Effect of Vitamin C Supplementation on *Ex Vivo* Immune Cell Functioning

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Abstract

Vitamin C is thought to be vital for the maintenance of proper immune system functioning. The purpose of the present study was to determine if and how vitamin C supplementation correlates with immune cell performance in white blood cells collected from healthy adults. Specifically, the ability of phagocytes to digest bacteria, the ability of lymphocytes to proliferate in response to PHA, the ability of monocytes to develop into mature antigen trained dendritic cells, and the ability of dendritic cell trained lymphocytes to lyse tumor cells were determined ex vivo. Phagocytic index, lymphocyte proliferation index, and mature dendritic cell yields (from monocytes) all decreased with donor age in a statistically significant fashion. However, once the effect of donor age is accounted for, immune cell performance was superior in cells from donors who supplemented with at least 1 g per day of vitamin C. The addition of 5 to 20 mg/dL sodium ascorbate to the growth medium during ex vivo assays improved the ability of phagocytes to digest bacteria and increased the tumor cell killing capabilities if dendritic cell trained lymphocytes. The use of vitamin C supplementation to improve immune cell function thus warrants further study.

Introduction

Vitamin C (ascorbic acid, ascorbate) is an essential water soluble antioxidant that plays rolls in immune collagen and carnitine synthesis and may be necessary for proper immune cell function. Since humans and other primates are incapable of producing vitamin C metabolically, they must obtain it from dietary sources. Historically, there has been some controversy concerning how much vitamin C the human body needs. The United States government recommends a daily allowance of 60 mg/day in order to prevent scurvy. Recently, Mark Levine and coworkers recommended increasing this allowance to 200 mg/day, based on pharmacokinetic experiments measuring the absorption and excretion of ascorbate in seven healthy men.[1] Linus Pauling, on the other hand, suggested that doses of one to ten grams per day would be more appropriate for optimal health.[2,3] This suggestion was based in part on the dietary ascorbate intake of ascorbate by primates, which on a per weight basis is ten to one hundred times the USRDA. In this regard, it is also interesting to note that United States government recommendations for guinea pigs, animals that share with primates the inability to produce vitamin C metabolically, are ten to twenty times above the USRDA for humans when considered on a per-weight basis.

Vitamin C has been recommended for preventing or treating colds, spurring interest in the effect ascorbate supplementation may or may not have on the immune system. Ascorbate appears to be important in neutrophil function and cell-mediated immunity,[4-6] particularly in elderly subjects who have low serum vitamin levels.[7,8] Clinical studies indicate that vitamin C supplementation can improve delayed hypersensitivity reactions,[7] increase T-lymphocyte counts and function,[9,10] reduce incidence of colds in stressed subjects,[11] enhance neutrophil function in subjects with chronic granulomatous disease,[12] and improve

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immune cell recovery from exposure to toxins.[13] The purpose of our study was to compare the functioning of immune cells isolated from donors who supplement with at least one gram/day of vitamin C to that of cells from donors who did not supplement with ascorbate. Specifically, we tested ex vivo the ability of lymphocytes to proliferate when stimulated by PHA, the ability of phagocytes to digest bacteria, and the ability of monocytes to develop into mature dendritic cells. Our results suggest that the ability of white blood cells to perform these functions decreases with age, but that the decrease is less dramatic in cells from donors who supplement with vitamin C.

Materials and Methods

Sample Collection: Blood samples (8 mL whole blood) were taken from 37 healthy donors between the ages of 19 and 82 for measurement of phagocytic index. In 25 of these patients, an additional sample (8 mL whole blood) was taken for measurement of lymphocyte proliferation. In all cases, samples were collected by venipuncture into heparin-containing vacuum tubes, and blood was processed immediately thereafter. Donors were also queried as to their age and their vitamin C supplementation habits. Moreover, 12 donors consented to giving larger volumes of blood (64 mL whole blood) for use in *ex vivo* growth of dendritic cells.

