Effects of ascorbic acid supplementation on immune status in healthy women following a single bout of exercise

Piyawan Bunpo (pbunpo@gmail.com)
Chiang Mai University https://orcid.org/0000-0001-9790-8530
Amonphat Chatarurk
Chiang Mai University Faculty of Associated Medical Sciences
Kodchaporn Intawong
Chiang Mai University Faculty of Associated Medical Sciences
Kananek Naosuk
Chiang Mai University Faculty of Associated Medical Sciences
Phennapha Klangsinsirikul
Chiang Mai University Faculty of Associated Medical Sciences

Research article

Keywords: exercise, ascorbic acid, immunonutrition, neutrophil phagocytic function, CD4+ T cell, CD8+ T cell

Posted Date: June 2nd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-32008/v1

Version of Record: A version of this preprint was published at Sport Sciences for Health on January 8th, 2021. See the published version at https://doi.org/10.1007/s11332-020-00726-3.

Effects of ascorbic acid supplementation on immune status in healthy women following a single bout of exercise

Piyawan Bunpo[1,*], Amonphat Chatarurk[1], Kodchaporn Intawong[1], Kananek Naosuk[1], Phennapha Klangsinsirikul[1]

[1]Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, 50200, Thailand.

*To whom correspondence should be addressed: Piyawan Bunpo, Ph.D., Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand, Tel: (+66) -(0)53-935085: E-mail; piyawan.b@cmu.ac.th

Email addresses:

Amonphat Chatarurk: amonncha@gmail.com

Kananek Naosuk: kananek_nao@cmu.ac.th

Kodchaporn Intawong: kodchaporn.intawong@gmail.com

17 Phennapha Klangsinsirikul: phennapha.k@cmu.ac.th

18 Piyawan Bunpo: piyawan.b@cmu.ac.th

19

20

21 Abstract

22 **Background:** This study was designed to determine the effects of ascorbic acid (AA)
23 supplementation on immune status following a single bout of exercise.

24 **Materials/Methods:** In a crossover design with a 1 week wash-out period, 20 healthy sedentary
25 women performed 30 minutes moderate-intensity cycling with (1,000AA) or without (0AA)
26 ingesting 1,000 mg of AA daily for 1 week. Blood samples were taken immediately before,
27 immediately after and 24 hours post-exercise to determine the oxidative stress markers,
28 hematological parameters, immunophenotyping of peripheral blood lymphocytes and neutrophil
29 phagocytic function with *Candida albicans*.

30 **Results:** Moderate-intensity exercise in subjects ranged in age from 21 to 23 years, showed no
31 significant changes in oxidative stress markers in both cohorts. Plasma total creatine kinase was
32 increased immediately after exercise and returned to baseline at 24 h post-exercise in both
33 cohorts. Subjects ingesting 1,000 mg AA demonstrated significant higher level of plasma AA at
34 pre-exercise and post-exercise as compared with the same time point in 0AA group. White
35 blood cell and absolute neutrophil counts were increased immediately after exercise and
36 returned to baseline at 24 h post-exercise in both cohorts. Exercise resulted in increased

lymphocyte count, CD4+ T cells, CD8+ T cells, CD45+/CD3-/CD4- cells, CD45+/CD3+/CD4- cells, CD45+/CD3-/CD8- cells, CD45+/CD3+/CD8- cells and CD45+/CD3-/CD8+ cells immediately after exercise ($p<0.05$) with a return to baseline at 24 hours in 0AA group. AA supplement mitigated effects of exercise on CD4+ T cells and CD45+/CD3-/CD8- cells. No significant change in neutrophil phagocytic function were observed when incubated with low or high concentrations of *C. albicans* in both cohorts.

Conclusion: A single bout of exercise induced muscle damage and transient changes in neutrophil count as well as lymphocyte subpopulations in sedentary women. Ascorbic supplementation does not show beneficial effect to the moderate-intensity exercise.

Keywords: exercise; ascorbic acid; immunonutrition, neutrophil phagocytic function, CD4+ T cell, CD8+ T cell

1. Background

Exercise has immunomodulatory actions. In humans, acute exercise increases the total numbers of circulating leukocytes and especially neutrophils which rise 2-3 fold within the first 3 h after a single bout of aerobic exercise (1). Even though total circulating numbers of lymphocytes are increased during recovery after prolonged exercise, measures of cell function are decreased. For example, CD4+ and 8+ T cells fail to migrate after a 2 h treadmill run (2) and T-cell proliferation decreases during and after exercise, as does the lymphocyte response to an antigen challenge. Furthermore, monocyte phagocytosis is impaired after exhaustive prolonged exercise (3), and 2 h of cycling at 80% VO_{2max} decreases the neutrophil oxidative burst (4). Interestingly, moderate exercise volume has an immunostimulatory effect for both neutrophil and monocyte phagocytosis are enhanced immediately after a submaximal prolonged exercise (5). These findings indicate that the immune response to exercise depends on intensity and duration, an idea consistent with the Open window hypothesis, proposed by Pedersen and Brunnsgaard (6), which proposes that endurance athletes are more vulnerable to illness after completing a race when numbers and function of immune cells are impaired.

67 Neutrophils play an important role in the innate immune system and are the most
68 abundant granulocyte, representing 40% to 75% of white blood cells. Neutrophils have many
69 ways of neutralizing microorganisms: namely, chemotaxis, phagocytosis, degranulation of
70 cytoplasmic granules, activation of the respiratory burst, and neutrophil extracellular traps (7).
71 Acute exercise has a profound impact on neutrophil count, potentially mediated by the
72 activation of catecholamines, growth hormones and cortisol (8, 9). In untrained humans and
73 animals, one bout of moderate or high intensity exercise may cause muscle damage, followed
74 by activation of neutrophils in response to inflammation and eventually resulting in muscle
75 soreness (10). The degree or severity of these types of immunomodulatory actions after
76 exercise is related to the generation of reactive oxygen species (ROS) and reactive nitrogen
77 species (RNS) during cell metabolism. Evidence indicates that mechanisms underlying
78 alterations in immune function following exercise are related to several factors such as
79 neuroendocrine system stimulations, metabolic factors (11), and cardiovascular factors.
80 Vitamin C or ascorbic acid (AA) is a water-soluble vitamin that is an essential dietary
81 component. The recommended daily intake for adult women is 75 mg per day and for adult men
82 is 90 mg per day. Our previous work demonstrates that supplementation of ascorbic acid prior

to exercise of moderate intensity over 3 months was associated with minor and inconsistent reductions in circulating superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase activities (12). Moreover, supplementation with 1,000 mg of ascorbic acid prior to moderate-intensity cycling improves antioxidant power but does not prevent muscle damage (13). It has been suggested that antioxidant supplementation may only improve performance when endogenous levels are already depleted, and after reaching normal concentrations, no further benefit is seen (14). However, vitamin C contributes to immune defense by supporting various cellular functions of both the innate and adaptive immune system. Vitamin C accumulates in phagocytic cells, such as neutrophils, and can enhance chemotaxis, phagocytosis, generation of reactive oxygen species, and ultimately microbial killing. Leukocytes, such as neutrophils, actively accumulate vitamin C against a concentration gradient, resulting in values that are 50- to 100-fold higher than plasma concentrations (15). The role of vitamin C in lymphocytes is less clear, but it is shown to enhance differentiation and proliferation of B- and T-cells, likely due to its gene regulating effects (16, 17). Vitamin C deficiency results in impaired immunity and higher susceptibility to infections (18, 19). Therefore, this study aimed to evaluate whether high dose ascorbic acid supplementation

99 before an acute bout of exercise modulates antioxidant defenses and immune responses

100 following a single bout of aerobic exercise in untrained healthy adults.

