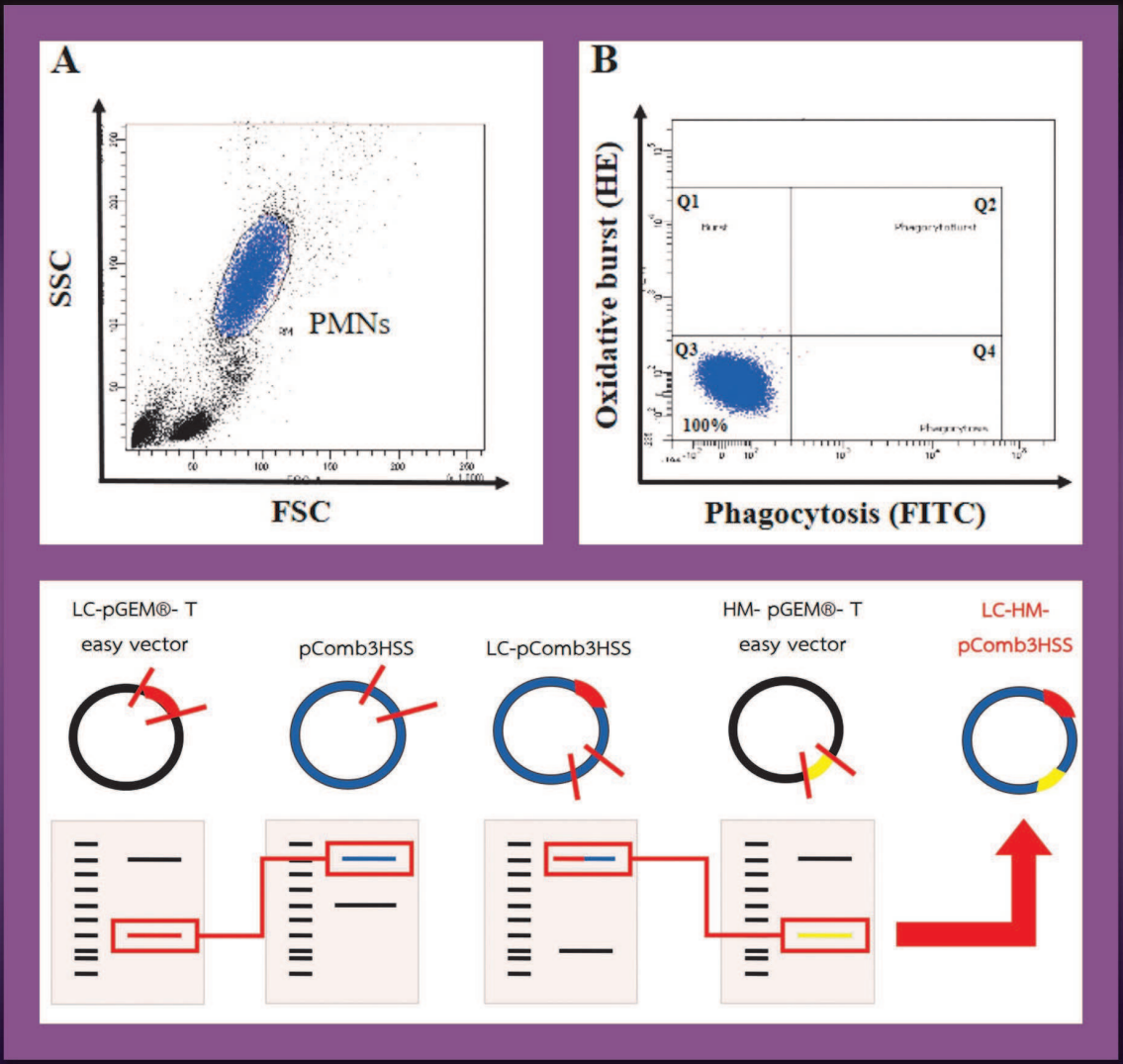
ARCHIVES OF ALLIED HEALTH SCIENCES

Archives of ALLIED HEALTH SCIENCES

Arch AHS
Volume 33
Issue 1
2021



Arch AHS
ARCHIVES OF ALLIED HEALTH SCIENCES

ISSN 2730-1990 (Print)
ISSN 2730-2008 (Online)

https://www.tci-thaijo.org/index.php/ams

Arch AHS
ARCHIVES OF ALLIED HEALTH SCIENCES

The **Archives of Allied Health Sciences (Arch AHS)** is an international peer-review multi-disciplinary journal published in English. It is owned by the Faculty of Associated Medical Sciences, Khon Kaen University, Thailand. The Arch AHS was formally known as *Journal of Medical Technology and Physical Therapy (JMTPT)*, which was founded in 1989. The title of the journal was changed to ***the Archives of Allied Health Sciences (Arch AHS)*** from 2020 (volume 32 issue 2: May - August) onward.

The Arch AHS aims to be a leading forum for research and knowledge in evidence-based practice relating to Allied Health Sciences. Contributions from all parts of the world and from different professionals in Allied Health Sciences are encouraged. Original articles, reviews, special reports, short communications, and letters to the editor are published 3 regular issues per year, online and in print.

Production and editorial assistance:
Arpassanan Wiyanad
Pakwipa Chokphukiao
Roongnapa Intaruk

All correspondence concerning manuscripts, editorial issues and subscription should be addressed to:
Editorial Officer: ArchAHS.TH@gmail.com
Faculty of Associated Medical Sciences,
Khon Kaen University, Thailand.

Publication information:
Arch AHS (ISSN: 2730-1990; eISSN: 2730-2008)
appears 3 issues a year.
Issue 1 January – April;
Issue 2 May – August;
Issue 3 September – December.

Indexing:
Arch AHS is indexed in Thai Citation Index (TCI tier 1) and ASEAN Citation Index (ACI) databases.

Manuscript preparation:
Please review author guideline for manuscript preparation: https://drive.google.com/drive/folders/1kO5FijEnuLSYwzgKcnQ9mxO74ZZ0wGRA

Link to website

Arch AHS
ARCHIVES OF ALLIED HEALTH SCIENCES

Contents

ORIGINAL ARTICLE

Arch AHS

Archives of Allied Health Sciences 2021; 33(1): 1-8.

Optimization of vitamin C concentration to boost PMN function in healthy individuals

Laongthip Ruknarong[1,2,3], Rujira Nonsa-ard[2,3], Nisa Chuangchot[1,2,3], Amonrat Jumnainsong[1,4], Naruemon Leelayuwat[3,5], Apinya Jusakul[1,4], Silvana Gaudieri[6,7,8], Chanvit Leelayuwat[1,4*]

[1] The Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

[2] Biomedical Sciences Program, Graduate School, Khon Kaen University, Khon Kaen, Thailand.

[3] Exercise and Sport Sciences Development and Research Group (ESRG), Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

[4] Department of Clinical Immunology and Transfusion Sciences, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

[5] Department of Physiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

[6] School of Human Sciences, University of Western Australia, Perth, Western Australia, Australia.

[7] Institute for Immunology and Infectious Diseases, Murdoch University, Perth, Western Australia, Australia.

[8] Division of Infectious Diseases, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, United States.

KEYWORDS
Vitamin C;
PMN function;
Neutrophils;
Phagocytosis;
Oxidative burst.

ABSTRACT

Vitamin C is an antioxidant agent and a promoter of biological functions including immune cells, particularly of neutrophils or polymorphonuclear (PMN) cells, which play a vital role in bacterial infection. The data of vitamin C impacting on PMN function during bacterial phagocytosis in healthy individuals are limited. This study aimed to investigate the optimal concentration and conditions of vitamin C that could enhance PMN phagocytosis and oxidative burst in healthy adult *ex vivo* using whole blood assay. Whole blood samples from healthy individuals were pre-incubated without and with vitamin C in various concentrations before co-culture with *Staphylococcus aureus* and detection of the PMN function by flow cytometry. Vitamin C at 20 mM significantly increased phagocytosis (p-value = 0.03) and had a trend to significantly induce oxidative burst (p-value = 0.06, p-trend = 0.04) at 15 minutes after the bacteria exposure (n = 3), compared to untreated, while vitamin C concentration of 30 mM induced significant increase in both phagocytosis (p-value = 0.04) and oxidative burst (p-value = 0.02). We further investigated the boosting effect of vitamin C at 20 mM, as a minimal concentration that can boost phagocytosis, on PMN function in additional 17 healthy participants and found that both phagocytosis and oxidative burst of PMN were significantly increased (p-value < 0.0001). In conclusion, vitamin C at 20 mM can enhance PMN function in healthy adults within 15 minutes after exposure of the bacteria. This condition may be beneficial for PMN to eliminate bacterial infection rapidly. Nevertheless, further research in the clinical trial and underlying mechanisms await further studies.

* **Corresponding author:** Chanvit Leelayuwat, MT, PhD. Department of Clinical Immunology and Transfusion Sciences and The Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand. Email: chanvit@kku.ac.th

Received: 21 April 2020/ Revised: 19 June 2020/ Accepted: 9 July 2020

Introduction

Vitamin C is an important element in human which must be received from exogenous, due to lack of enzyme, gulonolactone oxidase, using in vitamin C biosynthesis in the body[1]. The important properties of this element are antioxidants that can help to protect cells from exceeding oxidative stress by donation of electrons and vitamin C is a cofactor of several biological functions such as carnitine, catecholamines and collagen synthesis[1]. Vitamin C promotes immune cell activities by supporting various cellular functions, especially in neutrophil or polymorphonuclear (PMN) leukocytes[2]. This immune cell plays roles in acute inflammatory responses and defenses against bacterial infections mainly by phagocytosis and microbial killing[3]. Previous studies showed that vitamin C could enhance PMN functions including chemotaxis, phagocytosis and oxidative burst and could decrease the neutrophil extracellular trap (NET) production, thus reducing tissue injuries from inflammatory responses[2, 4]. Several previous studies indicated the increase in PMN function after receiving vitamin C, especially in patients who have low levels of blood vitamin C, but there is limited information in a healthy individual who has an adequate vitamin C level[2, 5]. To elucidate whether vitamin C boosts PMN phagocytosis and oxidative burst to *Staphylococcus aureus* (*S. aureus*) in healthy adults and find the optimal concentration of vitamin C for further study, we aim to set up *ex vivo* experiment for the optimization of vitamin C concentration to increase PMN function, phagocytosis and oxidative burst, using the flow-cytometry technique in whole blood assay.

Materials and methods

Study participants

Healthy adults were enrolled in the study with inclusion criteria as follows: lack of any chronic diseases and chronic infections such as HIV, hepatitis and TB, have no consumption of any supplementary for at least 3 months, no overnight meal before blood collection (fasting state) and live in Khon Kaen Province. All the subjects were recruited with written informed consent. This project was approved by the Human Ethical Committee of Khon Kaen University (HE571264 and HE611611) and was registered in Thai clinical trial with the registration number TCTR20191119002.

Materials

Culture reagents including RPMI-1640 without calcium and magnesium, fetal bovine serum (FBS) and 1x Phosphate-buffered saline (PBS) pH 7.4 were from Gibco (Thermo Scientific, USA). The BD FACS lysing solution for red blood cell lysis was from Becton Dickinson (BD Biosciences, USA). Fluorescein isothiocyanate (FITC) for bacteria staining was from Thermo Scientific, UK. Hydroethidine (HE) for detection of reactive oxygen species (ROS) was from Santa Cruz Biotechnology, USA. Phorbol 12-myristate 13-acetate (PMA) and vitamin C (L-Ascorbic acid A4544) were from Sigma Aldrich, USA. Blood parameter measurements were detected including lipid profiles by Refloton plus (Refloton plus, USA), fasting blood sugar by YSI 2300 glucose analyzer (YSI 2300 STAT plus, USA), complete blood count (CBC) by XT-2000i automated hematology analyzer (Sysmex XS-800i, Germany) and HbA1c by Cobas c513 (Roche, Japan).

Whole blood assay

To investigate whether vitamin C could enhance PMN function *ex vivo*, a whole blood culture was performed. Four ml of fasting blood was collected from healthy participants into a heparinized tube and was suspended in RPMI-1640 with 10% FBS at a concentration of $3x10^6$ neutrophils/ml calculated from the CBC parameter. The processes were performed within 2 hours after blood collection.

Vitamin C pre-incubation

Vitamin C was dissolved in RPMI-1640 freshly before use. $3x10^6$ neutrophils/ml was pre-incubated without or with vitamin C in various concentrations at 10, 20 and 30 mM for 1 hour in a multiwell plate at 37 °C, 5%CO_2[6].

Phagocytosis and oxidative burst

After the pre-incubation of vitamin C, neutrophils were co-cultured with *S. aureus*, ATCC 29223, which were labeled with 1mg/ml FITC for 15, 30 and 60 minutes at a multiplicity of infection of 30 (MOI30) for each condition. The oxidative

burst was simultaneously evaluated after added 3 ug/ml HE. 3 ug/ml of PMA and 1xPBS were used as a positive control of oxidative burst and negative control, respectively. The percentage of phagocytosis and oxidative burst of PMN from the whole blood assay was measured by a flow cytometer; BD FACSCanto II, and analyzed by the BD FACSDiva™ Software (BD Biosciences, USA). A representative pattern of phagocytosis and oxidative burst is shown in Figure 1. Percentage of phagocytosis was calculated by % total phagocytosis = Q2+ Q4 and percentage of oxidative burst by % total oxidative burst = Q1+ Q2 (Figure 1).

Statistical analysis

Distribution of data was tested by Shapiro-Wilk test. One-way ANOVA and pairwise by paired t-test were performed using SPSS v.19 (SPSS Inc., USA) and GraphPad Prism 5.0 (GraphPad Software Inc., USA) to compare between the vitamin C treatment and un-treatment groups. Trend analysis was examined by Jonckheere-Terpstra test. Significant levels determined at p-value < 0.05.

Results

The pattern of phagocytosis and oxidative burst in a whole blood assay using the flow cytometry method

The population of PMNs was firstly gated by side scatter (SSC) and foreword scatters (FSC) according to the properties of PMNs, large granulocyte and high granularity content (Figure 1A). Negative control was set as background (Figure 1B). Positive oxidative burst activated by PMA was greater than 90%, indicating PMNs can activate the function (Figure 1C). The pattern of PMN phagocytosis and oxidative burst clearly separated from non-phagocytosis cells that we could get the percentage of PMN function as represented in Figure 1D.

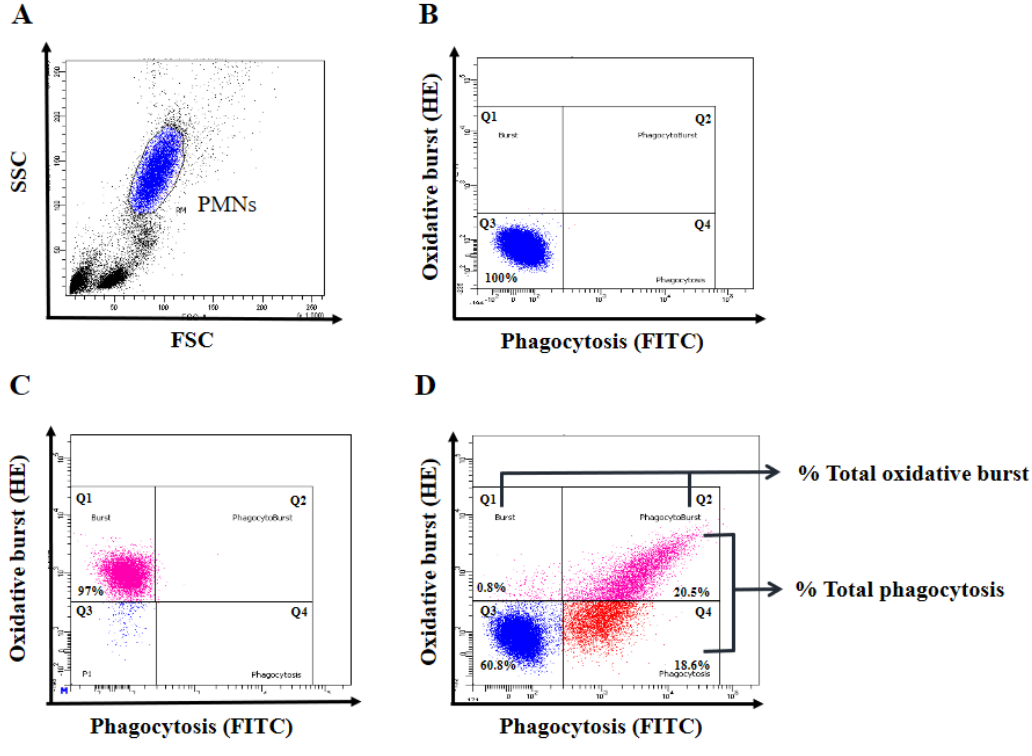


Figure 1 Flow cytometry analysis of PMN phagocytosis and oxidative burst from a whole blood assay. PMNs population (A), Un-stimulated PMNs (negative control) (B), PMA stimulated PMNs (positive control of oxidative burst) (C) and a test of two-color fluorescence FITC-HE assay represented phagocytosis and oxidative burst of a sample, respectively (D).

Optimal concentration of vitamin C boosting PMN function

To determine the optimal concentration of vitamin C that affected PMN function in an *ex vivo* experiment, we pre-incubated a whole blood sample with and without vitamin C and varied concentration of vitamin C from 10 mM to 30 mM for 1 hour before co-culture with the bacteria for 15, 30 and 60 minutes. The result showed that vitamin C concentration at 20 mM induced significantly increased phagocytosis (*p*-value = 0.03) and had a trend to significantly increase oxidative burst (*p*-value = 0.06, *p*-trend = 0.04) while vitamin C concentration at 30 mM showed a significant increase of both phagocytosis and oxidative burst (*p*-value = 0.04 and *p*-value =0.02, respectively) (Figure 2A and 2B). We noticed that the levels of phagocytosis and oxidative burst were not much different. Therefore, we selected the concentration of vitamin C at 20 mM as an optimal concentration which was a minimal concentration that can increase phagocytosis at exposure time of 15 minutes compared to the untreated condition.

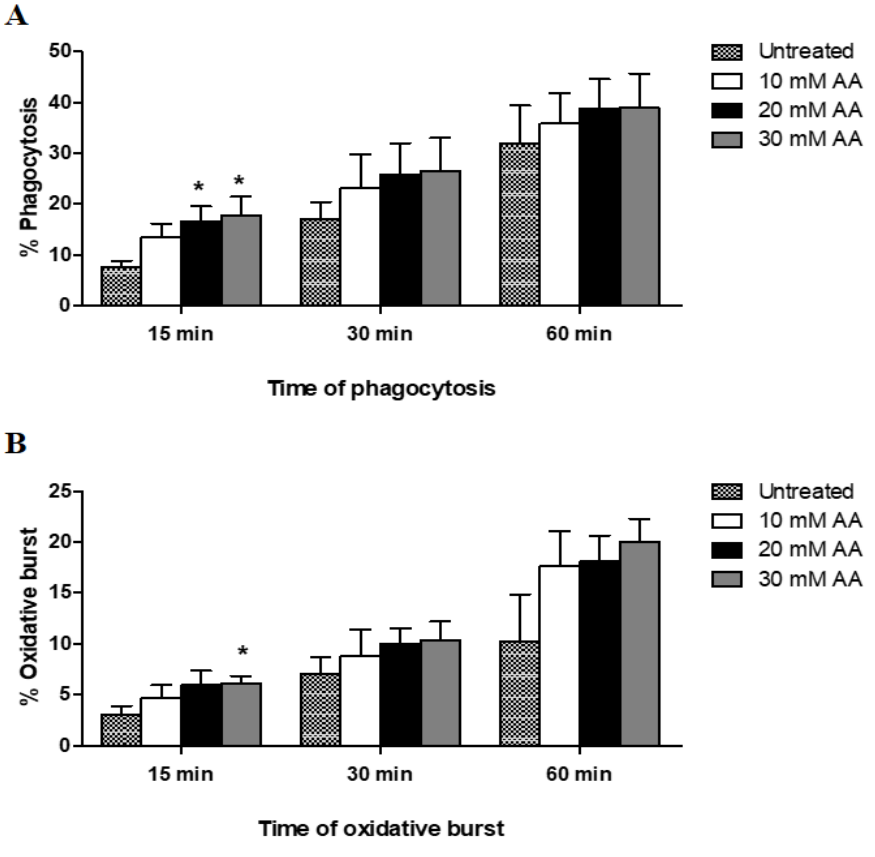


Figure 2 Determination of optimal vitamin C concentration in enhancing PMN function. Vitamin C concentration in enhancing phagocytosis (A) and oxidative burst (B) at 15, 30 and 60 minute of exposure time to the bacteria (n=3). *=*p*-value < 0.05, the mean difference is significant compared to untreated within group. AA; ascorbic acid or vitamin C, Untreated; without vitamin C adding or 0 mM AA, mM; millimolar.

Vitamin C at 20 mM enhances PMN function in healthy participants

To confirm the effect of 20 mM vitamin C treatment on PMN function, we enrolled 17 healthy adults in the study. The characteristics of the participants were shown in Table 1. Apparently, the vitamin C at 20 mM significantly increased PMN phagocytosis and oxidative burst in the whole blood assay compared with the untreated group with *p*-value < 0.0001 (Figure 3).

Table 1 Baseline characteristics of the participants included in this study

Baseline characteristics	Volunteers (n=17) (range)
Gender (female/male)	12/5 with the ratio of 70/30
Age (year)	39.4±13.4 (25-60)
BMI (kg/m^2)	24.0±3.9 (18.8-32.9)
HbA1c (%)	5.1±0.6 (4.4-5.9)
FBS (mg/dL)	85.3±6.3 (77.0-96.0)
Cholesterol (mg/dL)	186.5±41.2 (112.0-242.0)
Triglyceride (mg/dL)	119.5±57.4 (54.0-227.0)
HDL (mg/dL)	53.0±9.9 (35.0-70.0)
LDL (mg/dL)	109.6±40.7 (39.2-174.0)

Note: Data represent mean ± S.D. Abbreviations: BMI, body mass index; HbA1c, glycated hemoglobin; FBS, fasting blood sugar; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

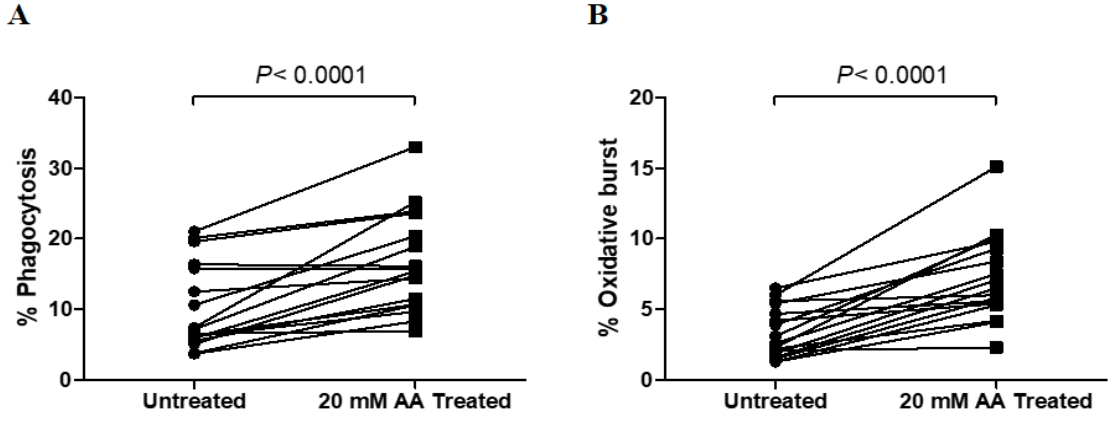


Figure 3 Vitamin C treatment enhanced PMN phagocytosis (A) and oxidative burst (B) in the whole blood culture assay at 15 minutes of exposure to the bacteria, compared to the untreated group (n = 17). AA; ascorbic acid or vitamin C, Untreated; without vitamin C adding or 0 mM AA, mM; millimolar.

Discussion

The current study showed that treatment with vitamin C could increase phagocytosis and oxidative burst of PMNs to *S. aureus ex vivo* in whole blood from healthy adults. The combination of the whole blood culture assay and flow cytometry is an easy method to investigate PMN function by measuring phagocytosis and oxidative burst that could be applied to clinical assessment. We hypothesized that vitamin C could boost PMN function *ex vivo*. The optimal concentration of vitamin C could be used for further study to investigate the molecular mechanisms affecting the PMN function. We varied vitamin C from 0, 10, 20, and 30 mM, and fixed time of pre-incubation at 1 hour according to the previous experiments as 1 hour was the approximate time for vitamin C to be accumulated highly intracellularly[6, 7]. The dose of vitamin C chosen in this study was not a normal physiological condition but was pharmacologic dosing[6, 8]. However, under physiological condition, intracellular vitamin C of PMN could be in millimolar range and reach up to 14 mM in the activated condition, indicating the important role of vitamin C in this cell type[9]. Interestingly, being treated by 20 mM and 30 mM of vitamin C significantly increased the PMN functions only within 15 min after stimulation with the bacteria compared to the untreated control, indicating vitamin C immediately activated PMN function. We chose the concentration at 20 mM to be further study because of being the lowest concentration that could enhance phagocytosis.

A recent study from Bozonet and colleagues showed that the physiological concentration of vitamin C (50-200 μM) could not induce significant increase in chemotaxis and superoxide generation of neutrophils isolated from healthy adults[4]. Moreover, incubated neutrophils with ascorbate could not increase intracellular ascorbate levels but only dehydroascorbic acid (DHA), the oxidized form of ascorbate, could be intracellularly taken up and converted to ascorbate inside the cells. This might be due to replete intracellular ascorbate[4]. The differences between the studies were the doses of vitamin C and the stimulator. We used a higher concentration of vitamin C and the Staphylococcus bacteria as a stimulator that might employ different pathways in inducing phagocytosis and oxidative burst. These processes required higher vitamin C concentration. However, the mechanism of this phenomenon needs further investigation.

During PMNs phagocytosis, oxygen consumption increases called "oxidative burst" producing ROS that is an antimicrobial substance. This is primarily due to the activation of a membranous NADPH oxidase (NOX2) and then catalyzed to hydrogen peroxide (H_2O_2) followed by hypochlorous acid (HOCl) production. This is the final substance which is extremely toxic to bacteria[10]. Vitamin C has been reported to enhance intracellular ROS by interaction with nitric oxide (NO) and peroxynitrite ($ONOO^-$) as well as NADPH oxidase leading to increased bactericidal activity of PMNs[11]. Moreover, vitamin C can easily undergo autoxidation due to being pH-dependent and the consequence of hydrogen peroxide production[12]. Furthermore, vitamin C can exhibit pro-oxidant properties due to the interaction of free metal such as ferric that can increase hydroxyl radicals (Fenton reaction)[13]. Thus, autoxidant or pro-oxidant of vitamin C may have an influence on PMN function by enhancing oxidative burst to kill the bacteria. However, the pro-oxidant activity may not be evident *in vivo* because there are iron-binding proteins in the body and prodrug property of vitamin C[12-14]. In addition, vitamin C could increase the microtubule movement[15]. However, the mechanisms of vitamin C enhancing phagocytosis are still unclear[16].

Limitation of the study is that we have not measured vitamin C concentration in the whole blood samples and PMNs in the condition of with and without vitamin C treatment to get more information. However, the dosage that we used was the same as others *in vitro* and *in vivo* studies showing decreased pro-inflammatory production and have been in phase I clinical trials [6, 8]. Investigating the other functions of PMNs such as NET formation may reveal more characteristic function of PMNs influenced by the vitamin C treatment in healthy individuals. We are

investigating the underlying mechanisms of vitamin C in boosting PMN function in healthy individuals.

Conclusion

We showed that vitamin C at 20 mM could boost phagocytosis and oxidative burst of neutrophils in healthy individuals *ex vivo*. This activation may be useful in case of emergency clinical failure such as patients with infections or sepsis to rapidly boost neutrophils to fight the bacterial infection. Nevertheless, further research in a clinical trial and molecular mechanisms underlying the PMN functions modulated by vitamin C await further studies.

Take home messages

Vitamin C at 20 mM can boost phagocytosis and oxidative burst of neutrophils in healthy adults within 15 min after bacterial exposure. This rapid activation may be applied to fight bacterial infection. However, further research in clinical trials and underlying mechanisms is therefore recommended.

Conflicts of interest

The authors declare no Conflicts of interest.

Acknowledgements

We would like to thank Exercise and Sport Sciences Development and Research Group (ESRG), Khon Kaen University for the sample and clinical data collection. This work is supported by The Royal Golden Jubilee (RGJ) Ph.D. Program (PHD/0045/2557), The Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Exercise and Sport Sciences Development and Research Group, and Faculty of Medicine, Khon Kaen University.

References

1. Mandl J, Szarka A, Banhegyi G. Vitamin C: update on physiology and pharmacology. Br J Pharmacol 2009; 157(7): 1097-110.
2. Carr AC, Maggini S. Vitamin C and immune function. Nutrients 2017; 9(11): 1211.
3. Rosales C, Neutrophil. A cell with many roles in inflammation or several cell types? Front Physiol 2018; 9: 113.
4. Bozonet SM, Carr AC. The role of physiological vitamin C concentrations on key functions of neutrophils isolated from healthy individuals. Nutrients 2019; 11(6): 1363.
5. Liugan M, Carr AC. Vitamin C and neutrophil function: findings from randomized controlled trials. Nutrients 2019; 11(9): 2102.
6. Bowie AG, O'Neill LA. Vitamin C inhibits NF-kappa B activation by TNF via the activation of p38 mitogen-activated protein kinase. J Immunol 2000; 165(12): 7180-8.
7. Nualart FJ, Rivas CI, Montecinos VP, Godoy AS, Guaiquil VH, Golde DW, et al. Recycling of vitamin C by a bystander effect. J Biol Chem 2003; 278(12): 10128-33.
8. Welsh JL, Wagner BA, van't Erve TJ, Zehr PS, Berg DJ, Halfdanarson TR, et al. Pharmacological ascorbate with gemcitabine for the control of metastatic and node-positive pancreatic cancer (PACMAN): results from a phase I clinical trial. Cancer Chemother Pharmacol 2013; 71(3): 765-75.
9. Washko PW, Wang Y, Levine M. Ascorbic acid recycling in human neutrophils. J Biol Chem 1993; 268(21): 15531-5.
10. Winterbourn CC, Kettle AJ, Hampton MB. Reactive oxygen species and neutrophil function. Annu Rev Biochem 2016; 85: 765-92.
11. Sharma P, Raghavan SA, Saini R, Dikshit M. Ascorbate-mediated enhancement of reactive oxygen species generation from polymorphonuclear leukocytes: modulatory effect of nitric oxide. J Leukoc Biol 2004; 75(6): 1070-8.
12. Du J, Cullen JJ, Buettner GR. Ascorbic acid: chemistry, biology and the treatment of cancer. Biochim Biophys Acta 2012; 1826(2): 443-57.
13. Carr A, Frei B. Does vitamin C act as a pro-oxidant under physiological conditions? Fed Am Soc Exp Biol 1999; 13(9): 1007-24.
14. Halliwell B. Vitamin C: poison, prophylactic or panacea? Trends Biochem Sci 1999; 24(7): 255-9.

15. Boxer LA, Vanderbilt B, Bonsib S, Jersild R, Yang HH, Baehner RL. Enhancement of chemotactic response and microtubule assembly in human leukocytes by ascorbic acid. J Cell Physiol 1979; 100(1): 119-26.

16. Stankova L, Gerhardt NB, Nagel L, Bigley RH. Ascorbate and phagocyte function. Infect Immun 1975; 12(2): 252-6.

ORIGINAL ARTICLE

Arch AHS

Archives of Allied Health Sciences 2021; 33(1): 9-18.

Monitoring heart rate, heart rate variability, and subsequent performance in team-sport athletes receiving hypoxic or normoxic repeated sprint training

Michael J. Hamlin[1*], Catherine A. Lizamore[2], Peter D. Olsen[2], Helen C. Marshall[2]

[1] Department of Tourism, Sport and Society, Lincoln University, Lincoln, Canterbury, New Zealand.

[2] Department of Applied Sciences and Social Practice, Ara Institute of Canterbury, Christchurch, Canterbury, New Zealand.

KEYWORDS
Altitude;
Rugby;
YoYo-Intermittent recovery;
Repeated sprint ability;
Autonomic nervous system.

