potentially significant in reducing cardiovascular risk[22]. Regarding the report of Law et al. (1994), the reductions of 26.1 mg/dL cholesterol in this study can reduce the incidence of ischemic heart disease by 60.8% at the age of 40 years, and reducing to 21.4% at 80 years[24]. The hypocholesterolemic effects can be explained by both direct and indirect mechanisms. These include increased cholesterol absorption, increased bile acid synthesis[25] (by activating the cytochrome P450-dependent enzyme cholesterol-7-apha-hydroxylase)[25], decreased hepatic lipoprotein secretion, and increased apolipoprotein B or E receptor activity and cholesterol content; these actions are associated with the increased clearance of cholesterol from the blood.

It is noted that after vitamin C treatment, the hypocholesterolemic effect was not observed when the patients performed the exercise. The reason why TC increased after the exercise remains unknown. No study explored the effect of low-intensity exercise on TC. Although there are previous studies found exercise could increase TC it is strenuous exercise[26,27]. However, even low physical activity such as a change in posture from lying to standing can increase TC concentration[28]. In response to the standing up, total plasma TC concentrations have been shown to be related to plasma noradrenaline concentrations[26]. The standing up is known to increase sympathetic nervous system activity[26]. Thus, the change in sympathetic nervous system activity may influence short-term changes in plasma total cholesterol levels, possibly due to haemodynamic changes.

Therefore, low-intensity exercise in this study which is higher activity than standing up, is likely to increase total plasma cholesterol concentrations. This result may attenuate the hypercholesteremic effect after the exercise in this study.

The increased fat oxidation rate during exercise in the vitamin C treatment arm in this study may possibly be due to the increased plasma ascorbic acid due to vitamin C intake (Supplementary Figure 1). Vitamin C is a cofactor of two enzymes in carnitine biosynthesis, namely, ε-N-trimethyl-L-lysine hydroxylase and γ-butyrobetaine hydroxylase[9,10]. The increased vitamin C intake may thus increase the levels of carnitine, leading to increased carnitine palmitoyltransferase, which transports fatty acids into the mitochondrial matrix and increases fat oxidation in the skeletal muscle. However, either the dose or duration of vitamin C treatment in this study may not be sufficient to cause the significant difference in all variables between the arms, except the resting plasma cholesterol and ascorbic acid (unpublished data). Further studies assessing either the higher dose or longer duration of oral vitamin C treatment may provide significant data. Additionally, the higher oxygen consumption, carbohydrate oxidation rates, and EE pre- and post-treatment during low-intensity exercise compared with baseline (at rest) in both treatment arms are not surprised. This finding is consistent with the study by Ghanassia and colleagues (2006) who showed that the lipid oxidation rate during exercise in patients with T2DM shifted toward carbohydrates as the predominant source [3]. Furthermore, in Thai subjects, the Thai diet, which has carbohydrates as the main component, is considered another factor that increases the carbohydrate oxidation rate at rest and during exercise in this study. This has been shown in observations made previously in non-diabetes Thai subjects[29]. Additionally, we have shown that low-intensity exercise increased oxygen consumption, resulting in increased EE. This result is considered beneficial when planning for weight reduction strategies. Thus, the data provide a beneficial and sufficient knowledge regarding exercise training for patients with T2DM.

Three groups of drugs were used in this study: hypoglycemic, antihypertensive, and lipid-lowering drugs. All of them improve blood glucose, insulin, and lipid concentrations and BP via their mechanisms of action. However, all patients in our study maintained their medication programs, including their medications' doses, types, and frequencies, suggesting that the drugs did not affect our results. Besides, we are aware of a potential food-drug interaction between oral vitamin C and hypoglycemic and anti-dyslipidemic drugs. Fortunately, there has been no report on the interaction in this study. Therefore, food-drug interactions may not affect our results.

This study has several limitations. First, glucose and free fatty acid kinetics were not measured, such as by muscle biopsies and stable isotope tracers. Therefore, a further study investigating both kinetics will disclose important pathophysiological effect of oral vitamin C in patients with T2DM. Second, we investigated Thai patients[17] who have different substrate utilization patterns from the western population[3]. Thus, generalizing our results with other populations is not possible. Third, our study was conducted for 6 weeks only. Finally, this study has a female-to-male ratio of 4:1. Since sex had an effect on substrate utilization[30], we cannot conclude our results to male patients.

Conclusion

The findings suggest that a daily treatment with 1000 mg vitamin C for 6 weeks decreased the resting plasma cholesterol concentrations in patients with poorly controlled T2DM. However, this effect was not observed during exercise. The mechanisms attributed to the hypocholesterolemic effects of vitamin C in patients with T2DM remain to be determined. Vitamin C treatment has no effect on substrate oxidation rate and other circulating substrates at rest and during exercise. Additionally, pre- and post-treatment, oxygen consumption, carbohydrate oxidation rate, and EE were higher during exercise than at rest without any effect from oral vitamin C treatment.

The hypocholesterolemic change in patients with poor controlled T2DM in this study seems to be alternative beneficial effect of the oral vitamin C on reducing the risk of cardiovascular complications. Elevated cholesterol is a strong risk factor for cardiovascular disease.

Take home messages

- Daily treatment with 1000 mg of vitamin C for six weeks reduced the resting cholesterol concentration in T2DM patients with poor control.
- Post-vitamin C treatment, fat utilization during exercise was higher than baseline.
- Low-intensity exercise increased carbohydrate oxidation rate in T2DM patients.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary

Table 1 Dietary composition, total EI and EE before and after treatment of T2D patients

	Placebo		Vitamin C	
	Before	**After**	**Before**	**After**
CHO (g/day)	241.0±13	261.4±13	228.1±13	210.7±23
Protein (g/day)	86.8±4	95.4±9	73.4±2	67.9±5
Fat (g/day)	55.2±4	62.3±2	78.1±2	79.3±5
Vitamin A (mg/day)	259.6±20	221.8±10	545.4±53	278.6±23
Vitamin C (mg/day)	341.3±21	407.9±18	235.9±17	308.2±20
Vitamin E (mg/day)	17.8±1.2	16.9±7.6	15.7±1.2	16.7±0.9
Dietary fiber (g/day)	188.8±8.2	193.6±6.3	184.5±12	135.2±8
Total EI (kcal/day)	1,808±47	1,988±64	1,909±68	1,828±82
Total EE (kcal/day)	1,492±38	1,500±33	1,495±38	1,490±38

Note: Values are means ± SE, n = 20 (14 females, 4 males). EI, Energy intake; EE, Energy expenditure; T2D, Type 2 diabetes mellitus; CHO, Carbohydrate.

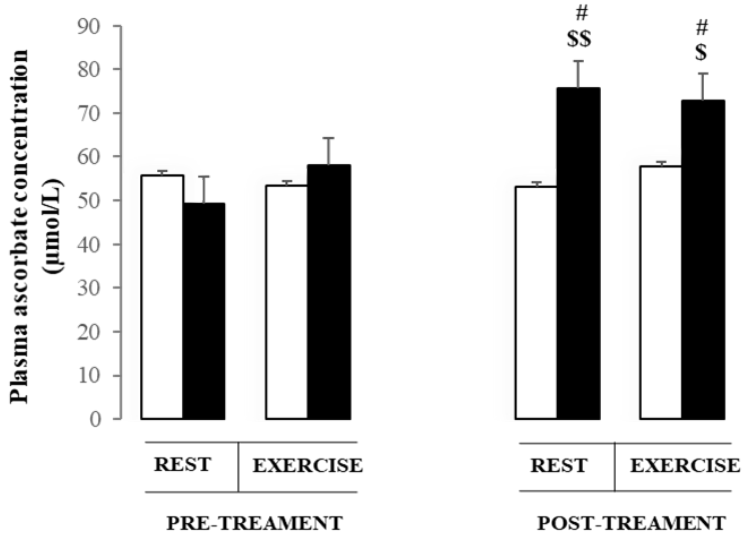

Figure 1 Plasma ascorbate concentration at rest and immediately after exercise pre- and post-treatment. Data are expressed as mean ± SD (n=20; 16 females, 4 males). $^{\$}$Significantly different from before treatment in the same treatment arm (p-value<0.05), $^{\$\$}$Significantly different from before treatment in the same treatment arm (p-value<0.01), $^{\#}$Significantly different from PLA treatment arm in the same condition (p-value<0.05).