Phagocytic Index Assay: The phagocytic index, defined as the percentage of neutrophils able to successfully engulf bacteria over a two hour period, is determined by exposing neutrophils to fluorescence-labelled bacteria and measuring their fluorescence via flow cytometry. White blood cells (including phagocytes, monocytes, and lymphocytes) were isolated by buoyant density using a Percoll gradient solution prepared using 2.4 mL de-ionized water, 1.25 mL concentrated (10X) PBS solution, and 8.85 mL Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden). Whole blood (8 mL) collected as described above was diluted roughly 1:1 with cold PBS, layered over Percoll gradient solution (4 mL diluted blood over 3 mL gradient solution per 15 cc conical centrifuge tube), and centrifuged at 200 G for 20 minutes. The resulting phagocyte-containing "buffy layer" was rinsed twice with PBS and diluted to 10 mL with PBS. The white blood cell concentration in this solution was determined using an EPICS XL-MCL flow cytometer (Coulter Corporation, Miami, FL). Neutrophils were distinguished from macrophages and lymphocytes by their larger size (forward scatter) and granularity (side scatter). The cells were then suspended at a concentration of 10^6 neutrophils per mL in Opti-MEM (Gibco BRL, Grand Island, NY) supplemented with 5% Bovine Calf Serum (HyClone, Logan, UT). Meanwhile, 10 mg BODPI labelled Escheriacha Coli bacteria (Molecular Probes, Eugene, OR) were dissolved in 1 mL PBS with 2 mM sodium azide. Samples for phagocytic index measurements were prepared by combining 0.5 mL of the white cell suspension with 0.25 mL Opti-MEM and 10mL fluorescent bacteria solution. Controls consisted of 0.75 mL Opti-MEM with 10 mL fluorescent bacteria solution. Some samples were supplemented with 5 to 200 mg/dL sodium ascorbate at this time. Samples and controls were incubated for two hours at 37° C with a gas phase of 5% CO_2 in air at 100% relative humidity. After this incubation, the cells were centrifuged and re-suspended in 150 mL PBS, with 350 mL 0.5% trypan blue being added to quench free bacteria. Cells were then analyzed on the flow cytometer, with the percentage of neutrophils (defined by forward scatter vs. side scatter gating) staining positive for BODPI being defined as the phagocytic index.

Lymphocyte Proliferation Index Assay: Lymphocyte proliferation index, defined as the percentage of lymphocytes that incorporate the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU), is determined by exposing lymphocytes overnight to BrdU,

labelling them with a fluorescence tagged BrdU antibody, and detecting the antibody using flow cytometry. Whole blood collected as described above was diluted two-fold in PBS, layered over Ficoll-Paque, and centrifuged at 200g for 20 minutes. The lymphocyte containing buffy layer was collected, rinsed twice in PBS, and suspended at a concentration of roughly 10^6 peripheral blood cells per ml in AIM-V (Gibco BRL, Grand Island, NY) supplemented with 1 ng/mL interleukin-2 (Sigma Chemical Co., St. Louis, MO) and 20 mM mercamptoethanol (Sigma Chemical Co., St. Louis, MO).

After incubating the peripheral blood cells overnight at 37° C with a gas phase of 5% CO_2 in air at 100% relative humidity (to let the cells adjust to *ex vivo* conditions and allow monocytes to attach to the cell culture dish), the floating lymphocytes and their growth medium was transferred to a separate cell culture dish, supplemented with 10 mM BrdU (Sigma Chemical Co., St. Louis, MO), and incubated an additional 24 hours. In some cases, the cells were also supplemented with 5 mg/dL sodium ascorbate during the incubation. After incubation, cells were labelled with FITC labelled anti-BrdU antibody (Becton Dickinson, San Jose, CA) according to the antibody manufacturer's instructions. Briefly, cells were washed, fixed in 70% ethanol, exposed to a denaturing solution (2 N HCl with 0.5% Triton 100X), washed with neutralizing solution (0.1M Borax) and suspended in Tween solution (PBS with 1% bovine serum albumen and 0.5% Tween 20) to permeablize their membranes. Cells were then exposed to FITC labelled anti-BrdU, rinsed, and stained with propidium iodide.

The cells were then analyzed by flow cytometry. Cells that were positive for propidium iodide (DNA stain) and FITC (anti-BrdU stained) were considered to have passed through S-phase during the overnight incubation, while cells that stained with propidium iodide only were considered to have been non-proliferative.