101

102

2. Materials and methods

2.1. Subjects and Design

Twenty healthy untrained female adults (age range 20-23 years) participated in this study. All the participants were informed of the purpose and demands of the study before giving their written consent to participate. The protocol was approved by the Ethical Committee of Associated Medical Sciences, Chiang Mai University. Volunteers were screened by interview. Exclusion criteria were cigarette smoking, use of regular medications. They were also asked to abstain from alcohol during the trial. Study participants were instructed to maintain their normal physical activity and dietary habits throughout the study. Before exercise, height and body weight were measured and body mass index (BMI) was calculated. Age-based maximum heart rate was predicted using the equation (220-age in years). A randomized and crossover study was conducted (Figure 1). The exercise program was performed on stationary bicycle, 30 min/session, at intensity corresponding to 65-75% of maximum heart rate with or without ascorbic acid supplementation. Before the first exercise bout, approximately half the subjects did not receive ascorbic acid (0AA, n=10) whereas the other half (1,000AA, n=10) received 1,000 mg of ascorbic acid (The Government Pharmaceutical Organization, Bangkok, Thailand)

119 daily for 7 days. Table 1 shows the main characteristics of participants in the study. The
120 subjects were sedentary and had not participated in any regular exercise training program for at
121 least 1 year. All subjects had no overt history of hepatic, renal, metabolic, cardiovascular, or
122 pulmonary diseases, or orthopedic limitations in the exercise tests.

123 Study subjects were fasted (2-4 h) before performing 30 min/session of indoor cycling
124 on a stationary bicycle at intensity corresponding to 65-75% of maximum heart rate. Subjects
125 were instructed not to perform any exercise for 1 wk (wash-out period) preceding the second
126 bout of cycling whereupon each subject received the opposite treatment before exercise. Blood
127 samples were collected pre-exercise, immediately after exercise and 24 hours post-exercise.
128 Heart rate (HR) was monitored continuously using pulse oximeter (P030, Beurer GmnH, Ulm,
129 Germany) during the exercise to estimate energy expenditure. During and after exercise, all
130 subjects were allowed to drink water according to their thirst.

131

132 2.2. Blood sampling and measurements

133 Blood samples were collected in the morning after fasting of at least 2-4 h prior to exercise bout
134 and into ethylenediaminetetraacetic acid (EDTA) tubes and heparinized tubes and then were

kept at 4°C. Plasma was obtained from the heparinized blood samples by centrifugation (15 min, 1,000 × g, 4°C) and were stored (at -20°C) before further analyses.

2.3. Determination of routine blood chemistry analysis and total creatine kinase (total CK)

Plasma glucose (glucose oxidase), protein (biuret) uric acid (uricase), alanine aminotransferase (ALT, International Federation of Clinical Chemistry (IFCC) without pyridoxal phosphate), creatinine (enzymatic method), total cholesterol (cholesterol oxidase) and total CK (IFCC) were determined by automate clinical chemistry analyzer (BioSystems BA400) after running control and calibration with the use of commercially available kits.

2.4. Determination of plasma TBARS

Malondialdehyde (MDA) level was determined by thiobarbituric acid (TBA) reactive substances (TBARS) in plasma (20). TBARS are a common way to measure lipid peroxidation products which can complement a more specific assay such as high-performance liquid chromatography. However, a variety of other compounds, such as oxidized lipids, saturated and unsaturated aldehydes, sucrose, and urea, interfere with the assay, causing overestimation

of MDA concentration. In the TBARS assay, one molecule of MDA reacts with two molecules of TBA and thereby produces a pink pigment with absorption peak at 532 nm. Results were compared using standard solution of 1,1,3,3-tetramethoxypropane and expressed as μmol per gram of protein.

2.5. Determination of total antioxidant status

Antioxidant quantification was done using 2,2' -azino-bis (3ethylbenzthiazoline-6-sulfonic acid) radical formation kinetics according to manufacturer instructions (Randox Laboratories, Ltd., Crumlin, UK). This assay was measured on automate clinical chemistry analyzer (BioSystems BA400) after running control and calibration.

2.10. Determination of plasma ascorbic acid

Plasma ascorbic acid was determined using ascorbic assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer instructions.

2.13. Determination of hematological parameters

Blood was collected from each participant into EDTA tube for complete blood count (CBC) analysis, done on the same day of collection by Sysmex KX-21, a three-part differential auto analyzer.

6.13. Assessment of peripheral neutrophil functions

The blood collected in the heparinized tubes was mixed with *Candida albicans* (Department of Medical Sciences Thailand; DMST 21424 kindly provided by Dr. Khajornsak Tragoolpua) to assess the phagocytic index. Briefly, *C. albicans* was grown on Sabouraud's 2% dextrose broth for 24-48 h at 37°C to obtain organisms in the yeast phase only. *C. albicans* suspended in normal saline solution (NSS) was adjusted with spectrophotometer at 620 nm to obtain 2.8×10^{8} CFU/mL. The yeast cells were mixed with the whole blood at ratio of 10:1 and 1:1 (neutrophil per yeast ratio), respectively, and incubated for 1 hour at 37°C with periodic agitation every 10 minutes. The whole assembly was centrifuged at 1,500 x g for 5 minutes. The equal volume of supernatant as in yeast solution added was then discarded and smears were prepared with the remaining whole blood, air dried and stained with Giemsa. One hundred neutrophils/slide were counted. Number of *Candida*-engulfed neutrophils were

counted as positive cells. Phagocytic activity = Number of positive cells/100 cells. Phagocytic index = (Total number of *Candida*/Positive neutrophils) x Phagocytic activity.

6.14. Immunophenotyping of peripheral blood lymphocytes

EDTA blood was used for immunophenotyping of peripheral blood lymphocytes. Lymphocytes subsets were analyzed on flow cytometer (Beckman Coulter, Cytomics FC 500) with the following three monoclonal antibody combinations (CYTO-STAT triCHROME CD45-FITC/CD4-RD1/CD3-PC5, Beckman Coulter Ireland Inc.) specific for CD45-FITC/CD4-RD1/CD3-PC5 (CD4+ T cell) and with the following three monoclonal antibody combinations (CYTO-STAT triCHROME CD45-FITC/CD8-RD1/CD3-PC5) specific for CD45-FITC/CD8-RD1/CD3-PC5 (CD8+ T cell). In brief, 100 µL of whole blood was mixed and incubated, in the dark, with 5 µL of each monoclonal antibody combinations in separate tubes at room temperature for 15 minutes. Red blood cells were then lysed by adding 500 µL of lysing solution (OptiLyse C lysis solution, Beckman Coulter Ireland Inc.), and the tubes were vortexed and incubated in the dark at room temperature for 15 minutes and finally centrifuged at 1,250 x g for 5 minutes. The pellet was

198 then washed 3 times with 2 mL of phosphate-buffered saline (PBS), resuspended in 1 mL of 1%

199 paraformaldehyde in PBS, and finally analyzed with software.