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Problems of lower limb loading symmetry during sit-to-stand in ambulatory individuals with stroke

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KEYWORDS

Rehabilitation;
Cerebrovascular accident;
Walking;
Stability.

ABSTRACT

Existing evidence on lower limb loading symmetry and movement stability of patients with stroke commonly involves data during standing and stepping, without clear evidence for sit-to-stand (STS) ability. This study investigated the lower limb loading during sit-to-stand (LLL-STS) in 39 ambulatory individuals with chronic stroke during usual and optimal conditions using digital load cells as compared to those found in 10 healthy individuals. During the tests, participants were instructed to perform a sit-to-stand movement in 2 conditions, including 1) at their usual manner, and 2) at their optimal manner with the attempt to put their body-weight on the lower limbs as symmetrically as they could. The findings indicated that the participants had maximal LLL-STS of 47% and 75% of their body-weight in the affected and non-affected limb, respectively, resulting in the LLL-STS symmetry of 62%, whereas the LLL-STS symmetry in healthy individuals were nearly 100%. However, the LLL-STS symmetry of stroke participants was significantly increased to 73% when they attempted to take body-weight onto both lower extremities equally. The findings suggested that the participants retained some capability that they did not usually access. The findings suggested the use of verbal commands as an alternative rehabilitation strategy to promote LLL-STS symmetry of individuals with chronic stroke.

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Introduction

The unilateral sensorimotor dysfunction following stroke commonly limit the ability of lower limb loading (LLL) of the affected limb that distorts movement stability, and ability to perform functional activities independently of the patients[1-3]. Based on the concept of task-specific practice, previous studies often emphasized on the LLL of the affected limb during standing or stepping[4-6]. In fact, a sit-to-stand (STS) task is a basic and pre-requisite ability for many daily activities that is mechanically demanding greater than that need for standing and walking[7]. Therefore, the amount of lower limb loading during sit-to-stand (LLL-STS) may be obviously deviated in such challenging task and subsequently impair ability to conduct other daily activities, levels of independence, and safety of these individuals.

The existing evidence reported that the mean LLL-STS on the affected leg of the patients was 37%[8] and there was 16% of the body-weight excess on the non-affected limb[9]. However, these data were derived from different studies[8, 9], thus it may not be clearly reflecting LLL-STS on the lower extremities and LLL-STS symmetry. Therefore, this study investigated the data relating to LLL-STS during usual and optimal conditions in ambulatory individuals with chronic stroke as compared to the data from healthy individuals who had gender and age matched (± 5 years). The findings would confirm the problems and effects of verbal commands on LLL-STS symmetry of ambulatory individuals with stroke.

Materials and methods

Participants

This cross-sectional study was conducted in ambulatory patients with the first chronic stroke episode because they were in a stable stage with difficult to make change according to any training tasks. The inclusion criteria were age at least 40 years with the ability to communicate or follow the commands used in this study, and ability of independent walking with or without a walking device over at least 10 meters with post-stroke duration more than 6 months. The exclusion criteria were any other neurological and medical conditions that might affect ability to participate in the study, such as uncontrolled underlying diseases (i.e. hypertension, heart disease, thyroid, etc.), visual deficits that were unable to be corrected using glasses or contact lens, pain with a score more than 5 out of 10 on a visual analog scale, and deformity in the joints of the lower extremities. The number of participants in this study was derived from a major study[10]. All procedures of the study were in accordance with the standards of the Khon Kean University Ethics Committee for Human Research (HE601350). All participants read and signed a written informed consent before participation in the study.

Research protocols

The eligible participants were interviewed and assessed for their demographics (i.e., age, gender, body weight and height), stroke characteristics (i.e., cause, post-stroke time, hemiplegic side), and ability of walking with or without a walking device over at least 10 meters. Then, they were evaluated for their LLL-STS using digital load cells (4 half-bridge weigh sensors, total rated load 200 kg, with standard calibration method based on UKASLAB 14: 2006, accuracy up to 0.1 kg and measurement uncertainty of ±0.082 kg/side)[10]. Participants sat on a standard armchair in a standard sitting position, with their back upright against the backrest of the chair, and feet placed flat on the digital load cells [11, 12]. Then they were instructed to stand up from the chair in 2 conditions including;

Usual condition: Participants were instructed to perform STS with or without using their arms as they normally performed in their daily living.

Optimal condition: Participants were instructed to perform STS with the attempt to adequately take their body-weight onto both feet as good as they could, with or without using their arms.

The data relating to LLL-STS (including minimum, maximum, average, and duration) were recorded automatically by the digital load cells when the participant's back moved away from the backrest of the chair until their back touching the backrest again after completing the test. The

average finding of each condition over 3 trials was used for data analysis.

Statistical analysis

Descriptive statistics were used to explain demographics, stroke characteristics, and findings of the study. The dependent samples t-test was applied to compare the difference of LLL-STS between the lower limbs of the participants. The independent samples t-test was utilized to analyze the LLL-STS symmetry between participants with stroke and healthy individuals. The level of significant differences was set at *p*-value < 0.05.

Results

Thirty-nine participants with chronic stroke were involved in the study. Most of them were male, with right hemiplegia, and used a walking device for daily living (Table 1). Their demographics showed no significant differences from healthy individuals (n = 10, *p*-value>0.05, Table 1).

Table 1 Demographic data of healthy individuals and stroke participants

Variable	Healthy individual (n=10)	Stroke participants (n=39)	P-Value
Demographic			
Age[a] (years)	60.8±9.7	59.4±7.4	*0.63*
	(53.9 – 67.7)	(57.0 – 61.8)	
Body mass index[a] (kg/m^2)	23.2±3.3	24.6±3.4	*0.26*
	(20.9 - 25.6)	(23.5 - 25.7)	
Gender[b]: male	6 (60.0)	31 (69.2)	*0.58*
Non-dominant leg/ Hemiplegic side[b]: Right side	9 (90.0)	16 (41.0)	*0.016*
Post-stroke time[a] (month)		41.97±21.23	
Walking device requirement[b]: Yes		19 (48.7)	

Note: [a] The data are presented using mean±SD (95% confidence interval), and the comparisons between the groups were analysed using the independent samples t test.
[b] The data are presented using number (percent), and the comparisons between the groups were analyzed using the Chi-square test.
Gender: male/female; Non-dominant leg/ hemiplegic side: right side/left side; Stage of stroke: chronic/ subacute; Walking device requirement: yes/no

Healthy individuals showed symmetrical LLL-STS of the lower extremities, that resulted in LLL-STS symmetry in nearly 100% for both usual and optimal conditions (*p*-value>0.05, Table 2). In contrast, participants with chronic stroke showed asymmetrical LLL-STS of the lower extremities, particularly in a usual condition, with the maximal LLL-STS in the affected and non-affected limb of 47% and 75% of their body weight, respectively (*p*-value<0.001). This resulted in their LLL-STS symmetry of approximately 62% (Table 2). However, these differences were significantly reduced when they attempted to take body weight onto both lower extremities equally (LLL-STS symmetry increased to 73%, *p*-value<0.001, Table 2).

Table 2 Amount of lower limb loading during sit-to-stand (LLL-STS) in usual and optimal condition of participants

Variable / Affected side		Usual condition		*P*-value Affected side	Optimal condition		*P*-value Usual condition	Loading symmetry		*P*-value
		Non-affected side			Non-affected side			Optimal condition		
Healthy individuals (n=10)	Minimal LLL-STS*	3.57±2.39	2.84±2.26	0.02	3.56±2.10	3.15±2.19	0.05	79.55	88.48	0.14
	Maximal LLL-STS*	58.31±5.22	58.16±5.23	0.95	59.42±5.90	56.49±5.59	0.34	99.74	95.07	0.31
	Average LLL-STS*	37.60±3.89	37.84±6.12	0.90	38.67±4.82	37.37±5.73	0.49	99.37	96.63	0.96
	Duration (s)	3.28±0.46			3.57±0.33					0.40
Stroke participants (n=39)	Minimal LLL-STS*	6.58 ±2.77	4.48 ±3.30	0.001	6.96 ±2.52	4.24 ±3.36	<0.001	68.08	60.92	0.72
	Maximal LLL-STS*	47.02 ±11.92	75.41 ±12.16	<0.001	52.56 ±10.52	71.57 ±10.81	<0.001	62.35	73.43	<0.001
	Average LLL-STS*	29.35 ±9.05	48.46 ±10.67	<0.001	33.51 ±7.96	48.43 ±8.01	<0.001	67.04	69.19	0.05
	Duration (s)	7.32 ±2.84			8.22 ±3.39			0.01		

Note: The data are presented using mean ±SD, *are presented using percent of their body weight.
The differences between affected and non-affected side, usual and optimal condition were analyzed using paired samples t test.
Healthy individuals were presented for the non-dominant side (affected side), and dominant side (non-affected side).