ABSTRACT

Repeated sprint training (RST) in hypoxia (RSH) is becoming more popular in team sport players owing to the potential for increases in high-intensity running performance without compromising power output during training. However, as the added hypoxic stimulus also increases training load, careful monitoring is needed to avoid overtraining. The first objective of this study was to determine whether resting heart rate (RHR) and resting heart rate variability (HRV) measured following 3 weeks of training was able to detect the increased training load in the group receiving hypoxic training. A second objective was to determine whether RHR and HRV measured after 3 weeks of training were associated with post-training normoxic running performance. Amateur club rugby players completed 3 weeks of twice-weekly RST (cycling) in either hypoxia (RSH, n = 9; 20.3 ± 2.1 years; 77.1 ± 10.2 kg; 173.9 ± 4.9 cm; FIO2: 14.5%) or normoxia (RSN, n = 10; 22.0 ± 4.1 years, 88.3 ± 14.1 kg; 177.9 ± 5.4 cm, FIO2: 20.9%). Resting heart rate and HRV were monitored during normoxic rest immediately before each training session over the 3 week training period. Pre- and post-intervention aerobic endurance (Yo-Yo Intermittent Recovery Level 1 (YYIR1)) and repeated sprint ability (RSA, running) were used as performance variables before and after the training intervention. Compared to RSN, RSH demonstrated possibly lower HRV (natural log of the root mean square of successive difference, RMSSD): -8.5, ± 19.1% and standard deviation of N-N intervals: -11.5, ± 25.0%; percent change, ± 90% CL), and higher RHR (3.2, ± 4.7 bpm) post-intervention. Week 3 RHR and HRV demonstrated strong, statistically significant correlations with post-intervention performances (YYIR1, RHR: -0.82, *p*-value = 0.02; RMSSD: r = 0.58, *p*-value = 0.17; low frequency: high frequency ratio (LF/HF): r = -0.85, *p*-value = 0.01; and RSA, RHR: r = 0.73, *p*-value = 0.06; RMSSD: r = -0.53, *p*-value = 0.22; LF/HF: r = 0.77, *p*-value = 0.05) in RSH, but not RSN. In conclusion, RHR and HRV were able to detect the increased training stimulus in hypoxic compared to normoxic repeated sprint training. In addition, RHR and HRV measured after 3 weeks of RSH were correlated with post-intervention performance whereby a lower RHR or increased HRV was associated with improved YYIR1 and RSA performance.

* **Corresponding author:** Michael J. Hamlin, Department of Tourism, Sport and Society, Lincoln University, Ellesmere Junction Road/Springs Road, 7674, Lincoln, Canterbury, New Zealand. E-mail: Michael.Hamlin@Lincoln.ac.nz

Received: 7 November 2020/ Revised: 28 January 2021/ Accepted: 22 March 2021

Introduction

Over the last few years the application of altitude training has extended beyond endurance athletes to include team sport athletes[1]. In particular, repeated sprint training in hypoxia may improve intermittent running performance more so than similar training performed in normoxia in team sport athletes[2]. However, while incorporating hypoxia into repeated sprint training protocols may result in improved running performance, the added hypoxic exposure increases the training stress and therefore increases the risks associated with overreaching[3]. Furthermore, not all athletes will respond to a standardized training programme in a similar fashion[4]. That is, for some of the athletes, the training programme will be well tolerated and yields performance-enhancing adaptation, while for others, the same training may result in mal-adaptation and performance decrement. The additional strain of training in hypoxia, as well as the array of individual responses anticipated from such training, means that monitoring the stress and fatigue of individual athletes is of paramount importance when it comes to ensuring optimal training and recovery, while preventing overtraining, fatigue, burnout and illness[5].

An ideal monitoring tool should be non-invasive, and able to provide readily available, time-efficient results[6]. One such tool is heart rate variability (HRV), which indicates the sympathetic and parasympathetic innervation of the heart[7]. A reduction in HRV implies reduced vagal stimulation of the sinus node and lower parasympathetic contribution while higher HRV indicates increased vagal activity and generally indicates improved health[7].

In a sporting context, typically, athletes with greater high frequency, or parasympathetic components in their post-intervention HRV measures demonstrate improved performances, such as decreased 800m swim time in elite triathletes[8]. Furthermore, HRV measured in normoxia[9] or at moderate altitude[10] has been used to provide valuable predictive information on climbers likely to develop acute mountain sickness[9, 10]. Therefore, using HRV appears to be a very useful monitoring tool during both athletic training and during altitude training. However, much of its use has been centered on endurance athletes, and more research is needed on the role of resting heart rate and heart rate variability measures in monitoring fatigue in team sport athletes receiving hypoxic training.

The primary objective of this study was to determine the effect of short, repeated sprint training intervals in hypoxia vs repeated sprint training in normoxia on HRV in a team sport population. Secondly, we wanted to determine the relationship between HRV taken at rest during the training period with subsequent performance.

Materials and methods

Participants

As described elsewhere[11], 19 amateur club rugby players performing similar pre-season training and fitness routines were matched for baseline repeated sprint ability (using the cumulative time for 8 x 20m sprints), and then randomly divided using randomization software (www.randomizer.org) into groups performing repeat-sprint training in hypoxia (RSH: n = 9, age: 20.3 ± 2.1 years; weight: 77.1 ± 10.2 kg; height: 173.9 ± 4.9 cm) or normoxia (RSN: n = 10, age: 22.0 ± 4.1 years, weight: 88.3 ± 14.1 kg; height: 177.9 ± 5.4 cm). The repeat sprint protocol was designed to be as sport-specific as possible, based on sprinting bouts observed during typical rugby matches[12]. All participants continued with similar training protocols throughout the study. Written informed consent was provided by the participants in accordance with the Declaration of Helsinki. The research was approved by the local University Human Ethics Committee (2015-46).

Procedures

This was a single-blind, randomised, placebo-controlled trial with similar numbers of participants divided into placebo and hypoxic groups. The lead researcher enrolled the participants, ran the random allocation sequence, and implemented the training protocols based on the randomised groups' particular intervention. All participants were blinded to their interventions (all believed they were receiving hypoxic air). No

alterations or amendments to the method were applied after the commencement of the trial. Recruitment into the study began in the second week of January, and the trial was started in the second week of February. All participants completed a familiarization session, followed 4 – 5 days later by their baseline testing session. Post-intervention testing occurred two days following the completion of the repeated sprint training. The study was concluded in the first week of April.

Participants were advised to arrive at testing sessions in a rested (no intense exercise for 24 hours) and well-hydrated stated. No caffeine was consumed for 12 hours prior to testing. Testing sessions were mirrored as closely as possible to each other to reduce the risk of the testing session or participant readiness and fatigue contributing to pre-post measurement differences. Each participant recorded their dietary intake before their baseline measurements for repetition prior to the post-intervention testing. Additionally, testing sessions were conducted at the same time of day (±1 h), and the measures were completed in the same order.

All tests were completed in a covered stadium on a slip-free floor under similar climatic conditions. Following a 15 min warm up (5-min slow jog and 5 – 10 mins of dynamic stretching), the participants completed a squat jump (data not reported here), repeated sprint test, and Yo-Yo Intermittent Recovery Level 1 test. Each test was separated by 10-15 mins of recovery.

Repeated sprint performance test

Participants completed 8 x 20 m maximal running-based sprints, timed to go every 20 s. Each sprint was recorded to the nearest 0.01 s using electronic speed-timing lights (Smartspeed, Fusion Sport, Ltd, Australia). Repeated sprint performance was assessed using mean sprint time.

Yo-Yo Intermittent Recovery Level 1 test

The Yo-Yo Intermittent Running Performance Test Level 1 is an intermittent, endurance-focused running test in which the athlete completes 2 x 20 m back-to-back shuttles interspersed by 10 s of active recovery. The test was externally paced using audible tones. As the test progressed, the tones got faster and the test was concluded when the athlete was unable to complete the shuttle before the tone sounds. The total distance (m) covered by the athlete was recorded.

Repeated sprint training

Repeated sprint training started one week after the baseline testing session. All participants completed cycling repeated sprint training twice a week for 3 consecutive weeks (total of 6 training sessions). Repeated-sprint training was performed in an upright, but seated position on a calibrated Wattbike (Wattbike Pro, Nottingham, UK) set up to participant-specific dimensions (saddle and handle bar height and position). The Wattbike was selected for its high reliability and low typical error of 2.6%[13]. In weeks 1 and 3, air brake and magnetic settings were adjusted to resistance level 3. Air brake resistance was increased to level 5 in week 2 to increase overload.

Training sessions started with a 5-min warm up at 50 W with a 5s sprint at the end of each minute. Participants completed 4 sets of 5 x 5-s repeated maximal-intensity sprints interspersed with 25 s between sprints and 5 min between sets of active recovery, slowly turning their legs over at 20-50 W. In total, training sessions included 35 minutes of exercise per session, and a total of 280 minutes over the course of the study (i.e. 280 mins of hypoxic exposure for the hypoxic group).

Hypoxic dosage during training

To improve blinding, all participants were informed that they would be receiving hypoxic air. Data that may indicate separate groups, such as blood oxygen saturation or heart rate, was also hidden from the participants. Prior to training, all participants were fitted with a face mask (Hans-Rudolph 8980, Kansas City, Missouri, USA) connected to one of 2 sets of 2x100L Douglas bags connected in series and containing either hypoxic (fraction of inspired oxygen (F_IO_2) of 14.5%, approx. 3000 m) or normoxic (F_IO_2 20.9%) air. The hypoxic or normoxic air was delivered to the participant via a one-way non-rebreathing valve (Hans-Rudolph 2700). Douglas bags were continually re-supplied with hypoxic or normoxic air using the GO_2Altitude® hypoxicator system (Biomedtech, Victoria, Australia).

An F_IO_2 of 14.5% was chosen as it has been found to increase physiological stress without impacting the typical attenuation in performance that normally occurs with such exercise[14].

Stress monitoring during training: Heart rate variability

Following arrival at the training session, participants were fitted with a heart rate monitor belt (WearLink, Polar, Kempele, Finland) and wrist watch (RS800CX, Polar, Kempele, Finland) and rested in a supine position in a dimly lit, quiet room for 10 mins. Heart rate variability was recorded on the wristwatch and later downloaded to a personal computer (HP Pavilion dv6 notebook, with Windows 10) using the Polar Protrainer software (Polar Protrainer 5™, Version 5.41.002) for HRV analysis. Data was filtered using the default error correction function (moderate filter power with a minimum protection zone of 6 beats per min). Identified errors were recorded, and all participants with an error rate of >5% were excluded from the dataset (RSH: n=1). On average, data was high quality (mean error percent 0.9 ± 1.1 %). The last 5 mins of the 10 min resting recording was used to determine resting heart rate (RHR) and time-domain analyses. Time domain analyses included the root mean squared of the successive difference (RMSSD) and the standard deviation of the N-to-N intervals (SDNN) as these time-domain measures are typically more reliable and display less variance than their frequency-domain counterparts[15]. Furthermore, the accuracy of HRV measures is vastly improved when the weekly average of heart rate variables is used compared to single "on the day" measurements[16]. Therefore, the weekly average of the HRV measurements taken before the training sessions was used. The low frequency to high frequency ratio was also included as this measurement has been associated with mountain climbers likely to develop acute mountain sickness[9].

Stress monitoring during training: Power output

Power output during training sessions was assessed by downloading and then averaging the peak power recorded by the Wattbike during each sprint in the sprint training (i.e. mean peak power of the 20 repeat sprints for each training day e.g. 4 sets of 5 reps).

Statistical analysis

A total of 17 participants were included in the analysis of this study. One participant in the RSH group was excluded due to outlying performance data indicating lower athletic ability than the rest of the cohort. A second participant in the RSH group was also excluded due to >5% errors detected in the filtering algorithm of the HRV analysis. Two researchers worked to examine and analyse the outcome measures in this study. All participants tolerated the interventions well, and none of the participants needed to stop their training at any stage.

Difference between hypoxic and normoxic repeat-sprint ability on stress markers

A pre-post parallel-groups spreadsheet[17] was used to assess the between-group differences in change scores from weeks 1 – 3 for measures of HRV. Cohen's value of 0.2 of the between-subject standard deviation was used to assess the smallest worthwhile change. Prior to analysis, data were log-transformed to reduce non-uniformity of error. Results are displayed as a percent change ± 90 % confidence interval, and qualitatively assessed using the clinical inference[18]. In this regard, an odds ratio of benefit: harm was only accepted if it was above 66%; if not, the effect was considered "unclear". For clear results, the magnitude of the change was reported using the following scale <0.5% = most unlikely; 0.5–5% = very unlikely; 5–25% = Unlikely; 25–75% = possibly; 75–95% = likely, 95–99.5% = very likely, >99.5% = most likely[18]. The direction of the change (increase, trivial or decreased) was determined, and interpreted according to the variable. In addition, *p*-values have been added to broaden the usefulness and scope of these results, particularly for those who do not use magnitude-based inferences.

Relationship between stress markers and post-intervention performance.

The athletes' week 3 HRV was correlated with the post-intervention performance using the Proc Corr procedure in the Statistical Analysis

System (Version 9.3; SAS Institute, Cary, NC). Correlations were interpreted using Cohen's scale of <0.10, 0.10, 0.30, 0.50, 0.70, and ≥0.90 for trivial, small, moderate, large, very large, and extremely large respectively[18].

Results

Blinding was effective as when questioned post-intervention only 20% of the participants in the control group suspected they were receiving either a higher oxygen dose or a placebo. All participants in the hypoxic group tolerated the hypoxia well, and no interruptions or cessations were necessary during the repeated sprint training in hypoxia.

Differences in stress markers in hypoxic vs normoxic groups

Compared to the participants who trained in a normoxic environment, the participants training in hypoxia had higher RHR and possibly lower HRV in week 3 compared to Week 1 (results are presented in Table 1). The higher LF/HF (%) values in the RSH group were driven by an increase in low frequency as well as a reduction in high frequency (ms) over weeks 1 – 3 (data not presented) in the RSH group compared to the RSN group.

Relationship between HRV variables and post-intervention exercise performance

The correlations between RHR, LnRMSSD and LF/HF ratio taken during Week 3 of the training period and post-intervention performance are presented in Figure 1. For the RSH group, measures with the strongest correlation with post-intervention performance change were resting heart rate, LF/HF ratio, and RMSSD. The correlation between heart measures and post-intervention performance were weaker in the RSN group.

There were likely trivial differences in the change in power output (-1.3, ± 7.5%, between-group difference in the week 3 – week 1 of the log-transformed change in performance) in the hypoxic (baseline: 824.4 ± 220.9 W to post-intervention: 826.3 ± 215.2 W) and normoxic (baseline: 990.2 ± 162.0 W to post-intervention: 1012.7 ± 203.0 W) repeated sprint training.

Table 1 The between-group differences in the physiological stress markers

			Week 1	Week 3	Between-group difference[a] (Week1-Week3)
HRV marker	Group	(n)	Mean ± SD	Mean ± SD	Change, ± 90% CL (%)
RHR (bpm)	RSN	10	74.8 ± 13.0	71.9 ± 10.1	3.2, ± 4.7% Possibly higher in RSH
	RSH	7	75.2 ± 12.1	74.9 ± 11.1	
RMSSD (ms)	RSN	10	48.2 ± 31.0	51.1 ± 27.1	-8.5, ± 19.1% Possibly lower in RSH
	RSH	7	37.0 ± 20.2	37.4 ± 21.0	
SDNN (ms)	RSN	10	73.7 ± 29.2	82.8 ± 25.9	-11.5, ± 25% Possibly lower in RSH
	RSH	7	69.2 ± 28.5	70.4 ± 26.9	
LF/HF (%)	RSN	10	301.7 ± 174.7	313.6 ± 151.8	-6.4, ± 42.3% Unclear
	RSH	7	469.9 ± 239.2	508.9 ± 301	

Note: [a]The between-group difference is the difference between RSN and RSH in week 1 compared to Week 3 using log-transformed means. CL, confidence limits; SD, standard deviation; RHR, Resting heart rate; HRV, heart rate variability; RSN, Group receiving a normoxic placebo during the repeated sprint intervention; RSH, group receiving hypoxic during the repeated sprint intervention; RMSSD, Root Mean Square of the Successive Differences; SDNN, Standard Deviation of the N-N interval; LF/HF, Low frequency to high frequency ratio expressed as a percent.

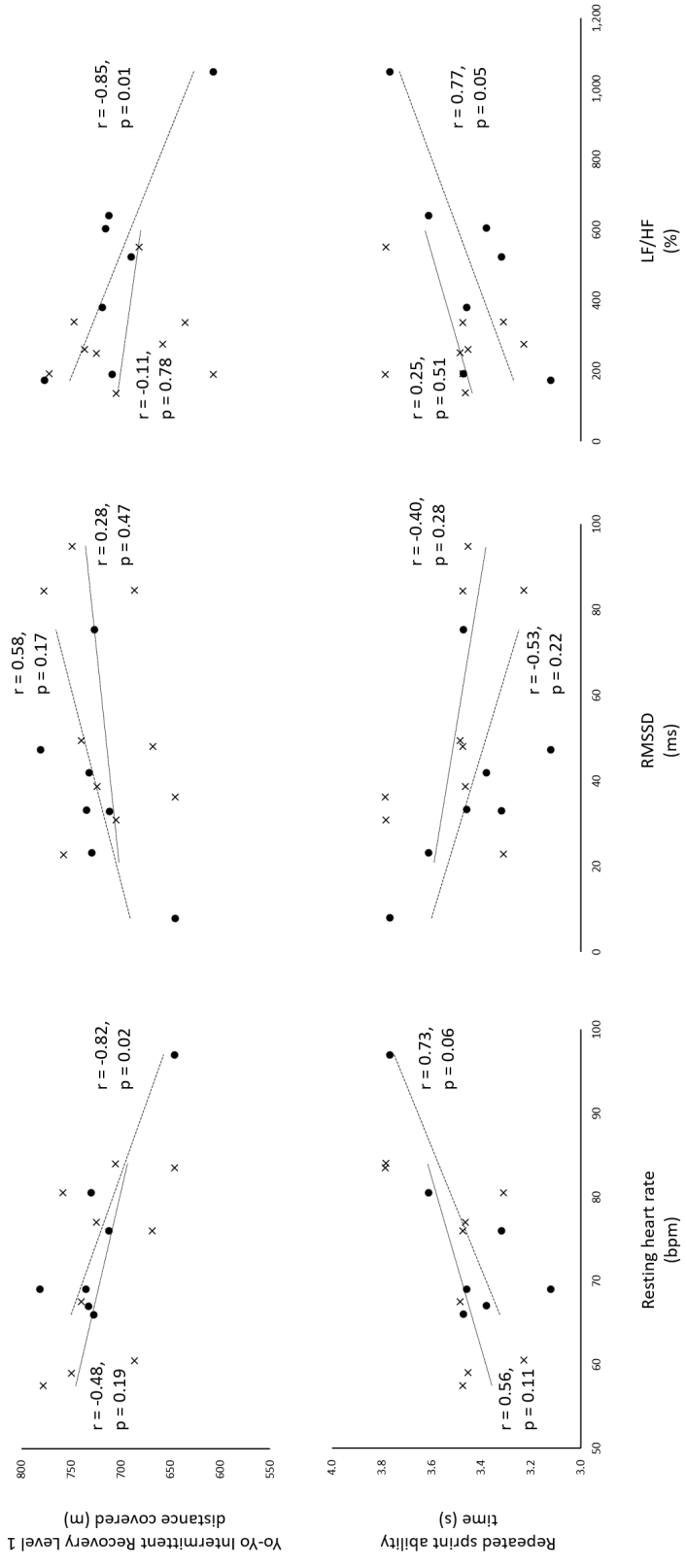


Figure 1 Correlations between heart rate and heart rate variability measurements and Yo-Yo Intermittent Recovery Level 1 and repeated sprint ability performances following repeated sprint training in hypoxia (circles, short dashes trendline) and normoxia (crosses; fine dots trendline). m, meters; s, seconds; bpm, beats per minute; RMSSD, root mean squared of the successive difference in heart beat intervals; ms, milliseconds; LF/HF, low frequency to high frequency ratio expressed as a percent.

Reliability of performance measures

The typical error between the familiarization and baseline tests for all participants for the RSA and YYIR were 0.7% (90% Confidence limit = 0.6 – 1.1%) and 7.5% (90% Confidence limit = 5.8 – 10.8) respectively.

Discussion

The objective of this study was first to determine the effect of short, repeated sprint training intervals in hypoxia vs repeated sprint training in normoxia on heart rate variability in a team sport population. Subsequently, the relationship between the post-intervention heart rate variability with subsequent performance was determined. Our study indicates that while RHR and HRV are sensitive enough to discriminate between participants receiving hypoxic training vs those receiving normoxic training, the effect of RSH on HRV is characterised more by an absence in HRV response, rather than an increase in HRV (suggesting increased parasympathetic innervation)[7] as was observed following RSN. Regarding the second objective, RHR and LF/HF had strong, statistically significant correlations with post-intervention aerobic and repeated sprint performance following RSH. These correlations were stronger than the correlations observed following RSN.

The absence of any change in HRV following RSH (in contrast to the increased HRV seen following RSN) may provide support for Schmitt and colleagues'[3] caution of an increased risk of overtraining with hypoxic repeated sprints compared to repeated sprint training in normoxia. That is, in the present study we increased the training stress in the RSH group but did not increase the recovery period more so than the RSN group, effectively increasing the overall workload and training stress[5,19] in RSH compared to RSN. As altitude training appears to be a particularly fatigue-inducing form of stress[3], this increased stress state would theoretically result in an increase in sympathetic innervation and a resultant reduction in HRV[7]. However, power output during training from week 1 to week 3 was either maintained or gradually increased indicating that over-reaching was avoided[8], and that it is unlikely that the training-stress was accumulating towards an elevated stress state[5]. What is likely is that the addition of hypoxia to the repeated sprint training intensified their normal preseason training, sustaining a higher level of training stress along the stress-adaptation continuum[19]. Continuation of such high-stress training over a longer timeframe may ultimately push the athletes further along the stress-adaptation continuum resulting in overtraining. Fortunately, the short time frame of the study, and low frequency of the RSH training allowed the hypoxic group to recover quickly and ultimately improve their repeated sprint ability compared to the normoxic group[11]. Indeed, following the cessation of the repeated sprint training intervention, and therefore with more relative rest, the hypoxic group continued to improve their repeated sprint ability compared to the control group[11] suggesting supercompensation occurred[5].

The RMSSD and SDNN values from the athletes in this study were in-line with healthy HRV values reported by Nunan et al.[20]. When taken together with the absence in power output drop-off during the repeat-sprint training in hypoxia, it is likely that RSH appeared to induce autonomic nervous system stress responses to a greater extent than when training in normoxia, but not to the extent that the additional physiological stress was detrimental to the athlete.

Correlation between HRV during training and post-training performance

The overall correlations between Week 3 HRV and post-intervention performance supports others who have found lower HRV to be associated with reduced performance[21]. In our study, the correlations between lower RHR, increased RMSSD and lower LF/HF values, all of which are typically due to increased vagal innervation[22], were all associated with improved performance in both the hypoxic and normoxic groups (see Figure 1).

Our findings support previous research which demonstrated an increase in HRV with an increase in YYIR1 performance following normoxic repeated sprint training in team sport athletes[23].

However, the findings in the present study indicate that an increase in HRV may not be evident immediately following repeated sprint training in hypoxia (see Table 1), possibly due to the increased training intensity in the hypoxic group[24]. Despite the hypoxic group's absence of improvement in HRV, the relationship between HRV variables and post-intervention performance was stronger compared to those receiving normoxic training (see Figure 1). This heightened relationship could possibly indicate the presence of responders and non-responders to hypoxic training[25]. That is, the response to hypoxic training can be quite binary, if the athlete tolerates the hypoxia well (and demonstrates increased HRV), they are likely to perform much better than if they had received similar training at sea-level. Conversely, if the hypoxic training is not tolerated, the athlete may enter a period of physiological distress which overwhelms the athlete's adaptive capabilities resulting in a reduction in resting HRV and worsened performances than had they trained at sea-level[8]. On the other hand, resting HRV during a period of repeated sprint training in normoxia, was not as strongly related to post-intervention performance as was noted in the hypoxic group. As the repeated sprint training was only held twice per week, it is possible that the normoxic training may have had less of an impact on the stronger or fitter athletes (yielding either a maintained or small increase in HRV). On the other hand, the weaker athletes in the normoxic group may have demonstrated a higher level of increased fitness and performance, with an associated increase in resting HRV. If this were the case, it may explain the overall increase in HRV seen in the RSN group (compared to the static HRV in the RSH group, see Table 1) as well as the weaker correlation between HRV and post-intervention performances in the RSN group (see Figure 1).

Of particular interest is that RHR had the strongest correlation with post-intervention performance. While more information[7] can be gleaned about a person's autonomic nervous system by means of HRV analysis, the more complex nature of the measurement, and the range in HRV outputs means that it is also more susceptible to breathing[26], technical and analytical[27] artefact compared to the more simple RHR measure. The increase in the risk of measurement artefact in the HRV outputs compared to the more straight-forward RHR measurement may be the reason for the higher correlation between RHR and post-intervention performance.

There were only small differences in the associations between the HRV variables and either repeated sprint ability and YYIR1. As repeated sprint ability and YYIR1 outcomes are not strongly associated with each other in team sport players[28], an improved autonomic balance appears to influence performance in a non-specific manner. For example, master and elite athletes typically have higher levels of HRV compared to similar-aged sedentary controls[29] regardless of whether the athlete are endurance- or sprint-athletes[30]. Therefore, it is unclear whether improved HRV is the driver of, or reflection of, generally improved performance.

Limitations

A correlation does not indicate causality, which, when combined with the small group sizes in our study, means that caution is needed inferring meaning from our results.

While the study of HRV is usually presented in a straight-forward manner, the results can be considerably more complex to interpret. This is particularly relevant in elite athletes where a decrease in HRV can be associated with improved performance[22]. The athletes in this study were all club-level athletes, as such, more research is needed before our findings can be asserted more confidently.

Conclusion

Our research supports the use of RHR and HRV measures to monitor training-related stress, particularly during repeated sprint training in hypoxia in club rugby players. Athletes in the RSH group demonstrated higher RHR and lower HRV during the training period when compared to the RSN group. While elevated RHR and lower HRV typically indicate higher levels of physiological stress, there were no reductions in training or

post-intervention performance in the hypoxic group compared to the normoxic group, and overtraining was likely avoided in RSH.

Our study demonstrated moderate to strong correlations between RHR and HRV measures taken immediately before training in Week 3, and both repeated sprint ability and YoYo intermittent recovery performance post-training. These findings support previous research in suggesting that higher HRV is predictive of better subsequent athletic performance, while extending the typical participant reach to include club-level rugby players receiving hypoxic exposure during repeated sprint training. Should future research support our findings, RHR and HRV could be useful tools in the prediction of post-training performance.

Take home messages

Monitoring resting heart rate and heart rate variability during the last week of repeated sprint training may predict YoYo-intermittent recovery and repeated sprint ability in club-level athletes after training. Lower resting heart rate and higher heart rate variability were associated with improved post-training performance.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors acknowledge the Lincoln University Research Fund (reference 2014-16E/INTF044) and Ara Institute of Canterbury for financial support for this project. The authors thank the player volunteers and their coaches for their assistance.

References

1. Billaut F, Gore CJ, Aughey RJ. Enhancing team-sport athlete performance: Is altitude training relevant? Sports Med 2012; 42: 751-67.
2. Hamlin MJ, Lizamore CA, Hopkins WG. The effect of natural or simulated altitude training on high-intensity intermittent running performance in team-sport athletes: A meta-analysis. Sports Med 2018; 48: 431-46.
3. Schmitt L, Regnard J, Coulmy N, Millet GP. Influence of training load and altitude on heart rate variability fatigue patterns in elite Nordic skiers. Int J Sports Med 2018; 39: 773-81.
4. Mann TN, Lamberts RP, Lambert MI. High responders and low responders: Factors associated with individual variation in response to standardized training. Sports Med 2014; 44: 1113-24.
5. Kellmann M. Preventing overtraining in athletes in high-intensity sports and stress/recovery monitoring. Scand J Med Sci Sports 2010; 20: 95-102.
6. Meeusen R, Duclos M, Foster C, Gleeson M, Nieman, D, Raglin J, et al. Prevention, diagnosis, and treatment of the overtraining syndrome: Joint consensus statement of the European College of Sport Science and the American College of Sports Medicine. Med Sci Sports Exerc 2013; 45: 186-205.
7. Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Circulation 1996; 93(5): 1043-65.
8. Hamlin MJ, Manimmanakorn A, Sandercock GR, Ross JJ, Creasy RH, Hellemans J. Heart rate variability in responders and non-responders to live-moderate, train-low altitude training. World Acad Sci Eng Technol 2011; 77: 936-40.
9. Sutherland A, Freer J, Evans L, Dolci A, Crotti M, Macdonald JH. MEDEX 2015: Heart rate variability predicts development of acute mountain sickness. High Alt Med Biol 2017; 18: 199-208.
10. Karinen HM, Uusitalo A, Vähä-Ypyä H, Kähönen M, Peltonen JE, Stein PK, et al. Heart rate variability changes at 2400 m altitude predicts acute mountain sickness on further ascent at 3000-4300 m altitudes. Front Physiol 2012; 3: 336-36.

11. Hamlin MJ, Olsen PD, Marshall HC, Lizamore CA, and Elliot CA. Hypoxic repeat sprint training improves rugby player's repeated sprint but not endurance performance. Front Physiol 2017; 8: 1-10.
12. Jones MR, West DJ, Crewther BT, Cook CJ, Kilduff LP. Quantifying positional and temporal movement patterns in professional rugby union using global positioning system. Eur J Sport Sci 2015; 15: 488-96.
13. Hopker J, Myers S, Jobson SA, Bruce W, Passfield L. Validity and reliability of the Wattbike cycle ergometer. Int J Sports Med 2010; 31: 731-36.
14. Bowtell JL, Cooke K, Turner R, Mileva KN, Sumners DP. Acute physiological and performance responses to repeated sprints in varying degrees of hypoxia. J Sci Med Sport 2014; 17: 399-403.
15. Kuss O, Schumann B, Kluttig A, Greiser KH, Haerting J. Time domain parameters can be estimated with less statistical error than frequency domain parameters in the analysis of heart rate variability. J Electrocardiol 2008; 41: 287-91.
16. Plews DJ, Laursen PB, Kilding AE, Buchheit M. Evaluating training adaptation with heart rate measures: A methodological comparison. Int J Sport Physiol 2013; 8: 688-91.
17. Hopkins W. Spreadsheets for analysis of controlled trials, with adjustment for a subject characteristic. Sportscience 2006; 10: 46-50.
18. Hopkins W, Marshall SW, Batterham AM, Hanin J. Progressive statistics for studies in sports medicine and exercise science. Med Sci Sport Exerc 2009; 41: 3-12.
19. Fry RW, Morton AR, Keast D. Overtraining in athletes: An update. Sports Med 1991; 12: 32-65.
20. Nunan D, Sandercock GRH, Brodie DA. A quantitative systematic review of normal values for short-term heart rate variability in healthy adults. Pacing Clin Electrophysiol 2010; 33: 1407-17.
21. Bellenger CR, Fuller JT, Thomson RL, Davison K, Robertoson EY, Buckley JD. Monitoring athletic training status through autonomic heart rate regulation: A systematic review and meta-analysis. Sports Med 2016; 46: 1461-86.
22. Plews DJ, Laursen PB, Stanley J, Kilding AE, Buchheit M. Training adaptation and heart rate variability in elite endurance athletes: Opening the door to effective monitoring. Sports Med 2013; 43: 773-81.
23. Soares-Caldeira LF, de Souza EA, de Freitas VH, de Moraes SM, Leicht AS, Nakamura FY. Effects of additional repeated sprint training during preseason on performance, heart rate variability, and stress symptoms in futsal players: a randomized controlled trial. J Strength Cond Res 2014; 28: 2815-26.
24. Plews DJ, Laursen PB, Kilding AE, Buchheit M. Heart-rate variability and training-intensity distribution in elite rowers. Int J Sport Physiol 2014; 9: 1026-32.
25. Chapman RF, Stray-Gundersen J, Levine BD. Individual variation in response to altitude training. J Appl Physiol 1998; 85: 1448-56.
26. Buchner T. HRV strongly depends on breathing. Are we questioning the right suspect? Eng Med Biol Soc 2011; 2011: 7739-42.
27. Kemper KJ, Hamilton C, Atkinson M. Heart rate variability: impact of differences in outlier identification and management strategies on common measures in three clinical populations. Pediatr Res 2007; 62: 337-42.
28. Chaouachi A, Manzi V, Wong DP, Chaalali A, Laurencelle L, Chamari K, et al. Intermittent endurance and repeated sprint ability in soccer players. J Strength Cond Res 2010; 24: 2663-69.
29. Sotiriou P, Kouidi E, Samaras T, Deligiannis A. Linear and non-linear analysis of heart rate variability in master athletes and healthy middle-aged non-athletes. Med Eng Phys 2013; 35: 1676-81.
30. Deus LA, Sousa CV, Rosa TS, Filho JMS, Santos PA, Barbosa LD, et al. Heart rate variability in middle-aged sprint and endurance athletes. Physiol Behav 2019; 205: 39-43.

ORIGINAL ARTICLE

Arch AHS

Archives of Allied Health Sciences 2021; 33(1): 19-33.