200

201 2.16. Statistical Analysis

202 At the end of the study there were n=20 subjects per cohort. Results were expressed as

203 mean ± SE. Data were processed by standard statistical software SPSS 18.0 (SPSS Software,

204 Thailand). Two-way repeated measures analysis of variance (exercise vs. supplement) were

205 used, differences among exercise (pre-, post- and 24 hours post-exercise) and supplements

206 (0AA and 1,000AA) were subsequently identified using a LSD *post-hoc* analysis with statistical

207 significance of $p<0.05$.

208

209 ## 3. Results

210 ### 3.1. Subject characteristics

211 The basic characteristics of the study subjects are shown in Table 1. Participants
212 ranged in age from 21 to 23 years. Height, body weights, body mass index (BMI), average heart
213 rate during exercise, percent of heart rate during exercise, distance and caloric expenditure
214 were not significantly different between 1,000AA and 0AA trials. Plasma creatinine, uric acid,
215 cholesterol and ALT levels were within the reference ranges, results did not substantially
216 change in 1,000AA versus 0AA. All study subjects with blood cholesterol in the normal range
217 had normal function of kidney and liver.

218 ### 3.2. Changes in levels of oxidative stress markers, muscle damage marker, glucose and
219 ascorbic acid

220 The acute responses in oxidative stress marker levels including TAS and MDA to a
221 moderate intensity of exercise are presented in Table 2. No significant changes were observed
222 in plasma TAS and MDA levels in both 1,000AA and 0AA cohorts. There was a main effect of
223 exercise but no interaction between exercise and supplements for total CK and glucose
224 ($p=0.002$, 0.009), respectively. Plasma total CK was increased immediately after exercise and

225 returned to baseline at 24 h post-exercise in both 1,000AA and 0AA cohorts. Glucose was
226 slightly reduced ($p<0.05$) immediately after exercise in 0AA but remained within the normal
227 range and returned to baseline values by 24 h post-exercise (Table 2). No significant change of
228 glucose in subjects receiving 1,000AA. There was a main effect of AA supplement ($p=0.0004$)
229 for plasma AA level. Subjects ingesting 1,000AA demonstrated significant higher level of
230 plasma AA at pre-exercise and post-exercise as compared with the same time point in 0AA. No
231 main effect of exercise was observed for plasma AA. As can be seen, AA basal levels increased
232 from initial 2.24±0.35 mg/L to 2.53±0.40 mg/L after 7 days of supplementation, indicating that
233 participants complied with given experimental protocol. Still, there was no significant difference
234 of total antioxidant status and MDA levels as a marker of lipid peroxidation between 0AA and
235 1,000AA supplementation, probably because the plasma AA was not sufficient to alter total
236 antioxidant status and MDA levels.

237 3.3. Changes in white blood cell count analysis

238 There was a main effect of exercise for white blood cell numbers and absolute
239 neutrophils ($p=0.0004$, 0.001), respectively. White blood cell count and absolute neutrophil were
240 increased immediately after exercise and returned to baseline at 24 h post-exercise in both 0AA

241 and 1,000AA cohorts. Subjects ingesting AA demonstrated significant lower absolute neutrophil
242 at pre-exercise as compared with the same time point in 0AA. No main effect of exercise or
243 supplement was observed for absolute lymphocyte and absolute monocyte, basophil and
244 eosinophil. However, moderate exercise resulted in increased lymphocyte count immediately
245 after exercise ($p<0.05$) with a return to baseline at 24 hours in 0AA cohort. No significant
246 change of lymphocyte count was observed in 1,000AA cohort.

247 3.4. Flow cytometric analysis of lymphocyte subpopulations

248 There was a main effect of exercise for CD45+/CD3+/CD4+ (CD4+ helper T cells,
249 $p=0.026$) and CD45+/CD 3+/CD8+ (CD8+ cytotoxic T cells, $p=0.006$). A significant main effect
250 of exercise was detected for other white blood cell subpopulations (B lymphocyte, natural killer
251 and monocyte cells), including CD45+/CD3-/CD4- cells ($p=0.001$), CD45+/CD3+/CD4- cells
252 ($p=0.007$), CD45+/CD3-/CD8- cells ($p=0.002$), CD45+/CD3+/CD8- cells ($p=0.023$) and
253 CD45+/CD3-/CD8+ cells ($p=0.002$). CD4+ helper T cells, CD8+ cytotoxic T cells and other
254 white blood cell subpopulations (CD45+/CD3-/CD4-, CD45+/CD3+/CD4-, CD45+/CD3-/CD8-,
255 CD45+/CD3+/CD8- and CD45+/CD3-/CD8+ cells) were increased immediately after exercise

256 ($p<0.05$) with a return to baseline at 24 hours. Ascorbic acid supplement mitigated effects of

257 exercise on CD4+ helper T cells and CD45+/CD3-/CD8- cells (Figure 2).

258 3.5. Neutrophil phagocytosis assay

259 Figure 3 demonstrated number of yeasts ingested by neutrophil. There was a main

260 effect of exercise for phagocytic index ($p=0.023$) when exposed with high concentration of *C.*

261 *albicans*. Neutrophil phagocytic function was slightly decreased at 24 h post-exercise as

262 compared with immediately after exercise in 0AA cohort. However, there was no difference of

263 neutrophil phagocytic function when compared with pre-exercise. No significant change in

264 neutrophil phagocytic function were observed when incubated with low concentration of *C.*

265 *albicans* in both 0AA and 1,000AA cohorts (Figure 4).

266

267

4. Discussion

The main findings in this study were that (1) moderate-intensity exercise induced muscle damage and transient changes in neutrophil number and function as well as CD4+ and CD8+ T cells in sedentary women. (2) Ascorbic supplementation does not alter antioxidant status, however, mitigated effects of exercise on CD4+ T cells and CD45+/CD3-/CD8- cells and slightly reduced changes in neutrophil phagocytic function. Since there was no apparent influence of ascorbic acid supplement, that is exercise with or without supplement showed similar results, then it is concluded that daily supplementation with ascorbic acid does not show beneficial effects to the moderate-intensity exercise.