Dendritic Cell Growth Assays: The dendritic cell (DC) growth process consists of exposing monocytes to cytokines, treating with tumor antigens, and then forming mature dendritic cells by exposing the cells to growth medium conditioned by gamma-globulin stimulated cells. The resulting DC can then be characterized by flow cytometry or mixed with T-lymphocytes for cytotoxicity assays. Dendritic cells were grown from peripheral blood monocytes using modifications of techniques described elsewhere.[14-16] Briefly, peripheral blood mononuclear cells were isolated from whole blood using density gradient centrifugation as described above. After rinsing with PBS, the resulting mixture of monocytes and lymphocytes were suspended in Opti-MEM. Half of these were added to tissue culture dishes containing gamma-globulin while the other half were added to tissue culture flask. The cells were incubated for 30 minutes to allow monocyte attachment.

The non-attaching lymphocytes were collected and re-suspended at roughly 107 cells/mL in AIM-V (Gibco BRL, Grand Island, NY) supplemented with one ng/mL interleukin-2 (Sigma Chemical Co., St. Louis, MO) and 20 mM mercamptoethanol (Sigma Chemical Co., St. Louis, MO). The attaching cells were supplemented with Opti-MEM containing 5% Bovine Calf serum and incubated overnight. After removing the floating lymphocytes, the remaining attached cells, mostly monocytes, were fed Opti-MEM containing 5% Bovine Calf serum, 1000 U/mL GM-CSF and 500 U/mL IL-4 (PeproTech Inc., Rocky Hill, NJ) and, in some cases, sodium ascorbate (5 to 20 mg/dL). Meanwhile, the monocyte conditioned medium (MCM) from the gamma globulin dish was collected, filtered at 0.45 microns, diluted two-fold with Opti-MEM containing 5% Bovine calf serum, and stored frozen for future use. After three days cytokine expo-

sure, the monocytes were 'pulsed' with tumor cell (PC-3 human prostate cells, ATCC, Rockville, MD) lysate by the method of osmotic lysis, as described elsewhere. After antigen pulsing, the dendritic cells were incubated in the presence of MCM for an additional three days. The cells were then characterized by three colour flow cytometry, using FITC stained antibodies to dendritic cell markers CD80 and CD86, PE stained antibody to the mature DC marker CD86, and PC5 stained antibodies to the monocyte and lymphocyte markers CD14, CD3, and CD19. All antibodies were supplied by Coulter Corporation (Miami, FL) except the CD86 antibody, which was obtained from Serotec Ltd. (Oxford, UK). Mature DC were defined as cells that were FITC and PE positive and PC5 negative. The percent DC yield was defined as the ratio of the final mature DC number to the initial monocyte number used.

Dendritic Cell Mediated Cytotoxicity Assays: In ten experiments, DC were mixed with lymphocytes to determine if they could "train" these cells to kill tumor cells. DC were added to lymphocytes at a 1:10 ratio, with the lymphocyte concentration being 10^7 cells/mL, and incubated for one week in AIM-V supplemented with 1 ng/mL interleukin-2, 20 mM mercamptoethanol and, in some cases, sodium ascorbate (5 to 20 mg/dL). Cell-mediated cytotoxicity was then assessed using the calcein release assay as described elsewhere. Briefly, PC-3 prostate cancer cells were stained with fluorescent calcein AM dye, rinsed, and then mixed with DC trained lymphocytes using an effector to target ratios between 30:1 and 50:1 (depending on the number of lymphocytes available). Lymphocytes were incubated with the tumor cells for three hours, and the amount of calcein dye released from the tumor cells was a measure of tumor cell killing. Specific tumor cell lysis was defined as the background subtracted level of calcein release (caused by lymphocytes) divided by the background subtracted maximal release (tumor cells lysed by Triton 100X).

Data Analysis: We used two methods to statistically test the hypothesis that *ex vivo* immune cell performance parameters differed depending upon donor age and vitamin C supplementation habits. We first divided the donors into four categories based on age (over or under fifty years old) and supplementation (daily supplements above or below one gram per day ascorbate) and used an F-test (analysis of variance, ANOVA) to test the null hypothesis that mean values within the individual categories did not differ significantly from the global mean. We also compared variances of three regression models: the null hypothesis (that the data are all described by a global mean), a single linear regression of the data on age, and two linear regressions of the data on age, one each for the supplementing and non-supplementing populations. In cases where sodium ascorbate was added during the *ex vivo* assays, Student's t-test with paired data (Microsoft Excel, Microsoft Inc.) was used to determine if there was a significant benefit to adding the vitamin at that time.

