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# Odorless Garlic Supplementation for Two Months Attenuates Exercise-induced Increases in Interleukin-6 : A Before and After Comparison Study

By

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**Summary** : Allicin, which is a primary compound in crushed garlic, inhibits exercise-induced interleukin (IL)-6 production in humans. However, the daily use of crushed garlic or stabilized allicin is not practical due to the strong odor of allicin. We therefore investigated the possibility that odorless garlic (OG) supplementation for 2 months attenuates exercise-induced IL-6 and other immunological responses. Six healthy untrained men (aged 22.0 ± 0.7 years) consumed 1 g of OG per day for 2 months. Participants underwent two 45-min periods of acute cycling exercise at 80% intensity of heart rate reserve (HRR). The first period was at baseline (control exercise ; CON-Ex) and the second after 2 months of OG supplementation (odorless garlic supplementation and exercise ; OG-Ex). To assess levels of IL-6, IL-10, leukocytes, neutrophils, lymphocytes, Natural Killer Cell Activity (NKCA) and cortisol, blood samples were collected before and immediately (0 min), and 30 and 60 min after each 45-min period of exercise. Two-way repeated measures analysis of variance (ANOVA) and paired t-test with Holm's method were performed, and the incremental area under the curve (iAUC) from before to 60 min after exercise was calculated. No significant differences in the pre-exercise levels of biochemical indices were observed between the CON-Ex and OG-Ex assessments. No significant interaction effects were observed in exercise-induced changes in leukocytes, neutrophils, lymphocytes, NKCA, IL-10, or cortisol. A significant interaction effect was observed in exercise-induced changes in IL-6 ($p = 0.011$). OG consumption also significantly decreased the iAUC of IL-6 (CON-Ex, 82.5 ± 12.2 min·pg/mL ; OG-Ex, 55.5 ± 14.2 min·pg/mL ; $p = 0.018$). Our findings support the hypothesis that two months of OG supplementation attenuates exercise-induced increases in IL-6 levels among untrained men. However, OG did not exert a similar effect on other immunological parameters. Further study is required to clarify the effect of OG on exercise-induced activation of the immune system.

***Key words*** : immunological responses, cytokine, cycling exercise

## Introduction

High-intensity exercise induces transient activation of the immune system[1], and highly trained athletes are reported to experience upper respiratory symptoms (URS) more frequently than recreational athletes or sedentary people[2,3]. Interleukin (IL)-6 is the first cytokine produced during exercise[4] and induces the production of other pro-inflammatory cytokines and cortisol[5]. COX *et al.* reported that illness-prone athletes, with four or more re-

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ported episodes of URS per year, exhibited excess production of IL-6 and of subsequent pro-inflammatory cytokines after acute intensive exercise[6]. Further, an association was found in highly trained athletes between a genotype conferring high levels of IL-6 expression and increased risk of URS[7]. In contrast, antioxidants such as vitamins C and E reportedly prevent IL-6 production after exercise[8,9], and vitamin C supplementation reduces URS episodes in athletes after a race[10]. Taken together, these findings suggest that antioxidants might prevent URS in athletes.

Recent studies have highlighted the positive health effects of garlic, including its antibacterial, anti-inflammatory, immunomodulatory, antithrombotic, antitumor, and hypolipidemic effects[11,12]. These positive health effects might be attributed to organosulfur compounds which are released when garlic is crushed[12]. The primary organosulfur compound in intact garlic is S-allyl-L-cysteine sulfoxide (alliin), which has no odor[11]. When garlic cloves are crushed or minced, alliinase is released and converts alliin into allicin. Although supplementation with stabilized allicin for two weeks reduces exercise-induced increases in IL-6 production in athletes[13], the daily use of crushed garlic or stabilized allicin is not practical due to the strong odor of allicin. Investigation into the protective effect of odorless supplementation on exercise-induced IL-6 is therefore required.