Discussion

The study investigated the data relating to LLL-STS in usual and optimal conditions in ambulatory individuals with stroke as compared to the data from healthy individuals. The findings indicated that participants with stroke showed obvious asymmetrical LLL-STS as compared to those found in healthy individuals. However, these differences were significantly reduced when they were instructed to take body-weight onto both lower extremities equally (p-value<0.001, Table 2).

Engardt et al.[8] reported that individuals with stroke could take their body weight onto the affected limb of approximately 37% of their body-weight. Brunt et al. [9] also found that stroke patients increased LLL-STS on the non-affected limb for 16% of the body-weight. However, these data were derived from different studies that may not clearly reflect LLL-STS symmetry. The current findings demonstrated that the LLL-STS differences of the lower extremities were approximately 28% of their body-weight. This resulted in asymmetrical LLL-STS (approximately 62%, Table 2) that was clearly greater than that found during stepping (LLL of the affected limb was 91.61±7.33% of their body weight as compared to that found in healthy individuals of 94.20±4.64% of their body-weight)[13-14]. The researchers [14] also reported the differences of LLL during stepping in stroke individuals for 5% of their body-weight that resulted in lower limb support symmetry during stepping of 94.The mark differences in LLL-STS between the lower extremities as compared to the data found during stepping may confirm the high demand of STS for stroke individuals.Subsequently, the decreasing use of the affected limb during such task could introduce the effects of learn non-used including muscular atrophy and bone density loss that could retard motor recovery. On the contrary, the increasing use of the non-affected limb as a compensatory strategy could enhance effects of overuse such as musculoskeletal pain and joint degeneration that further affect mobility [15].

Nonetheless, the degree of asymmetry LLL-STS between the lower extremities could significantly reduce when the participants were instructed to optimally take their body weight onto both legs equally (Table 2). This movement modification was demonstrated even in the participants with post-stroke time of 41.97±21.23 months (Table 1) that suggested benefit of verbal commands and remaining capability inherent in individuals with stroke. After stroke, patients commonly have impaired intrinsic information mechanisms that distort their ability to control an optimal movement. Thus they retain some ability that they are unable to generate by their own determination. Augmented verbal feedback plays a major role in helping the participants to become aware of how they perform, and correct any compensatory movements in order to optimize their movement control[16].Therefore, they showed significant improvement in their LLL-STS symmetry in an optimal condition (Table 2). Bach-Y-Rita and Kercel[17] indicated that the human movement system had a great capability to use alternative sources of input helping them to successfully carry out a required task. Then repetitive practice using verbal commands may promote functional ability of these individuals. The findings offer an alternative simple rehabilitation strategy that can be applied in various settings, such as hospital, clinical community and patients' home.

However, there are some limitations of the study. This study indicated participants at a chronic stage to clearly indicate effects of verbal command in those with less capability to be changed in their movement system. The cross-sectional data were unable to clearly indicate the importance of LLL-STS.Therefore, a further study that prospectively assesses the influence of asymmetrical LLL-STS on the occurrence of musculoskeletal injury and intervention study on the effects of verbal command for LLL-STS symmetry would strengthen the finding of this study.

Conclusion

Stroke individuals retained some capability that they did not usually access, even they had long post-stroke duration. The use of verbal commands could benefit LLL-STS symmetry of these individuals.

Take home messages

Ambulatory individuals with stroke could improve lower limb loading symmetry during sit-to-stand through verbal command. Thus, verbal command could be used to promote the use of retaining ability among individuals with stroke.

Conflicts of interest

The authors declare no conflict of interest.

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Differences in the level of functional ability between diabetic peripheral neuropathy patients both with and without functional limitations

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KEYWORDS

Diabetic mellitus; Diabetic peripheral neuropathy; Physical function; Functional limitation.

ABSTRACT

Diabetic Peripheral Neuropathy (DPN) is an important complication of diabetic mellitus. The primary symptom of DPN disrupts the integration of the somatosensory system contributing to both static and dynamic balance ability. Furthermore, muscles progressively decline in distal to proximal parts of the body. The combination of muscle weakness and balance impairment obviously leads to gait abnormality, then it is directly linked to functional limitations. The study aimed to compare the functional ability, both with and without functional limitations, in type 2-diabetes mellitus (T2DM) with diabetic peripheral neuropathy (DPN). This study was cross- sectionally collected the data in DPN patients from 25 sub-district health promoting hospital, Nakhon Phanom Province in the Northeastern of Thailand. They were diagnosed as DPN using the Michigan Neuropathy Screening Instrument (MNSI). Then, they were screened for functional limitation using the late-life function and disability instrument (Late-life FDI) questionnaire. The eligible participants were assessed by hand grip dynamometer (HGD), toe grip dynamometer (TGD), five times sit-to-stand test (FTSST), single leg stance test (SLS), timed up and go test (TUG), and 10-meter walk test (10MWT). Sixty-four eligible subjects were divided into two groups: a non- functional limitations group (45 subjects) and a functional limitations group (19 subjects). According to the findings, approximately 30% of the DPN patients were reported with functional limitations. The DPN patients with functional limitations were reported with significantly poorer muscle strength (hand grip and toe grip strength, and leg muscle strength), static and dynamic balance ability, and gait speed than the other group (p-value < 0. 05). The present study suggested the level of functional ability in these individuals could be meaningful for health practitioners in preventing severe complications and disability from functional limitation conditions.

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Introduction

Diabetic peripheral neuropathy (DPN) is one of the most significant complications of diabetes mellitus (DM)[1]. DPN occurs due to the progressive diffuse or focal degeneration of peripheral somatic and autonomic nerve fibers[1]. The chronic hyperglycemia of diabetes is related to long-term damage, dysfunction, and the failure of various organs[2]. Evidence reports a high prevalence of DPN in South East Asia[3], in which there was an estimated 34% prevalence of DPN patients in Thailand[4].

DPN invariably leads to several serious health problems. The primary symptom of DPN is abnormal sensation in the toes; then, it may extend to involve the feet and legs in a stocking distribution[5]. Consequently, DPN induces the risk of foot ulcers, which are the result of abnormal weight bearing on the feet due to the loss of sensation[6]. Motor and sensory neuropathy also results in abnormal foot muscle mechanics and developed structural changes in the foot, gangrene, and finally amputation[6]. These impairments disrupt the integration of the somatosensory system contributing to both static and dynamic balance ability. Particularly in severe or longer duration DPN, motor fibers are involved, and then motor functions (i.e., atrophy and weakness) are progressively decline in distal to proximal parts of the body[7]. The combination of muscle weakness and balance impairment obviously leads to gait abnormality[8]. The negative impacts of DPN influence the ability to perform daily activities such as bathing, shopping, and doing housework. The evidence confirms that DPN is directly linked to functional limitations, which impacts the quality of life in these individuals[7-9].

Although some of the previous studies investigated physical performance between DPN and non-DPN patients[10-12], they reported on some aspects of functional ability in these patients. Furthermore, none of the studies reported on functional ability in DPN both with and without functional limitations. Early detection of the functional limitations of DPN patients based on their significant impairments including distal muscle (hand grip and toe grip strength) and leg muscle strength, static and dynamic balance ability, and gait speed, is crucial in preventing severe consequences from the functional limitations in these individuals. These abilities can be quantified using the hand grip dynamometer (HGD), toe grip dynamometer (TGD), five times sit-to-stand test (FTSST), single leg stance test (SLS), timed up and go test (TUG), and 10- meter walk test (10MWT), respectively[13,14] . These functional tests are commonly used because their methods are simple, inexpensive, and easy to perform; they provide quantitative data and report an acceptable level of reliability in DM patients[15]. The information regarding the physical abilities of these patients assists both physical therapists and health practitioners in preventing severe complications and disability from functional limitation conditions. Therefore, this study aimed to compare functional abilities in terms of hand grip strength, toe grip strength, leg muscle strength, static and dynamic balance ability, and gait speed between DPN patients both with and without functional limitations.