Results

The mean phagocytic index for phagocytes from 37 healthy donors was 87.7 ± 5.5 %, suggesting that the vast majority of neutrophils were adept at digesting *Escheriacha coli*. The mean lymphocyte proliferation index for cells from 25 healthy donors was 56.2 ± 19.5 %. The fact that lymphocyte proliferation is less efficient than phagocytosis *ex vivo* is not surprising, since it is often difficult to grow lymphocytes in culture. Mature dendritic cell yields (29.2 ± 13.9 %) and specific lysis values (8.6 ± 7.3 %) were lower and more variable, reflecting the difficulty of the procedures involved. Table 1 (p.87) shows phagocytic, lymphocyte proliferation indices, and mature dendritic cell yields for cells from four categories of donors. In all

three cases, the F-value was sufficient to rule out the null hypothesis, suggesting some differences among values between the four donor categories. In the case of phagocytic index, it is obvious that values were comparatively low in cells from mature donors who did not supplement with vitamin C. For lymphocyte proliferation and mature dendritic cell yields, discerning which values differed significantly from the other was less straightforward. One can generally observe, though, that values for cells from mature donors were lower than values from young donors and that values for cells from vitamin C supplementing donors were higher, particularly for cells from mature donors. Specific lysis values (not shown) were not statistically different between the four donor categories.

Parameter values were correlated with age using linear regression and two ANOVA tests were conducted: one to determine if the use of a single linear regression of the data on age significantly improved accuracy over describing the data by the global mean, and a second to determine if using two regressions, one each for supplementing and non-supplementing donors, significantly improved accuracy over a single regression. The relationship between phagocytic index and age is shown in Figure 1 (p. 88). Statistically, the regression on age produced a significant decrease in variance (p=0.045) as did the use of separate regres-

Table 1. Mean phagocytic index, lymphocyte proliferation index, and mature DC yield values for cells from young (< 50 years old) or mature (> 50 years old) donors who either do (Yes) or do not (No) supplement with at least one gram per day of vitamin C. Errors are given as standard deviations.

Phagocytic Index			
Age Group	Supplementation	N	Index Mean (%)
Young	No	8	89.3 ± 7.0
	Yes	1	89.2 ± 4.1
Mature	No	7	82.0 ± 4.9
	Yes	11	88.3 ± 3.7
			p value = 0.011
Lymphocyte Proliferation Index			
Age Group	Supplementation	N	Index Mean (%)
Young	No	8	61.9 ± 15.4
	Yes	7	68.6 ± 10.9
Mature	No	3	35.7 ± 27.1
	Yes	7	45.9 ± 18.2
			p value = 0.021
Mature Dendritic Cell Yield			
Age Group	Supplementation	N	Index Mean (%)
Young	No	4	36.3 ± 5.1
	Yes	3	40.7 ± 4.0
Mature	No	3	10.7 ± 7.4
	Yes	2	25.5 ± 16.3
			p value = 0.006

sion lines for supplementing and non-supplementing donors (p = 0.003). Vitamin C supplementation affected the slope of the regression, suggesting that supplementation status becomes more important as the donor age increases. Figure 2 (p. 89) shows similar data for lymphocyte proliferation index. Again, the regression on age (p= 0.004) and the use of separate regression lines for supplementing and non-supplementing donors (p=0.033) significantly decreased the variance. In contrast to the phagocytic index data, donor vitamin C supplementation affected the y-intercept of the regression, suggesting that donor supplementation status was important regardless of donor age. Data with mature dendritic cell yields, shown in Figure 3, (p. 89) suggest that both the slope and the y-intercept of the regression are affected by donor vitamin C supplementation. However, the p-values for regression (0.128, 0.043) were not as low as in the other instances. Finally, specific tumor cell lysis showed no correlation with donor age or supplementation status.