Few studies have investigated the immunomodulatory effects of alliin in humans. In rats, alliin is absorbed in the intestines[14] and exerts preventive effects on isoproterenol-induced cardiotoxicity[15]. Further, alliin suppresses the production of IL-6 and other inflammatory cytokines induced by lipopolysaccharide in adipocytes and peripheral blood mononuclear cells[16,17]. These findings strongly suggest that alliin might also have an immunomodulatory effect in humans. To our knowledge, however, whether or not odorless garlic (OG) attenuates immunological responses induced by high-intensity exercise has not been studied in detail. Here, we examined the effects of daily OG supplementation for two months on exercise-induced IL-6 and other immunological responses in healthy untrained men.

## Methods

*Participants*

Six healthy untrained males (mean age ± standard deviation, 22.0 ± 0.7 years) who met the following criteria were enrolled : (a) no history of cardiac disease or other major chronic diseases, (b) no use of any medication or dietary supplements, (c) non-smoking, and (d) no habitual exercise (i.e. moderate-intensity exercise for < 150 min per week, or high-intensity exercise for 60 min per week[18]). The sample size was determined by considering effect size (0.8), significance level (0.05), and statistical power (0.8). All participants were instructed to avoid any products containing garlic or other dietary supplements and to maintain consistent dietary habits and levels of daily physical activity throughout the study period.

The study complied with the code of ethics of the World Medical Association (Declaration of Helsinki) and was approved by the ethics committee of the Tokyo University of Agriculture (No. 1113). Following an explanation of the purpose, associated demands, and risks of the study, all participants provided written consent to participate in the study and underwent questionnaire-based screening according to the above criteria.

*Study design and protocol*

In this before and after comparison study (Fig. 1), odorless garlic was supplemented for two months (from late July to late September), and participants completed an exercise loading protocol on two separate occasions, the first at baseline (control exercise ; CON-Ex) and the second after 2 months of OG supplementation (odorless garlic supplementation and exercise ; OG-Ex).

The day before exercise loading, participants visited the laboratory at 19 : 00 and ate a standardized meal. On the day of exercise loading, participants visited the laboratory at 07 : 40 following a 12-h period of overnight fasting. The following physical parameters were measured : height (digital automatic height-weight scale TBF-210 ; Tanita Co., Ltd., Tokyo, Japan), body weight and body fat percentage (Inner Scan 50VBC-621 ; Tanita Co., Ltd.), and heart rate at rest (digital automatic sphygmomanometer HEM-7070 ; Omron Healthcare Co., Ltd., Kyoto, Japan). Body mass index was calculated as weight (kg)/height $(m)^2$. After anthropometric measurements were taken, participants ate breakfast at 08 : 00. Pre-exercise blood samples were then taken at 10 : 00. Each 45-min period of cycling was initiated at 10 : 40. All participants followed the entire exercise loading protocol, and no problems were reported. Blood samples were also taken im-

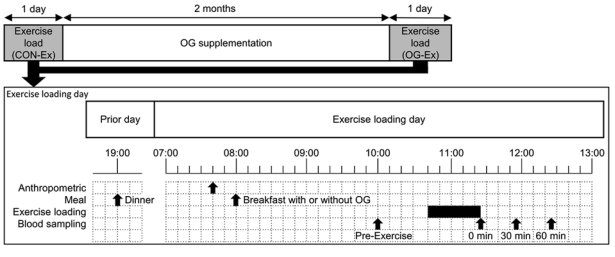


**Fig. 1** Experimental design. CON-Ex, control exercise ; OG-Ex, odorless garlic supplementation and exercise

mediately (0 min) and at 30 and 60 min after each 45-min period of cycling.