Prebiotic capsules containing anthocyanin, inulin and rice bran extracts increased plasma ascorbate of overweight or obese subjects

Panicha Pongnaratorn[1,2,3], Yupaporn Kanpetta[2,4], Ploypailin Aneknan[2], Juntanee Uriyapongson[5], Panakaporn Wannanon[6], Charnchai Panthongviriyakul[6], Naruemon Leelayuwat[1,2,6*]

[1] Graduate School, Khon Kaen University, Khon Kaen, Thailand.
[2] Sports and Exercise Science Program and Research Group, Khon Kaen University, Khon Kaen, Thailand.
[3] Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon Nakhon, Thailand.
[4] Department of Sports and Exercise Science, Khon Kaen University, Nong Khai, Thailand.
[5] Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand.
[6] Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

KEYWORDS
Antioxidant;
Dietary fiber;
Insulin resistance;
Leptin resistance;
Anthropometry.

ABSTRACT
Prebiotic foods have an important role in health well-being, especially for treating overweight or obesity by promoting gut microbiota to improve antioxidant activity and fat metabolism. We aimed to investigate the effect of prebiotic capsules containing anthocyanin, inulin and rice bran extract on antioxidant, leptin and insulin resistance, and fat metabolism of overweight or obese subjects. The research was a randomized double-blinded controlled trial. Sixty overweight or obese subjects were randomly allocated into two groups; 1) 350 mg/prebiotic capsule (prebiotic group) or 2) 350 mg/maltodextrin (placebo group), 3 capsules after each meal and before going to bed (4.2 g.day-1) for 30 days. Before and after 30 days of supplementation, anthropometry and body composition were measured. Blood samples were collected to analyze ascorbate, glucose, lipid profiles, leptin and insulin concentrations. An expired gas was collected for 5 min to determine substrate utilization. The results showed prebiotic capsules increased plasma ascorbate concentration (placebo group: before 34.6 ± 3.33 and after 45.2 ± 4.48 µmol/L; treatment group: before 35.7 ± 4.43 and after 62.3 ± 6.47 µmol/L, 95% CI 16.1(4.27-27.9), *p*-value < 0.05). Comparing within group, hip circumference, lipid profiles, and leptin and insulin concentrations were significantly decreased with prebiotic supplementation comparing to the baseline data (*p*-value < 0.05). However, there were no significant changes from baseline in fat and carbohydrate oxidation rates, plasma glucose, liver and kidney functions within and between groups (*p*-value < 0.05). In conclusion, supplementation of prebiotic capsule containing 1.89 g anthocyanin, 1.89 g inulin and 0.42 g rice bran per day for 30 days increased plasma ascorbate concentration in overweight or obesity subjects. Daily supplementation of 4.2 g prebiotics for 30 days did not cause liver and kidney dysfunctions. However, either higher dose or longer duration of the supplementation is warranted to show more variables those are significant and to confirm the safety.

* **Corresponding author:** Naruemon Leelayuwat, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.
E-mail: naruemon@kku.ac.th
Received: 7 May 2020/ Revised: 4 July 2020/ Accepted: 9 July 2020

Introduction

Worldwide prevalence of overweight and obesity has doubled increment since 1980 to nearly a third of the world's population[1]. According to the Asian-Pacific cutoff points, classification of overweight by body mass index (BMI) is between 23 and 24.9 kg/m^2, and obesity is higher than 25 kg/m^2[2]. BMI is demonstrated to be associated with oxidative stress, in consequence of impaired antioxidant status such as vitamin C[3], abnormal lipid metabolism[4] and gut microbiota[5]. Amount of short-chain fatty acids (SCFA) (such as acetate and l-lactate) was increased by fermentation of carbohydrates by microbiota[6]. The SCFA is used as metabolic substrates by the host cell to increase the energy determined by fatty acid oxidation. Besides, the microbiota dysfunction contributes to leptin[7] and insulin resistance[8]. Accordingly, overweight and obesity increase risks of many life-threatening diseases such as diabetes mellitus type 2[9]. Prebiotics are food components that induce the growth or activity of beneficial microbiota. Thus, prebiotic food is an alternative choice being developed for treating overweight and obesity[10].

Prebiotics used in this study are anthocyanin from black rice as an antioxidant and inulin from Jerusalem Artichoke and rice bran from rice mill as indigestible dietary fiber. The antioxidant activity has the potential to improve gut microbiota, which contributes to improved metabolic health. Many studies showed that the supplementation of anthocyanin extracted from black rice bran 1.8 g/day for 8 weeks displays very high antioxidant and anti-inflammatory activities resulting in a decrease in blood lipid concentrations and protecting the arteries[11-13]. Anthocyanin 2.5 g/day for 8 weeks also decreased abdominal fat, waist and hip circumferences of the people with overweight or obese[14,15].

The indigestible dietary fiber including inulin from Jerusalem artichoke and rice bran from Glutinous Rice RD6 dissolved in water increases viscosity leading to the delay of gastric emptying time. This dietary fiber can be digested and fermented by bacteria in the large intestine resulting in an increase of short-chain fatty acids (SCFAs)[6]. Dietary fiber also helps to increase the utilization of glucose and fat resulting in weight loss in overweight or obese people[16]. In addition, a previous study reported that a combination of inulin (4 g/day) and anthocyanin (163 mg/day) for 4 weeks can stimulate the growth of microorganisms in the digestive tract and increase satiety hormone and improves blood glucose tolerance[17]. Moreover, there are no previous studies exploring the effects of capsule containing all three prebiotics on various variables and on liver and kidney functions.

Taken together, we therefore aimed to investigate the effects of prebiotic capsule containing 1.89 g anthocyanin, 1.89 g inulin and 0.42 g rice bran extract per day for 30 days primarily on antioxidant, and subsequently on leptin and insulin resistance, and fat metabolism of overweight or obese subjects. We used plasma ascorbate to indicate antioxidant because it plays role in increased fat oxidation[18] which has been shown to improve insulin sensitivity[19]. Besides, it was demonstrated to improve lipid profile including cholesterol[20,21]. We hypothesized that the supplementation of the prebiotic capsule would improve the above variables for overweight or obese subjects.

Materials and methods

Subjects

All subjects signed an informed consent after being clarified by both verbal and writing information. Our research has been approved by the Khon Kaen University Ethics Committee on Human Research in accordance with the declaration of Helsinki (HE601277) and was registered in Thai Clinical Trials (TCTR20180317002) on March 14, 2018. The subjects were recruited if they met these criteria: males and females, aged 20-50 years with overweight (BMI between 23 and 24.9 kg/m^2), or obesity (BMI equal to or more than 24.9 kg/m^2)[2], no underlying diseases such as diabetes mellitus, cardiovascular, neuromuscular disorders, liver and kidney diseases. All subjects received the preliminary test of physical characteristics and anthropometry examinations including the height, mass, body

mass index (BMI), waist and hip circumferences, waist/hip ratio and electrocardiography (ECG). Their whole-body fat and muscle mass were assessed using Dual-Energy X-Ray Absorptiometry (DXA). Blood sample was collected to analyze anemia by measuring complete blood count, liver function by measuring serum glutamic-pyruvic transaminase (SGPT), kidney function by measuring blood urea nitrogen (BUN) and creatinine (Cr). Also, blood glucose, insulin and lipid profiles were analyzed. Moreover, the dietary intake and physical activity were recorded 3 days a week; 2 days on weekdays (Mon-Fri) and one day on weekends (Saturday or Sunday). The dietary data were reported to calculate total energy intake and the percent of three major nutrients (protein, carbohydrate, and fat) per day using the Inmucal-nutrients® version 3 software (Institute of Nutrition, Mahidol University, Thailand).

Study design

This research is a randomized, double-blinded, placebo, parallel controlled trial. Sixty subjects (51 females and 9 males) with overweight or obesity were divided into two groups; 1) 350 mg/ prebiotic capsule, 3 capsules 4 times a day for 30 days (4.2 g.day^{-1}) (prebiotic group) or 2) 350 mg/ maltodextrin (placebo group).

The sixty subjects including 15% dropout in this study were obtained based on the study of Parnell and Reimer, 2009[22]. The authors reported a statistically significant decrease in body weight 1.03 ± 0.4 kg due to the effects of oligofructose inulin. Therefore, this reported using researcher respect to the volunteers may have body weight decrease as 0.7 ± 0.43 kg. According to statistical calculation by program G' power 3.0, alpha concentration as 0.05 and power at 80% sample size of this study was 26 subjects and dropout 4 subjects in each group.

Protocol

After subjects passed the screening, they visited the laboratory for 3 occasions; visit 1, for pre-test and received the first half pack of the supplement capsules, visit 2, for blood sampling for liver and kidney functions and received the second half pack of the supplement capsules, and visit 3, for the post-test. All subjects did not change their daily dietary intake and physical activity throughout the experiment. Moreover, this study compliance was followed up by phone once a week. Before and after the supplementation. Five ml of blood samples were collected and divided into 5 of 1 ml tubes to measure glucose (sodium fluoride tube), ascorbic acid (lithium heparin tube wrapped with aluminum foil), insulin (SST gel tube), lipid profiles and leptin (EDTA tube). Before and after the supplementation, expired gas was collected for 5 min to determine oxygen uptake ($V\dot{O}_2$) (L/min) and carbon dioxide production ($V\dot{C}O_2$) (L/min) using a gas analyzer (Jaeger Oxycon Mobile; Jaeger, Germany). Carbohydrate and fat utilization were calculated based on $V\dot{O}_2$ and $V\dot{C}O_2$.

Preparation of prebiotic capsule containing anthocyanin, inulin and rice bran

Prebiotic capsule containing anthocyanin, inulin and rice bran extract (45:45:10) and placebo capsule containing maltodextrin were prepared under good manufacturing practices (GMP) in the Faculty of Food Technology, Khon Kean University, Thailand. Each prebiotic capsule contains 1.89 g anthocyanin, 1.89 g inulin, and 0.42 g rice bran extract. Placebo capsule contained 350 mg maltodextrin powder with the same color, size, and odor of the prebiotic. Both prebiotic extracts and placebo were in green color capsules.

The followings were the methods of the anthocyanin, inulin, and rice bran extracts and placebo production.

- Anthocyanin extract: black rice was boiled with hot water 85 ºC for 30 minutes and then the waste was separated. The liquid part was mixed to 20% maltodextrin and anthocyanin powder was produced by spray dryer machine at 160 ºC.
- Inulin extract: fresh Jerusalem artichoke was washed with clean water. Then it was cut into thin sheets and dried in a hot air oven at 60ºC for 12-14 hours until it had a moisture content of 9-10%. Then, it was boiled in hot water at 85-90 ºC for 30 minutes, after that the liquid was removed and mixed with 5% maltodextrin. The inulin powder was produced by spray dryer machine at 160 ºC.

- Rice bran (soluble dietary fiber) extract: glutinous rice RD6 was crushed and boiled in hot water with 95ºC for 20 minutes. After that, the liquid was removed by filtering. The dietary fiber powder was produced by spray dryer machine at 160 ºC.
- The prebiotic and placebo capsules were kept in zip lock bag labeled with expiration dates and the method required for oral ingestion. Both capsules were stored at 4 ºC in a refrigerator for maintaining quality of active ingredients.

All subjects took 350 mg/capsule 4 times a day (4.2 g/day) for 30 days. Contaminant level was measured for safety by the Central Laboratory, Thailand (Co., Ltd). The prebiotic and placebo capsules were kept in zip lock bags labeled with expiration dates and the method of how to ingest orally. All capsules were stored at 4 ºC in a refrigerator to maintain the quality of active ingredients until we use.

Outcome measurements

Plasma ascorbate

One mL of whole blood was centrifuged 3,000 x g at 4 ºC for 10 min. The upper plasma layer was separated and added with 1 mol/L $HClO_4$ to precipitate protein. Then, the tubes were centrifuged and wrapped with aluminum foil to be protected from sunlight. The upper solution was stored at -80ºC until analyzed. Plasma ascorbate concentration was analyzed by Zhang's assay[23]. In this assay method, Fe (III) was deoxidized to Fe (II) by ascorbate at pH 4.0 and Fe (II) reacted with potassium ferricyanide to form a blue product, soluble prussian blue [KFe^{III} [$Fe^{II}(CN)_6$]. The absorbance was measured using a spectrophotometer at 735 nm. The amount of ascorbate was calculated using the standard curve.

Plasma leptin

Plasma leptin concentration was measured using an enzyme immunometric assay (Human Leptin Enzyme Immunoassay Kit of SPI-Bio®, France) as a double-antibody sandwich technique. The wells of plate supplied in the kit were coated with a polyclonal antibody specific of human leptin. The plasma 100 µl and EIA buffer 100 µl was put on 96 wells. The plate was incubated in the dark at 20°C for 10 min to avoid being exposed to direct sunlight. Aluminum foil was recommended to be used to cover the plate. The activity developing color was stopped by adding 100 µL of stop solution and read the absorbance by ELISA plate reader at 450 nm within 5 min after addition of the stop solution.

Other biochemical analyses

Before and after the prebiotic supplementation, after 12 hours of overnight fasting blood, samples 3 mL were collected from the antecubital vein. Blood glucose concentrations were measured using the glucose oxidase method (YSI 2300 STAT Plus™, USA, which was auto calibrated in every five sample test) immediately after blood collection. Serum insulin concentration was analyzed using a radio-immunoassay kit (ImmuChem™ Coated Tube kit of MP Biomedicals®, USA). In addition, homeostatic model assessment for insulin resistance (HOMA-IR) was calculated using the equation of {HOMA-IR formula = Fasting glucose (mmol/L) x Fasting insulin (mIU/L)/22.5}. From 2 mL blood sample in EDTA-tubes, 32 µL was used to analyze plasma lipid profile, creatinine, BUN and SGPT by Reflotron strips (Reflotron® Plus, Boehringer Mannheim, German

Substrate oxidation rate

Carbohydrate and fat oxidation rates were calculated based on $\dot{V}O_2$ (L/min) and $\dot{V}CO_2$ (L/min) using the Peronnet and Massicotte equation ignoring protein oxidation rate[24].

Statistical analysis

Normal distributions were tested with the Kolmogorov-Smirnov test. Statistical analyses were performed using SPSS 19 package software. To compare within group difference (before and after), paired *t* test was used. The difference between the grouped volunteers was analyzed using an analysis of covariance (ANCOVA). Statistically significant difference was taken at *p*-value < 0.05. Data are expressed as mean ± SD except stated elsewhere.

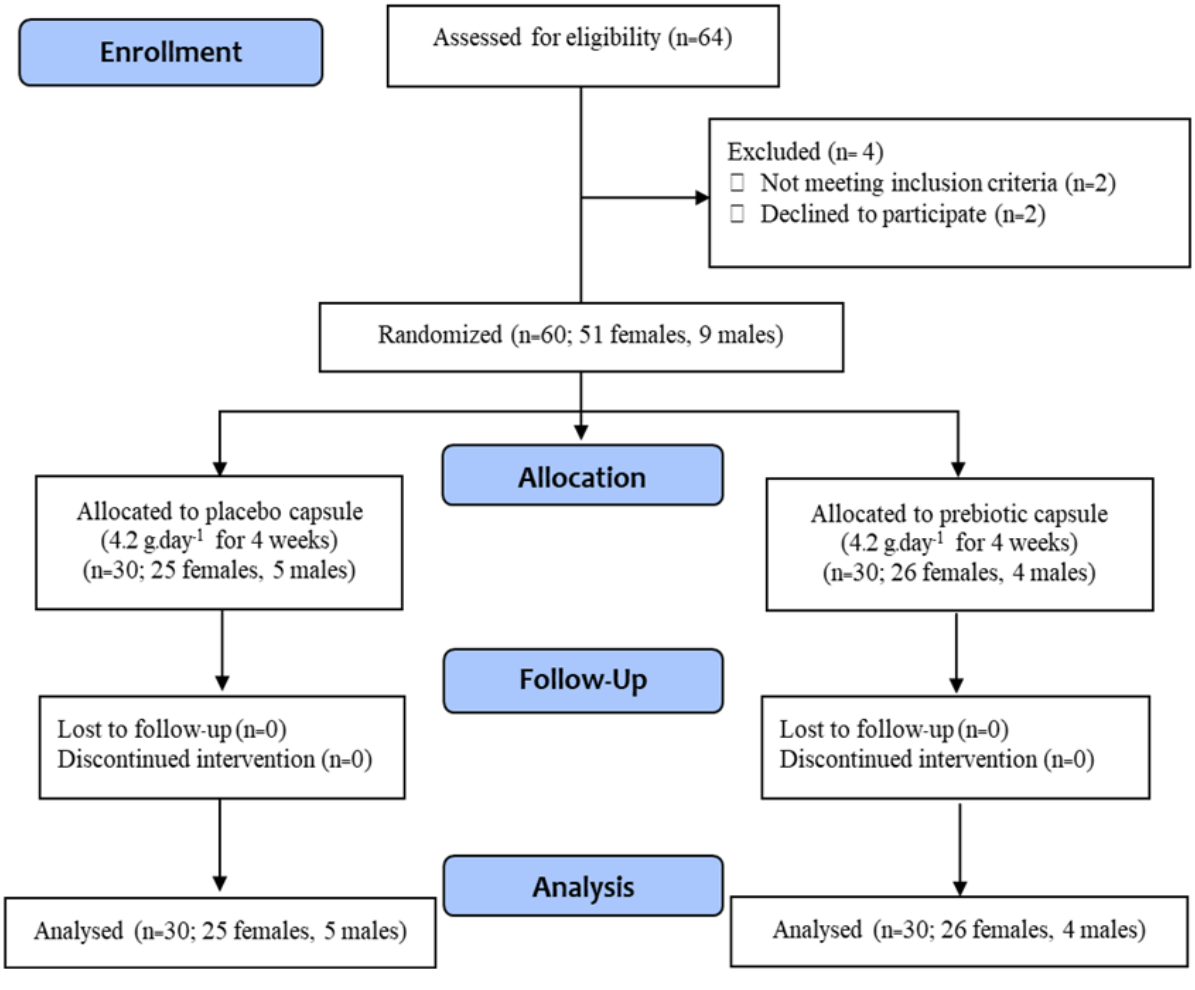


Figure 1 Flow chart of the trial protocol

Results

Originally this study had 64 subjects (54 females, 10 males). However, four subjects were excluded because they did not meet the inclusion criteria (n=2) and decided not to participate (n=2). Sixty subjects were separated into 2 groups with 30 persons each group, placebo group (25 females, 5 males) and prebiotic group (26 females, 4 males), whose data and samples completely collected with the study request in Figure 1.

Baseline physiological characteristics and energy intake and expenditure

The baseline physiological characteristics of all subjects were summarized in Supplementary Table 1. The characteristics of subjects including age, height, body mass, BMI, and blood pressure were not significantly different between placebo and treatment groups. Moreover, as shown in Supplementary Table 2, daily dietary components of energy intake, and energy expenditure did not differ between placebo and treatment groups (p-value > 0.05).

Anthropometry and body composition

After supplementation of placebo and prebiotic capsules, body mass, fat mass, lean mass, BMI, waist and hip circumferences, waist/hip circumference ratio were not significantly different (p-value > 0.05) before and after supplementation between both groups except hip circumference of the treatment group which was significantly (p-value < 0.05) lower than that of the baseline in Supplementary Table 3.

Plasma ascorbate

Plasma ascorbate concentrations after treatment was significantly higher in the prebiotic treatment group than those in the placebo group (p-value < 0.05). Both placebo and treatment groups showed an increase in ascorbic acid concentrations compared to that at the baseline (p-value < 0.05) in Table 1.

Plasma glucose, leptin, insulin, lipid profiles

While leptin and insulin concentrations of the placebo group increased after treatment, those of the prebiotic treatment group decreased from baseline after treatment (p-value < 0.05) in Table 1. Plasma leptin and insulin concentrations after supplementation did not significantly differ between the treatment and placebo groups.

Total cholesterol concentration was significantly decreased (p-value < 0.05) after prebiotics supplementation, whereas there were no changes in placebo group (Table 1). Insulin resistance was significantly increased in control group (p-value < 0.05), whereas it did not change after prebiotics supplementation. In addition, glucose, triglyceride, LDL, HDL, TC/HDL ratio, and LDL/HDL ratio were not significantly different within and between groups. Insulin, HDL, and leptin are tended to be lower than the control group in Table 1.

Substrate utilization

Carbohydrate and fat oxidation rates, as well as, the percentage of carbohydrate and fat contribution to total energy expenditure were not significantly different within and between groups in Table 2.

Liver and kidney functions

There were no significant changes in SGPT, BUN and creatinine concentrations before and after supplementation in both groups (Table 3). Also, SGPT, BUN, and creatinine concentrations were within normal range. These values confirmed the safety of the prebiotics capsule on liver and kidney functions.

Table 1 Blood variables of subjects in both groups

Variables	Placebo group		Prebiotic group		Mean difference	95% CI		*p*-value
	Before	After	Before	After		Lower	Upper	
Plasma glucose (mg/dL)	92.50 ± 3.90	94.20 ± 3.42	101.70 ± 4.88	98.00 ± 3.67	-2.89	-12.91	7.13	0.57
Insulin (µIU/mL)	5.29 ± 0.82	6.70 ± 0.86*	7.82 ± 1.77	7.07 ± 1.17*	1.81	-0.05	3.66	0.06
Insulin resistance (HOMA-IR)	1.21 ± 0.19	1.61 ± 0.10*	1.55 ± 0.20	1.32 ± 0.20	0.33	-0.11	0.77	0.14
TC (mg/dL)	202.20 ± 6.18	190.50 ± 8.07	212.10 ± 8.43	197.00 ± 8.03*	1.33	-14.71	17.36	0.87
TG (mg/dL)	116.50 ± 8.41	106.30 ± 7.68	118.20 ± 10.41	107.60 ± 7.88	-0.66	-20.29	18.96	0.95
LDL (mg/dL)	123.90 ± 5.50	112.50 ± 8.35	126.50 ± 7.37	119.70 ± 6.30	-1.83	-17.02	13.37	0.81
HDL (mg/dL)	54.90 ± 2.17	56.80 ± 4.06	57.40 ± 3.03	55.70 ± 3.32	6.82	-1.03	14.66	0.09
TC/HDL ratio	3.83 ± 0.18	3.76 ± 0.31	3.72 ± 0.22	3.67 ± 0.24	0.17	-1.22	1.56	0.81
LDL/HDL ratio	2.42 ± 0.14	2.29 ± 0.22	2.20 ± 0.18	2.17 ± 0.14	0.26	-1.08	1.59	0.70
Plasma ascorbate (µmol/L)	34.60 ± 3.33	45.20 ± 4.48*	35.70 ± 4.43	62.30 ± 6.47*#	16.08	4.27	27.88	0.01*
Plasma leptin (mg/mL)	4.40 ± 0.36	4.76 ± 0.37*	4.84 ± 0.32	4.66 ± 0.31*	0.47	-0.05	0.99	0.074

Note: Data are expressed as mean ± SE; n = 30 in each group (placebo group: 25 females, 5 males and prebiotic group: 26 females, 4 males); TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein; HDL, high density lipoprotein.
*Significantly different from before supplementation (*p*-value < 0.05).
#Significantly different from placebo group at the same condition (*p*-value < 0.05).

Table 2 Substrate utilization of subjects in both groups

Variables	Placebo group		Prebiotic group		Mean difference	95% CI		*p*-value
	Before	After	Before	After		Lower	Upper	
CHO oxidation rate (g/min)	0.12 ± 0.03	0.14 ± 0.02	0.12 ± 0.03	0.13 ± 0.02	0.01	-0.05	0.07	0.74
Fat oxidation rate (g/min)	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.01	-0.01	0.03	0.28
CHO oxidation rate (mg/kg.BM/min)	3.18 ± 0.77	3.61 ± 0.65	3.33 ± 0.63	3.49 ± 0.43	0.12	-1.51	1.76	0.88
Fat oxidation rate (mg/kg.BM/min)	2.00 ± 0.28	1.80 ± 0.20	1.86 ± 0.23	1.59 ± 0.20	0.03	-0.58	0.63	0.93
% CHO contribution to total energy expenditure	42.50 ± 8.87	45.20 ± 7.31	42.00 ± 8.82	50.70 ± 5.46	-4.02	-20.78	12.74	0.63
% Fat contribution to total energy expenditure	57.50 ± 8.87	54.90 ± 7.31	58.00 ± 8.82	49.30 ± 5.46	4.02	-12.74	20.78	0.63

Note: Data are expressed as mean ± SE; n = 30 in each group (placebo group: 25 females, 5 males and prebiotic group: 26 females, 4 males); CHO, carbohydrate.

Table 3 Blood variables determining liver and kidney functions of subjects in both groups

Variables	Placebo group		Prebiotic group		Mean difference	95% CI		*p*-value
	Before	After	Before	After		Lower	Upper	
SGPT (µ/L)	17.40 ± 1.65	17.90 ± 2.56	17.20 ± 2.18	17.20 ± 1.38	4.67	-2.44	11.78	0.19
BUN (mg/dL)	12.70 ± 0.44	13.30 ± 0.44	12.60 ± 0.51	12.80 ± 0.45	0.51	-0.63	1.64	0.38
Creatinine (mg/dL)	0.84 ± 0.03	0.78 ± 0.04	0.76 ± 0.04	0.78 ± 0.03	-0.03	-0.11	0.05	0.50

Note: Data are expressed as mean ± SE; n = 30 in each group (placebo group: 25 females, 5 males and prebiotic group: 26 females, 4 males); SGPT, serum glutamic-pyruvic transaminase; BUN, blood urea nitrogen.

Discussion

The present results show that supplementation of 4.2 g/day prebiotic capsule containing anthocyanin, inulin, and rice bran extract for 30 days increased fasting plasma ascorbate concentration. Also, prebiotic supplementation could decrease plasma triglyceride, insulin, and leptin concentrations and hip circumference. However, both prebiotic treated and placebo treated groups did not show any changes of substrate oxidation rates, blood glucose and insulin resistance. Prebiotic supplementation for 30 days did not affect liver and kidney functions.

We hypothesized that supplementation of the prebiotic capsule containing anthocyanin, inulin and rice bran extract would improve primarily on antioxidant, and subsequently on leptin and insulin resistance, and fat metabolism of overweight or obese subjects. The results partially supported our hypothesis since prebiotic supplementation significantly increased plasma ascorbate concentration, which has antioxidant activity[25]. For now, there have been no previous reports of the influence of any one of, or combination of, the three extracts on plasma ascorbate of overweight or obese subjects. Only

one previous work on those prebiotics was the research in healthy subjects[26]. Their results supported our findings although they used less anthocyanin amount than ours. They reported that 400 mg/50 kg body weight of anthocyanin-rich food increased plasma ascorbate concentration in healthy subjects with 50-65 kg body weight[26]. Anthocyanin also increased activity of another antioxidant such as superoxide dismutase (SOD). Intake of 750 mL bolus of anthocyanin-rich fruit juice containing 205.5 mg anthocyanin daily for 9 weeks significantly increased SOD activity[27]. On the other hand, Ellinger et al. (2012) did not find the antioxidant activity following 400 mL of fruit juice (27.9 mg anthocyanin) consisted of açai, camu-camu and blackberry in healthy nonsmokers, and this may be due to the insufficient dose of anthocyanin in the latter[28]. Another prebiotic, inulin, also has antioxidant activity in vitro and in vivo in animal and human studies. In vitro experiment showed that a DPPH radical scavenging activity of inulin at doses of 0.25 to 10 mg/mL increased linearly ($R2$ = 0.985, p-value<0.05)[29]. In animal experiment, inulin supplementation at the doses of 10, 15 and 20 g/kg increased antioxidant enzyme activities of SOD, catalase, and glutathione peroxidase and the total antioxidant capacity[29]. Likewise, supplementation of 10 g/day oligofructose-enriched inulin has antioxidant effect on women with type 2 diabetes[30]. In contrast, Hasan et al. (2018) could not find the antioxidant effect of intake of 30 g flavored-rice bran powder and 220 ml rice bran oil for 14 days in obese subjects[31], and this may be due to insufficient supplementation time. Further researches investigating the effects of the capsule in the present study on those antioxidants are needed.

In the present study, we found the significant improvement on total cholesterol, leptin and insulin concentrations, and hip circumference after prebiotic supplementation in prebiotic group and tended to be more improved than control group. There have not been any studies reporting the effects of the combination of three extracts on total cholesterol, insulin and leptin hormones and hip circumference. A review literature[32] reported that anthocyanin could reduce total cholesterol concentration. Studies on inulin at the dose of 7 g/day for 4 weeks to obese and 10 g/day for 8 weeks to diabetes type 2 subjects decreased total cholesterol[30,33]. A previous study reported also that plasma total cholesterol concentration of 12 healthy male subjects decreased after taking 50 g of cereals containing 18% inulin[34]. In this study, in agreement with Van der Beek et al.[35], plasma insulin concentration was reduced in the prebiotic supplemented group. Van der Beek et al.[35] investigated the effects of 24 g inulin in high-fat milk shake supplementation and found the decrease of plasma insulin concentration 3 hr after the consumption during 7 hr follow-up. Furthermore, 10 g/day inulin supplementation decreased plasma insulin concentration at 4 weeks in patients with type I and II diabetes mellitus[34,36]. No publication is available on the effect of anthocyanin, inulin, or rice bran on leptin concentration of people with overweight/obesity. In addition, anthocyanin-rich black soybean extracts (2.5 g/day) for 8 weeks was shown to decrease hip circumference but was not significantly different from control group in people with overweight/obese[14]. Administration of inulin 9 g/day for 18 weeks to obese dyslipidemia subjects could decrease hip circumference although it statistically was not different from control group[37]. No reference is available on the effect of consumption of rice bran on hip circumference.

In this study, other outcomes i.e. substrate oxidation rates, blood glucose, and leptin and insulin resistance were not affected by the supplementation of combined prebiotics. These results are inconsistent with previous studies. Administration of 11 g/day anthocyanin for 4 weeks was reported to improve plasma glucose concentration[38]. Similarly, rice bran at 10 g/day for 8 weeks also reduced hyperglycaemia of diabetes mellitus Types I and II patients[39]. The dose and duration of the prebiotics supplementation in this study may not be enough to cause the significant effects.

Theoretically, prebiotic supplements might augment their fermentation with gut microbiota resulting in the increase of SCFAs. The SCFAs can easily enter mitochondria leading to increase fat oxidation and providing energy for the cell. Inulin at doses of 23.5 and 24 g increased fat oxidation. rate of the people with overweight/obesity[35,40]. The increased fat oxidation rate resulted in the reduction of circulating FFA[35], lipid profile[11]. Likewise, 30 g inulin for 18 weeks to prediabetes patients reduced hepatic and muscle fat[41], and 1.8 g rice bran for 8 weeks to uncontrolled overweight/obesity could reduce body fat[11] and body mass[41]. The other mechanism of the effects of prebiotics is to improve *Lactobacillus* production which in turn controlling the TNF-α and leptin gene expression. Anthocyanin 200 mg/day consumption for 4 weeks significantly reduced blood leptin concentration[7], supporting our results of decreased leptin concentration in the prebiotic treated group. Besides, the previous study also showed anthocyanin could decrease body weight and BMI[7]. The body fat[11] and weight reduction[42] may contribute to decreased insulin resistance. The improved insulin resistance results in decreased blood glucose as shown in the previous studies of inulin supplementation[41] and HbA1c in healthy were significantly reduced after inulin supplementation[30].

In this study, prebiotic treatment of 4.2 g/day capsule for 30 days did not cause any damage of liver and kidney functions. Supportive of this, Mahadita et al. (2016) reported that anthocyanin 11 g/day for 4 weeks were safe on liver and kidney function measured by SGPT, SGOT, BUN, and Cr[38]. Besides, inulin 10 g/day for 2 months significantly decrease SGOT and SGPT but did not change Cr[43]. Moreover, 30 g/day rice bran for 6 months decreased SGOT and SGPT[44]. The doses of extracts and duration of the supplementation that we used in this study are lower[38,43,44] and shorter[43,44] than those in previous studies. Therefore, no damages in the liver and kidney were found in our participants.

There are several limitations of this study. Firstly, there is a lack of data on oxidants and other antioxidants of both enzymatic and non-enzymatic nature. The influence of the prebiotics used in this study on oxidants and other antioxidants should be investigated further. Another important missing investigation is the type and amount of microbiota and concentration of SCFA. The results may provide knowledge of the molecular mechanism explaining the effect of these prebiotics. The other limitation is the gender imbalance since a ratio of females vs males is 1:5 in control group and 1:6 in this study. A very recent study suggested that gender had an effect on the impact of dietary fiber on the gut microbiome[45]. Thus, the results of this study cannot be applied in male population. Besides, the future study should be taken on a higher dose and a longer duration because some data might be affected by higher doses and prolonged treatment. Moreover, the effects of the prebiotic capsule on proposed variables should be investigated in the population with high oxidative stress such as diabetes mellitus. This will yield beneficial effect of the prebiotic supplementation to more population, such as diabetes mellitus.

