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

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KEYWORDS

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

ABSTRACT

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

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Introduction

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

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

Materials and methods

Subjects

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

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

Research design and protocol

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

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

Blood sampling

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

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

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

Bacterial growth and colony forming units (cfu) counting

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

Labeling S.aureus with FITC fluorescence dye

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

Dose of multiplicity of infection (MOI) for phagocytosis

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

Assessment of PMN phagocytosis and oxidative burst

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

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

Flow cytometry analysis

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

Statistical analysis

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

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

Results

Optimization of the FITC fluorescence dye for bacterial labeling

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

Comparisons of PMN phagocytosis using different multiplicity of infection

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

Table 1 Characteristics of the patients with type 2 diabetes mellitus

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

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

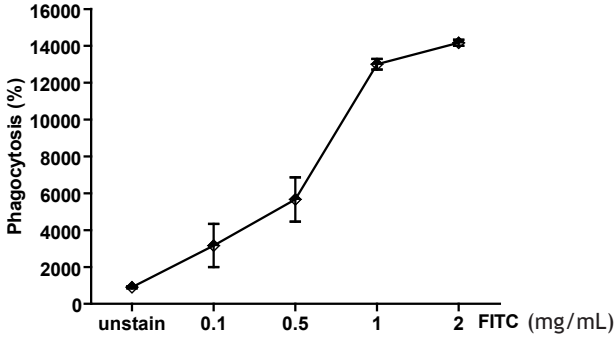

Figure 1 Variation of fluorescein-isothiocyanate (FITC) concentration for bacterial cell staining

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

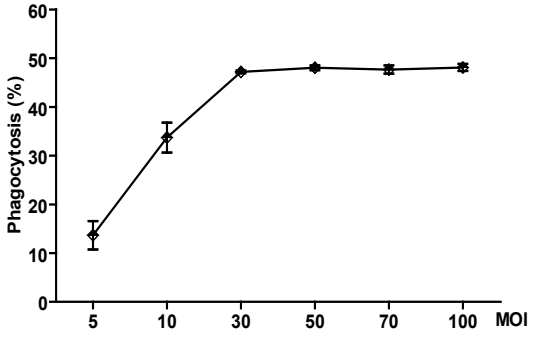

Figure 2 Effect of increasing dose of multiplicity of infection (MOI) on PMN phagocytosis

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

Effect of exercise on PMN cell function

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

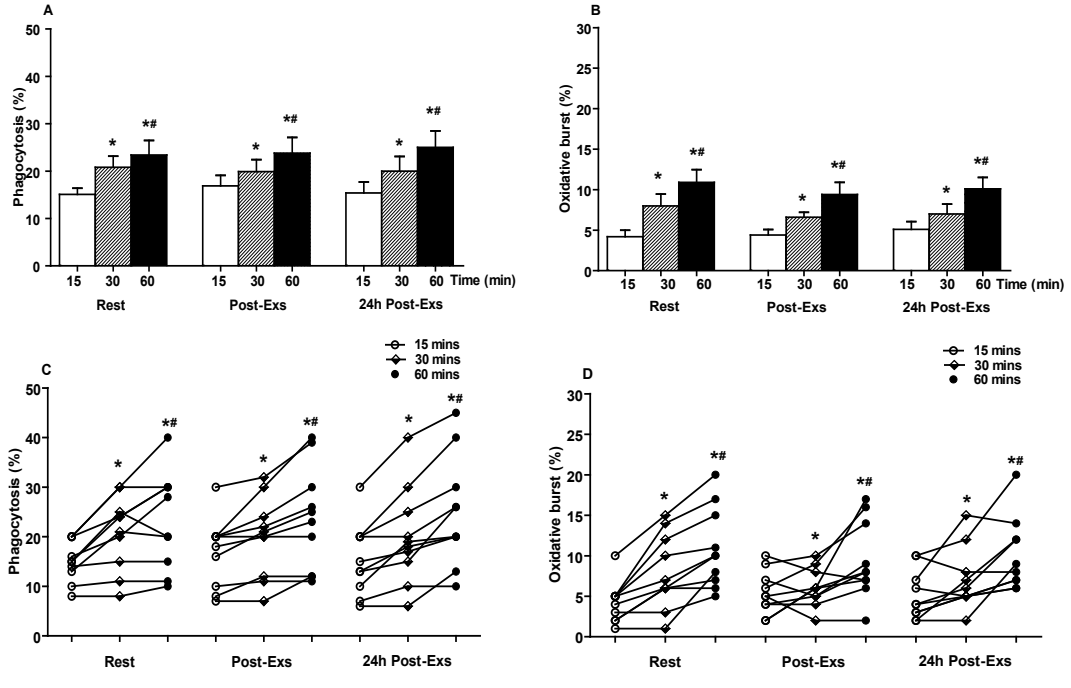

Figure 3 Effect of exercise on polymorphonuclear (PMN) phagocytosis and oxidative burst

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

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

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

Discussion

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

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

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

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

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

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

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

Conclusion

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

Take home messages

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

Conflicts of interest

All authors declare no conflicts of interest.

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