*Preparation and supplementation of OG*

OG (500 mg) was orally administered twice-daily after breakfast and dinner throughout the 2-month supplementation period (total of 1 g OG/day). OG was prepared by Fuji Sangyo Co., Ltd. (Kagawa, Japan) from intact raw garlic by boiling for 1 h at 90 ℃ and then crushing and extracting liquid. Extracted liquid was then spray-dried until the water content fell below 5%. A daily dose of 1 g OG is equivalent to 6 g (1 clove) of raw garlic, and contains 3.7 kcal of energy, 0.1 g of protein, 0 g of fat, and 0.8 g of carbohydrates. Each white gelatinous capsule (Size No. 2, Sunsho Pharmaceutical Co., Ltd., Japan) contained OG (167 mg) along with crystalline cellulose (57 mg ; CEOLUS FD-301, Asahi Kasei Chemicals Co., Ltd., Japan), sucrose fatty acid ester (5 mg ; DK-ester F-20W, Dai-ichi Kogyo Seiyaku Co., Ltd., Japan), and fumed silica (1 mg ; Aerosil 200FAD, Nippon Aerosil Co., Ltd., Japan).

*Dietary control*

Habitual energy and nutrient intakes two months before and during the supplementation period were estimated using a semi-quantitative food frequency questionnaire (Excel Eiyoukun Food Frequency Questionnaires)[19]. Participants were instructed to refrain from alcohol consumption for 7 days before exercise loading. The day before exercise loading, participants were also instructed to avoid caffeine, condiments, and high-fat meals.

To avoid the confounding effects of dietary factors on study outcomes, participants were provided dinner the day before exercise loading and breakfast on the day of exercise loading. Total energy requirement (kcal/day) was estimated using the Harris-Benedict equation[20] with an activity factor of 1.5, and energy intake for each meal was distributed depending on individual energy requirements (energy ratio of breakfast, lunch, and dinner was 1 : 1.5 : 1.5). Dinner consisted of 940 kcal of energy, 34.7 g of protein, 26.9 g of fat, and 134.5 g of carbohydrates, and breakfast consisted of 629 kcal of energy, 22.3 g of protein, 23.8 g of fat, and 84.5 g of carbohydrates.

*Exercise loading protocol*

Cycling exercise was performed using a bicycle ergometer (Computronic Aerobike 75XL ; Combi Wellness Co., Ltd., Japan). Exercise intensity was set at 80% heart rate reserve (HRR) as calculated using the Karvonen Formula[21], as follows :

$$80\%\ \mathrm{HRR} = 0.80 \times (\mathrm{maximal\ HR} - \mathrm{resting\ HR}) + \mathrm{resting\ HR}$$

where maximal HR = 220 − (age). As a significant relationship between percentage of HRR and percentage of oxygen consumption reserve was confirmed[22], %HRR was used as an indicator for classification of exercise intensity[23]. All participants received an explanation of the exercise loading protocol and practiced pedaling and the entire protocol before starting the intervention.

A single 45-min period of cycling consisted of 10 min of exercise with stepwise increases in rate up to 80% HRR, and 35 min of steady state exercise at 80% HRR. In addition, participants performed a 3-min warm-up at 50 W before exercise loading and a 1-min cool-down with unloaded pedaling immediately after exercise. Participants were required to maintain a pedaling rate of 60 rpm throughout the exercise protocol. HR was measured using an ear sensor included in the bicycle ergometer, and pedal rate per min was monitored. Water was permitted *ad libitum* during exercise loading.

*Blood analyses*

Peripheral venous blood samples were drawn from an antecubital vein with participants in a sitting position. Whole blood samples (11 mL total) were collected into three types of vacutainers, as follows : 4 mL, non-additive vacutainer ; 2 mL, vacutainer containing EDTA2K ; and 5 mL, vacutainer containing heparin sodium. Blood samples in non-additive vacutainers were centrifuged for 15 min at 3,000 rpm to obtain serum samples and stored at −80℃ until analysis.

Erythrocytes, hemoglobin (Hb), hematocrit (Ht), leukocytes, neutrophils, and lymphocytes in EDTA2K-containing blood were counted with an automated hematology analyzer (XE-2100 ; Sysmex Co., Ltd., Japan). Natural killer cell activity (NKCA) was determined using heparinized blood samples with K-562 target cells in a $^{51}$Cr-release assay (target cells : effector cells = 20 : 1)[24]. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using an automated biochemical analyzer (AU640 ; Olympus Corporation, Japan). Serum levels of IL-6 (IL-6 Human ELISA Kit ; Quantikine HS, R&D Systems, Inc., MN, USA), IL-10 (IL-10 Human ELISA Kit ; Quantikine HS, R&D Systems, Inc.), and cortisol (Cortisol Assay Kit Parameter ; R&D Systems, Inc.) were determined using enzyme-linked immunosorbent assays (IWAKI EZS-ABS Microplate Reader ; Asahi Techno Glass, Co., Ltd., Tokyo, Japan).