In our previous study with ascorbic acid, supplementation for 1,000 mg prior moderate-intensity exercise was effective in improving antioxidant power but does not prevent muscle damage (13). Our goal in this study was to determine whether supplementation of ascorbic acid daily for 1 week before a moderate-intensity exercise would improving antioxidant power and immune status but results were not supportive despite an increase in plasma ascorbic acid level. Our results showed that ingesting ascorbic acid led to the increased of plasma ascorbic acid level, however, daily supplement did not result in a measurable increase in antioxidant

power, implying that acute supplement is needed to allow the potential antioxidant influences of ascorbic acid to occur. Exercise caused significant increases in WBC, total CK, CD4+, CD8+ T cells, but only CD4+ T cell showed differences in the pattern of change that were measured between with or without ascorbic acid supplement.

Exercise causes a certain amount of stress to the body relative to the intensity and duration of the workout. Exercise involves the contraction of skeletal muscles which generate reactive oxygen species at a number of subcellular sites. The interaction between ROS in different sub-cellular compartments plays a crucial role in determining the beneficial or deleterious outcomes of ROS exposures on the muscle tissue and immune systems (21-23). There is a growing appreciation for immunonutrition as the use of specific nutritional elements to support and modulate the immune system in a way that benefits a certain condition of physiological stress, disease state, or injury (24, 25). Ascorbic acid, also known as vitamin C became very popular for its antioxidant properties and has been linked to impressive health benefits. Ascorbic acid is important in all stressful conditions that are linked to inflammatory processes and involve immunity. In the current study, the effect of exercise with or without ascorbic acid supplementation significantly increased plasma total CK. As reported in 2019, we

found that ascorbic acid supplementation at a dose of 1,000 mg prior exercise does little to prevent exercise-induced muscle damage (13). This indicated that supplementation with ascorbic acid before moderate-intensity exercise showed no beneficial outcome on muscle tissue.

During exercise, muscle tissue generate ROS which may promote the synthesis and secretion of pro-inflammatory and anti-inflammatory cytokines from myotubes (26). It has been previously noted that cytokines released from working muscle communicate with other tissues including immune cells in order to maintain normal physical condition (27). Typically, a lymphocytosis is observed during and immediately after exercise, with numbers of cells falling below pre-exercise levels during the early stages of recovery (28, 29). This pattern of mobilization is observed for T cells and to a lesser extent, B cells. Changes are proportional to exercise intensity and duration, although the effect of intensity is more marked (30). In our study, we showed that CD4+ T cells and CD8+ T cells which form a key part of the cell-mediated immune response, were increased immediately after exercise and ascorbic acid supplement mitigated effects of exercise on CD4+ T cells and CD45+3-8- cells. It should be noted that as the antioxidant supplement, ascorbic acid protect other immunoenhancing substances from

316 oxidation, it may also prevent signaling molecule such as free radicals generating during
317 exercise to mediate immune responses.

318 Metabolically, moderate exercise induces small, acute elevations in IL-6 that exert
319 direct anti-inflammatory effects, improving glucose and lipid metabolism over time (31). Immune
320 cells are tightly regulated by substrate availability, and exercise-induced decreases in glucose
321 and amino acid concentrations can contribute to immune system impairment. Prolonged and
322 intensive exercise has profound effects on innate immune function, including natural killer cell
323 function, macrophage cytokine-mediated response to viral infection, and granulocyte and
324 monocyte phagocytosis. Neutrophils are the first cells to translocate into damaged muscle
325 tissue following intensive exercise. In our study, absolute neutrophils were increased
326 immediately after exercise. Evidence indicates that mechanisms underlying exercise associated
327 with immune function alteration are related to several factors such as catecholamines, cortisol
328 and metabolic (i.e., carbohydrate, antioxidants, or prostaglandin) (11) as well as to cardiac
329 output, blood flow, blood pressure and shear forces. Some studies seem to suggest that the
330 acute effects of exercise such as apoptosis of some cells can stimulate a mobilization of
331 hematopoietic stem cells from bone marrow and of senescent immune cells from the peripheral

tissues to the circulation (8, 32, 33). Neutrophil phagocytic function was slightly decreased at 24 h post-exercise when exposed with high concentration of *C. albicans*, however, this change regarding exercise did not seem to affect the overall function of neutrophil. Ascorbic acid supplement appeared to stimulate neutrophil phagocytic function post-exercise. There is no strong evidence to prove that taking ascorbic acid supplement along with exercising increases neutrophil phagocytic function. However, subjects ingesting ascorbic acid at pre-exercise showed significant lower absolute neutrophil count. This suggested that antioxidant consumption is associated with reduced blood levels of neutrophils and monocytes and dampened inflammation. In accordance with another study in 2018, founded that an increased leukocyte count with an increase in neutrophil count in the peripheral blood is a typical response to inflammation. Participants who reported higher levels of exercise had increased antioxidant protein levels when compared to more sedentary subjects. Active participants exhibited lower circulating peripheral blood mononuclear cells than inactive subjects (34). In mice, N-acetyl cysteine antioxidant remarkably decreases inflammatory cytokines (IL-13, IL-5), neutrophil and eosinophil numbers (35). A couple of studies showed increases in both phagocytosis and oxidant generation by neutrophils following supplementation with vitamin C

348 (36, 37). However, other studies showed no improvement of *ex vivo* anti-fungal or anti-bacterial
349 activity in neutrophils isolated from chronic granulomatous disease or Chediak-Higashi
350 syndrome patients supplemented with ascorbic acid (36, 38).

351 Limitation of this study that should be emphasized is that these immune changes during
352 and after exercise occur in several compartments of the immune system and body including the
353 skin, upper respiratory tract mucosal tissue, lung, blood, muscle, and peritoneal cavity. Blood
354 sample was the only specimen of choice for this study. Furthermore, neutrophils isolated from
355 the circulation may have distinct functional properties compared with those immigrated into
356 tissue locations. Assays that reflect the activity of neutrophils at normal sites of function such as
357 the oral mucosa or respiratory tract should be particularly useful in this regard. Other limitation
358 was the lack of control for total calories, macronutrients and micronutrients.

359

360 ## 5. Conclusions

361 In summary, the present study reports that moderate-intensity cycling increases plasma
362 total CK level, in associated with elevations in white blood cell count, absolute neutrophil
363 numbers and lymphocyte subpopulations following acute exercise. Ingesting ascorbic acid for 7

364 days before a single bout of moderate intensity exercise does not alter antioxidant status or
365 change phagocytic function but instead mitigates the exercise-induced increase in CD4+ T
366 cells and CD45+/CD3-/CD8- cells. Further studies should investigate the possibility that
367 ascorbic acid supplement may alter the immune function during strenuous exercise.

368

369 **Declarations**

370 **Ethics approval and consent to participate**

371 This study was reviewed and approved by the Ethics Research Committee from Faculty of
372 Associated Medical Sciences, Chiang Mai University.

373 **Consent for publication**

374 Not applicable

375 **Availability of data and materials**

376 All data generated or analyzed during this study are included in this published article.

377 **Competing interests**

378 The authors declare no conflict of interest.

379 **Funding**

380 This work was supported by the Faculty of Associated Medical Sciences, Chiang Mai University.

381 The funders had no role in study design, data collection and analysis, interpretation of data and

382 preparation of the manuscript.