Materials and methods

Study design and population

The study was a cross-sectional data collection of the functional ability in DPN patients both with and without functional limitations. The number of sample size was calculated based on a pilot study (20 persons of both two groups) for detecting a difference between the means of two samples. In calculating the sample size, the α error and β error were set as 0.05 and 0.2, respectively. There were six parameters (HGD, TGD, FTSST, SLS, TUG, and 10MWT) in the study; therefore, the variance was ranged between 0. 28 to 11. 92, and the effect size was ranged from 0. 58 to 14. 89. Thus, the largest number of subject of all parameters was 19 persons/group. The subjects were male and female type 2-diabetes mellitus (T2DM) with DPN. They were from an out-patient diabetic clinic of 25 sub-district health promoting hospitals that are a network of Nakhon Phanom Hospital in the Northeastern Thailand.

The subjects had been diagnosed by a doctor as T2DM for at least 10 years. Their DPN was subsequently determined using the criteria of the Michigan Neuropathy Screening Instrument

(MNSI). The MNSI consisted of two separate parts: DPN was defined if they had a score of at least 7 out of 13 on the first part (self-administered questionnaire), and a score of at least 2.5 out of 10 on the second part (physical assessment)[16, 17]. To complete the protocol of the study, it was important that the subjects were able to understand simple commands. However, they were excluded if they had any abnormalities that might affect the study, including the following: (1) neurological diseases (e.g., cerebrovascular disease and Parkinson's disease) in which their impairments still interfered gait and balance ability, (2) open wounds on the weight-bearing surface of the foot, any deformities (foot or hand), or amputations that might affect the protocol, (3) inflammation of joints and muscles, or any musculoskeletal conditions such as osteoarthritis or gouty arthritis with a pain score of more than 5 out of 10 on a visual analogue scale, or any pain that affected the study, (4) a history of ankle, knee or hip joints replacement at least 6 months prior to participating in the study, or any residual effects in performing the test, (5) the use of drugs that had negative potential effects on cognition, alertness, and psychomotor function (e.g., opioids, antiepileptics, anxiolytics, antipsychotics, hypnotics, or sedatives), and (6) any signs or symptoms that might influence the study such as dizziness, visual and auditory impairments, angina pain, uncontrolled hypertension, and acute illness or injury. The protocol of the study was approved by Khon Kaen University Ethics Committee for Human Research (HE622004). Eligible individuals were required to sign a written informed consent prior to participation in the study.

Study protocol

The eligible subjects were screened for their demographic characteristics and health status, including the information regarding DM (i. e. , the duration of DM, the presence of symptoms, and the glucose level) using screening questionnaire. They also were interviewed functional limitations using the Late- life Function and Disability Instrument (Late- life FDI). This tool evaluates both the frequency of performing life- tasks and the limitations in capability to perform life-task. The functional components of the Late-life FDI include 7 items of the upper extremity and 25 items of the lower extremity. The dimensions of the upper extremity function include removing wrapping with the hands only and holding a full glass of water in one hand. The lower extremity functions consist of walking around one floor at home, picking up a kitchen chair, carrying while climbing stairs, and walking on a slippery surface[18]. The function component has a rating scale from 1 to 5; a score of 1 is defined when the subject feels completely limited in performing a particular task; whereas, a score of 5 is described as not at all limited in doing the activity. The total score of the function component was 160, which was calculated based on the summary scores of 32 items. The raters had to transform the raw data into the scaled scores (0-100). Scores of less than 53.2 out of 100 are considered a determined functional limitation[19]. Then, the eligible subjects were appointed to perform the functional tests.

The subjects were assessed for their muscle strength using the HGD, TGD, and FTSST. Their static and dynamic balance ability was examined using the SLS and TUG, respectively. Their gait speed was measured using the 10MWT. The details of the tests were explained as below:

HGD: The HGD was used to assess hand grip strength (HGS), which is the maximum power of the forceful flexion of all fingers. This test was reported high test-retest reliability (ICC = 0.95) in older adults. The subjects were asked to sit straight in a chair, without an armrest and with their arms hanging loosely at their sides. Their shoulder (on the dominant hand) was slightly abducted, at 15 degrees with elbow extension, the forearm in the neutral position, and the wrist with slight extension. They were instructed to squeeze the handle as hard as possible using the dominant hand and then hold for 3 seconds[20]. A 30-second rest was given between each of 3 trials and the average weight was recorded in kilograms.

TGD: The TGD represents toe grip strength (TGS), which is a complex motion involving several muscles in the foot, including the flexor hallucis brevis, flexor hallucis longus and lumbricals

muscles[21]. Toe grip strength was measured using the footedness test on the dominant foot, which is defined as the one with the toe used to kick a ball. The subjects were instructed to sit straight in an armless chair with their arms crossed over their chest, flexing their hip and knee joints at 90 degrees, and keeping their ankle joints in the neutral position. The first proximal phalanx of the foot was positioned on a grip bar and the heel position was fixed using a heel stopper and immobilization belt. Then, they were asked to grip the bar with maximum force and hold for 3 seconds. The test was measured in 3 trails with 30-second intervals, and the average value was recorded in kilograms.

FTSST: The FTSST was used to represent the strength of lower extremities. The subjects were asked to sit on an armless chair with a seat height of 43 cm. Then, their hips were flexed at 90 degrees, the ankle joints were kept 10 cm behind the knees, and their back touched the backrest. They were instructed to keep their arms crossed over their chest. Then, the time was recorded using a stopwatch. After the command "go", the subjects had to stand up and sit down 5 times continuously as quickly as possible and safe. The time was stopped when they completed 5 chair rise cycles with their back touching the backrest. The subjects were asked to perform 3 trials and the average time was recorded in seconds[14].

SLS: The SLS test is one of the most challenging tasks for the DPN patients, which evaluates the static balance by reducing base of support[9]. The subjects were instructed to choose the leg side that they preferred for the test. They were permitted to use the other leg if they had pain in the preferred leg. Then, they were instructed to keep their leg from touching and try to stand on one foot for as long as possible with their arms beside their trunk and their eyes open[11]. The time was started after the command "begin", and stopped when the subjects stepped out of position, lost balance control, or reached the 60-second time limit[11]. The average time for their ability to maintain the stances was calculated in seconds from 3 trials.

TUG: The TUG test is widely used to assess balance ability while changing position from sitting to standing, walking, and turning. The subjects were instructed to sit on an armless chair with a seat height of 43 cm. The starting position was set with their hips flexed at 90 degrees, ankle joints kept 10 cm behind the knees, and back touching the backrest[22]. Then, they were asked to keep their arms beside their body. After the command "go", they had to stand up from the chair without using their arms, walk around a traffic cone that was placed 3 meters from the chair, and return to sit down on the chair at the maximum and safe speed. The time was recorded after the "go" command and it was stopped when their back touched the backrest of the chair. The average time from 3 trials was calculated in seconds[22].

10MWT: The 10MWT is used to quantify gait speed; this measure represents health outcomes, including hospitalization, falls, disability, and mortality. Subjects were asked to walk with a comfortable pace and safe speed along a 10-meter walkway. They started walking after the command "go". Then, the time was recorded in the middle 4 meters using a stopwatch. The time was recorded when the participant's greater trochanter passed the marked tape on the floor at the first 3 meters. After that, the time was stopped when the same side of the greater trochanter passed at 7 meters. The subjects performed 3 trials and the average time was converted to gait speed in meters/second[23].

The subjects were able to rest as needed between the trials, for their vital signs (blood pressure, heart rate, and respiratory rate) to return to normal, which were monitored using the digital blood pressure monitoring. They could stop performing the tests if they had any abnormal signs or symptoms such as aggravated pain, acute illness, or injury. For safety and to prevent falls, the researchers stood or walked beside the subjects during the test, without interrupting their movement. In addition, the subjects were asked to wear sandal shoes and a safety belt that was prepared by the researchers. The sequence of the tests was random to minimize learning and fatigue effect. There were two raters

in the study: both were a physical therapist; they clarified their ability to use the tests by practicing a standard protocol compiled by an expert. The intra- and inter-rater reliability of all of the functional tests were reported at an excellent level in which the intraclass correlation coefficients (ICC) ranged between 0.856 and 1.00; *p*-value < 0.05.

Statistical analysis

The SPSS for Windows was used to analyze the collected data (SPSS Statistic version 17.0, IBM Corporation, 1 New Orchard Road Armonk, New York 10504-1722, USA, serial number: 5068054). Descriptive statistics were utilized to describe the demographic characteristics of the subjects in terms of mean, standard deviation, and percentage. The differences in the demographic characteristics and the functional abilities between the groups were compared using the chi-square test for the categorical variable and the independent t-test for the continuous variable. A *p*-value was set at less than 0.05.