*Statistical analysis*

Data are presented as mean ± standard deviation (SD). Differences in pre-exercise levels of parameters between CON-Ex and OG-Ex assessments were compared using paired t-tests. The effects of OG on exercise-induced

changes in cortisol and immunological parameters were analyzed using a 2 group (before or after supplementation) × 4 time (blood sampling points) repeated measures analysis of variance (ANOVA). When an interaction was significant, the difference between post-exercise levels at each sampling point of the CON-Ex and OG-Ex assessments were compared using a paired t-test with Holm's method[25]. Incremental areas under the curve (iAUCs) from pre-exercise levels were calculated for the CON-Ex and OG-Ex assessments and compared using the paired t-test. Statistical significance was set at $p<0.05$. Statistical analyses were performed using IBM SPSS Statistics ver. 20 (IBM Japan, Ltd., Tokyo, Japan).

## Results

*Comparison of pre-exercise parameters*

Mean compliance with OG intake throughout the two-month supplementation period was 99% ± 1%. None of the participants experienced poor health or dropped out during the study period. No significant differences were noted between CON-Ex and OG-Ex assessments for height, weight, body composition, energy intake, or nutrient intake. No significant differences were noted between the two assessments for leukocyte, neutrophil, or lymphocyte counts, NKCA, or IL-6, IL-10, or cortisol levels. Further, no significant differences were noted between the two assessments for erythrocyte count, or in Hb, Ht, AST, or ALT levels (data not shown).

*HR and accumulated work during exercise*

No significant differences in pre-exercise levels were noted between CON-Ex and OG-Ex assessments for HR (CON-Ex, 66 ± 12 bpm ; OG-Ex, 65 ± 10 bpm, $p=0.774$), in HR during exercise loading (CON-Ex, 172 ± 5 bpm ; OG-Ex, 169 ± 5 bpm, $p=0.123$), or in exercise intensity calculated by HRR (CON-Ex, 80 ± 5% ; OG-Ex, 79 ± 2%, $p=0.210$). Total accumulated work during exercise did not significantly differ between the two assessments (CON-Ex, 372 ± 33 kJ ; OG-Ex, 397 ± 99 kJ, $p=0.575$).

*Effects of OG on blood parameters after exercise*

Figures 2 and 3 show exercise-induced changes in blood leukocytes, leukocyte subsets, NKCA, IL-6, IL-10, and

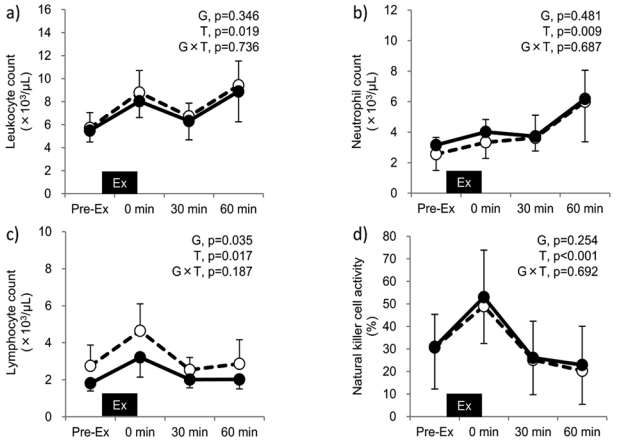


**Fig. 2** Exercise-induced changes in total circulating leukocyte, lymphocyte, and neutrophil counts and natural killer cell activity. Values are presented as mean ± SD (n = 6).
Open circle : CON-Ex, control exercise, Filled circle : OG-Ex, odorless garlic supplementation and exercise. a, Leukocyte count ; b, Neutrophil count ; c, Lymphocyte count ; d, Natural killer cell activity. p-values were analyzed by repeated two-way ANOVA (G = main effect of group, T = main effect of sampling time, G × T = interaction effect of group and time).