Conclusion

Our findings suggest that 30-day supplementation of prebiotic capsule containing 1.89 g anthocyanin, 1.89 g inulin and 0.42 g rice bran per day increased plasma ascorbate which is non-enzymatic antioxidant in individuals with overweight or obesity. However, it did not influence other outcomes. Either higher dose or longer duration of the supplementation may result in the significant effects. The prebiotics used in this study did not cause any serious toxic effects on liver and kidney.

Clinical implications

- Anthocyanin, inulin, and rice bran supplement increased plasma ascorbate concentration in overweight or obesity subjects.
- In prebiotic group, hip circumference, lipid profiles, and leptin and insulin concentrations were decreased.
- This 30-day prebiotic supplementation did not cause liver and kidney damage.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported by Research University Network, The National Research Council of Thailand, research grants from Exercise and Sport Sciences Development and Research Group, Khon Kaen University, and Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon Nakhon Campus, Thailand. We would like to acknowledge Prof. Yukifumi Nawa, for editing the manuscript via publication clinic Khon Kaen University, Thailand. The authors would like to thank all subjects.

References

1. Chooi YC, Ding C, Magkos F. The epidemiology of obesity. Metab Clin Exp 2019; 92: 6-10.
2. Pan WH, Yeh WT. How to define obesity? Evidence-based multiple action points for public awareness, screening, and treatment: an extension of Asian-Pacific recommendations. Asia Pac J Clin Nutr 2008; 17(3): 370-4.
3. Block G, Mangels AR, Patterson BH, Levander OA, Norkus EP, Taylor PR. Body weight and prior depletion affect plasma ascorbate levels attained on identical vitamin C intake: a controlled-diet study. J Am Coll Nutr 1999; 18(6): 628-37.
4. Wonisch W, Falk A, Sundl I, Winklhofer-Roob BM, Lindschinger M. Oxidative stress increases continuously with BMI and age with unfavourable profiles in males. Aging Male 2012; 15(3): 159-65.
5. Qiao Y, Sun J, Ding Y, Le G, Shi Y. Alterations of the gut microbiota in high-fat diet mice is strongly linked to oxidative stress. Appl Microbiol Biotechnol 2013; 97(4): 1689-97.
6. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006; 444(7122): 1027-31.
7. Tucakovic L, Colson N, Santhakumar AB, Kundur AR, Shuttleworth M, Singh I. The effects of anthocyanins on body weight and expression of adipocyte's hormones: Leptin and adiponectin. J Funct Foods 2018; 45: 173-80.
8. Anhe FF, Roy D, Pilon G, Dudonne S, Matamoros S, Varin TV, et al. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice. Gut 2015; 64(6): 872-83.
9. Stenholm S, Head J, Aalto V, Kivimaki M, Kawachi I, Zins M, et al. Body mass index as a predictor of healthy and disease-free life expectancy between ages 50 and 75: a multicohort study. Int J Obes (Lond) 2017; 41(5): 769-75.
10. Delzenne NM, Neyrinck AM, Backhed F, Cani PD. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. Nat Rev Endocrinol 2011; 7(11): 639-46.
11. Hongu N, Kitts DD, Zawistowski J, Dossett CM, Kopec A, Pope BT, et al. Pigmented rice bran and plant sterol combination reduces serum lipids in overweight and obese adults. J Am Coll Nutr 2014; 33(3): 231-8.
12. Oki T, Masuda M, Kobayashi M, Nishiba Y, Furuta S, Suda I, et al. Polymeric procyanidins as radical - scavenging components in red-hulled rice. J Agric Food Chem 2002; 50(26): 7524-9.
13. Hu C, Zawistowski J, Ling W, Kitts DD. Black rice (*Oryza sativa* L. indica) pigmented fraction suppresses both reactive oxygen species and nitric oxide in chemical and biological model systems. J Agric Food Chem 2003; 51(18): 5271-7.
14. Lee M, Sorn SR, Park Y, Park HK. Anthocyanin rich-black soybean testa improved visceral fat and plasma lipid profiles in overweight/obese Korean adults: a randomized controlled trial. J Med Food 2016; 19(11): 995-1003.
15. Jamar G, Estadella D, Pisani LP. Contribution of anthocyanin-rich foods in obesity control through gut microbiota interactions. Biofactors 2017; 43(4): 507-16.
16. Papathanasopoulos A, Camilleri M. Dietary fiber supplements: effects in obesity and metabolic syndrome and relationship to gastrointestinal functions. Gastroenterology 2010; 138(1): 65-72 e1-2.

17. Rebello CJ, Burton J, Heiman M, Greenway FL. Gastrointestinal microbiome modulator improves glucose tolerance in overweight and obese subjects: A randomized placeboled pilot trial. J Diabetes Complicat 2015; 29(8): 1272-6.
18. Kulkarni SS, Salehzadeh F, Fritz T, Zierath JR, Krook A, Osler ME. Mitochondrial regulators of fatty acid metabolism reflect metabolic dysfunction in type 2 diabetes mellitus. Metab Clin Exp 2012; 61(2): 175-85.
19. Daniele G, Xiong J, Solis-Herrera C, Merovci A, Eldor R, Tripathy D, et al. Dapagliflozin Data are expressed as mean ± SE; n=30 in each group (placebo group: 25 females, 5 males and prebiotic group: 26 females, 4 males). CHO; carbohydrate. Diabetes care 2016; 39(11): 2036-41.
20. Odumosu A, Wilson CW. Hypocholesterolemic effects of vitamin C, clofibrate and diosgenin in male guinea-pigs [proceedings]. Br J Pharmacol 1979; 67(3): 456P-7P.
21. Ginter E, Zdichynec B, Holzerova O, Ticha E, Kobza R, Koziakova M, et al. Hypocholesterolemic effect of ascorbic acid in maturity-onset diabetes mellitus. Int J Vitam Nutr Res Suppl 1978; 48(4): 368-73.
22. Parnell JA, Reimer RA. Weight loss during oligofructose supplementation is associated with decreased ghrelin and increased peptide YY in overweight and obese adults. Am J Clin Nutr 2009; 89(6): 1751-9.
23. Zhang H, Li J, Wang K, Du X, Li Q. A simple and sensitive assay for ascorbate using potassium ferricyanide as spectroscopic probe reagent. Anal Biochem 2009; 388(1): 40-6.
24. Peronnet F, Massicotte D. Table of nonprotein respiratory quotient: an update. Can J Sport Sci 1991; 16:23-9.
25. Zhou C, Na L, Shan R, Cheng Y, Li Y, Wu X, et al. Dietary vitamin C intake reduces the risk of type 2 diabetes in Chinese adults: HOMA-IR and T-AOC as potential mediators. PLoS One 2016; 11(9): e0163571.
26. Hassimotto NM, Pinto Mda S, Lajolo FM. Antioxidant status in humans after consumption of blackberry (Rubus fruticosus L.) juices with and without defatted milk. J Agric Food Chem 2008; 56(24): 11727-33.
27. Bakuradze T, Tausend A, Galan J, Maria Groh IA, Berry D. Antioxidative activity and health benefits of anthocyanin-rich fruit juice in healthy volunteers. Free Radic Res 2019: 1-11.
28. Ellinger S, Gordon A, Kurten M, Jungfer E, Zimmermann BF, Zur B, et al. Bolus consumption of a specifically designed fruit juice rich in anthocyanins and ascorbic acid did not influence markers of antioxidative defense in healthy humans. J Agric Food Chem 2012; 60(45): 11292-300.
29. Shang HM, Zhou HZ, Yang JY, Li R, Song H, Wu HX. In vitro and in vivo antioxidant activities of inulin. PloS one 2018; 13(2): e0192273.
30. Aliasgharzadeh A, Khalili M, Mirtaheri E, Pourghassem Gargari B, Tavakoli F, Abbasalizad Farhangi M, et al. A combination of prebiotic inulin and oligofructose improve some of cardiovascular disease risk factors in women with type 2 diabetes: a randomized controlled clinical trial. Adv Pharm Bull 2015; 5(4): 507-14.
31. Hasan M, Damayanthi E, Anwar F. Improvement of antioxidant status on adults obesity after intervention antioxidant drinks. IOP Conf Ser: Earth Env 2018; 196.
32. Liu C, Sun J, Lu Y, Bo Y. Effects of anthocyanin on serum lipids in dyslipidemia patients: a systematic review and meta-analysis. PloS one 2016; 11(9): e0162089.
33. Balcazar-Munoz BR, Martinez-Abundis E, Gonzalez-Ortiz M. Effect of oral inulin administration on lipid profile and insulin sensitivity in subjects with obesity and dyslipidemia. Rev med Chile 2003; 131(6): 597-604.
34. Ooi LG, Liong MT. Cholesterol-lowering effects of probiotics and prebiotics: a review of in vivo and in vitro findings. Int J Mol Sci 2010; 11(6): 2499-522.

35. Van der Beek CM, Canfora EE, Kip AM, Gorissen SHM, Olde Damink SWM, van Eijk HM, et al. The prebiotic inulin improves substrate metabolism and promotes short-chain fatty acid production in overweight to obese men. Metabolism Clin and Exp 2018; 87:25-35.
36. Jackson KG, Taylor GR, Clohessy AM, Williams CM. The effect of the daily intake of inulin on fasting lipid, insulin and glucose concentrations in middle-aged men and women. Br J Nutr 1999; 82(1): 23-30.
37. Castro-Sánchez FH, Ochoa-Acosta DA, Valenzuela-Rubio NG, Domínguez-Rodríguez M, FierrosValdez JA and Vergara-Jiménez MJ. Inulin effect on weight loss and associated parameters with the development of cardiovascular disease in obese dyslipidemic subjects. Austin J Nutr Metab 2017; 4(1): 1044.
38. Mahadita WG, Jawi M, Suastika K. Purple sweet potato tuber extract lowers malondialdehyde and improves glycemic control in subjects with type 2 diabetes mellitus. Glob Adv Res J Med Med Sci 2016; 5(7): 208-13.
39. Qureshia AA, Sami SA, Khan FA. Effects of stabilized rice bran, its soluble and fiber fractions on blood glucose concentrations and serum lipid parameters in humans with diabetes mellitus Types I and II. J Nutr Biochem 2002; 13(3): 175-87.
40. Van der Beek CM, Canfora EE, Ellen EB, Cornelis HD, Kaatje L. The prebiotic inulin enhances fat oxidation and improves metabolic parameter in overweight male. Metabolism 2018; 87: 25-35.
41. Guess ND, Dornhorst A, Oliver N, Bell JD, Thomas EL, Frost GS. A randomized controlled trial: the effect of inulin on weight management and ectopic fat in subjects with prediabetes. Nutr Metab 2015; 12: 36.
42. Chambers ES, Byrne CS, Morrison DJ, Murphy KG, Preston T, Tedford C, et al. Dietary supplementation with inulin-propionate ester or inulin improves insulin sensitivity in adults with overweight and obesity with distinct effects on the gut microbiota, plasma metabolome and systemic inflammatory responses: a randomised cross-over trial. Gut 2019; 68(8): 1430-8.
43. Farhangi MA, Javid AZ, Dehghan P. The effect of enriched chicory inulin on liver enzymes, calcium homeostasis and hematological parameters in patients with type 2 diabetes mellitus: a randomized placebo - controlled trial. Prim Care Diabetes 2016; 10(4): 265-71.
44. Shakib MC, Shreef G, Gamal G. Rice bran oil compared to atorvastatin for treatment of dyslipidemia in patients with type 2 diabetes. Open Access Maced J Med Sci 2014; 2(1): 95-102.
45. Tashiro H, Kasahara DI, Osgood RS, Brown T, Cardoso A, Cho Y, et al. Sex differences in the impact of dietary fiber on pulmonary responses to ozone. Am J Respir Cell Mol 2020; 62(4): 503-12.

Supplementary data

Supplementary table 1 Baseline characteristics of subjects

Variables	Control group	Prebiotic group	*p*-value
Gender (female/male)	25/5	26/4	0.73
Age (yr)	38.00 ± 9.48	38.00 ± 8.83	0.99
Height (m)	1.58 ± 0.09	1.58 ± 0.08	0.54
Body mass (kg)	73.70 ± 14.99	72.50 ± 10.39	0.71
BMI (kg/m^2)	29.50 ± 4.80	28.90 ± 3.30	0.97
Systolic (mmHg)	120.00 ± 13.58	123.00 ± 14.00	0.47
Diastolic (mmHg)	77.00 ± 12.62	79.00 ± 13.24	0.49
Heart rate (/min)	76.00 ± 8.28	78.00 ± 11.11	0.90

Note: Data are expressed as mean ± SD; n = 30 in each group; control group, prebiotic group; BMI, body mass index.

Supplementary table 2 Physical activity and dietary assessment of subjects

Variables	Control group	Prebiotic group	*p*-value
Protein (g)	87.30 ± 3.83	84.30 ± 4.52	0.62
Carbohydrate (g)	219.30 ± 9.58	232.10 ± 9.14	0.34
Fat (g)	67.60 ± 4.20	62.80 ± 3.02	0.36
Potassium (mg)	1456.50 ± 126.96	1359.50 ± 119.92	0.59
Sodium (mg)	3064.80 ± 377.36	3100.40 ± 353.06	0.95
Vitamin A	224.70 ± 38.36	271.30 ± 44.19	0.45
Thiamine (mg)	1.00 ± 0.13	1.07 ± 0.13	0.74
Riboflavin (m)	1.16 ± 0.10	1.08 ± 0.09	0.53
Vitamin B6 (mg)	0.39 ± 0.06	0.38 ± 0.07	0.67
Vitamin B12 (mg)	0.91 ± 0.18	1.19 ± 0.47	0.68
Vitamin C (mg)	35.96 ± 6.99	35.06 ± 5.56	0.88
Vitamin E (mg)	0.81 ± 0.12	0.74 ± 0.18	0.22
Crude fiber (g)	0.60 ± 0.09	0.69 ± 0.14	0.89
Dietary fiber (g)	6.67 ± 0.74	6.37 ± 0.87	0.79
Energy intake (Kcal/day)	1836.70 ± 11.06	1838.60 ± 9.80	0.90
Energy expenditure (Kcal/day)	1692.30 ± 84.66	1694.10 ± 76.07	0.70

Note: Data are expressed as mean ± SD; n = 30 in each group; control group, prebiotic group.

Supplementary table 3 Anthropometric and body composition of subjects

Variables	Control Group		Prebiotic Group		Mean difference	95% CI		*p*-value
	Before	After	Before	After		Lower	Upper	
BMI (kg/m^2)	29.50 ± 0.88	29.60 ± 0.87	28.90 ± 0.60	28.30 ± 0.83	0.69	-0.67	2.06	0.31
Body mass (kg)	73.70 ± 2.74	73.90 ± 2.70	72.50 ± 1.90	70.90 ± 2.35	1.84	-1.80	5.48	0.32
Fat mass (kg)	29.50 ± 1.45	29.60 ± 1.46	29.80 ± 1.41	31.20 ± 2.22	0.41	-0.82	1.65	0.50
% Fat android	48.30 ± 0.74	48.40 ± 0.82	50.30 ± 1.13	50.40 ± 1.12	-0.19	-1.34	0.97	0.75
% Fat gynoid	47.20 ± 1.31	47.80 ± 1.40	48.60 ± 0.97	47.80 ± 0.94*	0.86	-0.19	1.90	0.10

Note: Data are expressed as mean ± SE; n = 30 in each group; control group (25 females and 5 males), prebiotic group (26 females and 4 males); BMI, body mass index.
*Significantly different from before supplementation (p-value < 0.05).

Arch AHS

ORIGINAL ARTICLE

Archives of Allied Health Sciences 2021; 33(1): 34-43.

The genotypic distribution of drug resistant Mycobacterium tuberculosis strains isolated from Northern region of Myanmar

Waing Waing Moe Sann[1], Thyn Lei Swe[2], Kyi Kyi Swe[3], Thandar Thwin[3], Arunnee Sangka[4,5*]

[1] Medical Technology Program, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

[2] National TB Control Program, Yangon, Myanmar.

[3] National TB Control Program, Northern region of Myanmar TB Reference Laboratory, Mandalay Region, Myanmar.

[4] Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

[5] School of Medical Technology, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

KEYWORDS
Mycobacterium tuberculosis; Drug resistant; Beijing; Lineages; HGDI.

ABSTRACT

Myanmar is one of both 30 high TB and MDR-TB burden countries worldwide. While most studies have expressed distribution of Mycobacterium tuberculosis genotypes in Lower Myanmar, there has been little research in genetic diversity of M. tuberculosis in Northern region. The objective of this study was to determine the genotypic distribution of drug resistant M. tuberculosis strains isolated from Northern region of Myanmar. Sixty-five isolates were randomly collected from TB Reference Laboratory of Northern region of Myanmar between August 2016 and December 2017. All isolates were genotyped by using 24-locus Mycobacterial Interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing. The results showed MIRU-VNTR typing classified 64 different patterns: 63 isolates had unique MIRU-VNTR profile and 2 isolates were grouped into one cluster. We found that the most prominent strains were Beijing lineages (n = 58, 89.23%) and the other included EAI (n = 2, 3.08%), Delhi/CAS (n = 1, 1.54%), and Unknown strains (n = 4, 6.15%). The overall discriminatory power of all strains showed 0.9995. The allelic diversity of each locus was predictable by HGDI index. Mtub21, Qub2163b, MIRU 26, QUB26 showed HGDI > 0.6 that were recognized as highly discriminatory power. In conclusion, 24-locus MIRU-VNTR offered high discriminatory power within tested isolates. Our findings showed Beijing genotypes were dominant in Northern region of Myanmar. The analysis of 24-locus MIRU-VNTR typing might be useful for broader understanding of TB outbreaks and epidemiology in this region.

* **Corresponding author:** Arunnee Sangka, MT, PhD. Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand. E-mail: arusan@kku.ac.th

Received: 6 June 2020/ Revised: 7 July 2020/ Accepted: 13 July 2020

Introduction

Tuberculosis (TB) is a serious killer among the infectious diseases caused by *Mycobacterium tuberculosis* complex (MTB). Tuberculosis persists a major public health crisis all over the world[1]. Globally, 1.4 million people were predicted to lose their live with TB in 2015 and a 0.4 million of people living with HIV (PLHIV) died with TB. Additionally, the estimated 10.4 million was TB occurrence cases in 2015 worldwide[2]. In 2016, World Health Organization (WHO) reported that most anticipated TB incident cases were found within South East Asia region (45%), Africa (25%), Western Pacific (17%), Eastern Mediterranean (7%), Europe (3%), and the Americas (3%) respectively. Moreover, the top five countries including India, Indonesia, China, the Philippines and Pakistan which accounted for 56% of estimated cases[3]. Myanmar is one of both 30 high TB and MDR-TB burden countries[2]. It stood the fourth position with the higher prevalence rate, 525 cases per 100,000 populations compared with the global average of 178 cases per 100,000 populations in 2010. An anticipated 180,000 new cases occurred each year followed by 9,000 MDR-TB cases and 20,000 cases by TB co-infected HIV[4]. Myanmar is currently facing with the double burden of communicable diseases and non-communicable diseases. Diet style, less physical activity, tobacco usage and overdrinking alcohol are direct and indirect risk factors of TB and other health problems. Depletion of nutritional status such as protein energy malnutrition and micronutrient deficiencies are also the major causes of Tuberculosis in Myanmar. In recent years, Myanmar had increased the number of MTB strains which are resistant to drugs and co-infected with HIV[5].

The molecular epidemiology study of MTB is useful not only to examine the dispersion of tubercle bacilli in outbreaks but also to analyze the transmission of tuberculosis and to establish the risked aspects of tuberculosis among the community[6]. Spontaneously, there are several molecular typing methods now. The best approach to molecular method is whole genotype sequencing; however, WGS analysis is time consuming, costly and can examine only parts of the genome[7].

Other new developed molecular techniques for *M. tuberculosis* genetic classification are restriction fragment length polymorphism (RFLP)-IS*6110*, polymorphic GC-rich sequence (PGRS), pulsed field gel electrophoresis (PFGE), Spoligotyping (spacer oligo-nucleotide typing), ligation mediated (PCR), Mycobacterial interspersed repetitive unit (MIRU-VNTR) typing, amplification and sequencing of single nucleotide polymorphism (SNP) respectively[8]. MIRU-VNTR typing is currently used as standardized method and it is less laborious, has short time process, and the discriminatory power is similar in comparison with IS*6110*-RFLP typing, particularly if fully 24 loci are used[9]. The MIRU typing is based on variation in copy number of tandem repeat (VNTR) loci and it simply needs basic PCR and electrophoresis equipment. After introducing the standard sets of 12 loci and 15 loci MIRU-VNTR typing panels, 24 loci MIRU-VNTR typing is currently the best approach to discriminate strongly related strains[10]. Furthermore, it needs lower amount of DNA. Several reports were proved that 24-locus MIRU-VNTR typing is suitable for transmission of population-based studies[11].

In our study, MTB isolates were collected randomly from Northern region of Myanmar, TB reference Laboratory, Mandalay Region. These isolates were examined by 24-locus MIRU-VNTR typing to determine genetic diversity, to evaluate the discriminatory power of this method and to reveal transmission of some strains.

Materials and methods

Clinical isolates

A total of 65 drug resistant strain *M. tuberculosis* isolates were collected randomly from Northern region of Myanmar, TB reference laboratory between August 2016 and December 2017. All isolates were culture positive on Lowenstein Jensen medium and identified as MTB by using Capilia TB rapid test (MPB 64) and Niacin test (Biochemical test). Chromosomal DNA was extracted by using CTAB method for amplifying real time PCR[12]. This study protocol was approved by the Ethics Review Committee of the Department of Medical Research, Yangon, Myanmar (Ethics/DMR/2017/122) and the Khon

Kaen University Ethics Committee in Human Research, Khon Kaen, Thailand (Ethics number HE602220).

Drug susceptibility testing

Standard agar proportion method was used for susceptibility testing of first line anti-TB drugs performed on Lowenstein Jensen medium with the standard concentrations including ethambutol (ETB) 2.0 µg/ml, isoniazid (INH) 0.20 µg/ml, rifampicin (RIF) 40 µg/ml, and streptomycin (SM) 0.4 µg/ml[13]. Genotype MTBDRplus line-probe assay kit (Hain Lifescience, Nehren, Germany) was used for observation of resistant mutations for INH and RIF.

MIRU-VNTR genotyping

The 24-locus MIRU-VNTR typing was performed by PCR with specific primers including 24 loci that were described in Supply et al[14]. PCR premixes were prepared as following; one µl of DNA was added to a PCR master mix 49 µl (to the final volume of 50 µl). PCR master mixes 49 µl include 0.25 µl of Taq DNA polymerase (5 unit) (Invitrogen, USA), 8 µl of 1.25 mM dNTP (Sib enzyme), 5 µl of 10X PCR buffer, 5 µl of 10 µM each primer, and 1.5 µl of 50 mM $MgCl_2$. PCR was subjected to 40 cycles of conditions. The DNA of MTB genome H37RV was used as positive control and distilled water was used as negative control. PCR products were analyzed by electrophoresis on 1.5% agarose gels using 100 bp DNA ladder as standard size markers.

MIRU-VNTR analysis

Amplicon size was determined by Total Lab TL100 software, and obtained size was compared by applying online apparatus at (http://www.MIRU-VNTRplus.org) containing the allele for each locus by Supply et al. 2006. The number of alleles was filled into the indicated form according to the website instructor. The dendrogram was obtained using the UPGMA algorithm analysis.

Statistical analysis

The discriminatory power (the Hunter-Gaston discriminatory index [HGDI]) of each typing method was calculated according to a previously published method[15];

$$HGDI = 1 - \left[\frac{1}{N(N-1)}\sum_{j=1}^{s} xj(xj-1)\right]$$

Where D is the discriminatory power, N is the total number of isolates in the typing method, s is the number of distinct patterns discriminated by VNTR, and *j* is the number of isolates belonging to the *j*th pattern. The number of cluster strains in patients was used to calculate a rate of transmission, rather than progression to disease following infection in the past. Rate of transmission was calculated as follows[16]; (number of clustered strains-number of clusters)/ Total number of isolates x100. The percentage of clustering rate was calculated with following formula[17]; (c – c)/N x100, Where, N is the total number of cases in the sample, c is the number of clusters and *n*c is the total number of clustered cases.

Results

24-locus MIRU-VNTR

Among 65 isolates 24-locus MIRU-VNTR typing identified 64 different patterns. Four lineages were distributed containing Beijing (n = 58, 89.23%) and the other included EAI (n = 2, 3.08%), Delhi/CAS (n = 1, 1.54%), and Unknown strains (n = 4, 6.15%) (Table 1). Sixty-three isolates (96.92%) were unique (i.e., detected for only one strain) and only 2 isolates could be grouped into one cluster (Figure 1). The transmission rate was also evaluated by the formula as mentioned above in data analysis which showed 1.5% in this study.

Table 1 MIRU-VNTR fingerprinting patterns for 65 drug resistance *Mycobacterium* tuberculosis strains

[a]MIRU-VNTR pattern	Frequency	Lineages	[a]MIRU-VNTR pattern	Frequency	Lineages
2 4 4 2 4 2 3 5 2 5 3 4 2 2 5 1 5 3 3 5 3 7 2 3	1	[b]BJ	2 4 4 2 3 3 3 2 2 3 4 4 4 2 5 1 7 3 3 5 2 8 2 3	1	BJ
2 4 4 2 3 3 3 5 2 5 4 4 4 2 5 1 7 3 3 5 3 8 1 3	1	BJ	2 4 3 2 3 3 5 5 2 8 4 4 4 2 5 1 5 3 3 4 1 9 2 3	1	BJ
2 4 4 2 3 4 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 8 2 3	1	BJ	2 4 4 1 3 3 3 3 2 6 4 4 4 2 5 1 7 3 3 5 1 6 2 3	1	BJ
2 4 3 2 3 6 3 5 2 3 4 4 2 2 5 1 7 3 3 5 2 7 2 3	1	BJ	2 4 4 3 3 3 4 5 2 6 4 4 4 2 5 1 7 3 3 3 3 8 2 3	1	BJ
2 2 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 8 2 3	1	BJ	2 4 3 2 3 4 2 5 2 3 4 4 4 2 6 1 7 3 3 3 3 9 4 3	1	BJ
2 4 2 2 3 4 3 4 2 2 4 4 2 2 6 1 5 3 3 5 3 6 2 3	1	[c]Delhi/CAS	2 4 4 2 3 4 4 8 2 6 5 4 4 4 5 1 7 3 3 6 3 7 2 3	1	BJ
2 4 4 2 3 4 3 5 2 6 4 4 2 2 5 1 7 3 3 3 3 2 2 3	1	BJ	2 4 4 2 3 3 3 5 2 6 5 4 2 3 5 1 7 3 3 5 3 3 1 3	1	BJ
2 4 3 2 3 3 3 8 2 6 4 4 2 4 5 1 7 3 3 5 3 8 1 3	1	BJ	2 1 2 1 3 3 3 8 2 6 7 3 2 3 5 1 2 3 3 5 5 5 2 3	1	UK
2 4 4 2 3 3 3 5 2 6 5 4 4 4 5 1 7 3 3 5 3 3 4 3	1	BJ	2 4 4 1 3 3 3 3 2 6 5 4 3 2 5 1 5 3 3 5 3 7 2 3	1	BJ
2 4 4 2 3 2 3 3 2 6 3 4 2 2 5 1 7 3 3 5 3 8 1 3	1	BJ	2 4 4 2 3 2 4 5 2 6 4 4 3 2 5 1 5 3 3 5 3 7 1 3	1	BJ
2 2 4 2 3 3 2 2 2 6 5 4 2 2 5 1 5 3 3 3 3 5 2 3	1	[d]UK	2 4 4 3 2 3 3 5 2 6 8 4 2 3 5 1 2 3 3 5 3 6 2 3	1	UK
2 4 4 2 3 3 3 4 2 6 5 4 2 2 1 1 5 3 3 3 3 9 1 3	1	BJ	2 4 4 2 3 3 3 4 2 3 4 4 4 2 5 1 7 3 2 5 3 7 1 3	1	BJ
2 4 4 2 3 3 4 5 2 8 4 4 4 2 5 1 5 3 3 4 3 9 2 3	1	BJ	2 2 4 1 3 3 3 4 2 5 4 4 4 2 5 1 7 2 3 5 3 6 1 3	1	BJ
2 4 3 2 3 4 3 5 2 3 3 4 2 2 5 1 5 3 3 5 3 7 2 3	1	BJ	2 4 4 2 3 3 3 4 2 6 4 4 4 2 5 1 7 3 3 5 3 8 2 3	1	BJ
2 4 3 2 3 4 3 5 2 6 4 4 4 2 6 1 6 3 3 5 3 9 3 3	1	BJ	2 2 4 2 2 3 3 4 2 6 4 4 4 2 5 1 2 3 3 5 3 7 2 3	1	BJ
2 1 4 2 2 3 3 5 2 6 4 4 4 2 5 1 5 3 2 5 3 9 2 3	1	BJ	2 2 4 3 2 3 2 6 2 4 4 4 2 3 5 2 2 2 3 4 3 6 2 3	1	UK
2 4 6 2 3 3 3 4 2 6 4 4 4 2 5 1 7 3 2 5 3 7 1 3	1	BJ	2 4 4 2 3 3 2 5 2 4 4 4 4 2 5 1 5 3 3 5 3 8 2 3	1	BJ
2 4 4 2 3 3 3 5 2 8 4 4 4 2 5 1 5 3 3 5 2 7 3 3	1	BJ	2 4 4 2 3 4 2 5 2 6 4 4 4 2 5 1 7 3 3 5 3 8 2 3	1	BJ
2 2 4 3 3 4 3 7 2 3 6 3 4 2 6 1 2 3 3 3 4 6 1 3	1	[e]EAI	2 2 4 2 3 4 2 4 2 6 4 4 4 2 5 1 7 3 3 5 3 8 2 3	1	BJ
2 4 4 3 2 4 4 10 2 6 9 3 2 2 6 1 2 3 3 4 4 6 1 3	1	EAI	2 2 4 2 2 3 3 3 2 6 4 4 4 2 6 1 5 2 3 4 3 9 2 2	1	BJ
2 4 4 2 3 3 4 5 2 7 4 4 4 4 5 1 7 3 2 6 3 8 1 3	1	BJ	2 4 3 2 2 3 5 4 2 6 4 4 4 2 5 1 7 3 3 4 3 7 2 3	1	BJ
2 4 4 1 3 3 3 3 2 5 4 4 4 2 5 1 7 3 3 5 3 6 1 3	1	BJ	2 2 4 2 2 3 3 5 2 6 4 4 4 2 5 1 8 3 3 5 3 6 2 3	1	BJ
2 4 4 2 3 2 3 5 2 5 2 4 4 2 5 1 7 3 3 4 3 6 2 3	1	BJ	2 4 4 2 2 4 3 3 2 6 4 4 4 2 5 1 7 2 3 4 3 7 4 2	1	BJ
2 4 4 2 4 6 2 5 2 2 4 4 2 2 5 1 7 3 3 4 2 8 2 3	1	BJ	2 4 4 2 3 4 2 5 2 6 2 3 4 2 5 1 5 3 3 5 3 7 2 3	1	BJ
2 4 4 2 5 3 3 7 2 6 3 4 4 2 5 1 5 3 3 5 2 8 2 3	1	BJ	2 4 4 2 3 3 3 3 2 4 4 4 4 2 5 1 5 3 3 5 3 8 4 2	1	BJ
2 4 4 2 3 2 2 5 2 3 4 4 4 2 5 1 7 3 3 5 2 8 2 3	1	BJ	2 2 4 2 3 3 2 4 2 5 4 3 4 3 5 1 5 3 2 5 3 7 1 3	1	BJ
2 4 4 2 3 3 2 5 2 6 4 4 4 2 5 1 5 3 3 5 2 8 2 3	1	BJ	2 2 4 2 3 3 2 3 2 6 4 4 4 3 5 2 7 3 3 5 3 7 3 3	1	BJ
2 4 4 2 3 2 3 5 2 6 4 4 5 2 5 1 7 3 3 5 3 6 2 3	2	BJ	2 2 4 2 3 5 2 3 2 10 4 4 4 3 5 2 7 3 3 5 3 7 2 3	1	BJ
2 4 4 2 3 2 3 5 2 5 4 4 5 2 5 1 5 3 3 5 3 7 2 3	1	BJ	2 2 4 2 3 3 2 5 2 4 4 3 4 3 5 1 5 3 3 5 3 7 2 3	1	BJ
2 4 4 2 3 3 5 5 2 6 4 4 4 2 5 1 5 3 3 3 1 8 3 3	1	BJ	2 2 4 1 3 3 3 4 2 4 4 4 4 2 5 1 7 3 3 5 3 6 2 3	1	BJ
2 4 4 2 3 4 3 6 2 6 4 4 4 4 2 6 1 7 3 3 5 4 7 2 3	1	BJ	2 4 4 2 3 3 3 6 2 6 4 4 4 2 5 1 5 3 3 4 3 7 2 3	1	BJ
2 4 4 1 3 3 3 5 2 6 4 3 4 2 4 1 7 3 3 5 3 9 2 3	1	BJ			
2 2 4 2 3 3 3 2 2 3 4 4 4 2 5 1 7 3 3 5 2 9 2 3	1	BJ	**Total**	**64**	

Note: [a]MIRU pattern: MIRU 02, Mtub04, ETRC, MIRU 04, MIRU 40, MIRU 10, MIRU 16, Mtub21, MIRU 20, QUB2163b, ETRA, Mtub29, Mtub30, ETRB, MIRU 23, MIRU 24, QUB26, MIRU 27, Mtub34, MIRU 31, Mtub39, QUB26, QUB4156, MIRU 39. [b]Beijing strain. [c] Delhi-Central Asian strain. [d]Unknown strain. [e]East African Indian strain.