Results

A total of 661 DM patients agreed to participate in the study; however, 597 of them were excluded due to many reasons as shown in Figure 1. Therefore, 64 DM patients with DPN were involved in the study; all of them were interested and willing to participate the study. Therefore, they were divided into two groups: a non-functional limitation group (45 subjects) and a functional limitation group (19 subjects). There were 4 subjects in non-functional limitation group and 14 subjects in functional limitation group, who were unable to complete the FTSST and SLS due to aggravated pain and fear of fall during standing on one leg. Of the DPN patients, 30% reported functional limitations (MNSI score of 12.42 ± 1.73 scores). More than half of the subjects were female (73.44%). They also reported other underlying diseases, such as hypertension, dyslipidemia, and renal diseases. The average duration of DM was 15.30 ± 5.39 years for all of the subjects. In the last 6 months, 26.56% had experienced a fall; the number of falls ranged between 1 and 2. Around 21% of the functional limitations group reported using a gait device, including walker frame and tripod cane. Other demographic characteristics are shown in Table 1.

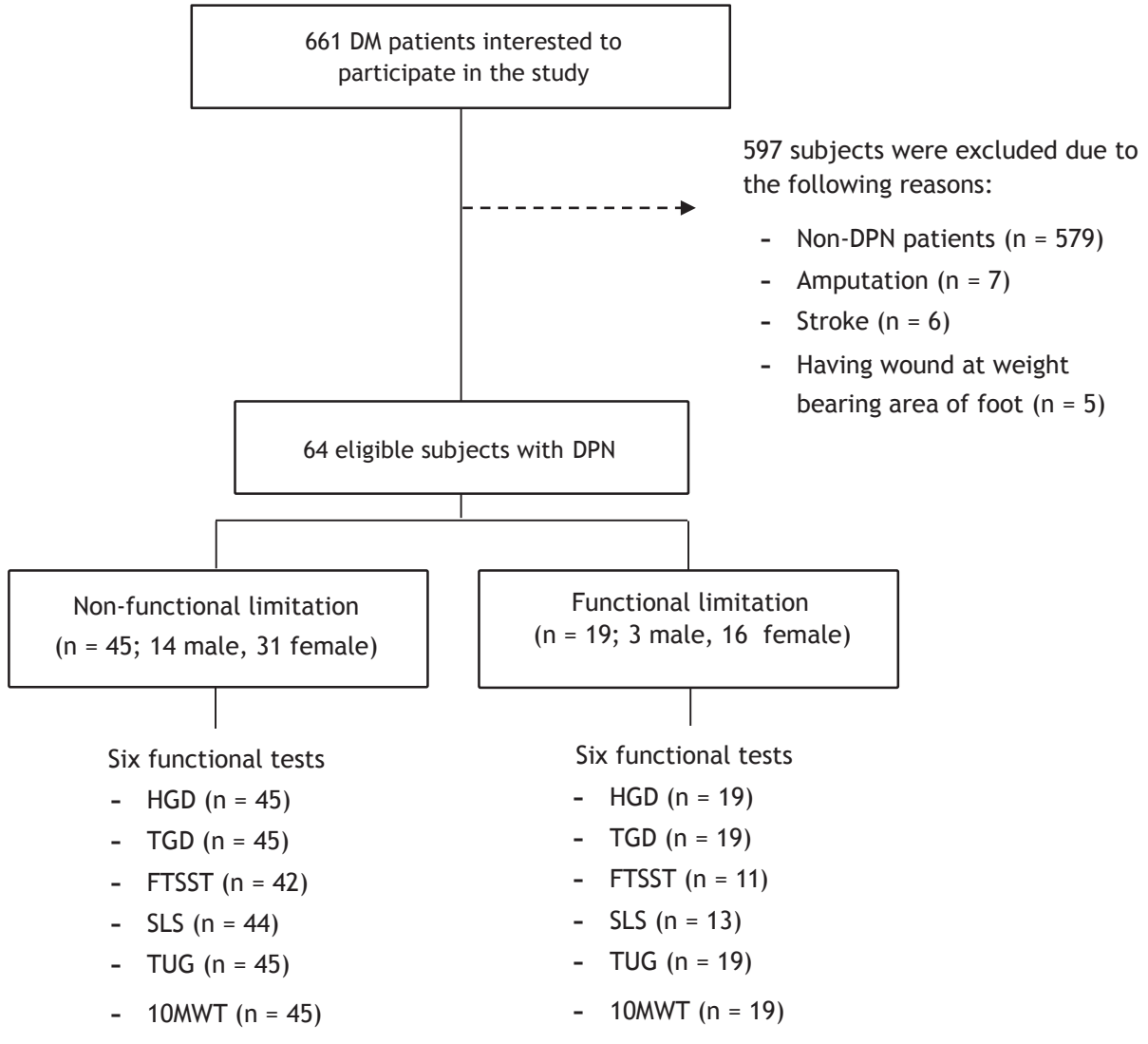

Figure 1 Subjects participation flow chart

Table 1 Demographic characteristics of the diabetic peripheral neuropathy patients with and without functional limitation (n = 64)

Variable	Non-functional limitation (n = 45)	Functional limitation (n = 19)	p-value
	Mean ± SD (95% CI)	Mean ± SD (95% CI)	
Gender: Female (n, %) [a]	31 (68.89)	16 (84.21)	0.205
Age (years) [b]	65.09 ± 9.81 (62.14 – 68.04)	68.11 ± 6.97 (64.75 – 71.46)	0.229
Weight (kg) [b]	61.47 ± 10.35 (58.36 – 64.57)	53.34 ± 11.81 (47.65 – 59.04)	0.008*
Height (cm) [b]	158.27 ± 8.48 (155.71 – 60.81)	152.94 ± 6.36 (149.88 – 156.01)	0.017*
BMI (kg/m^2) [b]	24.45 ± 3.05 (23.53 – 25.36)	22.62 ± 3.74 (20.82 – 24.42)	0.044*
Duration of DM (years) [b]	14.87 ± 5.19 (13.31 – 16.43)	16.32 ± 5.86 (13.49 – 19.14)	0.329
Underlying disease, except DM (numbers) [b]	1.18 ± 0.98 (0.88 – 1.47)	1.26 ± 0.81 (0.87 – 1.65)	0.739
Walking aid (n, %) [a]			
Do not use	45 (100)	15 (78.95)	0.001*
Use	0 (0)	4 (21.05)	
History of fall (n, %) [a]			
No	35 (77.78)	12 (63.16)	0.226
Yes	10 (22.22)	7 (36.84)	
MNSI (scores) [b]			
Total scores	11.80 ± 1.53 (11.34 – 12.26)	12.42 ± 1.73 (11.59 – 13.26)	0.159
Subjective part	7.22 ± 0.64 (7.03 – 7.41)	7.42 ± 0.96 (6.96 – 7.88)	0.333
Objective part	4.58 ± 1.36 (4.16 – 4.98)	5.00 ± 1.17 (4.44 – 5.56)	0.242

Note:
[a] Data are presented using the number of subjects (percentage of total subjects).
[b] Data are presented using the mean ± standard deviation (95% confidence intervals).
* indicates significant difference (p-value < 0.05).

The differences in the functional ability (measured using the HGD, TGD, FTSST, SLS, TUG, and 10MWT) between the groups are shown in Table 2. The findings showed a statistically significant difference in all of the functional abilities between the DPN patients, both with and without functional limitations (p-value < 0.001).

Table 2 Functional ability between the diabetic peripheral neuropathy patients who with and without functional limitation (n = 64)

Variable	Non-functional limitation (n = 45)	Functional limitation (n = 19)	*p-value*
HGD (kg)	21.61 ± 7.64 (19.32 – 23.91)	15.28 ± 6.37 (12.21 – 18.35)	0.002*
TGD (kg)	6.13 ± 4.23 (4.86 – 7.39)	2.79 ± 2.49 (1.59 – 3.99)	0.002*
FTSST (sec)	13.70 ± 4.25 (12.38 – 15.03)	21.28 ± 7.41 (16.30 – 26.26)	< 0.001*
SLS (sec)	11.15 ± 9.45 (8.28 – 14.02)	3.14 ± 2.12 (1.86 – 4.43)	0.004*
TUG (sec)	12.13 ± 2.94 (11.24 – 13.01)	24.52 ± 9.38 (20.00 – 29.05)	< 0.001*
10MWT (m/s)	0.93 ± 0.19 (0.88 – 0.99)	0.53 ± 0.21 (0.43 – 0.63)	< 0.001*

Note:
Data are presented using the mean ± standard deviation (95% confidence intervals).
* indicates significant difference (*p*-value < 0.05).