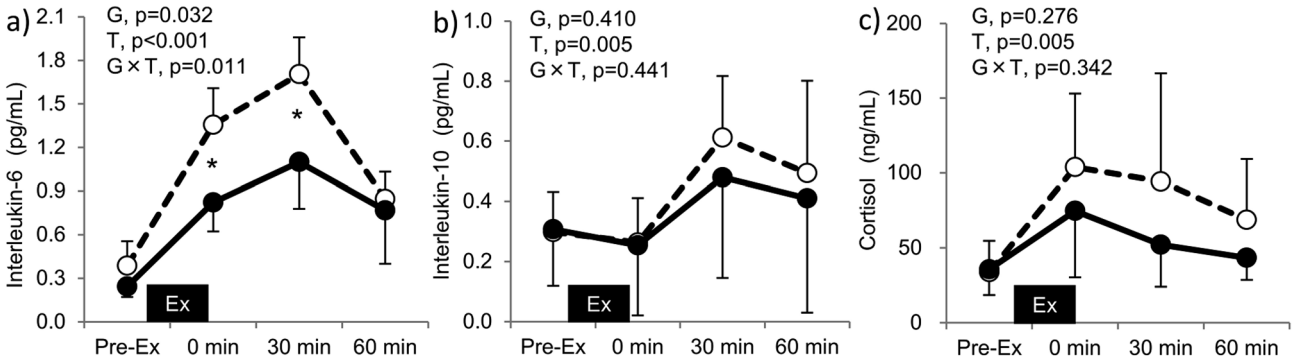


**Fig. 3** Exercise-induced changes in IL-6, IL-10, and cortisol levels. Values are presented as mean ± SD (n = 6). Open circle : CON-Ex, control exercise, Filled circle : OG-Ex, odorless garlic supplementation and exercise. a, Interleukin-6 ; b, Interleukin-10 ; c, Cortisol. p-values were analyzed by repeated two-way ANOVA (G = main effect of group, T = main effect of sampling time, G × T = interaction effect of group and time). *Denotes a significant difference (adjusted by Holm's method) between CON-Ex and OG-Ex assessments at the same sampling point.

cortisol levels. Although a significant main effect of group was noted for lymphocyte count ($p = 0.035$), we did not identify a significant interaction effect ($p = 0.187$). Additional analysis did not reveal any significant differences in the iAUCs between the OG-Ex and CON-Ex assessment of lymphocyte counts ($p = 0.541$). A significant interaction effect was noted for IL-6 levels ($p = 0.011$), and the significant main effect of time was noted for all parameters. IL-6 levels at 0 min and 30 min were significantly lower for the OG-Ex than CON-Ex assessment (0 min, $p = 0.015$ ; 30 min, $p = 0.018$). The iAUC of IL-6 was also significantly lower for the OG-Ex than CON-Ex assessment (CON-Ex, $82.5 \pm 12.2$ min・pg/mL, OG-Ex, $55.5 \pm 14.2$ min・pg/mL, $p = 0.018$).

## Discussion

OG was successfully administered throughout the study period without complaint and attenuated exercise-induced increases in IL-6 levels. However, OG did not exert a similar effect on other immunological or hormonal parameters such as NKCA, IL-10 and cortisol levels.