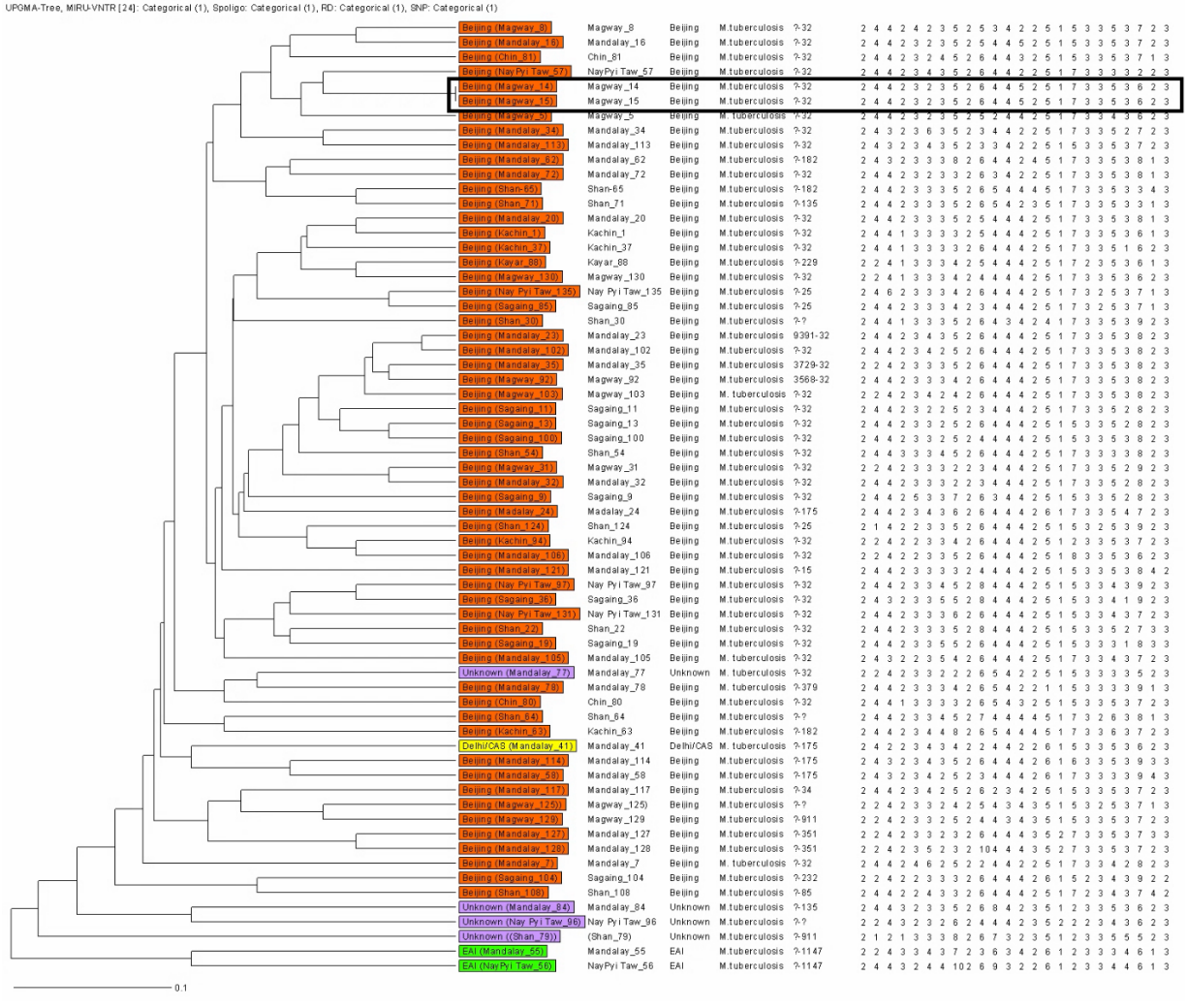


Figure 1 UPGMA tree show genetic relationship of all 65 drug resistant MTB strains including VNTR copy numbers and strain information. Large box indicated cluster isolates (MDR). Orange bars belong to Beijing lineages, Yellow (Delhi/CAS), Green (EAI), and Purple (Unknown).

Allelic profiles and discrimination

Allelic profiles and HGDI of 24-locus MIRU-VNTR for all MTB isolates in Northern region of Myanmar were summarized in Table 2. Allelic diversity was classified as highly discriminant (HGDI ≥ 0.6), moderately discriminant (0.3 < HDGI < 0.6), and poorly discriminant (HGDI ≤ 0.3)[18]. Mtub21, Qub2163b, MIRU 26, and QUB26 exceeded 0.6 that these were recognized as highly discriminatory power. Whereas 11 loci (Mtub04, MIRU 04, MIRU 40, MIRU 10, MIRU 16, ETRA, Mtub30, ETRB, MIRU 31, Mtub39, and Qub4156) were found moderately discriminative, the remaining 9 loci (MIRU 02, ETRC, MIRU 20, Mtub29, MIRU 23, MIRU 24, MIRU 27, Mtub34, MIRU 39) were found to be poorly discriminative. HGDI and cluster results based on the different set of MIRU-VNTR loci analysis of 65 MTB isolates from Northern region of Myanmar were shown in Table 3.

Table 2 Allelic diversity of 24 mycobacterial interspersed repetitive units (MIRUs) loci from 65 drug resistant tuberculosis strains

MIRU-VNTR locus	Allele number												Allelic diversity	Conclusion
	1	2	3	4	5	6	7	8	9	10	11	12		
MIRU 02		65											0.0	Poorly discriminant*
Mtub04	2	15	48										0.39	Moderately discriminant
ETRC		2	7	55		1							0.26	Poorly discriminant
MIRU 04	7	53	5										0.31	Moderately discriminant
MIRU 40		9	53	2	1								0.3	Moderately discriminant
MIRU 10		8	41	13	1	2							0.55	Moderately discriminant
MIRU 16		14	42	6	3								0.52	Moderately discriminant
Mtub21		3	9	11	33	3	2	3		1			0.68	Highly discriminant
MIRU 20		65											0.0	Poorly discriminant
QUB2163b		2	8	5	7	38	1	3		1			0.62	Highly discriminant
ETRA		2	4	49	6	1	1	1	1				0.41	Moderately discriminant
Mtub29			7	58									0.18	Poorly discriminant
Mtub30		15	2	45	3								0.46	Moderately discriminant
ETRB		53	8	4									0.31	Moderately discriminant
MIRU 23	1			1	56	7							0.23	Poorly discriminant
MIRU 24	62	3											0.07	Poorly discriminant
MIRU 26		6			22	1	35	1					0.58	Highly discriminant
MIRU 27		4	61										0.1	Poorly discriminant
Mtub34		5	60										0.13	Poorly discriminant
MIRU 31			7	10	46	2							0.45	Moderately discriminant
Mtub39	3	8	50	3	1								0.38	Moderately discriminant
QUB26		1	2		2	13	20	18	9				0.76	Highly discriminant
QUB4156	14	43	4	4									0.5	Moderately discriminant
MIRU 39		3	62										0.07	Poorly discriminant

Note: *Allelic diversity was classified as highly discriminant (HGDI ≥ 0.6), moderately discriminant (HGDI 0.3 < HDGI < 0.6), and poorly discriminant (HGDI ≤ 0.03).

Table 3 Hunter Gaston Discriminatory Index (HGDI) and cluster results based on different typing analysis of 65 drug resistant tuberculosis strains from Northern region of Myanmar

Typing methods	Total No. of patterns	No. of unique types	Total no. of clusters	Total no. of isolates in clusters (Cluster rate %)	Maximum no. of isolates in a cluster	HGDI
12 loci MIRU-VNTR	60 (N=65)	43	5	22 (33.8)	10	0.972
15 loci MIRU-VNTR	64 (N=65)	63	1	2 (1.5)	2	0.9995
24 loci MIRU-VNTR	64 (N=65)	63	1	2 (1.5)	2	0.9995

Drug resistance

Among all of 65 isolates, 31.4% (44/65 isolates) were accounted for resistant to both isoniazid and rifampicin and therefore, were known as MDR. 17 (26.15%) and 4 (6.15%) isolates were resisted to only isoniazid and rifampicin, respectively. Out of 44 MDR, 41 isolates were Beijing lineage, while only 3 were non-Beijing isolates (unknown strains). The MDR isolates were not found in EAI and Delhi/CAS lineage.

Discussion

24-locus MIRU-VNTR genotyping has developed a current standardized method and is presently useful for epidemiological study of *M. tuberculosis* thorough the world[19]. Total 65 isolates from Northern region of Myanmar between August 2016 and December 2017 were randomly collected. Sixty-one of 65 isolates (93.85%) could be classified into three lineages and the predominant genotype was Beijing (89.23%) which is highly prominent in Asian countries[20,21]. Remaining lineages distributed EAI (3.08%), Delhi/CAS (1.54%), and Unknown (6.15%). In contrast to a previous study, EAI lineage was the first predominance in Lower Myanmar, Yangon and the second was Beijing lineage[22]. The predominance of Beijing strains may be due to the human interaction with foreign countries such as China, India and Bangladesh.

The Beijing genotype is the most prominent genotype in Asia (Far East Asia, Middle East and Central Asia), Oceania and it is also emergent in other parts of the world[23]. The infection process of this lineage found to be related with immune response and it can control the macrophage-derived cytokines that was an important role in directing the immune response to a non-protective Th2 phenotype[24]. The relationship of Beijing genotype and drug resistance may have a certain tendency for acquiring drug resistance that were widely distributed (but not universal)[25]. In Myanmar, other previous studies described that the Beijing genotypes in MDR and XDR populations are highly prevalent[26,27]. Among Beijing isolates, 41 (70.69%) were infected with MDR, in which two strains were grouped into one cluster. Hence, Beijing strains in Northern region of Myanmar were highly correlated with Multi-drug resistance when compared with previous study in Yangon showed only 21.4%[22].

Allelic diversity of 24-locus MIRU-VNTR typing was analyzed by Hunter-Gatson Discriminatory Index. In the present study, alleles Mtub 21, Qub 2163b, MIRU 26 and Qub 26 found highly discriminatory power (HGDI ≥ 0.6). Some studies in China and India have expressed that those loci found highly discriminatory power[28,29]. A recent study in Myanmar has shown that Mtub21 found moderately discriminant (0.3 < HDGI < 0.6)[30]. On the other hand, the poorest discriminatory power was found in alleles MIRU 02, ETRC, MIRU 20, Mtub 29, MIRU 23, MIRU 24, MIRU 27, Mtub 34 and MIRU 39 (HGDI≤0.3). The remaining alleles Mtub 04, MIRU 04, MIRU 40, MIRU 10, MIRU 16, ETRA, Mtub 30, ETRB, MIRU 31, Mtub 39 and Qub

4156 showed moderately discriminant (HDGI ≥ 0.6). A previous study in Iran has found that MIRU 10 had high HDGI. However, it showed moderate discriminatory power in our study. HGDI of MIRU 20 and MIRU 02 were similar with the previous study of Iran[31]. The overall HGDI of total isolates in our study was 0.9995 that is similar to the previous studies[18,29].

In cluster analysis for all tested isolates, 64 different patterns were identified. One cluster of the obtained isolates shared MIRU patterns with two isolates, and 63 isolates (96.92 %) had unique patterns. Clustering rate of 65 isolates using 24 locus MIRU-VNTR typing showed only 3.1% belonged to Beijing strains. In previous study, the clustering rate of Beijing strains was nearly 80% compared with non-Beijing family that implies a high transmission rate[32]. Higher percentage of clustering rate indicates the possibility of related strains that are likely to spread other geographical areas. In our study, all resistant MTB isolates were genetically diverse and only one cluster was found. One cluster including two MDR strains (Beijing genotype) seemed to be acquired transmission of drug resistance strain. While tuberculosis is highly prevalent in Myanmar, the recent transmission rate in this study was 1.5%, which was lower than that described in some developed countries. For instance, one study in London stated that the recent transmission rate was 34%[33]. Another study in United States described that the proportion of TB cases attributable to recent transmission was 15%[34]. In China, the recent TB transmission rate was 13.34%[35]. The percentage of recent transmission was relatively low in our study, and this may indicate the higher possibility of recurrence in this study area.

Conclusion

This report describing the 24-locus MIRU-VNTR patterns and genetic diversity of *M. tuberculosis* genotypes from Northern region of Myanmar. Our findings revealed a diversity of strains and mode of transmission of some strains which can provide broader understanding of TB outbreaks and epidemiology. MIRU-VNTR typing is a useful tool to discriminate genetic diversity of MTB isolates in this area. Both 15 and 24-locus MIRU-VNTR typing show similar discriminatory power (HGDI 0.9995). The MIRU-VNTR locus that showed high discriminatory power (Mtub21, Qub2163b, MIRU 26, QUB 26 (h≥0.6)) can be applied as the first line locus for future studies. Beijing lineages isolates found to be MDR when compared with other lineage isolates. The lower clustering rate in our study indicates that acquired transmission occurred in this study period. Genotypic pattern proposes that the lower transmission rate may be due to higher possibility of reactivation cases in Northern region of Myanmar. Therefore, the information obtained from this study can be applied for future TB control studies.

Take home messages

The genotypic distribution of MDR-TB in Northern region of Myanmar was determined by 24-locus Mycobacterial Interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing. The most prominent strains were Beijing lineages and the other included EAI, Delhi/CAS, and Unknown strains. 24-locus MIRU-VNTR offered high discriminatory power (HGDI>0.6) within tested isolates.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors would be appreciating to Khon Kaen University Research Grant (project number 590038) and Centre for Research and Development of Medical Diagnostic Laboratories, Khon Kaen University. We thank the KKU Scholarship for ASEAN and GMS Countries' Personnel of Academic Year 2016, Khon Kaen University for providing a scholarship for Waing Waing Moe Sann. We are grateful to Kulrattana Rueangsak for her technical assistance.

References

1. Smith I. Mycobacterium tuberculosis pathogenesis and molecular determinants of virulence. Clin Microbiol Rev 2003; 16: 463-96.
2. World Health Organization. Global tuberculosis report 2016. WHO /HTM /TB /2016. Geneva, Swiftzerland: WHO, 2016.
3. World Health Organization. Global Tuberculsis Report 2017. WHO /HTM /TB /2017. Geneva, Swiftzerland: WHO, 2017.
4. Tuberculosis in Myanmar Progress, Plans and Challenges. 2015.
5. World Health Organization. Global Tuberculosis Report 2014. WHO /HTM /TB /2014. Geneva, Swiftzerland: WHO, 2014.
6. Narayanan S. Molecular epidemiology of tuberculosis. Indian J Med Res 2004; 120: 233-47.
7. Mostroin P, Cordon M, Sola C, Ridele M, Rastogp N. Methods used in the molecular epidemiology of tuberculosis. Clin Microbiol Infect 2002; 8: 694-704.
8. Rozo-anaya JC. Molecular tools for *Mycobacterium tuberculosis* genotyping. Revisita De Salud Publica 2010; 12: 510-21.
9. Maes M, Kremer K, Van Soolingen D, Takiff H, Waard JH. 24-Locus MIRU-VNTR genotyping is a useful tool to study the molecular epidemiology of tuberculosis among Warao Amerindians in Venezuela. Tuberculosis 2008; 88: 490-4.
10. Supply P. Proposal for Standardization of Optimized Mycobacterial Interspersed Repetitive Unit – Variable-Number Tandem Repeat Typing of Mycobacterium tuberculosi. J Clin Microbiol 2006; 44: 4498-510.
11. Dong H. Genetic diversity of Mycobacterium tuberculosis isolates from Tibetans in Tibet, China. PLoS One 2012; 7: 1-7.
12. Amaro A, Duarte E, Amado A, Ferronha H, Botelho A. Comparison of three DNA extraction methods for Mycobacterium bovis, Mycobacterium tuberculosis and Mycobacterium avium subsp. avium. Soc Appl Microbiol 2008; 47: 8-11.
13. Stinson KW, Eisenach K, Kayes S, Matsumoto M, Siddiqi S, Nakashima S. Mycobacteriology Laboratory Manual. 5th ed. CDC; 2014.
14. Supply P. Multilocus Variable Number Tandem Repeat Genotyping of Mycobacterium tuberculosis technical guide. Lille, France: National Institute of Health and Medical Research Unit 629 Institute ofBiology /Pasteur Institute of Lille, 2005.
15. Hunter P. Reproducibility and indices of discriminatory power of microbial typing methods. J Clin Microbiol 1990; 28: 1903-5.
16. Shemko M, Yates M, Fang Z, Gibson A, Shetty N. Molecular epidemiology of *Mycobacterium tuberculosis* in patients of Somalian and white ethnic origin attending an inner London clinic. Int J Tuberc Lung Dis 2004; 8: 186-93.
17. Augusto CJ. Comparative study of RFLP-IS6110 and MIRU-VNTR from Mycobacterium tuberculosis isolated in the state of Minas Gerais, Brazil. Brazilian J Microbiol 2018; 334: 6-11.
18. Sola C. Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs: Association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. Infect Genet Evol 2003; 3: 125-33.
19. Pan X. A quantitative and efficient approach to select MIRU -VNTR loci based on accumulation of the percentage differences of strains for discriminating divergent *Mycobacterium tuberculosis* sublineages. Emerginh Microbes Infect 2017; 1-8.
20. Liu Y. Genotypic diversity analysis of *Mycobacterium tuberculosis* strains collected from Beijing in 2009, using spoligotyping and VNTR typing. PLoS One 2014; 9(9): e106787.
21. Van SD. Predominnance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. J Clin Microbiol 1995; 33: 3234-8.
22. Phyu S. Predominance of *Mycobacterium tuberculosis* EAI and Beijing Lineages in Yangon, Myanmar J Clin Microbiol 2009; 47: 335-44.

23. Brudey K. Mycobacterium tuberculosis com plex genetic diversity: Mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol 2006; 6: 1-17.
24. Manca C. Differential Monocyte Activation Underlies Strain-Specific Mycobacterium tuberculosis pathogenesis differential monocyte activation underlies strain-specific mycobacterium tuberculosis pathogenesis. Infect Immun 2004; 72: 5511-4.
25. Glynn JR,Whiteley J, Bifani PJ,Kremer K,Van Soolingen, D. Worldwide occurrence of Beijing/W strains of Mycobacterium tuberculosis: A systematic review. Emerg Infect Dis 2002; 8: 843-9.
26. Tun T. Genotypic diversity of Mycobacterium tuberculosis strains in Myanmar. Infect Dis(Auckl) 2017; 49: 237-9.
27. Ei PW, Aung WW, Nyunt WW, Swe TL, Htwe MM, Win SM, et al. Extensively drug- resistant tuberculosis in Myanmar : burden and mutations causing second-line drug resistance. Int J Tuberc lung Dis 2018; 22: 47-53.
28. Devi KR. Genetic diversity of *Mycobacterium tuberculosis* isolates from Assam, India: Dominance of beijing family and discovery of two new clades related to CAS1-Delhi and EAI family based on spoligotyping and MIRU-VNTR typing. PLoS One 2015;10: 1-20.
29. Liu HC. Molecular Typing Characteristic and Drug Susceptibility Analysis of *Mycobacterium tuberculosis* Isolates from Zigong, China. Biomed Res Int 2016; 1-7.
30. San LL, Aye KS, Oo NAT, Shwe MM, Fukushima Y, Gordon SV, et al. Insight into multidrug-resistant Beijing genotype *Mycobacterium tuberculosis* isolates in Myanmar. International Journal of Infectious Diseases 2018; 76: 109-19.
31. DeHaas PE, Khosravi AD, Feizabadi MM, Jolodar A. Study of genetic diversity in *Mycobacterium tuberculosis* by using mycobacterial interspersed repetitive unit: Variable number tandem repeat typing in African J Microbiol Res 2011; 5: 1549-56.
32. Iwamoto T. Genetic Diversity and Transmission Characteristics of Beijing Family Strains of *Mycobacterium tuberculosis* in Peru. PLoS One 2012; 7(11): e49651.
33. Hamblion EL, Le Menach A, Anderson LF, et al. Recent TB transmission, clustering and predictors of large clusters in London, 2010-2012: Results from first 3-years of universal MIRU-VNTR strain typing. Thorax 2016; 71(8):749-56.
34. Moonan PK, Ghosh S, Oeltmann JE, Kammerer JS, Cowan LS, Navin TR. Using genotyping and geospatial scanning to estimate recent *Mycobacterium tuberculosis* transmission, United States. Emerg Infect Dis 2012; 18(3): 458-65.
35. Xu G, Mao X, Wang J, Pan H. Clustering and recent transmission of *Mycobacterium tuberculosis* in a Chinese population. Infect Drug Resist 2018; 11: 323-30.

Arch AHS

ORIGINAL ARTICLE

Archives of Allied Health Sciences 2021; 33(1): 44-54.

A comparison of treadmill and overground running on physical performance in sedentary individuals

Krueakaew Tiaprapong[1], Kanok Tiaprapong[1*]

[1] Department of Physical Therapy, School of Allied Health Sciences, Walailak University, Nakhon Si Thammarat, Thailand.

KEYWORDS
Exercise training;
Muscle strength;
Balance;
Agility.

ABSTRACT

This study aimed to compare the effects between treadmill and overground running on lower extremity muscle strength, balance, and agility. Forty-six sedentary healthy participants with a mean age of 20.59±1.44 years were randomly allocated into treadmill, overground, or control groups. The participants in treadmill and overground groups completed 4 weeks of training sessions, while control group did not participate in any training. Results showed that there was a significant increase in lower extremity muscle strength and balance in both treadmill and overground groups (p-value<0.05), but only treadmill group showed a significant increase in agility (p-value=0.001) when compared with pre-intervention values. When comparing between treadmill and overground groups, there were no significant differences found in all parameters (p-value>0.05) after receiving intervention. Nevertheless, treadmill group tended to show more balance and agility than overground group. This was because treadmill group showed a significantly higher balance (posterolateral direction) and agility than control (p-value=0.017 and p-value=0.020, respectively), while overground group did not (p-value=0.069 and 0.196, respectively). Four weeks of both treadmill and overground running could improve physical performance. Interestingly, treadmill running might be a more beneficial training than overground running among sedentary healthy individuals.

* **Corresponding author:** Kanok Tiaprapong, PT, MSc. Department of Physical Therapy, School of Allied Health Sciences, Walailak University, Nakhon Si Thammarat, 80160, Thailand. Email: kanok_tia@hotmail.com
Received: 26 October 2020/ Revised: 25 January 2021/ Accepted: 22 March 2021

Introduction

At present, people tend to have more sedentary lifestyle, which includes prolonged sitting or sustained postures in any position for a long time. Previous studies reported that sedentary lifestyle has negative effects on person's health, including impairment to cardiovascular system[1] and musculoskeletal system[2]. For this propose, the World Health Organization (WHO) suggested that people should have moderate intensity of physical activity for at least 150 minutes per week. Thus, the simple exercise as running is an interesting method to increase physical activity in general public.

Running is one type of physical activity or exercise in this trend, and it is also a basic skill in most sports. Statistics in 2016 showed that there were 18.2% of all people in Thailand who exercised by running[3]. These statistics indicated that running is a popular choice for exercise in Thailand. Moreover, running is a low-cost exercise because it requires only a few pieces of equipment. Additionally, people can choose the location of running according to their convenience, such as a public park or stadium. People who cannot go to these places may choose to run on a treadmill that is located in a house or at a nearby fitness center instead. Running has many benefits and requires low-skill performance. Therefore, running is an appropriate exercise to reduce risk of disease and improve people's health[4, 5].

Previous studies reported that running could improve physical performance on musculoskeletal system includes an increase in muscle strength[6] and an improvement to the balance system[7]. Nevertheless, overground running (OG) and treadmill running (TM) have differences in some aspects. Firstly, there were differences between OG and TM in biomechanical analysis, such as kinematic, and kinetic parameters[8, 9]. Moreover, the people who undergo TM had to adjust their strategy of movement (e.g., cadence, and step length) when compared with OG. Lastly, many participants reported that they have to keep balance during running on a treadmill because of feeling unstable[10]. Therefore, the OG and TM might demonstrate the difference effects on physical performance.

There were many studies that reported on the effect of running on the cardiovascular system with a training period of at least 6 weeks. To the author's knowledge, there were few studies that reported on the effect of running on other physical performance. Moreover, no study has compared the effects of training between TM and OG on physical performance before. Therefore, the aim of this study was to compare the effects between TM and OG training for 4 weeks on lower extremity muscle strength, balance, and agility.

Materials and methods

Participants

This study employed a randomised controlled trial. The population of this study was 46 sedentary healthy individuals of both males and females. The participants were randomised into three groups that included TM group, OG group, and control group. All of them were measured for baseline data and post-data again after 4 weeks of intervention. This study was approved by the ethics committee of Walailak University (WUEC-19-008-01). The sample size was calculated by the G*Power (version 3.1.9.4) from the relevant study[7], was set to 0.05, was set to 0.95, the effect size was equal to 1.665, and estimated 20% dropout rate.

Participants who were between the ages of 18-25 years old, had a body mass index (BMI) between 18.5-22.9 kg/m^2, had good cooperation, and able to follow instructions were included in this study. On the other hand, Participants who had at least a moderate intensity of physical activity (3 Metabolic Equivalents Task: METs) for more than 150 minutes per week, pain in the lower extremity or trunk, a recent history of trauma or fracture, underlying diseases such as cardiovascular disease, and vertigo were excluded from the study.

Procedure

Randomization

All participants signed an informed consent prior to participation in this study. Participants were randomly allocated into three groups (i.e., TM, OG, and control) using simple randomization.

Intervention

For intervention group, participants were trained during 12 sessions over a period of 4 weeks. The schedule of training was set at 2-4 sessions per week, and participants who were absent for 2 consecutive days of training session were excluded from the study. Before training, participants had to warm up with dynamically stretch the lower extremities through such as hip flexors, hip extensors, knee flexors, knee extensors, and ankle plantarflexors for 5 minutes, and cool down with statically stretch for 5 minutes after each training session. For TM, participants performed all training sessions on a treadmill (Lode: Valiant-17745 model). The intensity of training was increased progressively with each consecutive week. In the first week, participants ran at 2 kilometers for 20 minutes. In the second week, participants ran at 2.8 kilometers for 25 minutes. In the third week, participants ran at 3.6 kilometers for 30 minutes. In the last week, participants ran at 4.4 kilometers for 35 minutes[6]. For OG, participants performed all training sessions on a standard 400-m oval running track. The intensity of training was the same as the treadmill group, and participants were asked to control intensity by time. For example, if they were slower than expected, they would be encouraged to run quickly.

For control group, participants were asked to perform their activities regularly during a period of 4 weeks, and they were asked to avoid any intense activities and sports during this period. After 4 weeks, the intensity of each participant's activities or sports in each week were inquired and recorded.

Assessments

To evaluate the effect on physical performance of the intervention, all outcome measures were assessed before and after 4 weeks of intervention.

Outcome measures

Lower extremity muscle strength

The lower extremities strength was measured by a hand-held dynamometer (HDD) (JTECH Commander Powertrack Muscle Dynamometer MMT: JT-AA104 model). This equipment had high intra- and inter-rater reliability (ICC=0.949-0.992)[11]. The measurements included hip flexor, knee flexor, ankle dorsiflexor, and ankle plantarflexor strength. Hip flexors and knee flexors were conducted in a sitting position with hip and knee joints flexed to 90°. The HDD was placed at the anterior aspect above the knee 2 inches for hip flexors and at the posterior aspect above the heel 2 inches for knee flexors. Ankle dorsiflexors were conducted in a supine position with the ankle in a neutral position, and the HDD was placed at the metatarsophalangeal joint (MTPJ) of the dorsal aspect of the foot. Lastly, ankle plantarflexors were conducted in a prone position with the ankle in a neutral position, and the HDD was placed at the MTPJ of the plantar aspect of the foot[12]. After that, participants exerted the maximum effort against the HDD for all muscle groups. Each muscle group was measured 2 times with a 1-minute rest between each contraction and between each muscle group to avoid fatigue. The maximum value for each of the muscle group was recorded.

Balance

Dynamic balance was measured by the modified Star Excursion Balance Test (mSEBT). This test had high intra- and inter-rater reliability (ICC=0.87-0.94)[13]. There were three directions which consisted of the anterior, posteromedial, and posterolateral direction. Firstly, the testing leg was placed at the center of the grid line on the floor with three reach lines (in Y-shape), while both hands were maintained at the waist. The participants were allowed to practice 2 times, and then they were instructed to reach their leg as far as possible along each of the three lines, touch the line with their big toe, and return the reaching leg back to the center without swaying or displacing their hands from their waist[14]. Each direction was measured 3 times with 15 seconds of rest between each reach and 2 minutes of rest between each direction. The average value for each direction was recorded.

Agility

Agility was measured by the Illinois agility test. This test had high intra-rater reliability (ICC=0.80-0.89)[15]. The length of the test was 10 meters, and the width was 5 meters. Four cones were placed as the starting, finishing, and two turning points, and another four cones were placed at the center of the area at 3.3 meters apart. Participants were instructed to run as fast as possible from the starting to the finishing point, where the time was started after the word "go" and stopped when the participants reached the finishing point. Between trials participants were instructed to avoid any contact with the cones or cross the cones. In this test, participants had to accelerate, decelerate, run in different angles, and turn in different directions[16]. This test was measured for two times, and the best value was recorded.

Statistical analysis

Baseline characteristics were summarised as mean and standard deviation (SD). The Shapiro-Wilk test was used to verify the normality of the data. One-way analysis of variance (ANOVA) was used to compare the baseline data between groups. Paired t-test was used to compare outcome variables at baseline with 4 weeks of intervention within a group. Since the randomised allocation did not confirm that the baseline characteristics would be the same between groups, analysis of covariance (ANCOVA) was performed. If ANCOVA detected the difference of outcome variables, the Bonferroni test would be performed to identify this difference between pairs. Outputs of the paired t-test and ANCOVA were reported as mean and adjusted mean with *p*-value. All data were analysed using the SPSS (version 17).

Results

One hundred potential participants were assessed for eligibility. Of these participants, 46 met the inclusion and exclusion criteria (14 males at 30.43%, and 32 females at 69.57%) and signed the informed consent. Sixteen participants were randomly allocated to TM group, 14 participants were randomly allocated to OG group, and another 16 participants were randomly allocated to control group.

Demographic and baseline characteristic data

The details of the demographic and baseline characteristics are shown in Table 1. The average age of the participants was 20.59±1.44 years old. The average BMI of the participants was 20.18±1.6 kg/m^2. The comparison of all variables is also shown in Table 1, and there were differences between groups in age and lower extremity muscle strength variables.

Comparison within a group

All of the parameters were normally distributed. In TM group, there was a significant increase in all lower extremity muscle strengths, all directions of balance, and agility (*p*-value<0.05) after training for 4 weeks when compared with pre-intervention values. For the OG group, there was a significant increase in all lower extremity muscle strengths and all directions of balance (*p*-value<0.05) after training for 4 weeks when compared with pre-intervention values, but no significant difference was found in agility (*p*-value=0.183). In contrast, no statistical differences were found in control group, except for balance in the anterior direction (Table 2).