Abbreviations:
HGD = hand grip dynamometer; TGD = toe grip dynamometer; FTSST = five times sit-to-stand test; SLS = single leg stand test; TUG = timed up and go test; 10MWT = 10-meter walk test.

Discussion

This study informed the differences in functional ability in the DPN patients both with and without functional limitations. Functional ability was investigated in terms of hand grip strength, toe grip strength, leg muscle strength, static and dynamic balance, and gait speed using the HGD, TGD, FTSST, SLS, TUG, and 10MWT, respectively. Approximately 30% of the DPN patients reported functional limitations. The findings showed a statistically significant difference in all of the above functional abilities between the groups (*p*-value < 0.05, Table 2).

The evidence indicated that approximately 30 to 50% of the patients with DM developed DPN. Interestingly, the present study found that around 30% of the DPN patients progressed to functional limitations. The findings additionally confirmed a loss of muscle strength of the intrinsic muscles of hand and foot, and of the leg muscles in DPN patients with functional limitations using the HGD, TGD, and FTSST (15.28 ± 6.37 kg, 2.79 ± 2.49 kg, and 21.28 ± 7.41 sec; respectively; *p*-value = 0.002 and < 0.001, Table 2). A previous study reported hand grip strength in male and female DPN patients (27.0 ± 9.4 and 21 ± 4.1 kg, respectively)[24], although the present findings were not separated by gender, the values were close the DPN patients with non-functional limitations (21.61 ± 7.64 kg). Furthermore, the hand grip strength in the DPN patients with functional limitations was significantly less than that in those with non-functional limitations, eventually manifesting in toe grip strength which are the intrinsic muscle as well, which are the intrinsic muscles (Table 2). Patients with loss of

toe grip strength should be suggested to wear the appropriate shoes to reduce diabetic foot ulcers and prevent falls. DPN patients have a decrease in muscle strength, while more severe degrees of DPN further exacerbate this decline. Although the present study did not divide the patients by severity of DPN, the average duration of DM in the present study was reported as 15.30 ± 5.39 years, which was longer than that in the previous studies[25]. However, both for clearer findings and to lead to a proper intervention plan, further investigation should concern the severity of the DPN.

When focusing on leg muscle strength, the present study applied the FTSST because it is commonly used in clinical and community settings with a high correlation to knee extension force and leg press force. However, some subjects were unable to complete the task due to aggravated pain (Figure 1) and the researchers asked them to rest and stop the test. Although there were no severe injuries, attentiveness should be a concern in future investigation. The findings reported that the subjects with functional limitations took a significantly longer time to perform the test than those with non-functional limitations (21.28 ± 7.41 sec and 13.70 ± 4.25 sec, respectively; *p*-value < 0.001, Table 2). Previously, there were no any studies reporting the FTSST for describing leg muscle strength in DPN patients. Goldberg and colleagues reported the cut-off score of the FTSST as 14.2 seconds for discriminating the fall risk in the elderly[26]. Therefore, the DPN patients with functional limitations might take a risk of fall, as they reported their fall experience as 36.84% in the past 6 months. The evidence shows that deficits in lower limb muscles reduce functional capacity and contribute to impaired balance and gait in DPN patients.

The present study investigated both static and dynamic balance in these DPN patients, using the SLS and TUG. The subjects with functional limitations were able to stand on one leg for a very short duration (3.14 ± 2.12 sec) in comparison to the non-functional limitations group (11.15 ± 9.45 sec) (Table 2). Similarly, Bohannon and colleagues reported the average time of SLS in the elderly with functional limitations as 3.2 ± 3.3 seconds[27]. The possible reasons might occur due to the similar characteristics of the subjects after considering the age range and gender. A previous study reported a cut-off score of the SLS as 30 seconds to indicate a decrease in accurate proprioceptive information from the lower extremities, which resulted in postural instability in the DPN patients[13]. Therefore, all of the subjects in the present study were a frailty group who had the impairment of balance ability. Although evidence supported that SLS is easy to perform with high sensitivity[27], they still reported fear of falling when performing the SLS test. Therefore, the application of this test in the DPN patients should take their fear of falling while standing on one leg into consideration.

In the measurement of dynamic balance using the TUG, the DPN patients with functional limitations were reported with twofold poorer balance ability than the other group (24.52 ± 9.38 sec and 12.13 ± 2.94 sec; *p*-value < 0.001). Another previous study reported that a time greater than 13.5 seconds to complete the TUG determined risk of fall in the DPN patients[12]; this value is lower than that of those with functional limitations in the present study. This might have occurred due to the difference in the DM duration, which was longer in the present than the previous study. Further instability of postural control is caused by the destruction of the somatosensory, visual, and vestibular systems by the pathologic condition of DPN[28]. Moreover, the complications of DPN were not only caused by motor dysfunction but also by impaired sensory function, which increased the risk of falls due to the loss of static and dynamic balance. Consequently, it might be a reason for the fall in these patients. While the present study reported the incidence of fall as 22.22%, the rate might increase to 36.84% in DPN with functional limitations (Table 1). Evidence has shown that focusing on muscle strength and balance ability is an effective method of determining the degree of autonomy in walking in these patients[29].

The present study also investigated gait speed in DPN patients; these findings showed a statistically significant difference between the functional and non-functional limitation groups

(0.53 ± 0.21 m/s and 0.93 ± 0.19 m/s, respectively; *p*-value < 0.001). A previous study reported that a gait speed of less than 0.8 m/s indicated a high risk of frailty and disability in the elderly[30]. A poor gait speed in a DPN patient with functional limitations might relate to a loss of plantar cutaneous sensation, which induced a slow gait speed in these patients. DPN patients are always reported as having an incorrect pressure distribution on the foot, increased time in the stance phase, and shorter steps, which also reveal a slow gait speed. Prolonged DPN results in a significant loss of muscle strength, which contributes to balance impairment and altered gait speed.

Nevertheless, there were some limitations of the study. First, the subjects were not analyzed separately as male and female. For clearer findings and to verify the effect of gender on the level of functional ability in these individuals, further study should analyze the data by gender. Second, the present study did not divide the patients by severity of DPN. The results in the early stage of DPN include a deficit of sensory, which may lead to a major dysfunction of the neuromuscular system. However, in the severe stage, the combination of sensory deficits and motor dysfunction may occur and contribute to decreased functional capacity, impaired mobility, altered gait, and increased fall risk[7]. Further study should consider an assessment that indicates the severity of the DPN. This finding might help health practitioners in detecting an early degree of DPN for proper management.

Conclusion

The present findings suggested the level of functional ability in DPN patients; individuals with functional limitations were reported with significantly poorer functional ability (in terms of muscle strength, balance ability, and gait speed) than those with non-functional limitations. These abilities might affect the capability to perform the activities in daily living independently; therefore, knowledge of the functional ability level could be beneficial for health practitioners in preventing any severe complications from functional limitations in the DPN patients.

Take home messages

Approximately 30% of DPN patients were reported functional limitations with significant poor muscle strength, balance ability, and gait speed. These deteriorations induced risk of fall in these individuals; therefore, physical therapists and health practitioners could suitably plan to prevent further complications from functional limitations in DPN patients.

Conflicts of interest

The authors declare no conflict of interest.

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Validity of the footprint assessment method using contrast imaging method

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KEYWORDS

Footprint;
Contrast imaging;
Foot parameters.

ABSTRACT

Foot-print recording is a popular method of foot parameter measuring as it offers ease and convenience amid use. Radiographic images are both highly reliable and accurate amid clinical diagnosis, though this method incorporates a relatively high cost in regards to evaluating the contrast imaging validity of a foot print. Twenty-nine healthy participants with 57 normal feet were assessed for demographic data. Participants' foot parameters were assessed via dry footprint and contrast imaging method. For contrast imaging, participants were instructed to stand in a clear box containing black poster colouring. The assessor then took a photo of the normal foot. Study parameters included arch width, plantar arch index (PI), Clarke's angle, Chip index, and Staheli index. The assessor calculated footprint parameters using a ruler in addition to contrast imaging (kinovea program). Pearson correlation coefficients were applied to calculate the validity of both tools. Significant correlations existed between PI (r=0.50, *p*-value=0.0001), Chip index (r=0.41, *p*-value=0.001), and Staheli index (r=0.50, *p*-value =0.0001). Moreover, there were no significant correlations in regards Clarke's angle (r=-0.2, *p*-value=0.13) and arch width (r=0.22, *p*-value= 0.096). Evaluation of PI, Chip index, and Staheli index may be performed via footprint parameters and the contrast imaging method. Clinical measurements pertaining to PI, Chip index, and Staheli index provide valid information in relation to the footprint method and contrast imaging.