383 Authors' contributions

384 PB conceived and designed the study. PB, AC, KI, KN and PK carried out all the experimental

385 work and statistical analysis. PB participated in the manuscript design, interpretation and

386 preparation of the manuscript. All authors read and approved the final manuscript.

387 Acknowledgements

388 We acknowledge the superior technical assistance of Suwatsin Kittikunnathum, Hataikan

389 Siwamoke and Sukanya Dechying. The authors would also like to thank Dr. Tracy Anthony for

390 helpful discussions during the writing of this manuscript.

391

392 References

393 1. Nakaya M, Xiao Y, Zhou X, Chang JH, Chang M, Cheng X, et al. Inflammatory T cell
394 responses rely on amino acid transporter ASCT2 facilitation of glutamine uptake and mTORC1
395 kinase activation. Immunity. 2014;40(5):692-705.

396 2. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, et al. Position
397 statement. Part one: Immune function and exercise. Exerc Immunol Rev. 2011;17:6-63.

398 3. Bishop NC, Walker GJ, Gleeson M, Wallace FA, Hewitt CR. Human T lymphocyte
399 migration towards the supernatants of human rhinovirus infected airway epithelial cells:
400 influence of exercise and carbohydrate intake. Exerc Immunol Rev. 2009;15:127-44.

401 4. Ortega E. Neuroendocrine mediators in the modulation of phagocytosis by exercise:
402 physiological implications. Exerc Immunol Rev. 2003;9:70-93.

403 5. Pyne DB, Baker MS, Smith JA, Telford RD, Weidemann MJ. Exercise and the neutrophil
404 oxidative burst: biological and experimental variability. Eur J Appl Physiol Occup Physiol.
405 1996;74(6):564-71.

406 6. Pedersen BK, Bruunsgaard H. How physical exercise influences the establishment of
407 infections. Sports Med. 1995;19(6):393-400.

7. Pyne DB. Regulation of neutrophil function during exercise. Sports Med. 1994;17(4):245-58.

8. Bruunsgaard H, Pedersen BK. Special feature for the Olympics: effects of exercise on the immune system: effects of exercise on the immune system in the elderly population. Immunol Cell Biol. 2000;78(5):523-31.

9. Pedersen BK, Bruunsgaard H, Klokker M, Kappel M, MacLean DA, Nielsen HB, et al. Exercise-induced immunomodulation--possible roles of neuroendocrine and metabolic factors. Int J Sports Med. 1997;18 Suppl 1:S2-7.

10. Moflehi D, Kok LY, Tengku-Kamalden TF, Amri S. Effect of single-session aerobic exercise with varying intensities on lipid peroxidation and muscle-damage markers in sedentary males. Glob J Health Sci. 2012;4(4):48-54.

11. Pedersen BK, Toft AD. Effects of exercise on lymphocytes and cytokines. Br J Sports Med. 2000;34(4):246-51.

12. Bunpo P, Anthony TG. Ascorbic acid supplementation does not alter oxidative stress markers in healthy volunteers engaged in a supervised exercise program. Appl Physiol Nutr Metab. 2016;41(2):175-80.

13. Yimcharoen M, Kittikunnathum S, Suknikorn C, Nak-On W, Yeethong P, Anthony TG, et al. Effects of ascorbic acid supplementation on oxidative stress markers in healthy women following a single bout of exercise. J Int Soc Sports Nutr. 2019;16(1):2.

14. Peake JM, Suzuki K, Coombes JS. The influence of antioxidant supplementation on markers of inflammation and the relationship to oxidative stress after exercise. J Nutr Biochem. 2007;18(6):357-71.

15. Washko P, Rotrosen D, Levine M. Ascorbic acid transport and accumulation in human neutrophils. J Biol Chem. 1989;264(32):18996-9002.

16. Nikolouli E, Hardtke-Wolenski M, Hapke M, Beckstette M, Geffers R, Floess S, et al. Alloantigen-Induced Regulatory T Cells Generated in Presence of Vitamin C Display Enhanced Stability of Foxp3 Expression and Promote Skin Allograft Acceptance. Front Immunol. 2017;8:748.

17. Sasidharan Nair V, Song MH, Oh KI. Vitamin C Facilitates Demethylation of the Foxp3 Enhancer in a Tet-Dependent Manner. J Immunol. 2016;196(5):2119-31.

18. Bakaev VV, Duntau AP. Ascorbic acid in blood serum of patients with pulmonary tuberculosis and pneumonia. Int J Tuberc Lung Dis. 2004;8(2):263-6.

19. Bharara A, Grossman C, Grinnan D, Syed A, Fisher B, DeWilde C, et al. Intravenous Vitamin C Administered as Adjunctive Therapy for Recurrent Acute Respiratory Distress Syndrome. Case Rep Crit Care. 2016;2016:8560871.

20. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95(2):351-8.

21. McCarthy DA, Macdonald I, Grant M, Marbut M, Watling M, Nicholson S, et al. Studies on the immediate and delayed leucocytosis elicited by brief (30-min) strenuous exercise. Eur J Appl Physiol Occup Physiol. 1992;64(6):513-7.

22. Robson PJ, Blannin AK, Walsh NP, Castell LM, Gleeson M. Effects of exercise intensity, duration and recovery on in vitro neutrophil function in male athletes. Int J Sports Med. 1999;20(2):128-35.

23. Peake JM. Exercise-induced alterations in neutrophil degranulation and respiratory burst activity: possible mechanisms of action. Exerc Immunol Rev. 2002;8:49-100.

24. Chow O, Barbul A. Immunonutrition: Role in Wound Healing and Tissue Regeneration. Adv Wound Care (New Rochelle). 2014;3(1):46-53.

455 25. Bermon S, Castell LM, Calder PC, Bishop NC, Blomstrand E, Mooren FC, et al.
456 Consensus Statement Immunonutrition and Exercise. Exerc Immunol Rev. 2017;23:8-50.
457 26. Paulsen G, Benestad HB, Strom-Gundersen I, Morkrid L, Lappegard KT, Raastad T.
458 Delayed leukocytosis and cytokine response to high-force eccentric exercise. Med Sci Sports
459 Exerc. 2005;37(11):1877-83.
460 27. Pedersen BK. Muscles and their myokines. J Exp Biol. 2011;214(Pt 2):337-46.
461 28. Nieman DC, Groen AJ, Pugachev A, Vacca G. Detection of Functional Overreaching in
462 Endurance Athletes Using Proteomics. Proteomes. 2018;6(3).
463 29. Peake JM, Della Gatta P, Suzuki K, Nieman DC. Cytokine expression and secretion by
464 skeletal muscle cells: regulatory mechanisms and exercise effects. Exerc Immunol Rev.
465 2015;21:8-25.
466 30. Shek PN, Sabiston BH, Buguet A, Radomski MW. Strenuous exercise and immunological
467 changes: a multiple-time-point analysis of leukocyte subsets, CD4/CD8 ratio, immunoglobulin
468 production and NK cell response. Int J Sports Med. 1995;16(7):466-74.
469 31. Pedersen BK. Anti-inflammatory effects of exercise: role in diabetes and cardiovascular
470 disease. Eur J Clin Invest. 2017;47(8):600-11.