Increased production of reactive oxygen species (ROS) during exercise causes increased production of IL-6 following exercise[8,9,26,27]. Kosmidou *et al.* examined whether or not ROS stimulate IL-6 production from skeletal myocytes using differentiated C2C12 murine skeletal muscle cells called myotubes and reported that IL-6 production by these myotubes was dependent on the concentration of ROS[27]. Further, Vassilakopoulos *et al.* reported that a combination of ROS scavengers (vitamins A, E and C and *N*-acetylcysteine) and inhibitors of ROS-producing enzymes (allopurinol) attenuated exercise-induced IL-6, IL-1$\beta$, and TNF-$\alpha$ production in sedentary men[8]. Moreover, in physically active non-athletes, vitamin C and E supplementation for 28 days reduced exercise-induced IL-6 production via inhibition of IL-6 release from contracting skeletal muscle[9]. In untrained healthy men, a high-polyphenol diet of purple sweet potato leaves for 7 days was recently reported to attenuate exercise-induced increases in IL-6 levels compared to a low-polyphenol diet[26]. These observations strongly suggest that food or a diet rich in antioxidants can attenuate exercise-induced IL-6 production.

Alliin is reported to inhibit the production of IL-6 and other inflammatory cytokines stimulated by lipopolysaccharide in adipocytes and peripheral blood mononuclear cells[16,17]. Further, in isoproterenol-treated rats, oral administration of alliin for 35 days inhibited plasma thiobarbituric acid-reactive substances and lipid hydroperoxides production, and increased plasma vitamin C and $\alpha$-tocopherol levels[15]. Although few studies have investigated the effects of alliin in humans, from these previous study results, we speculate that alliin exerts antioxidative effects in humans. In addition, Su *et al.* reported that 80 mg of allicin supplementation for 14 days before and 2 days after exhaustive running on a downhill treadmill attenuated exercise-induced IL-6, LDH, and CK production[13]. Allicin is reported to exert antioxidative effects[28,29], and allicin supplementation was found to increase pre-exercise levels of total antioxidant capacity (TAC). Thus, Su *et al.* note that this attenuation of exercise-induced IL-6 production was partly due to higher pre-exercise TAC levels as radical scavengers because high intensity exercise increases oxidative stress in skeletal muscle cells. Another speculation by the authors is that a higher glycogen content in skeletal muscles in the allicin group led to the low exercise-induced IL-6 levels[13]. Garlic can have hypoglycemic effect[30,31], which may contribute to the transport of glucose into muscle cells, where it is converted to glycogen. In the present study, approximately 10 mg of alliin was contained in 1 g of OG supplement (data not shown), but we did not investigate whether a dose response relationship exists between the amount of alliin intake and levels of exercise-induced IL-6 production. In addition, it was unclear if 10 mg of alliin elicits the same level of effect induced by 80 mg of allicin. However, as alliin is the precursor of allicin and this previous study is consistent with our finding that OG supplementation results in the attenuation of exercise-induced increases in IL-6 levels, it may indicate that this attenuation might be explained by similar mechanisms to that noted for allicin supplementation.

Although OG attenuated exercise-induced IL-6 production, other immunological and hormonal parameters between pre- and post-exercise levels were unchanged. Administration of aged garlic extract for three months improves NKCA in patients with advanced cancer[32]. Garlic extract is also reported to increase IL-10 production from placental explant culture[33], and recombinant IL-6 infusion increased plasma IL-10 and cortisol levels in humans[5]. Given that both previous and present findings have shown that OG attenuates IL-6 production after exercise, we consider NKCA to increase before exercise and levels of IL-10 and cortisol to decrease after exercise. However, oral administration of OG did not exert any immunomodulatory effect on NKCA, IL-10, or cortisol levels. Further studies are required to investigate the effect of OG administration on other immunological parameters.

Several limitations of the present study warrant mention. First, we did not establish a placebo control group. However, we recruited participants with no habitual exercise and strictly prohibited them from exercise throughout the study period and accordingly observed no significant differences in pre-exercise IL-6 levels