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Introduction

In the feet we can observe a reduced medial longitudinal arch condition which potentially leads to flat foot and internal rotation[1] especially during weight-bearing. Factors related to flat feet are many, including genetics, shoe type, obesity and over-use[2] i.e. standing and walking over extended periods. In addition, flat-feet result in fatigue to the soles of the feet, the feet and ankles, the lower extremities, and back alignment. Currently, clinical assessment methods for flat feet diagnosis are available, such as observation for Valgus Heel – Calcaneovalgus, flattened medial longitudinal arch and forefoot abduction. Methods of observation encompass qualitative data, thus making them less reliable[3] due to human-error[4]. Nevertheless, there are advantageous methods which are simply applied. Clinical evaluation of footprint imaging offers ease of use and convenience in that numerous indicators may be read, for instance, arch index (AI) which renders 86.26% sensitivity. Notwithstanding, as mentioned, the disadvantages are that it takes time to prepare the equipment, and recording surface, moisture of color, and fabric must be appropriate. If the moisture of color is inappropriate, image-clarity and parameters will be negatively affected. Medical assessment methods comprise of radiographic images which are highly reliable (ICC=0.98)[5] in terms of great accuracy amid clinical diagnosis[6]. However, this method poses difficulties and a relatively high cost.

Image processing is the use of a computer to process digital images through an algorithm. Accordingly, the image is converted into a gray scale and filtered. This technique enhances gray scale image contrast[7]. Image-based processing- methods are employed to detect the "standard" in wound healing which may present the disadvantages of the necessity for high cost equipment along with an algorithm program[8]. Such disadvantages may also be associated with image processing systems widely applied to monitor the repair of wounds in diabetic patients[9].

A gap in the study arose from photography with a mobile phone or camera imaging which was then run through an application to observe the pressure point or wound healing[8], as well as wound repair in diabetic patients, where a narrower image is witnessed. The image used to calculate the area is observed on a gray scale[9]. which is highlighted through a filter to produce a black and white image. This principle incorporated in this research is based on the principle of contrast of black and white imaging clearly being able to indicate boundary areas amid the measuring of foot parameters. As a consequence, the latest foot-measuring method (contrast imaging) may be a clinical foot assessment option in the future. Hence, the aim of this particular study was to evaluate contrast imaging and foot-print validity.

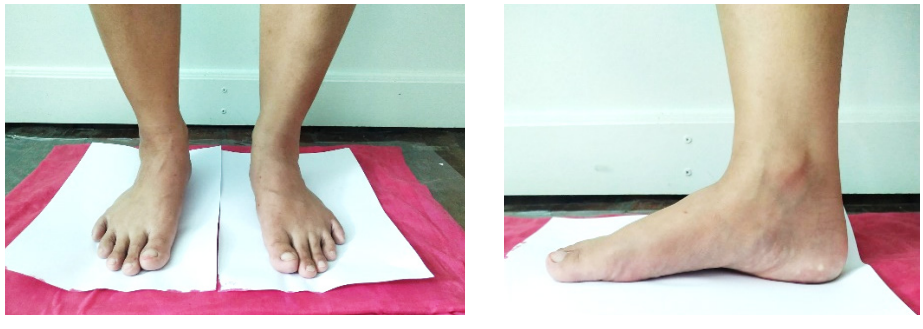
Materials and methods

The study was observational in design and conducted from June 1 to July 31, 2019 at the physical therapy laboratory, Burapha University, Thailand. The present study was approved by the Burapha University Ethics Committee in Human Research. All participants provided their consent through written consent. The study recruited 29 healthy participants from Muang District, Chonburi Province. Inclusion criteria consisted of being aged between 15-25 years, no pain at the lower extremities during standing, no injury at the ankle or foot within 6 months prior, no history of surgery at the lower extremities within the previous 6 months, no wounds to the feet or ankles, no skin diseases, and no poster coloring or food coloring allergies. Exclusion criteria consisted of the difference in the length of both legs being greater than 2 centimeters, pathological conditions of the musculoskeletal system affecting weight bearing during standing, and any pathological conditions concerning the nervous system affecting balance[5,10-12]. Participants were assessed for demographic data including age and gender, BMI Navicular Drop Test (NDT), leg length discrepancies, and bodily pain. Measured while standing and utilizing Vernier calipers NDT is employed to measure the difference in the height of the navicular bone position during sitting and loading. So, NDT was measured in both feet in each subject. An ND of ≥ 10 mm indicated

flat-feet[10]. Skin allergies were assessed via the patch test whereby the researcher applied poster colouring and foot colouring to participants' skin in order to observe for any skin irritation over the course of 30 minutes. If the volunteer presented no allergies, they then proceeded to the footprint and novel method measurement taking. Participants' foot parameters were then assessed via dry footprint and contrast imaging methods. Sample size was calculated through a computer program (Sigma stat). Determined correlation coefficient was 0.5, power 0.95, and alpha 0.05. The number of volunteers rendered 47 feet as decreased with a 20 percent drop-out rate from an original total of 57 feet.

Intervention

For dry footprint recording (Figure 1) participants had their footprint taken while in the standing position. For contrast imaging (Figure 2), participants were instructed to stand in a clear box containing black poster coloring. The box was set approximately a foot from the ground. The assessor then took a photo of the foot. Participants were given 5 minutes rest between tests. Study parameters integrated arch width, plantar arch index (PI), Clarke's angle, Chip index and Staheli index. Boasting 6 years of clinical orthopedic experience, the physical therapist calculated footprint parameters using a ruler for distance and degree, with contrast imaging conducted using the kinovea program. For each subject, approximately 10 minutes were required. Subsequently, tool validity was calculated using Pearson correlation coefficients (normal distribution) or Spearman rank correlation coefficients (non-normal distribution).



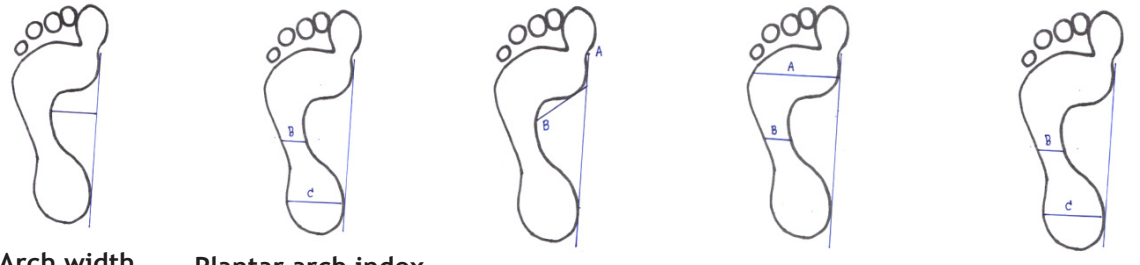
A B

Figure 1 Dry footprint method A: Anteroposterior view, B: Mediolateral view

Figure 2 Contrast imaging method and calibration procedure

Measurement outcomes

Outcomes of the treatment methods applied in this study (figure 3).



Arch width Modified from Pranati et al., 2017

Plantar arch index Modified from Pranati et al., 2017

Clarke's angle Modified from Salvador et al., 2014

Chippaux-Smirak index Modified from Salvador et al., 2014

Staheli index Modified from Salvador et al., 2014

Figure 3 Foot parameters

Arch width signifies the width of the soles of the feet. We measured the distance in centimeters[13]. Plantar arch index (PI) accounts for the width ratio of A and B. Read in centimeters, A is the width of the center of the footprint. B is the width of the heel amid the footprint[13]. Measured in degrees, Clarke's angle is the angle that passes through the metatarsal bone and the tangent line of the foot[14]. Chip index (Chippaux–Smirak index) is the ratio of the length of line B, to the length of line A which is on the same parallel line. Line B is the narrowest point of the foot arch. A is the widest line of the foot arch (B/A x 100, %) with the distance measured in centimeters[14]. Staheli index is the ratio of the length of line B, to the length of line C. C is the widest line of the heel. Line B is the narrowest point of the foot arch. Readings are taken in centimeters (B/A x 100, %)[14].