471 32. Cao Dinh H, Beyer I, Mets T, Onyema OO, Njemini R, Renmans W, et al. Effects of
472 Physical Exercise on Markers of Cellular Immunosenescence: A Systematic Review. Calcif
473 Tissue Int. 2017;100(2):193-215.
474 33. Navalta JW, Mohamed R, El-Baz A, McFarlin BK, Lyons TS. Exercise-induced immune
475 cell apoptosis: image-based model for morphological assessment. Eur J Appl Physiol.
476 2010;110(2):325-31.
477 34. Busquets-Cortes C, Capo X, Bibiloni MDM, Martorell M, Ferrer MD, Argelich E, et al.
478 Peripheral Blood Mononuclear Cells Antioxidant Adaptations to Regular Physical Activity in
479 Elderly People. Nutrients. 2018;10(10).
480 35. Eftekhari P, Hajizadeh S, Raoufy MR, Masjedi MR, Yang M, Hansbro N, et al. Preventive
481 effect of N-acetylcysteine in a mouse model of steroid resistant acute exacerbation of asthma.
482 EXCLI J. 2013;12:184-92.
483 36. Bozonet SM, Carr AC, Pullar JM, Vissers MC. Enhanced human neutrophil vitamin C
484 status, chemotaxis and oxidant generation following dietary supplementation with vitamin C-rich
485 SunGold kiwifruit. Nutrients. 2015;7(4):2574-88.

37. de la Fuente M, Ferrandez MD, Burgos MS, Soler A, Prieto A, Miquel J. Immune function in aged women is improved by ingestion of vitamins C and E. Can J Physiol Pharmacol. 1998;76(4):373-80.

38. Foroozanfar N, Lucas CF, Joss DV, Hugh-Jones K, Hobbs JR. Ascorbate (1g/day) does not help the phagocyte killing defect of X-linked chronic granulomatous disease. Clin Exp Immunol. 1983;51(1):99-102.

Table 1. Characteristics of female subjects before exercise.

Parameter	0AA	1,000AA
Age, years	22.4±0.1 (21-23)	
Ethnicity/race, n	Southeast Asian (Thai) 20	
Height, m	1.59±0.09 (150-164)	
Weight, kg	52.4±1.7	52.5±1.7
BMI	20.8±0.6	20.8±0.6
Average heart rate (beat/min)	133±1.6	135±2.2
% of heart rate	67±0.8	68±1.1
Distance, km	7.6±0.1	7.3±0.1
Calories, kcal	113±1.4	109±0.8
Creatinine, mg/dL	0.55±0.02	0.54±0.02
Uric acid, mg/dL	5.3±0.2	4.9±0.2
Cholesterol, mg/dL	190±6.4	184±5.6
ALT, U/L	9±1.4	8±1.0

Values are means ± SE; n=20 per cohort. 0AA, 0 mg of ascorbic acid; 1,000AA, 1,000 mg of ascorbic acid; m, meter; kg, kilogram; BMI, body mass index; min, minute; km, kilometer; kcal, kilocalories; mg, milligram; dL, deciliter; ALT, alanine aminotransferase; U, unit; L, liter

Table 2. Antioxidant status, muscle damage marker and glucose measured in the peripheral blood of females with or without ingesting ascorbic acid.

Test	0AA			1,000AA			P value		
	Pre-exercise	Post-exercise	24 h post-exercise	Pre-exercise	Post-exercise	24 h post-exercise	Effect of exercise	Effect of supplement	Effect of exercise × supplement
TAS, mM Trolox equivalent	1.00±0.05	0.98±0.04	0.98±0.04	0.97±0.05	0.96±0.05	1.00±0.05	0.615	0.720	0.122
Plasma TBARS, μmol/g protein	0.09±0.01	0.08±0.01	0.08±0.01	0.08±0.01	0.07±0.01	0.07±0.01	0.130	0.230	0.974
Total CK, U/L	72±3.9	77±4.6 *	70±3.8	73±5.3	78±5.6 *	74±4.0	0.002	0.532	0.724
Glucose, mg/dL	84±1.6	79±1.6 *	87±1.5	88±2.8	84±2.0	89±2.1	0.009	0.080	0.431
Ascorbic acid (mg/L)	2.24±0.35	2.46±0.43	2.36±0.33	2.53±0.40 $	2.91±0.40 $	2.77±0.40	0.262	0.0004	0.278

The exercise bout was performed indoors on a stationary bicycle for 30 min at intensity corresponding to 65-75% of maximum heart rate. Values are means ± SE; n=20 per cohort. The *p*-values indicate the results of a LSD *post-hoc* analysis. *, $p<0.05$ compared with pre-exercise value (within group). $, $p<0.05$ compared with the same time point (in between group). 0AA, 0 mg of ascorbic acid; 1,000AA, 1,000 mg of ascorbic acid; TAS,

total antioxidant status; TBARS, thiobarbituric acid reactive substances; U/g protein, unit per gram of protein; Total CK, total creatine kinase; U/L, unit per liter; mg/dL, milligram per deciliter.

Table 3. White blood cell differential parameters in females with or without ingesting ascorbic acid.

Parameter	0AA			1,000AA			P value		
	Pre-exercise	Post-exercise	24 h post-exercise	Pre-exercise	Post-exercise	24 h post-exercise	Effect of exercise	Effect of supplement	Effect of exercise × supplement
WBC (10^3 cell/μL)	6.3±0.3	6.9±0.3 *	6.0±0.3	6.2±0.3	6.6±0.3 *	6.2±0.3	0.0004	0.382	0.165
Absolute lymphocyte (10^3cell/μL) (%)	2.2±0.1 (35.1±1.6)	2.4±0.1 * (35.2±1.8)	2.2±0.1 (37.3±1.5)	2.3±0.1 (37.7±1.6)	2.3±0.1 (36.5±1.8)	2.3±0.1 (37.7±1.9)	0.113	0.632	0.517
Absolute neutrophil (10^3cell/μL) (%)	3.6±0.2 (56.3±1.4)	3.9±0.2 * (56.2±1.9)	3.3±0.2 (53.4±1.6)	3.3±0.2 $ (52.7±1.8)	3.6±0.2 * (54.6±2.1)	3.4±0.2 (52.8±2.1)	0.001	0.137	0.246
Absolute monocyte, basophil, eosinophil (10^3cell/μL) (%)	0.5±0.04 (8.6±0.5)	0.6±0.05 (8.6±0.6)	0.6±0.07 (9.2±0.6)	0.6±0.05 (9.4±0.6)	0.6±0.05 (8.6±0.6)	0.6±0.04 (9.1±0.7)	0.702	0.659	0.308

The exercise bout was performed indoors on a stationary bicycle for 30 min at intensity corresponding to 65-75% of maximum heart rate. Values are means ± SE; n=20 per cohort. The *p*-values indicate the results of a LSD *post-hoc* analysis. *, $p<0.05$ compared with pre-exercise value (within group) $, $p<0.05$ compared with the same time point (in between group). 0AA, 0 mg of ascorbic acid; 1,000AA, 1,000 mg of ascorbic acid.