among our population. One previous study investigated seasonal variation in blood concentrations of IL-6 and found no significant differences between summer and autumn, which were the seasons when our study was conducted[34]. Therefore, our results were not likely affected by daily physical activity or seasonal variation. Further, all participants ate standardized meals (dinner and breakfast) before the two exercise loadings, and no significant differences were noted in potentially confounding nutritional factors (i.e. carbohydrates[35], or vitamins C, A, or E[8]) throughout the study period. SU *et al.* did not serve standard meals[13], and VASSILAKOPOULOS *et al.*[8] and FISCHER *et al.*[9] served only breakfast before exercise loading, so serving standardized meals in our study was appropriate to control the effects of dietary intake before exercise loading. We therefore do not consider our results to have been affected by dietary intake. Second, as all participants were untrained sedentary males, our findings cannot be directly applied to highly trained athletes. However, excessive exercise has been reported to result in high IL-6 production in athletes, and URS has been observed more frequently during periods of high-intensity training[36]. Taking these findings into account, OG appeared to attenuate exercise-induced IL-6 and URS during periods of high-intensity training in athletes. A recent epidemiology study investigated the effect of antioxidants on exercise-induced IL-6 production and URS in athletes[37]. Further studies using placebo controls are therefore required to verify the effect of OG on exercise-induced IL-6 production and URS in athletes.

## Conclusion

Two months of OG supplementation attenuated exercise-induced increases in IL-6 levels in non-athletic men, but not in pre-exercise levels. However, this supplementation did not exert an immunomodulatory effect, particularly on NKCA, IL-10, or cortisol levels.

## Author contributions

A.S. and Y.K. designed the study, secured funding, collected and analyzed data, and prepared the manuscript. H.T., S.K., and T.K. designed the study, prepared and controlled the quality of the OG supplement, and collected data. M.S-S collected data and critically reviewed the manuscript. Y.T. and A.H. assisted with statistical analysis and critically reviewed the manuscript. All authors read and approved the final manuscript.

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## Conflicts of interest

There were no conflicts of interest with respect to this study.

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# 無臭ニンニク 2 か月間の摂取は運動誘発性 IL-6 上昇を抑制する：前後比較試験

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**要約**：ニンニクに含まれるアリシンはヒトで運動誘発性インターロイキン（IL）6 産生を抑制することが報告されている。しかし，アリシンは特有の強い臭気を持つため，実生活を考えると日常的に使用することが難しい。我々はそこで，無臭ニンニク（odorless garlic ; OG）2 か月間摂取が運動誘発性 IL-6 産生と他の免疫応答を減弱する可能性について検討することを目的とした。日頃からあまり活動的でない 6 名の健康な男性（22.0 ± 0.7 歳）を対象に一日当たり 1 g の OG を 2 か月間にわたって摂取させた。研究での運動負荷条件は推定最大心拍数の 80％強度の自転車運動とし，45 分間実施した。運動負荷は，実験開始前（control 群 ; CON-Ex）とニンニク摂取 2 か月後（odorless garlic supplementation and exercise 群 ; OG-Ex）に実施した。血液サンプルは運動負荷を行う前，運動負荷直後（0 min），30 分後，60 分後に採取し，IL-6，IL-10，白血球数，好中球数，リンパ球数，Natural Killer Cell Activity（NKCA），コルチゾールを測定した。運動負荷前から運動負荷 60 分後までの上昇曲線下面積（iAUC）を算出し，統計解析には繰り返しのある二元配置分散分析（ANOVA）および Holm's method を用いた対応のある t 検定を行った。運動負荷前の生化学値には CON-Ex と OG-Ex の間に有意な差は見られなかった。運動負荷後の白血球数，好中球数，リンパ球数，NKCA，IL-10，およびコルチゾールに交互作用は見られなかったものの，IL-6 には交互作用が見られた（$p = 0.011$）。OG-Ex 群の運動誘発性 IL-6 iAUC 値は CON-Ex 群に比べて有意な低下がみられた（CON-Ex, 82.5 ± 12.2 min･pg/mL ; OG-Ex, 55.5 ± 14.2 min･pg/mL ; $p = 0.018$）。今回の結果は 2 か月間の OG 摂取が運動誘発性 IL-6 上昇を抑制するという仮説を支持する結果となった。しかしながら，OG 摂取は他の免疫指標に影響を及ぼすことはなかった。OG 摂取が運動誘発性の免疫機能に及ぼす影響については，今後のさらなる研究が必要である。

**キーワード**：免疫応答，サイトカイン，自転車運動

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