Statistical analysis

Descriptive statistical analysis was employed to describe participants' demographic and clinical characteristic data with mean (standard deviation). The Shapiro-Wilk test was employed to explore the 5 normalities of all variables. Statistical significance was set at p-value<0.05. Pearson correlation was applied to test the correlation between both methods. Correlation level may be divided into excellent level r = 0.75 - 1, moderate level, r = 0.5 - 0.74, average level, r = 0.25 - 0.49, and low level, r = 0 - 0.24[9].

Results

Fifty-seven feet of 29 participants (25 female, 4 male) with normal feet were recruited. Participants' demographic data are shown in Table 1.

Table 1 Participant characteristics

Variables	Values
Age (years)	20.29 ± 0.59
Gender (Female/Male)	25 / 4
NDT (mm.) (Right side)	4.71 ± 0.27
NDT (mm.) (Left side)	4.33 ± 3.16
BMI (kg/m^2)	21.97 ± 4.23
Leg length (cm.) (Right side)	82.58 ± 4.59
Leg length (cm.) (Left side)	82.77 ± 4.77
Dominant foot (Right side/ Left side)	29/0

Note: *NDT=Navicular Drop Test; BMI=Body Mass Index

Mean and standard deviation for the footprint and contrast imaging measurement parameters are demonstrated in Table 2. There were significant differences between groups in all parameters except for PI and Chip index. The correlation of parameters between the footprint and contrast imaging methods are shown in Table 3. Significant correlations existed between the PI, Chip index, and Staheli index. The validity test for the PI, Chip index, and Staheli index exhibited moderate values (r=0.50, 0.41 and 0.50, respectively). Moreover, the validity test showed no difference in arch width and Clarke's angle between the 2 techniques. An example of the results taken from the contrast imaging method employing the kinovea program is shown (Figure 4).

Table 2 Dependent Variable Group Means (Mean ± SD)

Variable	Footprints	Contrast imaging	p[a]
Arch width (cm)	3.88 ± 687	4.43 ± 0.63	0.01*
Plantar arch index (PI)	0.67 ± 0.15	0.63 ± 0.12	0.02*
Clarke's angle (°)	41.26 ± 9.45	29.84 ± 7.33	0.0001*
Chip index	0.35 ± 0.08	0.33 ± 0.06	0.03*
Staheli index	0.67 ± 0.15	0.63 ± 0.12	0.02*

Note: * Significant difference (*p*-value<0.05)
P[a] p-value of Independent t- test
cm=Centimetre, -^{0}=Degree

Table 3 Pearson r correlation values among foot print and contrast imaging methods

Foot print method	Contrast imaging methods				
	Arch width	Plantar arch index (PI)	Clarke's angle (°)	Chip index	Staheli index
Arch width	0.22	-	-	-	-
P[b]	0.96				
Plantar arch index (PI)	-	0.50	-	-	-
P[b]		0.0001*			
Clarke's angle (°)	-	-	-0.20	-	-
P[b]			0.14		
Chip index	-	-	-	0.41	-
P[b]				0.001*	
Staheli index					0.50
P[b]					0.0001*

Note: P[b] p-value of Pearson correlation coefficient
* Significant difference (*p*-value <0.05)

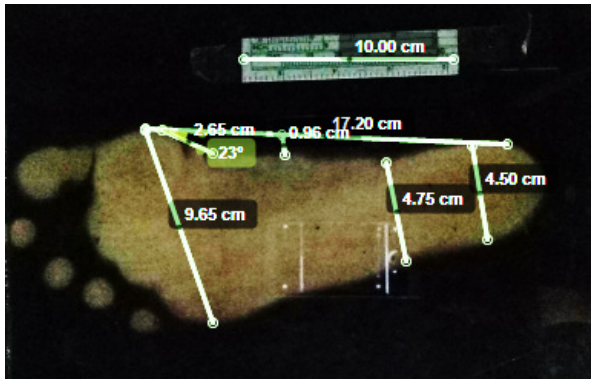


Figure 4 Results from contrast imaging (kinovea program) cm=Centimetre, -^{0}=Degree

Discussion

The purpose of this study was to investigate measurement validity amid foot print measuring and contrast imaging recording. Significant correlations existed between the PI, Chip index and Staheli index. Meanwhile, arch width and Clarke's angle did not demonstrate acceptable validity.

The strength of these findings lies within the reliability of the "gold standard" of foot print measurement taking. Reliability level was at a moderate level. Strength of agreement was poor if correlation ranged from 0-0.40; fair to moderate if the correlation ranged from 0.40-0.75, and excellent if the correlation ranged from 0.75-1.00 [15].

Arch width in the original dry-foot footprint assessment was conducted on firm ground while stepping forward into the sagittal plane[16]. Hence, weight distribution center of mass was transferred from the posterior to anterior aspect. So, without controlled pressure by individual participants the foot arch tended to be lower (increased NDT test score: main reference to flatfoot diagnosis[17]. when compared with the contrast imaging measurement. Contrast imaging employed the water fill principle with the foot placed on the bottom of the acrylic box, with water then filled into the empty space under the foot which represented the non-contact space[18]. The length between the foot arch (arch width) amid contrast imaging was much greater than the arch width method which represented more real contact. Due to the fact that the arch width method included a towel under paper it did not represent firm ground. The standard foot-print measurement operated under the same principle. The degree of contrast imaging was less than Clarkes's angle as water fill and firm ground contact cause the medial arch to move closer to the bottom of the acrylic box. In regards to the difference in starting position between the two measurement methods, for the foot-print test participants stepped on one foot and then stepped on the other. For the contrast imaging method participants stood with their feet side by side (one side on the stool the other on the acrylic box). The above maintained participant safety during standing on the acrylic box.

From this point, foot-arch contrast imaging measurement is applied more so amid clinical use as it represents real pressure and flat and firm contact in the same principle as the navicular drop test. The difference in starting position of the two methods may affect weight bearing amount taken during measurement. However, it is possible that produced less weight during the contrast imaging measuring method due to the fact that they shifted their body weight to the left foot to avoid bearing weight on the unstable plastic box. Notably, this could affect the results.

Resultant of numerous causes, different methods attempt to discover the easiest process in addition to novel ideas to explain changes in the foot. From this study, contrast imaging measurement outcomes reported moderate correlations existing between plantar index, Chip index, and Staheli index which are evenly applied to the clinical field for assessment of flat-foot representing any foot region[19]. Accordingly, the measurement of variables may be used amid any method. Hence, the clinical implication of this study is that the clinician can evaluate foot parameters in real time using a smart phone or laptop computer. Clinical practice may be used interchangeably and digital files for electronic chart collection may be included which are easily recalled.

All 3 foot parameters demonstrated significant correlation (p-value<0.05) amid both methods, yet arch width and Clarke's angle were insignificant.

The footprint method is a fast, easy, simple, non-invasive technique, though image quality may be ineffective[20] as image clarity is dependent on poster coloring or ink suitability. What's more, reading of the values obtained from imaging may be inaccurate as footprint values require the use of a ruler to calculate distance and angle. Meanwhile, the advantage of the contrast imaging method is the same as that of the footprint method, though contrast imaging may render superior image clarity. Reading the values obtained from images may be accurate because the calculation of data from contrast imaging utilises the kinovea program. It uses a computer to open the file which can zoom in to focus on the specific area and adjust picture contrast. Moreover, value reading from a PC allows for a calibration distance of 10 cm prior to calculating foot parameter distance. The calibration procedure is able to measure the length of the ruler (10 cm) which is attached under the acrylic box.

This study took place with normal subjects. Future research ought to study flat-feet and incorporate comparisons with other methods. For the contrast imaging method, data calculation may be improved by using a mobile phone application. Hence, this method may present greater advantages for the user.

Conclusion

Results suggest that PI, Chip index, and Staheli index should be used as a common or standard technique amid foot assessment.Thus, the proposed method may be employed by clinicians as an easily applied tool as it's repeatable and offers electronic file collection amid the patient charting quantifying method.

Take home messages

Clinician can evaluate foot parameters in real-time using a smart phone or laptop computer by the contrast imaging measurement. Clinical practice may be used interchangeably and digital files for electronic chart collection may be included which are easily recalled.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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