Table 1 Demographic and baseline characteristic data, and comparison of the baseline data between all three groups (ANOVA)

Parameters		All (n=46)	TM (n=12)	OG (n=14)	CON (n=16)	*p*-value
Gender [n(%) female)]		32 (69.57)	11 (68.75)	10 (71.43)	11 (68.75)	-
Age (yr)		20.59±1.44	20.38±1.45	19.79±1.19	21.5±1.15*†	0.002
Height (cm)		161.57±5.97	163.69±6.2	160.57±5.5	160.31±5.88	0.213
Weight (kg)		52.8±6.23	55.5±5.29	52.07±6.79	50.75±5.97	0.082
BMI (kg/m^2)		20.18±1.6	20.70±1.48	20.15±1.91	19.69±1.31	0.202
Leg length (cm)	Left	82.16±3.9	83.96±3.46	81.25±4.07	81.16±3.74	0.069
	Right	81.92±3.89	83.5±3.45	81.14±4.07	81.03±3.87	0.132
Hip flexor strength (lb)	Left	19.62±5.86	23.34±7.29	16.57±3.41*	18.56±3.87*	0.003
	Right	21±5.49	24.38±6.43	18.21±4.01*	20.06±3.84	0.004
Knee flexor strength (lb)	Left	20.37±6.72	24.22±8	17.18±2.8*	19.3±6.17	0.009
	Right	22.97±7.15	27.25±8.91	19.54±3.3*	21.69±5.68	0.006
Ankle dorsiflexor (lb)	Left	19.9±6.47	22.06±7.75	15.32±2.2*	21.75±5.76†	0.004
	Right	20.53±6.56	22.97±8.66	16.93±2.08*	21.25±5.59	0.033
Ankle plantarflexor (lb)	Left	31.77±12.34	36.47±15.35	24.61±5.99*	33.34±10.78	0.022
	Right	31.61±12.37	38.81±15.35	23.79±5.8*	31.25±9.04	0.002
Balance (cm)	A	63.23±5.38	63.79±4.71	64.03±4.49	61.98±6.68	0.519
	PM	63.92±9.96	62.49±8.73	60.32±9.41	68.5±10.38	0.059
	PL	76.22±7.4	76.13±8.06	75.49±6.53	76.94±7.84	0.871
Agility (s)		25.13±2.05	24.75±2.29	25.57±2.21	25.13±1.67	0.560

Note: All continuous parameters were reported in mean±SD; Gender parameter was reported in number (percentage). TM, treadmill; OG, overground; CON, control; A, anterior; PM, posteromedial; PL, posterolateral. *difference from TM (*p*-value<0.05); † difference from OG (*p*-value<0.05).

Table 2 Comparison of the parameters between pre-intervention and post-intervention of the three groups (paired t-test)

Parameters		Groups	Pre-intervention	Post-intervention	*p*-value
Hip flexor strength (lb)	Left	TM	23.34±7.29	28.97±6.19	<0.001*
		OG	16.57±3.41	27±6.12	<0.001*
		CON	18.56±3.87	18.31±3.42	0.745
	Right	TM	24.38±6.43	30.56±7.11	<0.001*
		OG	18.21±4.01	28.32±7.07	<0.001*
		CON	20.06±3.84	19.07±3.87	0.221
Knee flexor strength (lb)	Left	TM	24.22±8	33.75±10.07	<0.001*
		OG	17.18±2.8	29.11±5.71	<0.001*
		CON	19.3±6.17	18.53±4.6	0.585
	Right	TM	27.25±8.91	37.75±11.28	<0.001*
		OG	19.54±3.3	33±6.42	<0.001*
		CON	21.69±5.68	20.44±5.25	0.254
Ankle dorsiflexor (lb)	Left	TM	22.06±7.75	35.19±11.29	<0.001*
		OG	15.32±2.2	31.71±5.73	<0.001*
		CON	21.75±5.76	21.94±5.8	0.841
	Right	TM	22.97±8.66	36.94±11.16	<0.001*
		OG	16.93±2.08	33±5.14	<0.001*
		CON	21.25±5.59	21.22±5.69	0.972
Ankle plantarflexor (lb)	Left	TM	36.47±15.35	58.97±19.08	<0.001*
		OG	24.61±5.99	50.46±13.58	<0.001*
		CON	33.34±10.78	32.63±8.84	0.577
	Right	TM	38.81±15.35	61.72±20.92	<0.001*
		OG	23.79±5.8	50.32±13.49	<0.001*
		CON	31.25±9.04	31.78±7.97	0.581
Balance (cm)	Anterior	TM	63.79±4.71	68.39±5.58	0.003*
		OG	64.03±4.49	71.23±6.18	<0.001*
		CON	61.98±6.68	66.04±6.47	0.002*
	Posteromedial	TM	62.49±8.73	72.36±6.58	<0.001*
		OG	60.32±9.41	70.6±6.39	<0.001*
		CON	68.5±10.38	66.04±8.59	0.168
	Posterolateral	TM	76.13±8.06	87.01±7.27	0.001*
		OG	75.49±6.53	85.56±6.71	<0.001*
		CON	76.94±7.84	79.33±9.27	0.329
Agility (s)		TM	24.75±2.29	23.63±2.63	0.001*
		OG	25.57±2.21	24.71±2.05	0.183
		CON	25.13±1.67	25.44±1.59	0.312

Note: All parameters were reported in mean±SD. TM, treadmill, OG, overground, CON, control.

* significant difference at *p*-value<0.05.

Comparison between groups

Because the baseline characteristics were not equal, ANCOVA was therefore performed to manage these differences. There were significant differences found in all parameters (p-value<0.05), except for balance in the anterior direction (p-value=0.101) when comparing between all groups. In lower extremity muscle strength, all variables of both TM and OG showed higher values than the control group (p-value<0.05). In balance, the posteromedial direction of both TM and OG showed higher values than the control group (p-value=0.001 and p-value=0.030, respectively), whereas the posterolateral direction of TM only showed higher values than the control group (p-value=0.017). Lastly, the agility of TM showed lesser values than the control group (p-value=0.020) (Table 3).

Table 3 Comparison of mean post-intervention measures between all three groups after adjustment for differences in baseline values (ANCOVA)

Parameters		Treadmill (n=16)	Overground (n=14)	Control (n=16)	*p*-value
Hip flexor strength (lb)	Left	26.31 (1.06)	29.18 (1.1)	19.07 (0.98)	<0.001*
	Right	27.54 (1.18)	30.81 (1.22)	19.91 (1.09)	<0.001*
Knee flexor strength (lb)	Left	30.65 (1.43)	31.67 (1.49)	19.39 (1.35)	<0.001*
	Right	34.02 (1.6)	35.99 (1.65)	21.55 (1.49)	<0.001*
Ankle dorsiflexor (lb)	Left	33.26 (1.62)	35.8 (1.85)	20.29 (1.61)	<0.001*
	Right	35 (1.61)	35.87 (1.77)	20.65 (1.57)	<0.001*
Ankle plantarflexor (lb)	Left	55.3 (2.95)	56.06 (3.25)	31.4 (2.88)	<0.001*
	Right	55.55 (3.24)	57.03 (3.47)	32.09 (3.01)	<0.001*
Balance (cm)	Anterior	68 (1.22)	70.78 (1.31)	66.9 (1.23)	0.101
	Posteromedial	72.99 (1.51)	72.2 (1.65)	64.01 (1.57)	<0.001*
	Posterolateral	87.03 (1.92)	85.76 (2.05)	79.12 (1.92)	0.013†
Agility (s)		23.91 (0.38)	24.39 (0.41)	25.44 (0.38)	0.020†

Note: All parameters were reported in adjusted mean (SE). TM, treadmill; OG, overground. * significant difference between control and both TM and OG (p-value<0.001); † significant difference between control and TM (p-value<0.05).

Discussion

The aim of this study was to compare the effects between TM and OG training for 4 weeks on lower extremity muscle strength, balance, and agility. Even though mean age of all groups was different, age range of participants was young adulthood[17]. Therefore, this difference did not influence the effect of training for both TM and OG. The main finding of this study was that the different types of running elicited similar outcomes of physical performance in lower extremity muscle strength, balance, and agility. However, there were minor differences in balance and agility when compared with untrained participants.

The present study revealed that the lower extremity muscle strength was found to increase for both TM and OG groups after training. This result was supported by previous studies that reported that training had positive effects on muscular strength[6, 18]. This improvement was in accordance with the results of Franks et al. (2012), which reported that treadmill running for 4 weeks improved strength in quadriceps and hamstrings in non-running individuals. This improvement could be explained by the effects of training, where the running consisted of concentric and eccentric contraction of the leg muscles. Previous study reported that contraction of muscle could increase muscle strength and muscle size in healthy untrained subjects[19]. The effect of training on

muscle strength could be explained by these mechanisms: muscle protein synthesis, exercise-induced muscle damage (EIMD), enhanced extra-cellular matrix remodeling, and gene responses and cellular signaling pathways[20]. In addition, another previous study found that muscle protein synthesis could be observed in acute condition (4.5 hours after exercise) and also in chronic condition (within the period of 4 weeks)[21]. When comparing the effects of training on lower extremity muscle strength, non-significant differences were found between TM and OG (*p*-value>0.05). Although, there were differences in the biomechanics, OG showed a higher ROM for knee flexion and ankle dorsiflexion than TM. In kinetic analysis, OG showed a higher knee flexion moment, ankle dorsiflexion moment, anterior ground reaction force (GRF), and medial GRF than TM[8, 9], these differences were not enough to alter muscle strength between TM and OG. In contrast, the study of Fellin et al. (2010) reported that the kinematic curves of the lower limb during the running were similar between TM and OG[22]; therefore, this previous study may explain the similar effects of TM and OG in this study. Consequently, lower extremity muscle strength could be improved after 4 weeks for both TM and OG.

In regard to balance, the present study found that after training by TM and OG, balance was improved in all directions. This study was supported by previous studies that reported that training has positive effects on balance[7,23]. The improvement of dynamic balance was in accordance with the results of Pirouzi et al. (2014), where treadmill training for 4 weeks improved balance and the six-minute walk test (6MWT) in elderly people. In addition, this finding was also in accordance with the study of Asl et al. (2014), where running on a treadmill for 6 weeks improved balance in elementary students. In humans, the postural control (balance) consisted of three main systems including the visual, proprioceptive, and vestibular systems. The running could improve postural control, which especially stimulates the somatosensory or proprioceptive system, resulting in increased balance in this study. Additionally, the improvement of balance could be affected by the raised lower extremity muscle strength. Previous studies reported that lower limb strengthening exercise could improve balance[24,25]. For example, the study of Mohammadi et al. (2012) reported that 6 weeks of lower limb strength training could improve both static and dynamic balances in young male athletes. Therefore, the improvement of balance of both TM and OG in this study could be explained by the enhancement of proprioception and lower limb strength. In contrast, this study revealed that there was no significant difference in the anterior direction of dynamic balance between all groups. In the control group, there was a significant increase after intervention (*p*-value=0.003). The possible explanation was that the feedback mechanism played a major role in this situation. In anterior direction of mSEBT, participants received visual feedback from the reaching leg as they reached and observed the score in each trial, whereas the other directions could not be observed[26]. Therefore, participants made an effort to reach further than the latest trial, until they reached a plateau in this direction. Because this study allowed the participants to practice mSEBT for only 2 times in each direction, this might not be enough to reach the plateau value of the participants in anterior direction among control group. A previous study recommended that participants should perform six practice trials before recording the values of mSEBT[27]. There were no significant differences between TM and OG in all directions of balance. Nevertheless, TM tended to show more effects than OG when compared with the control. Only TM showed significantly greater posterolateral balance than control (*p*-value=0.017). The differences between TM and OG might be explained by the different proprioceptive input in TM, which included the following: the belt always pulled legs which resulted in instability while running and disturbance of the proprioceptive sense[28], and the compelled speed on the narrower path on TM[29]. As mentioned above, TM required more adjustment to the strategy of postural control than OG and tended to have more balance in this study.

The present study revealed that agility was significantly increased only in TM after training

(*p*-value=0.001), whereas OG was not (*p*-value =0.183). In addition, there were no significant differences between TM and OG in this parameter. However, TM tended to show more effect than OG when compared with the control. After training, TM showed a higher agility than the control (*p*-value=0.020), whereas OG showed not (*p*-value =0.196). So far as we know, there was limited literature about the effect of running on agility. However, the slightly higher agility in TM might be influenced by dynamic balance that tended to be higher in TM than OG because dynamic balance helped to improve ability to change directions resulting in improved agility. The present study was in accordance with a previous study reported that 4 weeks of dynamic balance training improved agility in basketball players[30]. Moreover, as running on a treadmill could increase cadence and step length, it may improve speed which is a component that influences agility. The previous study reported that treadmill running improved speed due to increased neuromuscular response[31]. In addition, 4 weeks of training was enough for agility improvement with TM. Interestingly, this was the first study that revealed the effects of running on agility.

The present study had certain limitations. One limitation was the lack of blinding the assessor, which might have led to the risk of bias. Therefore, future studies should consider blinding the assessor to reduce the risk of bias. Another limitation was that we measured only strength of flexor muscles of the lower extremity. Therefore, future studies should consider about the extensor muscle strength of the lower extremity.

Conclusion

Both TM and OG revealed an improvement of lower extremity muscle strength and dynamic balance, while agility was only improved in TM after training. When comparing between TM and OG, TM tended to show more dynamic balance and agility than OG. In addition, TM showed a higher dynamic balance and agility than the control, whereas OG did not. Therefore, our results may suggest that only 4 weeks of both TM and OG could improve physical performance, and TM might be a more beneficial training than OG among sedentary healthy individuals.

Take home messages

Treadmill running help to improve muscle strength of lower extremities, balance, and agility among sedentary people. Thus, running on a treadmill regularly may result in enhanced physical performance.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

No.

References

1. Mainous AG, Tanner RJ, Rahmanian KP, Jo A, Carek PJ. Effect of sedentary lifestyle on cardiovascular disease risk among healthy adults with body mass indexes 18.5 to 29.9 kg/m^2. Am J Cardiol 2019; 123(5): 764-8.
2. Shad BJ, Wallis G, van Loon LJC, Thompson JL. Exercise prescription for the older population: the interactions between physical activity, sedentary time, and adequate nutrition in maintaining musculoskeletal health. Maturitas 2016; 93: 78-82.
3. Rakpuang S. Marathon in Thailand: social network and challenges in 21st Century. Romphruek J 2019; 37(1): 8-17.
4. Lee D, Pate RR, Lavie CJ, Sui X, Church TS, Blair SN. Leisure-time running reduces all-cause and cardiovascular mortality risk. J Am Coll Cardiol 2014; 64(5): 472-81.
5. Lavie CJ, Lee D, Sui X, Arena R, O'Keefe JH, Church TS, et al. Effects of running on chronic diseases and cardiovascular and all-cause mortality. Mayo Clin Proc 2015; 90(11): 1541-52.
6. Franks KA, Brown LE, Coburn JW, Kersey RD, Bottaro M. Effects of motorized vs non-motorized treadmill training on hamstring/quadriceps strength ratios. J Sports Sci Med 2012; 11(1): 71-6.

7. Asl FA, Sokhanguei Y, Memar R. The effect of the aerobic activities on dynamic and static balance in elementary boy students. Eur J Exp Biol 2014; 4(1): 440-7.
8. Rozumalski A, Novacheck TF, Griffith CJ, Walt K, Schwartz MH. Treadmill vs. overground running gait during childhood: a qualitative and quantitative analysis. Gait Posture 2015; 41(2): 613-8.
9. Riley PO, Dicharry J, Franz J, Della Croce U, Wilder RP, Kerrigan DC. A kinematics and kinetic comparison of overground and treadmill running. Med Sci Sports Exerc 2008; 40(6): 1093-100.
10. Yang F, King GA. Dynamic gait stability of treadmill versus overground walking in young adults. J Electromyogr Kinesiol 2016; 31: 81-7.
11. Kim S-G, Lim D-H, Cho YH. Analysis of the reliability of the make test in young adults by using a hand-held dynamometer. J Phys Ther Sci 2016; 28(8): 2238-40.
12. Arnold CM, Warkentin KD, Chilibeck PD, Magnus CRA. The reliability and validity of handheld dynamometry for the measurement of lower-extremity muscle strength in older adults. J Strength Cond Res 2010; 24(3): 815-24.
13. van Lieshout R, Reijneveld EAE, van den Berg SM, Haerkens GM, Koenders NH, de Leeuw AJ, et al. Reproducibility of the modified star excursion balance test composite and specific reach direction scores. Int J Sports Phys Ther 2016; 11(3): 356-65.
14. Clagg S, Paterno MV, Hewett TE, Schmitt LC. Performance on the modified star excursion balance test at the time of return to sport following anterior cruciate ligament reconstruction. J Orthop Sports Phys Ther 2015; 45(6): 444-52.
15. Stewart PF, Turner AN, Miller SC. Reliability, factorial validity, and interrelationships of five commonly used change of direction speed tests: reliability of field-based CODS tests. Scand J Med Sci Sports 2014; 24(3): 500-6.
16. Alanazi H. RELATIONSHIPS Between illinois agility test and reaction time in male athletes. Swed J Sci Res 2015; 2: 28-33.
17. McDonagh JE, Ambresin A-E, Boisen KA, Fonseca H, Jakobsson Kruse P, Meynard A, et al. The age of adolescence and young adulthood. Lancet Child Adolesc Health 2018; 2(4): e6.
18. Sung E. The effect of treadmill-based and track-based walking training on physical fitness in ankle-sprain experienced young people. J Exerc Rehabil 2017; 13(1): 84-8.
19. Jones DA, Rutherford OM. Human muscle strength training: the effects of three different regimens and the nature of the resultant changes. J Physiol 1987; 391: 1-11.
20. Franchi MV, Reeves ND, Narici MV. Skeletal muscle remodeling in response to eccentric vs. concentric loading: morphological, molecular, and metabolic adaptations. Front Physiol 2017; 8: 447.
21. Franchi MV, Wilkinson DJ, Quinlan JI, Mitchell WK, Lund JN, Williams JP, et al. Early structural remodeling and deuterium oxide-derived protein metabolic responses to eccentric and concentric loading in human skeletal muscle. Physiol Rep 2015; 3(11): 1-11.
22. Fellin RE, Manal K, Davis IS. Comparison of lower extremity kinematic curves during overground and treadmill running. J Appl Biomech 2010; 26(4): 407-14.
23. Pirouzi S, Motealleh AR, Fallahzadeh F, Fallahzadeh MA. Effectiveness of treadmill training on balance control in elderly people: a randomized controlled clinical trial. Iran J Med Sci 2014; 39(6): 565-70.
24. Mohammadi V, Alizadeh M, Gaieni A. The Effects of six weeks strength exercises on static and dynamic balance of young male athletes. Procedia - Soc Behav Sci 2012; 31: 247-50.
25. Lee W-Y, Kang H-J. Effects of TaeKwonDo with lower limb strengthening exercises on quadriceps muscle strength and balance in female university students. Off J Korean Acad Kinesiol 2017; 19(4): 11-7.

26. Coughlan GF, Fullam K, Delahunt E, Gissane C, Caulfield BM, Sci M. A Comparison between performance on selected directions of the star excursion balance test and the y balance test. J Athl Train 2012; 47(4): 366-71.
27. Gribble PA, Hertel J, Plisky P. Using the star excursion balance test to assess dynamic postural-control deficits and outcomes in lower extremity injury: a literature and systematic review. J Athl Train 2012; 47(3): 339-57.
28. Bayat R, Barbeau H, Lamontagne A. Speed and temporal-distance adaptations during treadmill and overground walking following stroke. Neurorehabil Neural Repair 2005; 19(2): 115-24.
29. Dingwell JB, Cusumano JP, Cavanagh PR, Sternad D. Local dynamic stability versus kinematic variability of continuous overground and treadmill walking. J Biomech Eng 2001; 123(1): 27-32.
30. Saraswat A, Malhotra D, Sicaram C. Effect of dynamic balance training on agility in male basketball players. Int J Physiother 2015; 2(5): 798-803.
31. Brown LE. Treadmill running to improve speed. Strength Cond J 2002; 24(3): 27-9.

ORIGINAL ARTICLE

Arch AHS

Archives of Allied Health Sciences 2021; 33(1): 55-61.

Effects of a hip-core warm-up protocol on Q-angle during single-leg drop jump in 18–35 years old healthy female runners

Teerapat Laddawong[1*], Supachok Jiaranaikitchai[1], Anna Ritdhate[1], Jinwara Danrasri[1], Chuanpis Boonkerd[1], Thanawat Kitsuksan[1]

[1] Department of Physical Therapy, Faculty of Allied Health Sciences, Thammasat University, Pathumthani, Thailand.

KEYWORDS
Q-angle;
Warm-up;
Single leg drop jump;
Running.

ABSTRACT
Changing of Q-angle could detect abnormal biomechanics while running especially lower extremity. Increasing the angle can cause knee pain in various runners. Weakness and fatigue of hip-core muscles during exercises are the main factors that alter the angle and contribute to knee pain. Maintaining muscle performance through the running race and improving their strength are beneficial to athletic performance and decrease the injury rate. Unfortunately, there is no evidence of effectiveness of hip–core warm-up affects the Q-angle. The study aimed to investigate the effects of a hip–core warm-up protocol on the Q-angle during single-leg drop jump in 18–35 years old healthy female runners. Twenty-eight healthy female runners at the age of 18–35 participated and were randomly assigned to the exercise and control groups, fourteen in each. All runners performed pre-warm-up, post-warm-up and post-exercise single-leg drop jump test and the Q-angle was recorded by video cameras. The exercise group was assigned to have the hip–core warm-up protocol and the warm-up protocol for runners, while the control group was assigned to have only the warm-up protocol for runners. The results were compared within testing conditions and between two groups. In the exercise group, the result demonstrated that the Q-angle after warm-up programs was significantly decreased and was slightly increased after the 30-minute treadmill running. In the control group, this angle did not show any change. However, after 30-minute treadmill running, the angle showed a considerable increase and reached more than both pre-warm-up and post-warm-up angles. When compared between groups, the Q-angle was not altered significantly in any comparable. In conclusion, the hip-core warm-up protocol could immediately decrease the Q-angle and maintain the angle though 30-minute running. In contrast, the warm-up routine program could not decrease the angle, and it was higher than pre-warm up after finishing treadmill running. So, professionals should advise the hip-core warm-up protocol to reduce the risk of knee injuries in the runner

* **Corresponding author:** Teerapat Laddawong, PT, MSc. Department of Physical Therapy, Faculty of Allied Health Sciences, Thammasat University, Pathumthani, Thailand. Email: teerapat.l@allied.tu.ac.th

Received: 16 September 2020/ Revised: 15 January 2021/ Accepted: 14 March 2021

Introduction

The patellofemoral angle (Q-angle) represents the vector of pull exerted by the quadriceps muscles on the patella[1-2]. Ten percent increasing the angle during the single-leg landing phase could rise lateral tracking force on the patella and 45% increased compression force on the patella-femoral joint[3]. Abnormal forces could cause patellofemoral pain syndrome (PFPS), found in runners at a 25% prevalence rate[1-2]. Single-leg landing is an important mechanism which continuously impacts a patellofemoral joint force during running. The Q-angle measurement can detect weakness and fatigue of hip-core muscles that play an essential role in controlling runners' movement[4]. Paz AG et al. 2018[5] found that the Q-angle increased significantly during single-leg landing and was related with proper hip–core muscle activation.

The previous study found that the Q-angle changed by 0.216° when these muscles were 1% stronger[6]. In 2015[7], two rehabilitation programs were compared to increase the muscles' strength around the hip-core and the muscles around the knee joint in PFPS for six weeks. The results showed that both programs could reduce pain and increase muscle strength, but the hip joint-core exercises provided faster results and increased overall muscle strength. Nevertheless, some studies show proper warm-up programs before exercise, stimulating muscle readiness and increasing physical fitness by 79% and reducing the injury rate. Besides, they can stimulate a motor unit recruitment that allows the muscles to work more efficiently[8]. So, the hip–core warm-up protocol might immediately stimulate those muscles to control runners' movements and last long until they finish exercises to decrease the risk of knee injuries. However, there was no evidence to support the hypothesis. This study's objective was to study the effects of a hip–core warm-up protocol on Q-angle during single-leg drop jump in healthy female runners at the age of 18 - 35 years.

Materials and methods

Participants

The pilot study results were used to calculate the sample size by G-power program, which was twenty-eight total. Healthy female runners with a normal BMI and 18 - 35 years and practised at least 32 - 40 km per week participated in this study. Volunteers were randomly assigned to two groups, with 14 participants in each group.

The exercise group followed a hip–core warm-up protocol and warm-up routine for runners, while the control group performed only a warm-up routine for runners. None of the participants felt pain or any discomfort of the lower extremities or had a history of leg or back operation. In the three days before the study, participants were asked to abstain from treatments, including massage and anti-inflammatory drugs (NSAID). The Ethic Review Sub-Committee Board for Human Research Involving Sciences, Thammasat University, No. 3 (ECScTU) approved this study (approval number 076/2562).

Protocol

The volunteers who had met the inclusion and exclusion criteria participated in three vertical drop jump tests comprising pre-warm up, post-warm up and post-exercise jump test. Before the tests, reflexive markers were attached to the subjects to locate six bony prominences in the testing leg: anterior superior iliac spine (ASIS), mid-point of the patella, tibial tubercle, greater trochanter, lateral epicondyle of the femur, and lateral malleolus. The researcher (A), who was blinded to the participants' condition, also placed two video cameras, 50 cm. high, 3 m. away from a wooden box with a height of 30 cm. The first camera took pictures in the frontal plane, and the second camera took pictures in a sagittal plane. Volunteers stood on the wooden box with both hands on the waist. The volunteer extended the test leg (dominant leg) forward. Then, the volunteer jumped onto the ground with one leg and was able to stand on the test side. Three jump tests were performed with 30 seconds rest between the jumps.

Warm-up protocols

Volunteers received a warm-up program according to groups under supervision by the researcher (B). The exercise group received the hip-core warm-up protocol as shown in Table 1 in conjunction with the warm-up routine for runners as shown in Table 2, which took approximately 15 minutes while the control group received only a warm-up program. The hip-core warm-up protocol contains ten warm-up poses, according to Costa PB. in 2011[9]. The warm-up routine for the runner features seven warm-up moves, according to Runner world magazine in 2014[10].

Table 1 The hip-core warm-up protocol[9]

Exercises	Intensity
Lunges with unilateral trunk rotation	8 rep / set 3 sets
Lunges with unilateral trunk rotation	10 sec / set 3 sets
Elbow extension (sprawl)	10 rep / set 3 sets
Superman	10 rep / set 1 set
Static crunches with hip abduction (2 sides)	10 sec / set 3 sets
Static crunches with the hip flexed and trunk rotation (2 sides)	10 rep / set 1 set
Static crunches with trunk rotation lying down (2 sides)	10 rep / set 3 sets
Static crunches with rotation standing up (2 sides)	10 rep / set 1 set
Static elbow extension with unilateral knee flexion (2 sides)	10 rep / set 3 sets
Standing from the guard (2 sides)	10 rep / set 1 set

Table 2 The warm-up routine for the runner[10]

Exercises	Intensity
Active hamstring stretches	10 rep / set 3 sets
Walking straight leg kick up	10 rep / set 3 sets
Walking quad stretch	10 sec / set 3 sets
Walking leg cradle	10 rep / set 3 sets
Walking in-step stretch	10 rep / set 3 sets
Walking single leg RDL	10 rep / set 3 sets
Walking inchworm	10 rep / set 3 sets

Exercise program

Volunteers ran on a treadmill according to the researcher defined program for 30 minutes with the researcher adjusting the speed and timing for each interval.

Data processing

The recorded data were imported into the Kinovea motion analysis program (Kinovea.org, version 0.9.3) to measure maximal knee flexion during landing phase in the sagittal plane. The Q-angle was measured at the peak knee flexion angle. The mean value obtained from three jumps was used for statistical analysis.

Statistical analysis

Fragmentation of the data was analysed in SPSS version 24 by using the Shapiro-Wilk normality test. Comparison of the Q-angle while jumping with one leg before and immediately after warming up and after running on the treadmill within the groups and comparing the groups was done using two-way ANOVA and the least significant difference (LSD) test. This study

defined the degree of statistical significance at *p*-value < 0.05.

Results

The general participant characteristics included average age, weight, height and body mass index (Table 3) were statistically not significantly different between exercise group (n = 14) and control group (n = 14).

Table 3 General participant characteristics

Characteristics	Exercise group (Mean ± SD)	Control group (Mean ± SD)
Age (year)	21.07 ± 1.14	21.71 ± 0.91
Weight (kg)	52.71 ± 4.30	52.48 ± 4.96
Height (cm)	159.64 ± 3.81	161.11 ± 6.78
BMI (kg/m^2)	20.71 ± 1.80	20.20 ± 2.08

Figure 1 demonstrates the difference of Q-angle between each single-leg drop jump test in the exercise and the control groups. The exercise group showed a significant decrease after performing the warm-up protocol (16.43 ± 3.61˚) with *p*-value of 0.028 and slightly increased after the 30-minute treadmill running. The post-running Q-angle (17.5 ± 4.20˚) remained lower than before performing the warm-up program (18.29 ± 3.75˚). Besides, warming up programs in this group could decrease the angle immediately and throughout the 30-minute treadmill running. Additionally, the control group was not different when compared between the pre-warm-up (17.36 ± 8.53˚) and post-warm-up angle (17.00 ± 7.66˚). However, after 30-minute treadmill running, the angle showed a considerable increase (18.71 ± 8.14˚) (*p*-value 0.041) and reached more than both pre-warm-up and post-warm-up angles.

Figure 2 demonstrates a comparison of Q-angle differences between exercise and control groups in each single-leg drop jump test. The analysis found no significant difference in the angle between groups. However, even pre-warm up angle in the exercise group showed higher than the other. However, after warm-up, the angle was lower in both immediately post warm-up and after treadmill running.

Discussion

The study investigated the hip–core warm-up protocol on Q-angle during single-leg drop jump in twenty-eight, 18 - 35 years old healthy female runners. Participants were randomly assigned to the exercise and control group and performed three single-leg drop jump test, including pre-warm up, post-warm up, and post-exercise.

The main results demonstrated that the hip–core warm-up protocol could significantly decrease the angle and control it until finishing 30-minute treadmill running. Changing of the Q-angle resulted from the effectiveness of the hip-core muscles. The ability to control the hip and knee joint, associated with Q-angle changing, was related to the core muscles and the muscles surrounding the hip joint [4, 6]. Those were classified as stabiliser muscles[11, 12]. Studies have shown that a muscle-stimulating warm-up program can help muscles to work more efficiently [7, 15]. Stickler et al. in 2014[5] found that the hip and core muscles affected Q-angle when performing a single-leg squat. Also, when these muscles had been appropriately activated, it could decrease the Q-angle. A decline in the angle can lead to less compressive force on the patellofemoral joint[3]. So, it would help to decrease the risk of knee injuries caused by PFPS. Besides, after the

30-minute treadmill running, this group's angle showed slightly increased but remained lower than before performing the warm-up program. The increase could be the result of hip-core muscles fatigue that used to control movement. Studies have shown that 30-minutes running exercise can reduce muscle efficiency[13, 14]. Nevertheless, adding the hip-core protocol to regular warm-up programs could maintain the Q-angle for 30 minutes after running on the treadmill.

On the contrary, only the warm-up routine for runner protocol performed in the control group could not instantly decrease the angle. It was significantly higher after treadmill running. This warm-up program focused on lower limb muscle flexibility and functional activation, which targets prime movers muscles function during running[9]. According to specific muscle exercises, effective stimulation or strengthening of muscles was required to use a position that stimulates them directly[16]. Besides, the programs did not focus on hip-core stabiliser muscles which show directly affect the Q-angle. So, only the warm-up routine for runner protocol may not be enough to decrease the angle before exercise. After 30 - minute treadmill running, this group's angle showed higher than both pre and post-warm up due to failure of stabiliser muscles to control body mechanics during running, which caused by muscle fatigue[13, 14]. Thus, runners who prepare themselves by the warm-up routine for runner protocol only may impact the patellofemoral joint force more than combining with the hip-core warm-up protocol. In 2015, Stickler et al.[6] found that the Q-angle of hip and core muscle strengthening programs was not a statistically significant difference from the knee muscle strengthening programs in subjects with anterior knee pain. However, the hip-core exercise group was more likely to reduce the Q-angle and improve anterior knee pain.

The results suggested that the hip–core warm-up protocol that targets stabiliser muscle can reduce the Q-angle immediately and maintain through 30-minute treadmill running. Thus, therapists should advise this program to patients or athletes to reduce knee injury risk while running. Before applying for the program, athletes should be trained to follow the program properly to make their warm-up more effective. However, the study collected biomechanic data via two 2D cameras, which could not analyse the whole-body movement. Besides, gender difference may affect the results due to all participants were female. So, it would be a limitation. In further studies, due to humans' complex movement, the 3D motion analysis system would be further covered. Moreover, ground reaction force and foot pressure distribution may expand many points of view.