The above data describe the effect of donor vitamin C supplementation on white cell performance when no ascorbate was added during the ex vivo tests. The effect of *ex vivo* sodium ascorbate on immune cell performance is shown in Table 2, (p. 90) where mean values with and without vitamin C are compared. Sodium ascorbate improved the ability of neutrophils to digest bacteria but did not affect lymphocyte proliferation significantly. It also enhanced the ability of DC trained lymphocytes to lyse tumor cells, and may improve the ability of monocytes to develop into mature DC (p-value=0.096). The effect of sodium ascorbate on phagocytic examined more closely in Table 3, (p.90) where values for each of the four donor categories are shown as a function of sodium ascorbate concentration during *ex vivo* co-mingling of neutrophils with bacteria. Interestingly, phagocytes from mature non-supplementing donors were more responsive to added so-

Figure 1. Phagocytic Index as a function of age for cells from donors who did (Yes, solid line, solid boxes) or did not (No, dashed line, open boxes) supplement with at least one gram per day vitamin C. Regression lines are y = 98.4–0.28 x (r = -0.69) and y = 93.6–0.09 x (r = -0.33) for non-supplementing and supplementing donors, respectively.

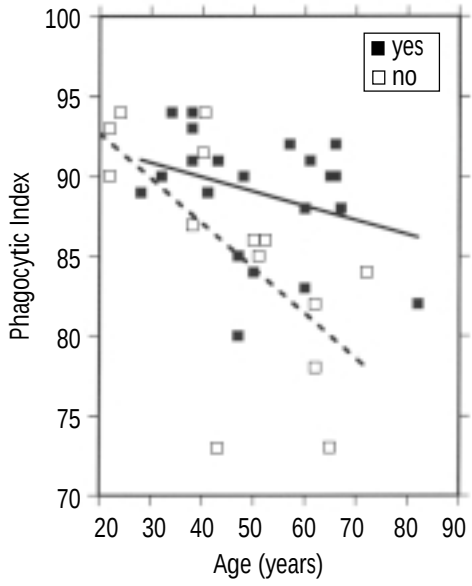


Figure 2. Lymphocyte proliferation index as a function of age for cells from donors who did (Yes, solid line, solid boxes) or did not (No, dashed line, open boxes) supplement with at least one gram per day vitamin C. Regression lines are y = 95.7 – 1.12 x (r = -0.65) and y = 108.6 – 1.00 x (r = -0.83) for non-supplementing and supplementing donors, respectively.

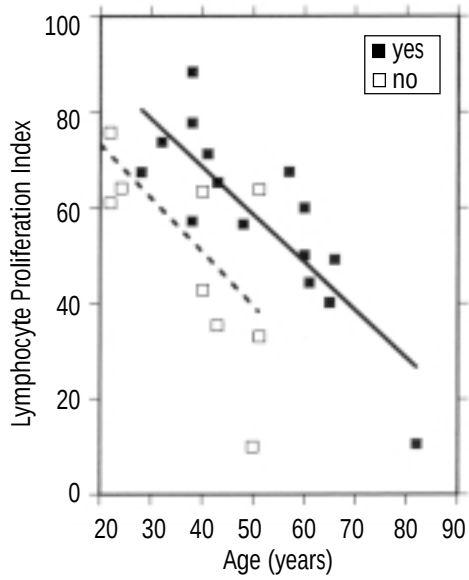
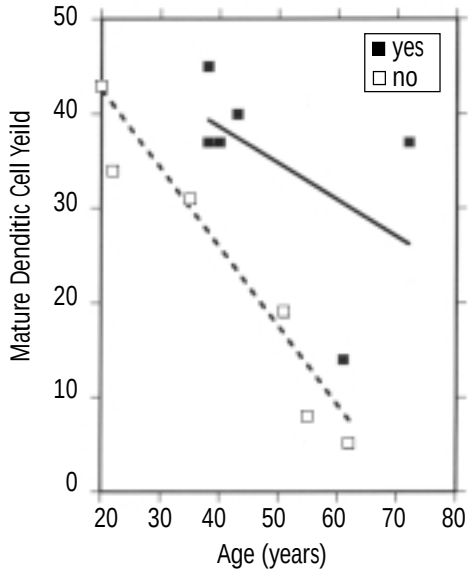


Figure 3. Mature dendritic cell yield as a function of age for cells from donors who did (Yes, solid line, solid boxes) or did not (No, dashed line, open boxes) supplement with at least one gram per day vitamin C. Regression lines are y = 59.3 – 0.83 x (r = -0.92) and y = 54.2 – 0.39 x (r = -0.50) for non-supplementing and supplementing donors, respectively.



dium ascorbate, showing statistically significant enhancement at concentrations as low as 5 mg/dL and showing greater increases in phagocytic index at higher sodium ascorbate concentrations.