Figure legends

Figure 1. Illustration of the design and analysis of a crossover trial. Female subjects ingested 2 capsules containing 0 mg (0AA) or 1,000 mg of ascorbic acid (1,000AA) daily for 7 days. The exercise bout was performed indoors on a stationary bicycle for 30 min at an intensity corresponding to 65-75% of maximum heart rate. A 1-week wash out period separated the two exercise bouts.

Figure 2. Flow cytometric analysis of lymphocyte subpopulations in females ingesting 0 mg (0AA) or 1,000 mg of ascorbic acid (1,000AA). Values are means ± SE; n=20 per cohort. The *p*-values indicate the results of a LSD *post-hoc* analysis.

Figure 3. Effects of ascorbic acid supplement on neutrophil phagocytic function after single bout of exercise. Arrows point to *C.albicans* engulfed by neutrophil.

Figure 4. Effects of ascorbic acid supplement on neutrophil phagocytic function after single bout of exercise. Values are means ± SE; n=20 per cohort. *= $p<0.05$.

Figures

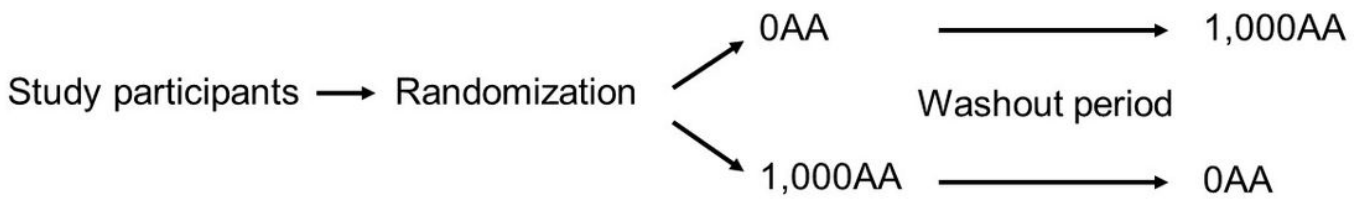
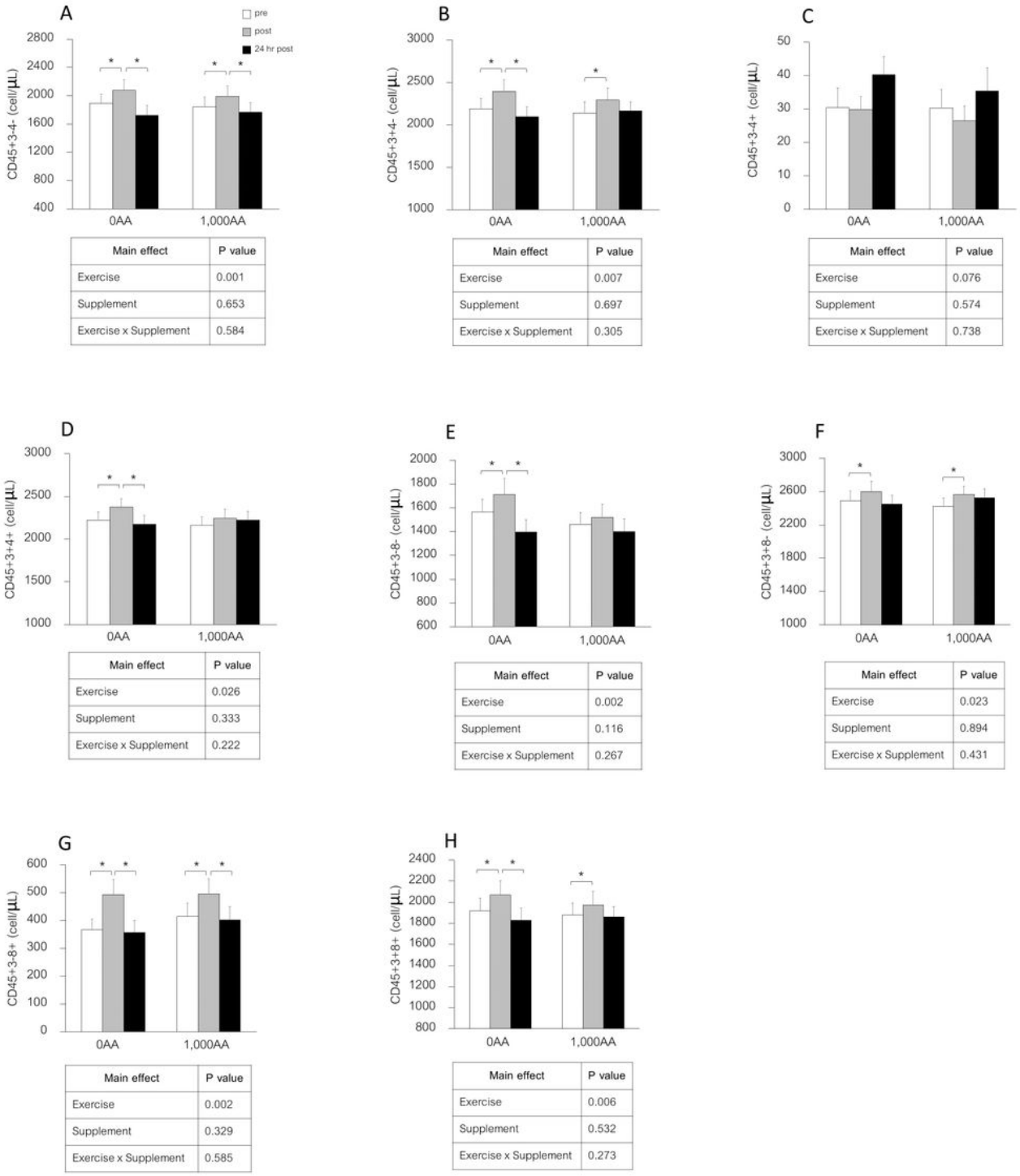


Figure 1

Illustration of the design and analysis of a crossover trial. Female subjects ingested 2 capsules containing 0 mg (0AA) or 1,000 mg of ascorbic acid (1,000AA) daily for 7 days. The exercise bout was performed indoors on a stationary bicycle for 30 min at an intensity corresponding to 65-75% of maximum heart rate. A 1-week wash out period separated the two exercise bouts.



Main effect	P value
Exercise	0.001
Supplement	0.653
Exercise x Supplement	0.584

Main effect	P value
Exercise	0.007
Supplement	0.697
Exercise x Supplement	0.305

Main effect	P value
Exercise	0.076
Supplement	0.574
Exercise x Supplement	0.738

Main effect	P value
Exercise	0.026
Supplement	0.333
Exercise x Supplement	0.222

Main effect	P value
Exercise	0.002
Supplement	0.116
Exercise x Supplement	0.267

Main effect	P value
Exercise	0.023
Supplement	0.894
Exercise x Supplement	0.431

Main effect	P value
Exercise	0.002
Supplement	0.329
Exercise x Supplement	0.585

Main effect	P value
Exercise	0.006
Supplement	0.532
Exercise x Supplement	0.273

Figure 2

Flow cytometric analysis of lymphocyte subpopulations in females ingesting 0 mg (0AA) or 1,000 mg of ascorbic acid (1,000AA). Values are means ± SE; n=20 per cohort. The p- values indicate the results of a LSD post-hoc analysis.