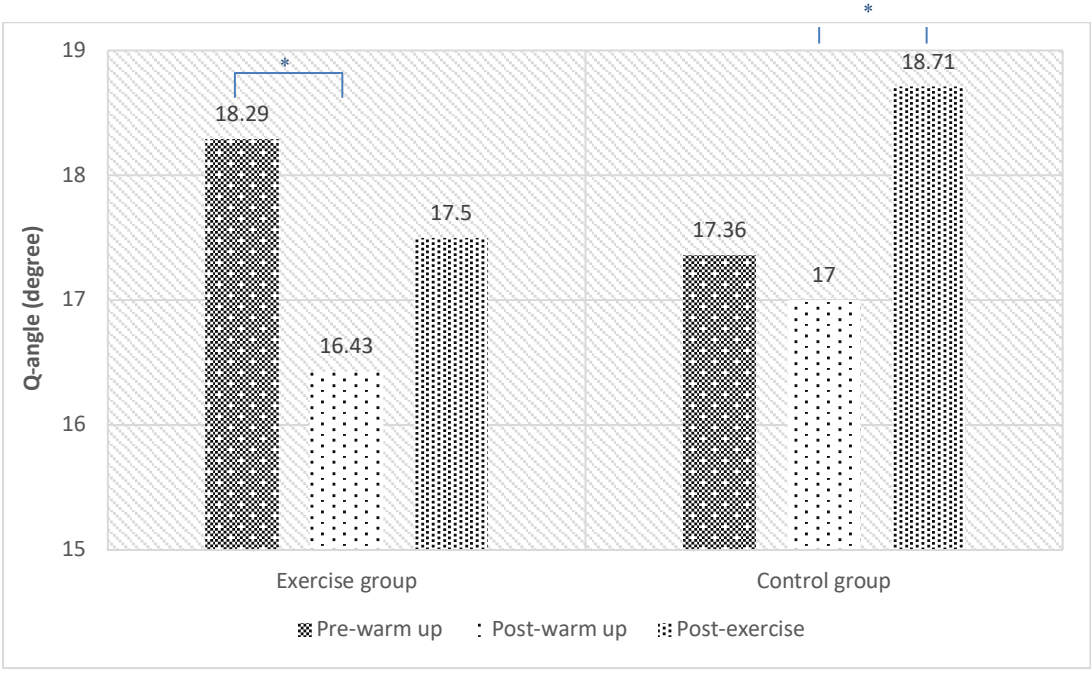


Figure 1 Q-angle differences between single-leg drop jump tests of the two groups
Note: *Significant level at *p*-value < 0.05

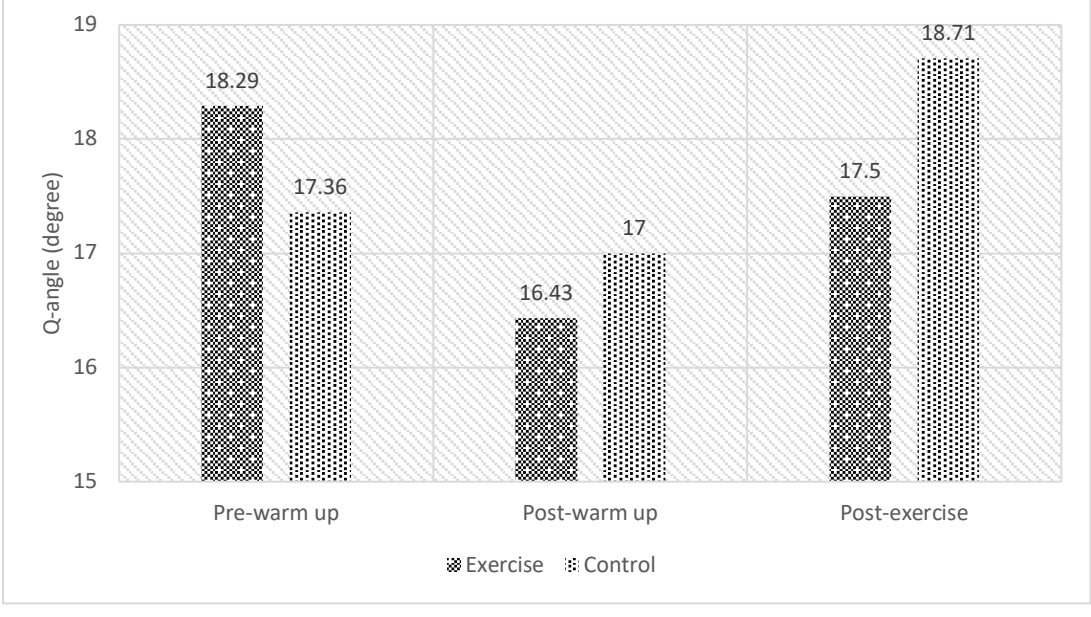


Figure 2 Q-angle between the exercise and control groups in each single-leg drop jump test.

Conclusion

The hip-core warm-up protocol could immediately decrease the Q-angle and could maintain the angle though 30 minutes running. In contrast, the warm-up routine program could not decrease the angle, and it was higher than pre-warm up after finishing treadmill running.

Take home messages

The hip-core warm-up protocol significantly reduced the Q-angle during single-leg drop jumps and could maintain the beneficial effect though 30-minute treadmill running.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors gratefully acknowledge the financial support provided by the Faculty of Allied Health Sciences, Thammasat University Research Fund, Contact No. TU (Special issue) 1/2562.

References

1. Cook C, Mabry L, Reiman MP, Hegedus EJ. Best tests/clinical findings for screening and diagnosis of patellofemoral pain syndrome: a systematic review. J Physiother 2012; 98(2): 93-100.
2. Roush JR, Curtis Bay R. Prevalence of anterior knee pain in 18-35-year-old females. Int J Sports Phys Ther 2012; 7(4): 396-401.
3. Huberti HH, Hayes WC. Patellofemoral contact pressures. The influence of q-angle and tendofemoral contact. J Bone Joint Surg Am 1984; 66(5): 715-24.
4. Hollman JH, Ginos BE, Kozuchowski J, Vaughn AS, Krause DA, Youdas JW. Relationships between knee valgus, hip-muscle strength, and hip-muscle recruitment during a single-limb step-down. J Sport Rehabil 2009; 18(1): 104-17.
5. Paz GA, de Freitas Maia M, Santana HG, Miranda H, Lima V, Willson JD. knee frontal plane projection angle: a comparison study between drop vertical jump and step-down tests with young volleyball athletes. J Sport Rehabil 2019; 28(2): 153-8.
6. Stickler L, Finley M, Gulgin H. Relationship between hip and core strength and frontal plane alignment during a single leg squat. Phys Ther Sport 2015; 16(1): 66-71.
7. Ferber R, Bolgla L, Earl-Boehm JE, Emery C, Hamstra-Wright K. Strengthening of the hip and core versus knee muscles to treat patellofemoral pain: a multicenter randomized controlled trial. J Athl Train 2015; 50(4): 366-77.
8. Fradkin AJ, Zazryn TR, Smoliga JM. Effects of warming-up on physical performance: a systematic review with meta-analysis. J Strength Cond Res 2010; 24(1):140-8.
9. Costa PB, Medeiros HBO, Fukuda DH. Warm-up, stretching, and cool-down strategies for combat sports. Strength Cond J 2011; 33(6): 71-9.
10. Rice A. Top five marathon warm-up races. Runner's World. 2014.
11. Saez Saez de Villarreal E, Gonzalez-Badillo JJ, Izquierdo M. Optimal warm-up stimuli of muscle activation to enhance short and long-term acute jumping performance. Eur J Appl Physiol 2007; 100(4): 393-401.
12. Skof B, Strojnik V. The effect of two warm-up protocols on some biomechanical parameters of the neuromuscular system of middle-distance runners. J Strength Cond Res 2007; 21(2): 394-9.
13. Dierks TA, Manal KT, Hamill J, Davis IS. Proximal and distal influences on hip and knee kinematics in runners with patellofemoral pain during a prolonged run. J Orthop Sports Phys Ther 2008; 38(8): 448-56.
14. Kellis E, Liassou C. The effect of selective muscle fatigue on sagittal lower limb kinematics and muscle activity during level running. J Orthop Sports Phys Ther 2009; 39(3): 210-20.
15. Gorassini M, Yang JF, Siu M, Bennett DJ. Intrinsic activation of human motoneurons: reduction of motor unit recruitment thresholds by repeated contractions. J Neurophysiol 2002; 87(4): 1859-66.
16. Lin CY, Tsai LC, Press J, Ren Y, Chung SG, Zhang LQ. Lower-limb muscle-activation patterns during off-axis elliptical compared with conventional gluteal-muscle-strengthening exercises. J Sport Rehabil 2016; 25(2): 164-72.

Arch AHS

ORIGINAL ARTICLE

Archives of Allied Health Sciences 2021; 33(1): 62-69.

Involvement of red blood cell on calcium oxalate crystal growth and aggregation in vitro

Mohamed Shahid[1,2], Nattaya Sae-ung[2,3], Porntip Pinlaor[2,3], Patcharee Boonsiri[4], Wichien Sirithanaphol[5], Vitoon Prasogwattana[4], Ratree Tavichakorntrakool[2,3*]

[1] Graduate School, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.
[2] School of Medical Technology, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.
[3] Centre for research and development of medical diagnostics Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.
[4] Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.
[5] Division of Urology, Department of Surgery, Faculty of Medicine, Khon Kaen University, Thailand.

KEYWORDS
Red blood cell;
Calcium oxalate;
Crystal growth;
Crystal aggregation.

ABSTRACT
Cell membranes and their components may play an important role in stone formation. Hematuria is one of the most common manifestations in kidney disease. We therefore extensively investigate the involvement of intact red blood cell (iRBC) and red blood cell membrane fragments (fRBC) on CaOx crystal growth and aggregation. Intact RBC and fRBC were prepared from healthy blood samples. Calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) crystals were investigated for crystal growth and aggregation in the condition without or with iRBC or fRBC. Crystal growth and aggregation were analyzed from crystal area and the number of crystal aggregation, respectively, and also confirmed by using spectrophotometric oxalate depletion assay and calcium oxalate crystal aggregation-sedimentation assay, respectively. The results showed that only COM crystal with fRBC was significantly increased the crystal area and the number of crystal aggregation as compared to control conditions (p-value = 0.035 and p-value = 0.011, respectively) while COD crystals did not have any significant change to the crystal area or the number of crystal aggregates with both types of RBC. These data indicated that fRBC might promote the growth and aggregation of COM crystals. However, the molecular mechanism involvement of fRBC with COM crystals still needs to be studied.

* **Corresponding author:** Ratree Tavichakorntrakool, MT, PhD. Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand. E-mail: ratree.t@kku.ac.th
Received: 20 July 2020/ Revised: 11 September 2020/ Accepted: 20 October 2020

Introduction

Kidney stones consist of crystalline and non-crystalline (organic matrix) compositions. The organic matrix is a supporting structure of stones[1] and it is responsible for calcium stone[1, 2]. Which some macromolecules (lipids, proteins, glycosaminoglycans and carbohydrates) of stone matrix can play an important role in the promotion or inhibition of stone formation[3]. Urinary macromolecules may associate with calcium oxalate (CaOx) stone formation in two ways: 1) heterogeneous nucleation of CaOx crystals in low concentration of urinary salts leading to homogenous nucleation and 2) nucleated crystals further precipitate on the surface acting as an adhesive to bind more crystals leading to crystal aggregation and growth[4].

The previous evidence suggested that cell membranes and their lipids in urine play a role in calcium stone formation. The organic matrix of CaOx stone formers had a significant amount of acidic phospholipids compared to other types of stones[5]. Calcium-binding molecules on the inner surface of the red blood cell (RBC) membrane, particularly trans-membrane proteins may associate with crystal aggregation to red blood cell membrane fragments (fRBC)[5, 6]. Hematuria, RBC in urine, is one of the most common manifestations in urological and kidney disease and evidence of crenated red blood cells (RBC) in CaOx stone core matrix[7]. It is considered as effect of kidney stone disease. However, idiopathic hematuria with urinary metabolic abnormality (hypocitraturia, hypomagnesuria, hyperuricosuria, hypercalciuria and hyperoxaluria) showed increased promoters and decreased inhibitor concentration in the absence of nephrolithiasis[8]. These metabolic abnormalities were also risk factors for CaOx stone. A recent study showed that normal RBC does not promote calcium oxalate dihydrate (COD) crystal growth and aggregation[6]. However, the crenated RBC appeared to participate in the COD crystal growth[7] observed under a series of electron microscopic. Moreover, membrane lipid was found inside stone matrices of CaOx stones[5, 9].

We therefore extensively investigated the involvement of intact RBC (iRBC) and fRBC from healthy subjects on the growth and aggregation of calcium oxalate monohydrate (COM) and COD crystals in vitro.

Materials and methods

Selective criteria for healthy subjects

Healthy subjects were selected according to the results of nine parameters; hemoglobin (Hb) > 13.0 g/dl, hematocrit (HCT) > 39%, total RBC count 4.18 - 5.48 × 10^6 /µl, MCV > 90 fl, MCH > 27 pg, WBC count 4.6 - 10.6 × 10^3/µl, platelet count 150 - 400 × 10^3/ul, normal RBC morphology, and negative screening for hemoglobin E with dichlorophenol indophenol precipitation test.

Sample collection and preparation of iRBC and fRBC

This study was approved by the institutional ethic committee of Khon Kaen University (HE 621366). Blood samples from healthy subjects were collected into 2 ml tube containing ethylene diamine tetraacetic acid from Sirinagarind Hospital, Khon Kaen University. The blood samples were centrifuged at 2,500 rpm for 5 min. After that, 500 µl of RBCs from the bottom pallet was resuspended in 5 ml of an isotonic buffer containing 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl. RBCs were washed for 5 times with isotonic buffer at 2,500 rpm. After RBC washing, RBC count was done with a hemocytometer and 10^5 cells/ml were used per assay. 10^5 cells/ml RBCs were treated with deionized water to lyse RBCs. When RBCs were lysed completely, the fRBC were separated by centrifugation at 10,000 g for 5 min. iRBC and fRBC were analyzed using a UriSed3 urine analyzer with UriSed3 Pro software (UriSed3 analyzer, Hungary) to check the purity of RBC, fragments, and RBC morphology.

Evaluations of iRBC and fRBC on COM and COD crystal growth and aggregation

To investigate the involvement of iRBC and fRBC on COM and COD crystal growth and aggregation, COM and COD crystals were generated as a previous study[6]. All assay conditions were performed in each well of a 24-well plate (Thermo Fisher Scientific, East Grinstead, UK). For control condition, COM crystals were prepared with

a final concentration of 5 mM $CaCl_2 \cdot 2H_2O$ and 1.0 mM $Na_2C_2O_4$ in a buffer containing 10 mM Tris-HCl and 90 mM NaCl (pH 7.4) while COD crystals were prepared with a final concentration of 6.27 mM $CaCl_2 \cdot 2H_2O$ and 1.6 mM $Na_2C_2O_4$ in a buffer containing 9.6 mM $C_6H_5Na_3O_7 \cdot 2H_2O$, 11.6 mM $MgSO_4 \cdot 7H_2O$, and 63.7 mM KCl (pH 6.5). For treatment condition, 105 cells/ml of iRBC or fRBC were added in each well. The solutions were incubated at 25°C for 1 h and then examined under the inverted light microscope (CKX41, Olympus; Tokyo, Japan) connected to a digital camera. Minimum 10 high power fields (HPF) were examined and pictures were captured. Crystal aggregates were defined as two or more crystals that adhered together. Secondly, crystal size was analyzed with Image J software (National Institutes of Health, USA). Minimum 100 individual COM crystals and 10 individual COD crystals were analyzed in each experiment. All experiments were done in triplicate for each sample.

Spectrophotometric oxalate-depletion assay of COM crystal growth

The spectrophotometric oxalate-depletion assay was carried out according to Nakagawa et al.[6, 10] to confirm the COM crystal growth with iRBC and fRBC. For control condition, COM crystal seeds (160 µg/ml) were added to 1-ml equilibrated solution containing 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $Na_2C_2O_4$, 10 mM Tris-HCl (pH 7.4) and 90 mM NaCl. For treatment conditions, iRBC or fRBC was added to the equilibrated solution to a final concentration of 10^5 cells/ml before adding crystal seeds. The depletion of free oxalate ions in a solution containing $CaCl_2$ and $Na_2C_2O_4$ due to the growth of seeded crystals were detected and monitored at 214 nm by UV-visible spectrometer (Eppendorf 6137000015 BioSpectrometer Fluorescence, Germany). The rate of free oxalate depletion was calculated using baseline value and the value after 60 min incubation with or without RBC (iRBC or fRBC) equivalent to 10^5 cells per assay. The relative percentage of oxalate depletion was calculated.

CaOx crystal aggregation-sedimentation assay of COM crystal aggregation

The effects of iRBC and fRBC on COM crystal aggregation were confirmed by CaOx crystal aggregation-sedimentation assay[11, 12]. For control condition, COM crystal seeds (100 µg/ml) was added to 1 ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $Na_2C_2O_4$, 10 mM Tris-HCl (pH 7.4) and 90 mM NaCl. The reduction of turbidity of the solution due to crystal aggregation was monitored by a UV-visible spectrophotometer at 620 nm for 1 h. For treatment conditions, iRBC or fRBC were added to the equilibrated solution to a final concentration of 10^5 cells/ml before adding crystal seeds. Crystal aggregation was evaluated by comparing the turbidity slope in the presence and the absence of iRBC or fRBC at the end of 1 h assay time. Besides, the morphology of the aggregates was observed under an inverted light microscope to reconfirm the crystal aggregation.

Statistical analysis

Statistics were calculated by SPSS program version 17.0 (IBM Corp, Armonk, NY, USA). Multiple comparisons were analyzed using variance with Turkey's post hoc test. Quantitative data were shown as the mean and standard error of the mean (mean ± SEM). A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Preparation of iRBC and fRBC

To confirm the purity and homogeneity of iRBC and fRBC, the samples were analyzed by UriSed3 urine analyzer with UriSed3 Pro software before the experiment. The purity of iRBC isolated was greater than 99.8%. RBCs were completely lysed and fragmentation occurs approximately 99% of the RBC.

Involvement of iRBC and fRBC on COM crystal growth and aggregation

To investigate the effect of iRBC and fRBC on COM crystal growth, the COM crystals were generated in the absence and presence of iRBC or fRBC. Crystal areas in pixel unit (mean ± SEM) were compared to the control, iRBC and fRBC (4296.6 ± 50.0, 4834.2 ± 58.3, and 5850.4 ± 60.8), respectively. The fRBC significantly increased crystal size as compared to control condition (*p-value* = 0.035) (Table 1).

The number of crystal aggregates significantly increased with fRBC. Crystal aggregates in control, iRBC, and fRBC were (0.23 ± 0.08, 0.46 ± 0.12, and 1.27 ± 0.18), respectively. The fRBC significantly increased COM crystal aggregation as compared to control (*p-value* = 0.011) and compare to iRBC (*p-value* = 0.029) (Table 1).

Table 1 Involvement of iRBC and fRBC on COM crystal growth (size in pixel unit) and aggregation (number of crystal aggregates/HPF)

Experiment conditions	Control (C)	iRBC (I)	fRBC (F)	*p*-value
Crystal area (pixel unit)	4296.60 ± 50.00	4834.20 ± 58.30	5850.40 ± 60.80	a
Number of crystal aggregates/HPF	0.23 ± 0.08	0.46 ± 0.12	1.27 ± 0.18	b, c

Note: Quantitative data presented from 3 individual experiments as mean ± SEM. Each experiment was done in triplicate.
a; significant difference between F and C (*p-value* = 0.035).
b; significant difference between F and C *(p-value* = 0.011).
c; significant difference between F and I (*p-value* = 0.029).

Spectrophotometric oxalate depletion assay was performed to confirm the COM crystal growth. The assay was monitored for oxalate depletion with iRBC or fRBC for 60 min. At the end of 60 min, the amounts of oxalate depletion were 39.3 ± 1.4% of iRBC and 44.6 ± 1.4% fRBC.

To confirm the crystal aggregation with iRBC and fRBC, the CaOx crystal aggregation-sedimentation assay was performed (Figure 1). The fRBC significantly increased the crystal aggregation as compared to control and iRBC. At the end of 60 min, fRBC (78.0 ± 3.7%) decreased turbidity of reaction as compared to the control condition (59.3 ± 3.4%). The iRBC had no any significant effects on COM crystal aggregation.

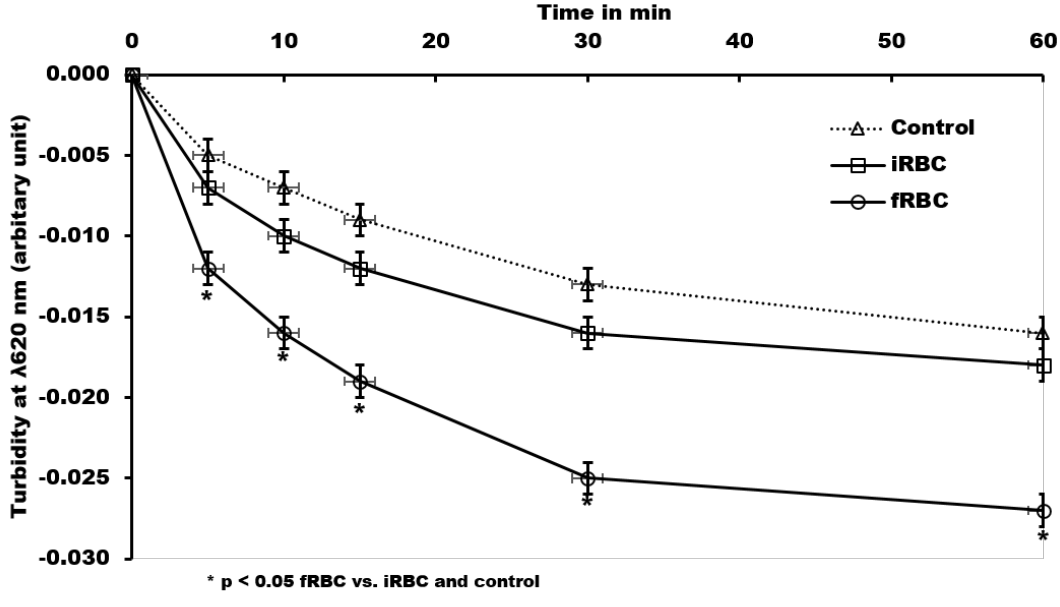


Figure 1 CaOx crystal aggregation-sedimentation assay.
Note: The reduction of turbidity from reactions was monitored at 620 nm for 60 min in control, iRBC and fRBC. Quantitative data presented as mean±SEM from 3 individual experiments. Each experiment was performed in triplicate.

The aggregated products were generated from CaOx crystal aggregation-sedimentation assay after 60 min (Figure 2). Aggregates induced by fRBC were much larger and firmer than those induced by control and iRBC.

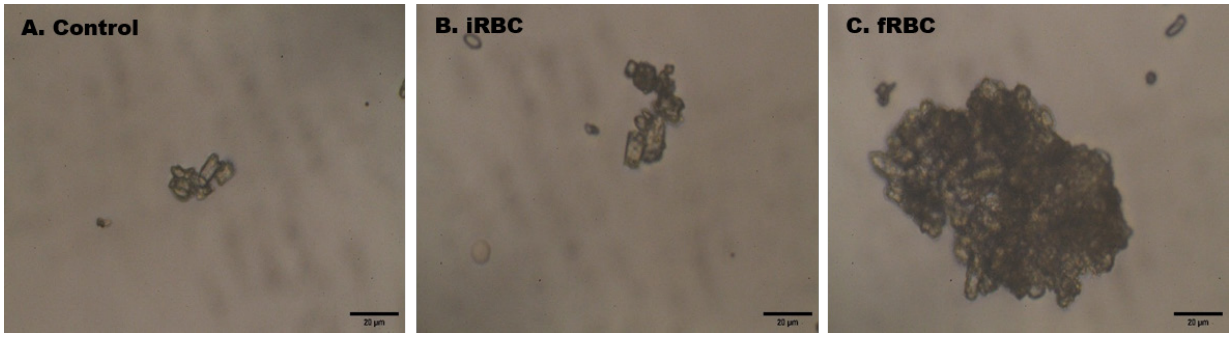


Figure 2 Aggregated product of CaOx crystals ((A) Control, (B) iRBC and (C) fRBC) by aggregation-sedimentation assay at 60 min (original magnification ×400).

Involvement of iRBC and fRBC on COD crystal growth and aggregation

To investigate the involvement of iRBC and fRBC on COD crystals, the generation of COD crystals was analyzed. Neither iRBC nor fRBC had a significant effect on COD crystal growth or crystal aggregation (Figure 3).

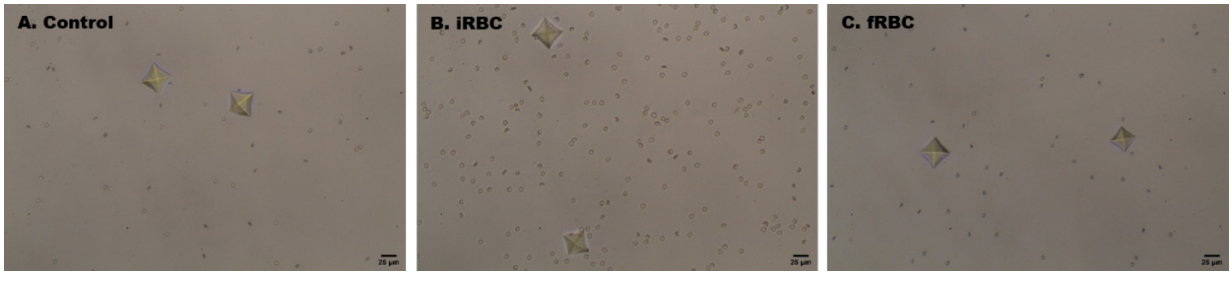


Figure 3 Effects of iRBC and fRBC on COD crystal growth and aggregation ((A) Control, (B) iRBC and (C) fRBC) under light microscope (original magnification ×400).

COD crystal growth represented as size area (pixel) of COD crystal were 4527.5 ± 206.1, 4645.2 ± 400.3 and 4356.5 ± 325.0 in control condition, iRBC and fRBC, respectively. The number of COD aggregates were 0.11 ± 0.003, 0.28 ± 0.006 and 0.36 ± 0.006 in control condition, iRBC and fRBC, respectively (Table 2).

Table 2 Involvement of iRBC and fRBC on COD crystal growth and aggregation

Experiment conditions	Control (C)	iRBC (I)	fRBC (F)	*p*-value
Crystal area (pixel unit)	4527.50 ± 206.10	4645.20 ± 400.30	4356.50 ± 325.00	d
Number of crystal aggregates/ HPF	0.11 ± 0.03	0.28 ± 0.06	0.36 ± 0.06	e

Note: Quantitative data presents as mean ± SEM from 3 individual experiments. Each experiment was performed in triplicate.
d; no significant difference between groups (F vs. C *p-value* = 0.904, F vs. I *p-value* = 0.858 and I vs. C *p-value* = 0.765).
e; no significant difference between groups (F vs. C *p-value* = 0.231, F vs. I *p-value* = 0.690 and I vs. C *p-value* = 0.397).

Discussion

Kidney stone formation is multifactorial such as genetic[3, 13-14] and environmental factors[3, 8, 15-17]. Some metabolic abnormalities (hypocitraturia, hypomagnesuria, hypercalciuria, hyperoxaluria, hyperuricosuria and potassium depletion in skeletal muscle)[3, 15-16] and reactive oxygen species[17] are risk factors for kidney stone formation. The three most common chemical compositions of stone were CaOx mixed with phosphate, pure CaOx and uric acid[18]. The organic matrix of CaOx stone formers had a significant amount of acidic phospholipids compared to other types of stones[5]. Hematuria is the most common manifestatios in kidney stone disease and evidence of crenated RBC in CaOx stone core matrix[7]. Therefore, in vitro experiments were performed to investigate the involvement of iRBC and fRBC on the growth and aggregation of CaOx crystals.
We carefully evaluated the morphology and purity of RBC that is suitable for the crystal analysis. Our result demonstrated that iRBC had no significantly effect on COM crystal growth or crystal aggregation. While fRBC promoted COM crystal growth approximately 36% and promoted COM crystal aggregate number approximately 2.5-fold as compare to control condition. This effect may be caused by some reasons. Firstly, calcium-binding molecules on the inner surface of the RBC membrane, particularly trans-membrane proteins associated with crystal aggregation to fRBC[5-6,9]. Secondly, cell membrane lipids may promote the stone formation. Some evidences found that the organic matrix of CaOx stone formers had a significant amount of acidic phospholipids compared to other types of stones examined[5]. The stone formers found an abnormal arachidonic acid content in RBC membrane[19].

Our results from healthy subjects were consistent with the previous study[6] which showed that iRBC had no significant effect on COM crystal, while fRBC showed 75% increase in size of the COM crystals and 2.5-fold increase in COM crystal aggregates number. The different percentages of crystal size may have effect from the source of blood samples. For the effect of iRBC on COD crystal growth and crystal aggregation, in this study was no statistically significantly effect similar to that of the Chutipongtanate and Thongboonkerd[6]. However, Kim s' study[7] showed that the crenated RBC appeared to participate in the COD crystal growth by observed under a series of electron microscopic in human urinary stones. For further studies should be analyzed the involvement of RBC of kidney stone formers on CaOx formation.

Conclusion

Our data indicated that fRBC significantly promotes CaOx crystal growth and aggregation. The fRBC may have involvement in CaOx crystal growth and aggregation. However, further studies are required to study the involvement of molecular mechanisms in fRBC with COM crystals.

Take home messages

Fragmented red blood cells significantly promoted calcium oxalate monohydrate crystal growth and aggregation in healthy subjects.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The study was supported by the Thailand International Cooperation Agency (TICA) and Faculty of Associated Medical Sciences, Khon Kaen University, a research grant from Khon Kaen University (I62 – 01 - 16) and Thailand research fund (RSA6180032). Urised3 analyzer was supported by Firmer Co., Ltd Thailand.

References

1. Boyce WH, Carolina N. Organic matrix of human urinary concretions. Am J Med 1968; 45: 673-83.
2. Warpehoski MA, Buscemi PJ, Osborn DC, Finlayson B, Goldberg EP. Distribution of organic matrix in calcium oxalate renal calculi. Calcif Tissue Int 1981; 33: 211-22.
3. Alelign T, Petros B. Kidney stone disease: an update on current concepts. Adv Urol 2018. doi: 10.1155/2018/3068365.
4. Ryall RL. The scientific basis of calcium oxalate urolithiasis: predilection and precipitation, promotion and proscription. World J Urol 1993; 11: 59-65.
5. Khan SR, Glenton PA, Backov R, Talham DR. Presence of lipids in urine, crystals and stones: implications for the formation of kidney stones. Kidney Int 2002; 62: 2062-72.
6. Chutipongtanate S, Thongboonkerd V. Red blood cell membrane fragments but not intact red blood cells promote calcium oxalate monohydrate crystal growth and aggregation. J Urol 2010; 184: 743-9.
7. Kim KM. Mulberry particles formed by red blood cells in human weddelite stones. J Urol 1983; 129: 855-7.
8. Valavi E, Nickavar A, Aeene A. Urinary metabolic abnormalities in children with idiopathic hematuria. J Pediatr Urol 2019; 15: 165e1-4.
9. Khan SR, Kok DJ. Modulators of urinary stone formation. Front Biosci 2004; 9: 1450-2.
10. Nakagawa Y, Abram V, Parks JH, Lau HS, Kawooya JK, Coe FL. Urine glycoprotein crystal growth inhibitors: evidence for a molecular abnormality in calcium oxalate nephrolithiasis. J Clin Invest 1985; 76(4): 1455-62.
11. Chutipongtanate S, Sutthimethakorn S, Chiangjong W, Thongboonkerd V. Bacteria can promote calcium oxalate crystal growth and aggregation. J Biol Inorg Chem 2013; 18: 299-308.
12. Amimanan P, Tavichakorntrakool R, Fong-Ngern K, Sribenjalux P, Lulitanond A, Prasongwatana V, et al. Elongation factor Tu on *Escherichia coli* isolated from urine of kidney stone patients promotes calcium oxalate crystal growth and aggregation. Sci Rep 2017; 7: 2953.
13. Sritippayawan S, Borvornpadungkitti S, Paemanee A, Predanon C, Susaengrat W, Chuawattana D, et al. Evidence suggesting a genetic contribution to kidney stone in northeastern Thai population. Urol Res 2009; 37: 141-6.
14. Sayer JA. Progress in understanding the genetics of calcium-containing nephrolithiasis. J Am Soc Nephrol 2017; 28: 748-59.
15. Bovornpadungkitti S, Sriboonlue P, Tavichakorntrakool R, Prasongwatana V, Suwantrai S, Predanon C, et al. Potassium, sodium and magnesium contents in skeletal muscle of renal stone-formers: a study in an area of low potassium intake. J Med Assoc Thai 2000; 83(7): 756-63.
16. Prasongwatana V, Tavichakorntrakool R, Sriboonlue P, Wongkham C, Bovornpadungkitti S, Premgamone A, et al. Correlation between Na, K-ATPase activity and potassium and magnesium contents in skeletal muscle of renal stone patients. Southeast Asian J Trop Med Public Health 2001; 32(3): 648-53.

17. Khan SR. Reactive oxygen species, inflammation and calcium oxalate nephrolithiasis. Transl Androl Urol 2014; 3: 256-76.
18. Tavichakorntrakool R, Prasongwattana V, Sungkeeree S, Saisud P, Sribenjalux P, Pimratana C, et al. Extensive characterizations of bacteria isolated from catheterized urine and stone matrices in patients with nephrolithiasis. Nephrol Dial Transplant 2012; 27: 4125-30.
19. Baggio B, Gambaro G, Zambon S, Marchini F, Bassi A, Bordin L, et al. Anomalous phospholipid n-6 polyunsaturated fatty acid composition in idiopathic calcium nephrolithiasis. J Am Soc Nephrol 1996; 7: 613-20.