Discussion

The data in this study suggest that *ex vivo* immune cell performance decreases with donor age but increases with vitamin C supplementation. This confirms data cited in the introduction suggesting that cell mediated immunity and phagocytosis may be improved with vitamin C supplementation, and that the improvement is particularly important in elderly populations. These data also expand upon previous reports in two ways: they show that the ben-

Table 2. Effect of ex vivo vitamin C supplementation (average of data from 5 to 20 mg/dL) on phagocytic index, lymphocyte proliferation index, mature dendritic cell yield, and percent lysis of cancer cells by DC trained lymphocytes. Parameter means for all donors with (YES) and without (NO) added vitamin C are given with standard deviations and p-values is given for the null hypothesis that ex vivo vitamin C had no effect on the parameter mean.

Parameter	N	Vitamin C Added NO	Vitamin C Added YES	Null p value
Phagocytic Index	37	87.7 ± 5.5	91.8 ± 3.5	3×10^{-6}
Lymphocyte Proliferation Index	25	56.2 ± 19.5	61.2 ± 18.0	0.899
Mature Dendritic Cell Yield	12	29.2 ± 13.9	38.1 ± 21.0	0.096
Specific Tumor Cell Lysis	10	8.6 ± 7.3	2.4 ± 10.6	0.037

Table 3. Mean phagocytic index values as a function of vitamin C concentration during *ex vivo* phagocytosis assay for cells from young (<50 years old) or mature (>50 years old) donors who either do (Yes) or do not (No) supplement with at least one gram per day of vitamin C. Errors are given as standard deviations.

	Phagocytic Index (%)			
Ascorbate Added	Young NO	Young YES	Mature NO	Mature YES
0 mg/dL	89.3 ± 7.0	89.6 ± 4.1	82.0 ± 4.9	88.3 ± 3.7
5 mg/dL	93.1 ± 2.3	91.4 ± 4.7	88.7† ± 2.4	89.7 ± 6.5
20 mg/dL	94.0 ± 1.9	90.5 ± 7.2	89.1† ± 3.2	90.7 ± 3.2
50 mg/dL	94.5† ± 1.7	92.8† ± 3.5	90.3† ± 4.1	92.4† ± 2.1
100 mg/dL	95.1† ± 1.9	93.7† ± 2.3	92.7† ± 3.9	91.6†± 3.6
200 mg/dL	95.2† ± 1.8	92.6 ± 5.4	90.0† ± 5.9	91.3 ± 5.3

†=p value < 0.05 for comparison with 0 mg/dL ascorbate

efits seen in unhealthy or vitamin deficient populations may also apply toward healthy populations; and they provide the first indications that ascorbate supplementation may enhance the development of dendritic cells and the ability of T-cells trained by these dendritic cells to kill tumor cells. In short, these data suggest that vitamin C supplementation of at least 1 g per day is correlated with improved immune cell function. Two caveats concerning these data, however, need to be mentioned. First, the data concerning donor age and donor vitamin C supplementation establish correlation, but not necessarily causation. This was not a controlled study where random selected populations were treated identically except for the introduction of vitamin C. It is possible that the donors who supplement with vitamin C are more health conscious and are doing other things (vitamin supplementation, healthy diet, exercise, stress reduction) to improve immune health. Vitamin C supplementation in this study may thus serve as an indicator of a healthy lifestyle rather than a causal agent. Secondly, we do not know the precise meaning of the parameters we evaluated. Obviously, each of these *ex vivo* assays measure functions that are important to the immune system. Phagocytosis is crucial in the control of bacterial infections, and lymphocyte proliferation is a key part of adaptive immune response. Our understanding of dendritic cells is still developing, but evidence thus far suggests they play an important role in immune surveillance, especially in regards to cancer. However, we do not know how *ex vivo* data relate to *in vivo* data nor do we have a quantitative understanding of what a certain change in parameter values might mean in terms of donor health. Even with these caveats, the data presented in this article suggest that the possibility of improving immune function by daily intake of vitamin C above the USRDA warrants further study.

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