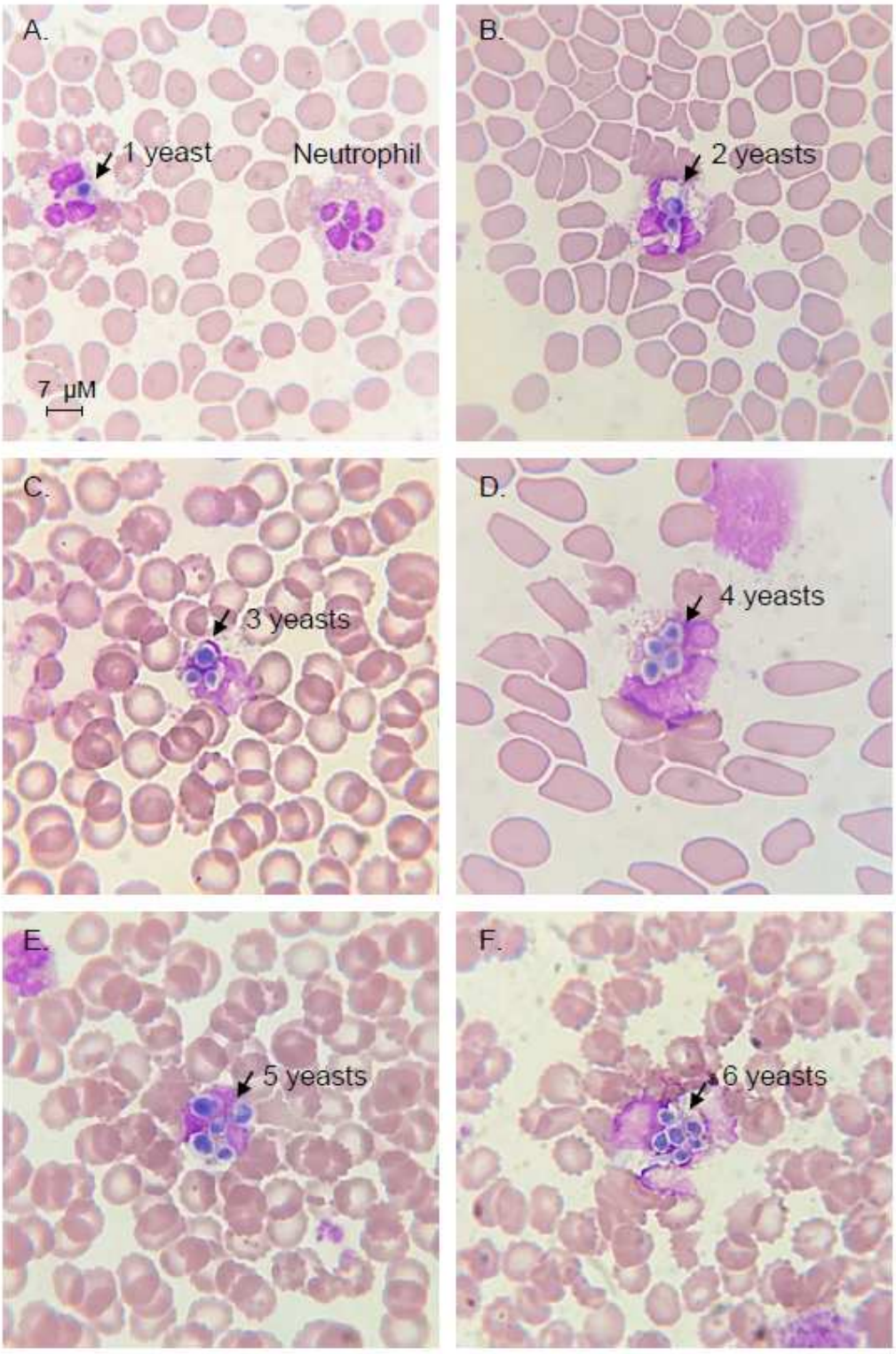
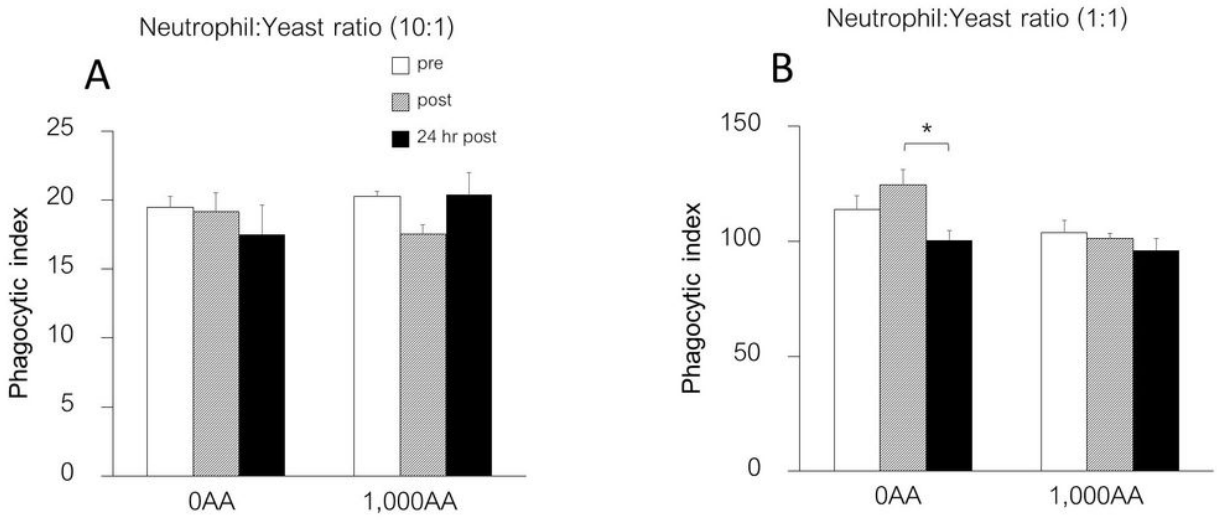


Figure 3

Effects of ascorbic acid supplement on neutrophil phagocytic function after single bout of exercise. Arrows point to C.albicans engulfed by neutrophil.



Main effect	P value
Exercise	0.156
Supplement	0.144
Exercise x Supplement	0.684

Main effect	P value
Exercise	0.023
Supplement	0.894
Exercise x Supplement	0.431

Figure 4

Effects of ascorbic acid supplement on neutrophil phagocytic function after single bout of exercise. Values are means ± SE; n=20 per cohort. *= $p<0.05$.