Arch AHS

ORIGINAL ARTICLE

Archives of Allied Health Sciences 2021; 33(1): 70-78.

Design and optimization of primer for the cloning of the mouse Fd immunoglobulin M for antibody phage display technology

Sitthinon Siripanthong[1], Achara Phumyen[2], Anchalee Techasen[2, 3], Paiboon Sithithaworn[3, 4], Prasert Saichua[5], Chanvit Leelayuwat[2], Amonrat Jumnainsong[2*]

[1] Biomedical Sciences, Graduate School, Khon Kaen University, Khon Kaen, Thailand.
[2] School of Medical Technology, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.
[3] Liver Fluke and Cholangiocarcinoma Research Institute, Khon Kaen University, Khon Kaen, Thailand.
[4] Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.
[5] Tropical Medicine Graduate Program, Academic Affairs, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

KEYWORDS
Opisthorchis viverrini; Antibody; Phage display technology; Primer.

ABSTRACT
Hybridoma technique is worldwide used for antibody production. However, there are many issues of using this technique such as hybridoma instability, chromosome loss, mutation and short haft-life that lead to loss of hybridoma genome and loss of antibody production. The technique which displays antibody on phage particle can solve this problem. Therefore, antibody phage display was used to sustain the hybridoma technique to produce antibodies. In this technique, it is needed to amplify antibody sequence by a primer that is specific to antibody isotype and have cloning site to clone antibody sequence into a phagemid vector. Unfortunately, there was no reverse primer for amplifying mouse Fd immunoglobulin M (IgM) and clone into the pComb3HSS phagemid vector. The aim of this study was to design the new reverse primers to amplify mouse Fd immunoglobulin M antibody gene by polymerase chain reaction (PCR) using KKU505 hybridoma as a model. The KKU505 hybridoma can produce anti-Opisthorchis viverrini (O. viverrini) monoclonal antibody (mAb). The results demonstrated that this new primer could amplify the Fd immunoglobulin M of anti-Opisthorchis viverrini mAb and construct into pComb3HSS phagemid vector. This technology could preserve antibody gene and use for more stable antibody production.

* **Corresponding author:** Amonrat Jumnainsong, MT, PhD. Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand. E-mail: amonrat@kku.ac.th
Received: 31 March 2020/ Revised: 6 May 2020/ Accepted: 19 June 2020

Introduction

Antibody phage display technology is a technique that displays antibody fragments that still have the antigen-binding ability on the phage particles. This technique was introduced in 1990 by George P. Smith who won the 2018 Nobel Prize in chemistry[1-3]. This technique can be used for monoclonal antibody-producing, selection, and purification of the specific antibody and improvement of affinity antibody using mutagenesis[4-6]. This technique is more effective and stable in monoclonal antibody production than conventional hybridoma techniques[1, 2, 4, 7]. Since, this technique needs to clone the antibody coding gene in the phagemid vector; half-life of the vector is longer and more stable than a cell line such as the hybridoma cell line[8, 9]. This study focuses on the Fab antibody format which is a stable format to construct a phage vector with anti-*O. viverrini* mAb sequence. This vector can be used for producing anti-*O. viverrini* mAb using phage display technology.

One of the steps in antibody phage display technology is to amplify the antibody fragment gene for cloning into a phagemid vector[10, 11]. The forward primer should be variety like degenerate primers (Zhongde Wang, 2000)[12]. The reverse primer, which binds in the 3' end constant region, should be specific to antibody fragment format, isotype, and species of the interested genome. Both forward and reverse primers have to contain enzyme restriction sites used in processing to clone antibody genes into a phagemid vector. Unfortunately, there has no reverse primer to amplify mouse Fd immunoglobulin M for pComb3HSS phagemid vector construction nowadays[10, 11].

The aim of this article was to design and optimize the optimal condition for new reverse primers, which was applied for combination with degenerate forward primers from the Zhongde Wang's study. Both forward and reverse primers were used to amplify mouse Fd immunoglobulin M antibody gene by polymerase chain reaction (PCR), constructed to pComb3HSS phagemid vector with antibody gene for antibody phage display technology. KKU505 hybridoma cell line which produces IgM specific to *Opisthorchis viverrini* was used as model.

Materials and methods

Cell culture

KKU505 hybridoma cells producing anti-*O. viverrini* mAb were validated isotype antibody using a mouse monoclonal antibody isotyping using by Isostrip kit test (mouse monoclonal antibody isotyping kit, Roche, cat. 1493027) according to the manufacturer's instructions, which provided by the liver fluke and cholangiocarcinoma research center, faculty of medicine, Khon Kaen University, Khon Kaen, Thailand. The cell was cultured in RPMI 1640 medium (Gibco) with 10% fetal bovine serum (FBS) (Gibco) and streptomycin (100 µg/ml), and was maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Escherichia coli and vectors

E. coli strains, XL-1 Blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F' proAB+, LacIq, ZdelM15, Tn10]) (Stratagene, USA) and TG-1 (K-12 supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, (rK-mK-)) were used as host for phage display. The pGEM®- T easy vector (Promega, USA) was used for the pGEM®- T easy vector (promega) system and the pComb3HSS phagemid vector, which was kindly provided from Carlos Barbas, was used for the pComb3HSS system.

Primer design

The new reverse primers were designed with a restriction site using IgM isotype mouse 3' end from the constant region (sequence ID are AJ851868.3, S79401.1, AH003148.2, and V00818.1) as templates. The heavy chain Fd fragment amplification using these new reverse primers is combined with degenerate forward primers from the Zhongde Wang's study. This primer set also was optimized annealing temperature and magnesium concentration for an optimal condition of heavy chain Fd fragment amplification. The design of this primer was the following characteristics of population-based methods from Li-Yeh Chuang's study[13].

Amplification of KKU505 Fab gene

Total RNA was extracted from KKU505 hybridoma cells using TRIzol® (invitrogen) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by cDNA synthesis kits (Promega, USA) to the manufacturer's instructions from 2 µg total RNA using a random forward primer and reverse primer. Resulting in the first-strand cDNA was used as a template for further PCR amplification of Fab fragments. IgM-specific variable heavy (VH) and light (VL) chain Fab gene from purified KKU505 cDNA was amplified using PCR system (Bio-active, Thailand) for 30 cycles in the first round (at 94°C for 60 sec, at 45°C for 60 sec, at 72°C for 90 sec and 10 min at 72°C for final extension), with each forward and reverse oligonucleotide primers set.

Results

Primer design and alignment

IgM isotype mouse 3' end from the constant region (sequence ID are AJ851868.3, S79401.1, AH003148.2, and V00818.1) as a template for design new reverse primers as MUHCM1-4 with cloning enzyme restriction sites for pComb3HSS phagemid vector construction (Figure 1). The specific sequence of IgM constant region was in the 3' end of these primers. The cloning enzyme restriction site is in the 5' end of these primers that have no effect for template-primer binding and all primer sequences in this study are shown in Table 1.

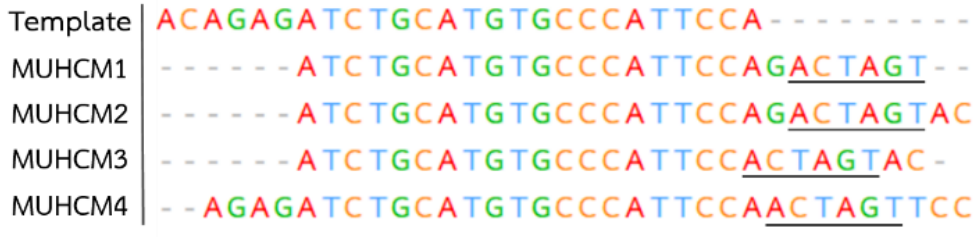


Figure 1 Alignment of new primer sequences of different 4 heavy chain reverse primers compared with the template. The single-letter nucleotide codes were colored. Adenine (A), thymine (T), cytosine (C) and guanine (G) are red, blue, yellow and green, respectively. The template was IgM isotype mouse 3' end from the constant region. The letters with underline represent cloning enzyme restriction sites.

Polymerase chain reaction using these reverse primers

All four new reverse primers (MUHCM1-4) combined with two heavy chain high degenerated forward primers (MH1-2) which are shown in Table 1 were used to amplify the heavy chain Fd sequence of KKU505 cDNA. The specific band is 600-700 bp. The specific band was shown in all reactions. The intensity of the interaction bands from MUCHM4 was highest and clearest combined with two heavy chain high degenerated forward following MUCHM2, MUCHM1 and MUCHM3, respectively (Figure 2).

Table 1 Sequences of PCR primers used in this study

Name	Sequences
Heavy chain high degenerated forward primers (ref 10)	
MH1	5'-CTT ctc gag SAR GTN MAG CTG SAG SAG TC-'3
MH2	5'-CTT ctc gag SAR GTN MAG CTG SAG SAG TCW GG-'3,
Heavy chain low degenerated forward primers (ref 10)	
MH3	5'-ctt ccg gaa ttc CAG GTT ACT CTG AAA GWG TST G-'3
MH4	5'-ctt ccg gaa ttc GAG GTC CAR CTG CAA CAR TC-'3
MH5	5'-ctt ccg gaa ttc CAG GTC CAA CTV CAG CAR CC-'3
MH6	5'-ctt ccg gaa ttc GAG GTG AAS STG GTG GAA TC-'3
MH7	5'-ctt ccg gaa ttc GAT GTG AAC TTG GAA GTG TC-'3
Heavy chain reverse primers (New primers from this study)	
MUHCM1	5'-act agt CTG GAA TGG GCA CAT GCA GAT-'3
MUHCM2	5'-GTa cta gtC TGG AAT GGG CAC ATG CAG AT-'3
MUHCM3	5'-GTa cta gtG GAA TGG GCA CAT GCA GAT-'3
MUHCM4	5'-GGA act agt TGG AAT GGG CAC ATG CAG ATC TCT-'3
Light chain forward primers (ref 8)	
MULC1	5'-CCA GTT CCg agc tcG TTG TGA CTC AGG AAT CT-3'
MULC2	5'-CCA GTT CCg agc tcG TGT TGA CGC AGC CGC CC-3'
MULC3	5'-CCA GTT CCg agc tcG TGC TCA CCC AGT CTC CA-3'
MULC4	5'-CCA GTT CCg agc tcC AGA TGA CCC AGT CTC CA-3'
MULC5	5'-CCA GAT GTg agc tcG TGA TGA CCC AGA CTC CA-3'
MULC6	5'-CCA GAT GTg agc tcG TCA TGA CCC AGT CTC CA-3'
MULC7	5'-CCA GTT CCg agc tcG TGA TGA CAC AGT CTC CA-3'
Light chain reverse primer (ref 8)	
MULK3	5'-GCG CCG tct aga ATT AAC ACT CAT TCC TGT TGA A-3'

Note: The reverse primers for heavy chain sequence were designed in this study. Small letters represent cloning or enzyme restriction sites, *XhoI* (ctcgag), *XpeI* (actagt), *SacI* (gagctc) and *XbaI* (tctaga).

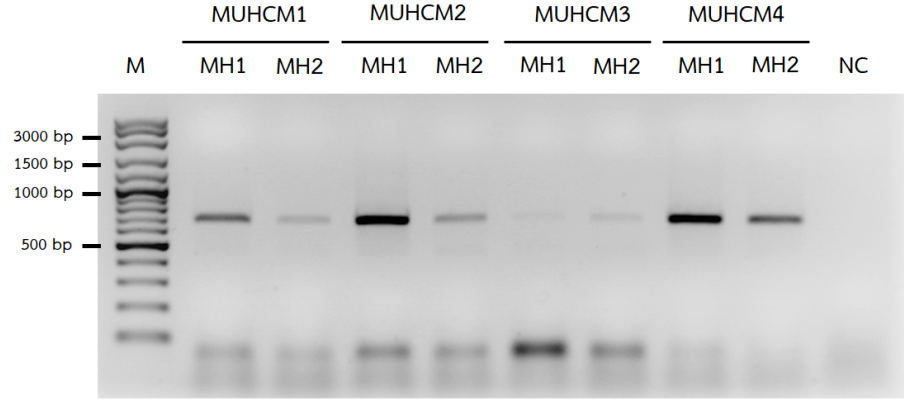


Figure 2 Gel analysis of PCR of difference 4 reverse primers with degenerated forward primers. MUHCM1-4 was new designed reverse primers. MH1-2 was degenerated forward primers. The heavy chain Fd fragments were 600-700 bp, M was DNA marker, Bioline 100 bp.

The optimal condition of MUCHM4 reverse primer

MUCHM4 reverse primer combined with two heavy chain high degenerated forward primers (MH1-2) were used to optimize the condition. Annealing temperature and magnesium concentration were optimized for heavy chain Fd fragment amplification. The result suggested that the optimal annealing temperature was 48 ºC and optimal magnesium concentration was 2.5 mM. This optimal condition was used to amplify the heavy chain Fd sequence of KKU505 cDNA (Figure 3).

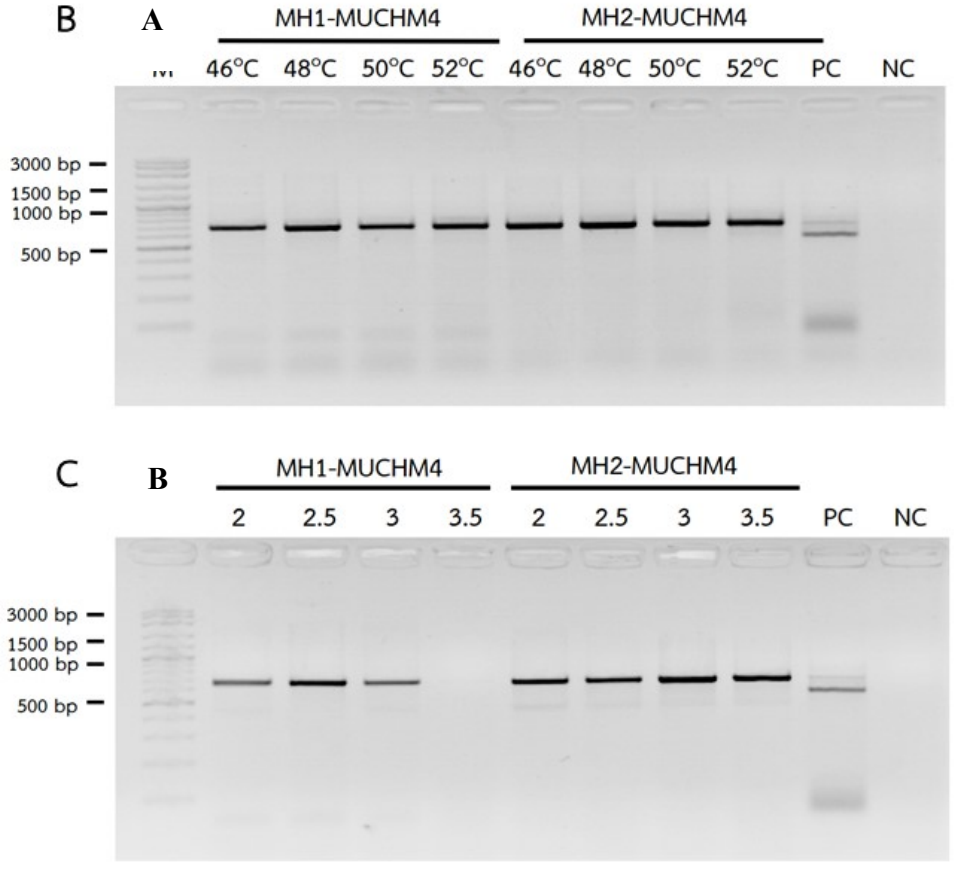


Figure 3 Optimization for heavy chain Fd fragment amplification. **A.** The heavy chain Fd fragment amplification was optimized annealing temperature and the optimal temperature was 48 ºC. **B.** The heavy chain Fd fragment amplification was optimized magnesium concentration and optimal magnesium concentration was 2.5 mM. M was DNA marker, Bioline 100 bp. PC was positive control. NC was negative control.

Construction of the pComb3HSS phagemid vector carrying KKU505 Fab sequence

MUCHM4 reverse primer with all heavy chain degenerated primers (MH1-7) and light chain reverse primer (MULK3) with all light chain forward primers (MULC1-7) which are provided in Table 1 were used to amplify heavy chain Fd and light chain sequence of KKU505, respectively. The specific band for heavy chain Fd and light chain fragments were about 600-700 bp. The KKU505 heavy chain Fd sequence was successfully amplified using MUCHM4 and degenerated forward

primers No. 1 and 2 (MH1 and MH2). The light chain was successfully amplified using forward primers No. 3, 4, 5, 6 and 7 (MULC3-7) (Figure 4). Heavy chain Fd and light chain fragment were eluted from electrophoresis gel before ligated into pGEM®- T easy vector and then heavy chain Fd and light chain fragments were digested from pGEM®- T easy vector to insert into pComb3HSS phagemid vector. The simulation diagram of construction the pComb3HSS phagemid vector with heavy chain Fd and light chain fragment is provided in Figure 5. After that, inserted pComb3HSS phagemid vector was transformed to *E.coli*. The colonies from transformed *E.coli* were selected to extract phagemid vector and validated by restriction enzyme to ensure that the vector was carrying the KKU505 heavy chain Fd and light chain Fab sequences. Heavy chain Fd fragment was digested with XhoI/SpeI and Light chain fragment was digested XbaI/SacI. The result showed that colony number C.HM.LC.1, 2, 3, 4 and 8 were successful to demonstrate the heavy chain Fd and light chain Fab fragment (Figure 6). The sequences from C.HM.LC.1, 2, 3, 4 and 8 were confirmed again by sequencing analysis. The result showed that all of these sequences were heavy chain Fd and light chain sequence and found regions of similarity between biological sequences. This suggested that the heavy chain Fd and light chain Fab sequences were carried in pComb3HSS phagemid vector.

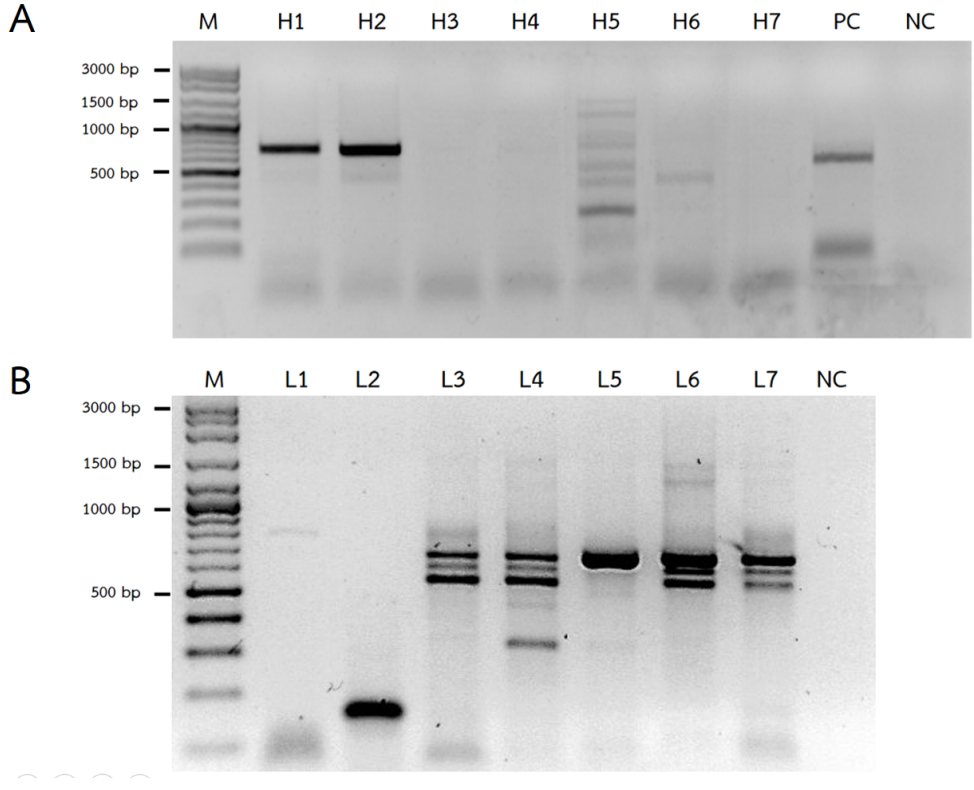


Figure 4 Gel analysis of PCR of KKU505 heavy chain Fd and light chain fragment. The heavy chain Fd and light chain fragments were amplified by different primers and the specific band was shown at 600-700 bp. **A.** For heavy chain Fd fragment amplification, the primers that could amplify this fragment were reverse primer; MUHCM4 with forward primers; MH1 and 2. **B.** For light chain fragment amplification, the primers that could amplify this fragment were reverse primer; MULK3 with forward primers; MULC3, 4, 5, 6 and 7. M was DNA marker, Bioline 100 bp. NC was negative control (PCR buffer without DNA template.

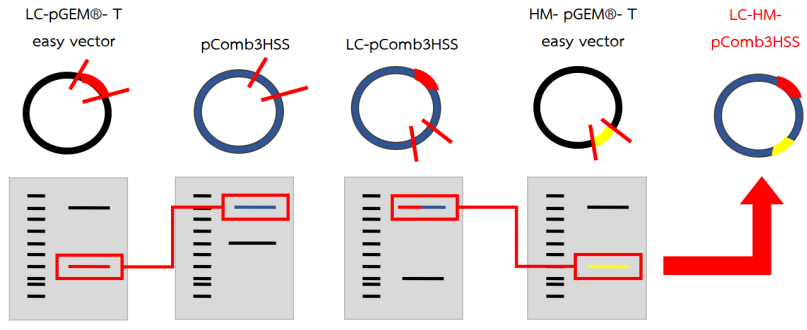


Figure 5 Simulation diagram and experiment for respectively construct the pComb3HSS phagemid vector with KKU505 Fab sequence. Simulation diagram showed the construction method for the pComb3HSS phagemid vector with KKU505 Fab sequence.

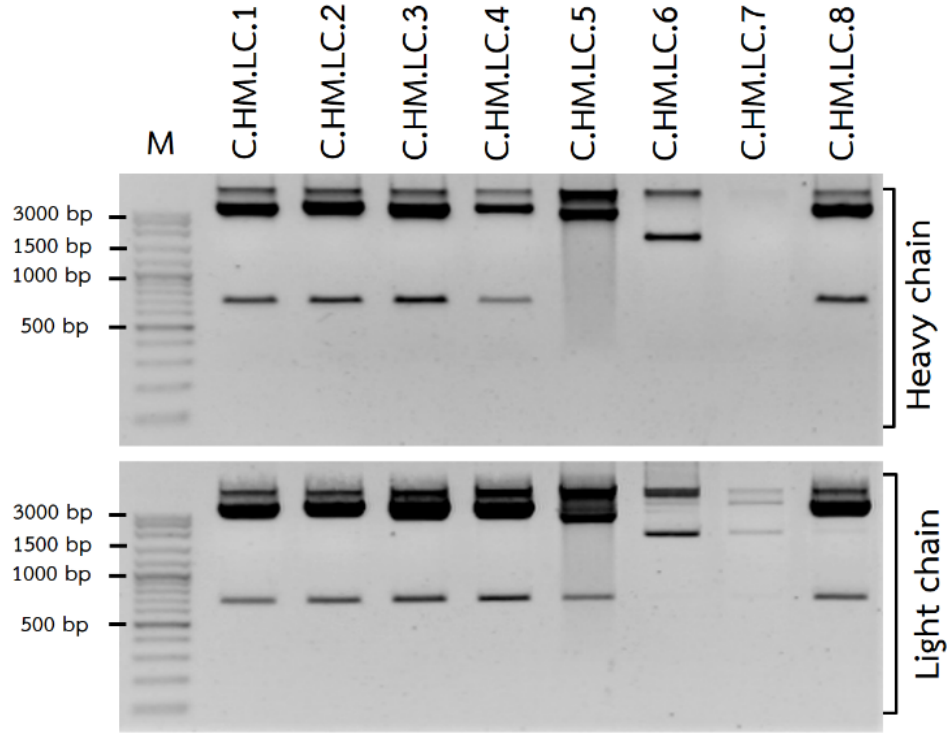


Figure 6 Verification of KKU505 Fab sequences insertion into the pComb3HSS vector. The heavy chain Fd and light chain sequences, which were inserted into the expression vector pComb3HSS were confirmed by restriction method. *XhoI*/*SpeI* restriction site for heavy chain Fd fragment and *XbaI*/*SacI* restriction site for light chain fragment. The specific band size is 600-700 bp. M was 100 bp Bioline DNA marker.

Discussion

An initial objective of the project was to preserve anti-*O. viverrini* antibody sequence from KKU505 hybridoma cell line, which can be translated to antibody-protein which can be utilized for *O. viverrini* infection detection in a urine sample for diagnosis[14]. In Chanika study, the anti-*O. viverrini* antibody was produced using hybridoma technique. The antibody from this technique is not stable. Several studies have

reported that the hybridoma technique had many limitations; there are no practical ways to alter the properties or improve antibody produced, and the cell line has a short half-life that can lead to cell death and genome loss[15, 16]. This study was designed to preserve the antibody sequence and maintain antibody production. For solving the hybridoma cell half-life issue, Phage display has been introduced as a technology to sustain hybridoma technology for antibody production[1, 2, 4, 7], because this technique has to construct phagemid harboring antibody sequence. Antibody sequence preservation in phagemid or nucleotide form has longer half-life and is more stable than hybridoma cell because nucleic acid is more stable than cell line[8].

We have developed the new reverse primers for using with degenerate forward primers for amplifying mouse IgM to the construction of phagemid vector carrying anti-*O. viverrini* Fab. For reverse primer design, the new primers were designed with a restriction site using mouse 3' end from constant region templates (sequence ID are AJ851868.3, S79401.1, AH003148.2, and V00818.1). The new primers were combined with degenerated forward primers[12], which were used for binding the complementary sequences of mouse heavy chain FR1 region or the 5' end of the variable region. This study successfully designed new reverse primers that is specific to amplify mouse IgM and had restriction site to the construction of phagemid vector carrying anti-*O. viverrini* Fab. In addition, the PCR products from this amplification were successfully constructed into pComb3HSS phagemid vector that might be used to produce the antibody specific to *O. viverrini* antigen. However, the antibody from this technique needs to be characterized in the future.

The expression of antibody on the surface of phage particles represents a powerful way for the isolation of monoclonal antibody with bio-panning that can serve for design and construct of antibody fragments[3, 17]. Therefore, antibody phage display can be applied to improve the affinity of antibody using mutagenesis technique and is easy to isolate a single clone from the mutagenesis technique to select the better phage display antibody. Several experiments could explain this idea, antibody against major histocompatibility complex class I[18] and a human monoclonal antibody against the third hypervariable loop of human immunodeficiency virus were improved for binding activity by phage display technology[19]. This finding broadly supports that the anti-*O. viverrini* antibody affinity improvement can be increased *O. viverrini* detection in urine for better diagnosis with more sensitivity and specificity in the future.

Conclusion

The present study has demonstrated new reverse primers for using with degenerate forward primers for amplify mouse IgM to construction of phagemid vector carrying anti-*O. viverrini* Fab.

Take home messages

Antibody phage display was used to produce the antibody and sustain the antibodies from hybridoma cell line. However, there was no reverse primer for amplifying mouse Fd immunoglobulin M (IgM) and clone into the pComb3HSS phagemid vector. This study could design and demonstrate the efficiency of the primer for mouse IgM amplification for phage display technology.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

S. Siripanthong has been holding an MSc scholarship by the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. The authors are grateful to C. Worasith for supporting KKU505 hybridomas cell line and R. Nutalai for PCR amplification and cloning assistances.

References

1. Hammers CM, Stanley JR. Antibody phage display: technique and applications. J Invest Dermatol 2014; 134(2): 1-5.
2. Azzazy HM, Highsmith WE, Jr. Phage display technology: clinical applications and recent innovations. Clin Biochem 2002; 35(6): 425-45.
3. Finlay WJ, Bloom L, Grant J, Franklin E, Shuilleabhain DN, Cunningham O. Phage display: a powerful technology for the generation of high-specificity affinity reagents from alternative immune sources. Methods Mol Biol 2017; 1485: 85-99.
4. Saeed AF, Wang R, Ling S, Wang S. Antibody engineering for pursuing a healthier future. Front Microbiol 2017; 8: 495.
5. Marvin DA. Filamentous phage structure, infection and assembly. Curr Opin Struct Biol 1998; 8(2): 150-8.
6. Frei JC, Lai JR. Protein and antibody engineering by phage display. Methods Enzymol 2016; 580: 45-87.
7. Carmen S, Jermutus L. Concepts in antibody phage display. Brief Funct Genomic Proteomic 2002; 1(2): 189-203.
8. Nguyen HH, Park J, Park SJ, Lee CS, Hwang S, Shin YB, et al. Long-term stability and integrity of plasmid-based DNA data storage. Polymers (Basel) 2018; 10(1): 1-10.
9. Gravier R, Dory D, Laurentie M, Bougeard S, Cariolet R, Jestin A. In vivo tissue distribution and kinetics of a pseudorabies virus plasmid DNA vaccine after intramuscular injection in swine. Vaccine 2007; 25(39-40): 6930-8.
10. Barbas CF, 3rd. Recent advances in phage display. Curr Opin Biotechnol 1993; 4(5): 526-30.
11. Barbas CF. Synthetic human antibodies. Nat Med 1995; 1(8): 837-9.
12. Wang Z, Raifu M, Howard M, Smith L, Hansen D, Goldsby R, et al. Universal PCR amplification of mouse immunoglobulin gene variable regions: the design of degenerate primers and an assessment of the effect of DNA polymerase 3' to 5' exonuclease activity. J Immunol Methods 2000; 233(1-2): 167-77.
13. Chuang LY, Cheng YH, Yang CH. Specific primer design for the polymerase chain reaction. Biotechnol Lett 2013; 35(10): 1541-9.
14. Worasith C, Kamamia C, Yakovleva A, Duenngai K, Wangboon C, Sithithaworn J, et al. Advances in the diagnosis of human opisthorchiasis: development of opisthorchis viverrini antigen detection in urine. PLoS Negl Trop Dis 2015; 9(10): e0004157.
15. Hnasko RM, Stanker LH. Hybridoma technology. Methods Mol Biol 2015; 1318: 15-28.
16. Tomita M, Tsumoto K. Hybridoma technologies for antibody production. Immunotherapy 2011; 3(3): 371-80.
17. Tan Y, Tian T, Liu W, Zhu Z, C JY. Advance in phage display technology for bioanalysis. Biotechnol J 2016; 11(6): 732-45.
18. Phumyen A, Jumnainsong A, Leelayuwat C. Improved binding activity of antibodies against major histocompatibility complex class I chain-related gene A by phage display technology for cancer-targeted therapy. J Biomed Biotechnol 2012; 2012: 597647.
19. Thompson J, Pope T, Tung JS, Chan C, Hollis G, Mark G, et al. Affinity maturation of a high-affinity human monoclonal antibody against the third hypervariable loop of human immunodeficiency virus: use of phage display to improve affinity and broaden strain reactivity. J Mol Biol 1996; 256(1): 77-88.

REHAB MEDICAL CO., LTD.
Better together

บริษัท รีแฮบ เมดิคอล จำกัด
REHAB MEDICAL CO., LTD

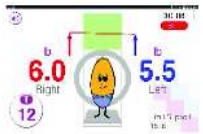
จำหน่ายเครื่องมือกายภาพบำบัด ผลิตภัณฑ์ที่มีคุณภาพจากต่างประเทศและในประเทศ

1201/104 ซ.ลาดพร้าว94 ถนนศรีวรา แขวงพลับพลา เขตวังทองหลาง กรุงเทพมหานคร 10310

TEL : 02-559-2429-31 FAX : 02-530-4461 E-Mail : Sales@rehabmed.co.th

LiteGait — mobility research

The balance and gait trainer in one



LiteGait PGI, Base34 with Gait Keeper

Continuous Passive Motion

Maestra: Hand&Wrist CPM

kinetec®

Centura : Shoulder CPM

Spectra : Knee CPM

6080 : Elbow CPM

FOR A LIFE IN MOTION — THERA TRAINER

ACTIVE-PASSIVE BICYCLE

TIGO

Group Therapy Software

THERA-soft game

ELECTROTHERAPY — gymna®

Gymna 200 serie

- Combined, ES, US and Laser
- ES with EMG biofeedback (Myo200)
- Han-stim current
- 2 independent channels

Gymna 400 serie

- Combined, ES, US and Laser
- 10.4 full color touch screen
- GTS technology (Anatomical library, Evidence-based therapy)
- Han-stim current
- 2 independent channels

Balance & Gait trainer

End effector gait trainer

THERA TRAINER

Static & Dynamic Balance trainer

LYRA

BALO

BEMO

THE SMALL & HANDY ACTIVE-PASSIVE FOR ARMS & LEGS

MOBI540

(Active&Passive bicycle)

ACTIVO574

Active bicycle)