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

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KEYWORDS
Vitamin C;
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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.

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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.

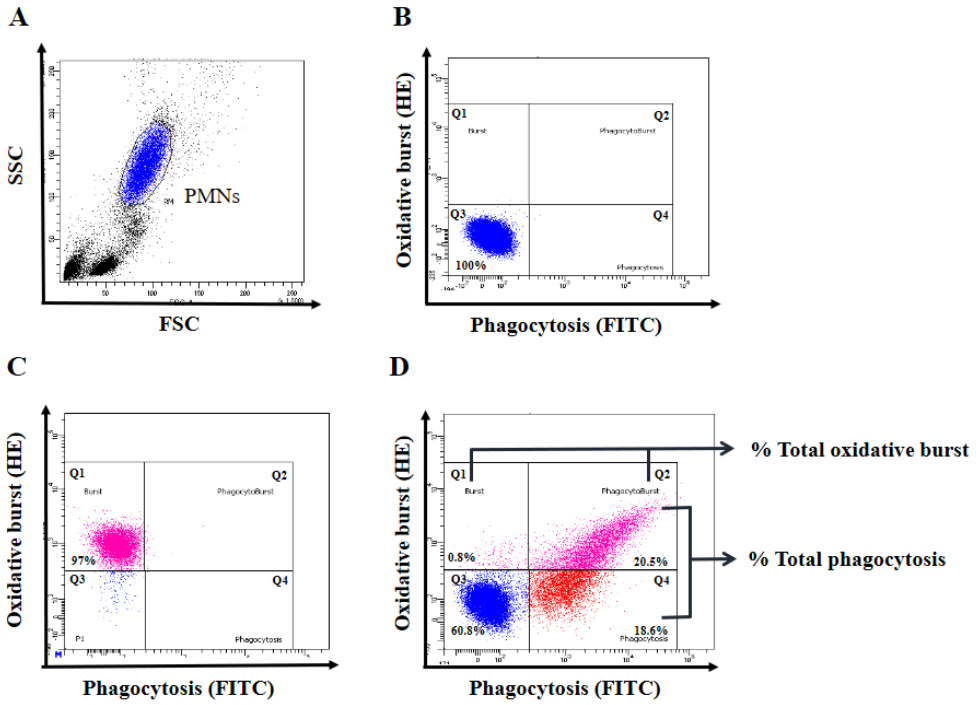

Figure 1 Flow cytometry analysis of PMN phagocytosis and oxidative burst from a whole blood assay. PMNs population (A), Un-stimulated PMNs (negative control) (B), PMA stimulated PMNs (positive control of oxidative burst) (C) and a test of two-color fluorescence FITC-HE assay represented phagocytosis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

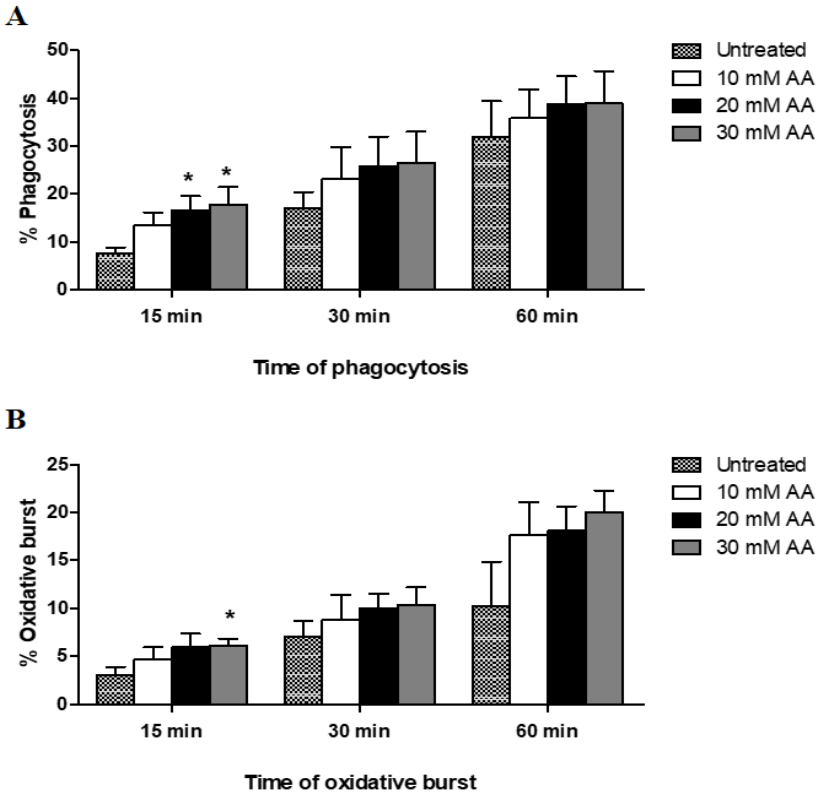

Figure 2 Determination of optimal vitamin C concentration in enhancing PMN function. Vitamin C concentration in enhancing phagocytosis (A) and oxidative burst (B) at 15, 30 and 60 minute of exposure time to the bacteria (n=3). *=*p*-value < 0.05, the mean difference is significant compared to untreated 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.

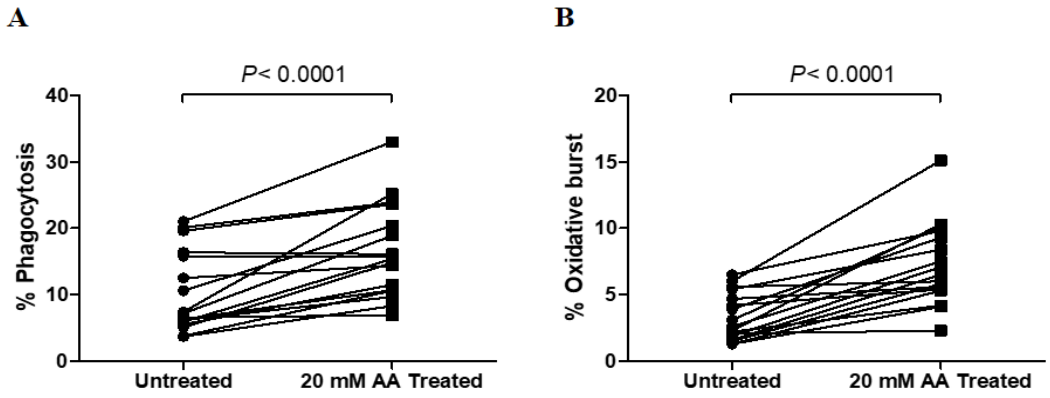

Figure 3 Vitamin C treatment enhanced PMN phagocytosis (A) and oxidative burst (B) in the whole blood culture assay at 15 minutes of exposure to the bacteria, compared to the untreated group (n = 17). AA; ascorbic acid or vitamin C, Untreated; without vitamin C adding or 0 mM AA, mM; millimolar.

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

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