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ORIGINAL ARTICLE: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Co-administration of L-cystine and L-theanine enhances efficacy of influenza vaccination in elderly persons: Nutritional status-dependent immunogenicity

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Aim: The immune response to influenza vaccine is attenuated in elderly persons, though they are at greatest risk for morbidity and mortality by influenza virus infection. Experimental studies demonstrate that co-administration of L-cystine and L-theanine enhanced antigen-specific production of immunoglobulin in aged mice infected with influenza virus. We thus investigated the effect of L-cystine and L-theanine on antibody induction by influenza vaccines in elderly persons.

Methods: Residents in a nursing home were randomly allocated to L-cystine and L-theanine ($n = 32$) or placebo ($n = 33$). The test substances were administered p.o. for 14 days before immunization. Serum influenza virus antibody titers were measured before and 4 weeks after vaccination.

Results: Vaccination significantly elevated hemagglutination inhibition (HI) titers for all the three strains of influenza viruses (A/New Caledonia [H1N1], A/New York [H3N2] and B/Shanghai) in both groups. HI titers after vaccination were not significantly different between the two groups for either strain. Also, the seroconversion rate was not significantly different between the two groups in the aggregate. A stratified analysis showed that the rate of seroconversion was significantly greater in the L-cystine and L-theanine group compared with the placebo group for influenza virus A (H1N1) among subjects with low serum total protein (63% vs 10%, $P < 0.05$) or low hemoglobin (71% vs 9%, $P < 0.05$).

Conclusion: Co-administration of L-cystine and L-theanine before vaccination may enhance the immune response to influenza vaccine in elderly subjects with low serum total protein or hemoglobin.

Keywords: antibody, influenza, L-cystine, L-theanine, vaccine.

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Introduction

Influenza viruses spread rapidly from person to person with abrupt seasonal epidemics, causing a spectrum of respiratory symptoms. As a consequence, influenza virus infection increases morbidity and mortality from

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pneumonia and cardiovascular complications in the community, particularly in elderly people. Vaccination has a pivotal role in the prevention of influenza infection and its complications. Several clinical studies have shown the efficacy of vaccination in preventing hospitalization and death among persons at high risk, including the elderly.[1–4] Influenza immunization is, therefore, encouraged for all individuals 65 years of age or older, or residents of nursing homes to prevent local epidemics and secondary complications.[5–8] However, a reduced or variant antibody response after vaccination is documented in elderly persons compared with young adults because of an impaired immune response to stimulation by influenza antigen.[3,9–12] This attenuated response leaves the elderly still susceptible to influenza infection. Thus, strategies to increase the efficacy of vaccination in elderly persons are important and should be explored.

The plasma levels of glutathione and interleukin-2 decreases with advancing age, leading to impaired immune function and insufficient protection against viral infection in aged people.[9,13,14] A recent report demonstrated that co-administration of the amino acids L-cystine and L-theanine enhanced antigen-specific immunoglobulin (Ig)M and IgG production through augmentation of glutathione and cytokine-mediated immune response.[15,16] These findings indicate that an enhanced immune response after vaccination would be achieved through co-administration of L-cystine and L-theanine (C/T). The current clinical study was designed to evaluate the effect of co-administration of C/T on the immune response after influenza vaccination in the elderly.

Methods

Subjects

Sixty-seven healthy elderly residents (≥65 years old) of a nursing home (Nagoya Kosein) participated in this study. They did not have any past or current conditions of the kidney, liver or heart requiring medical therapy, or allergy to influenza vaccine. None of the subjects had a history of immune dysfunction or was treated with corticosteroids or immunosuppressive agents. All participants ingested the same diet of 7500–8400 kJ/day in which the energy ratio was 15%, 20% and 65% for protein, fat and carbohydrate, respectively, and roasted tea containing a little L-theanine. The subjects were investigated for a history of influenza vaccination, laboratory-confirmed influenza infection, or influenza-like illness (ILI) in the preceding season. ILI was defined as an acute onset of respiratory illness with fever (≥38°C) and one or more of the following: cough, sore throat, arthralgia, myalgia. A survey of individual charts on clinical symptoms disclosed four subjects with ILI in the preceding season. All of them belonged to C/T group. The protocol was approved by the Ethical Committee of Nagoya Kosein Hospital. All subjects provided written informed consent to participate in the study.

Administration of test substances

The subjects were randomly allocated to the control or C/T group. The C/T group was administered p.o. 700 mg L-cystine and 280 mg L-theanine once/day, while the control group received 930 mg cellulose and 50 mg sodium glutamate. The amount of test substances used in this study was based on the following data. The mean value of L-cystine ingestion is reported as 1000 mg/day with maximum of 2200 mg/day.[17,18] An experimental analysis revealed the weight ratio of L-cystine to L-theanine of 5:2 was best to achieve the effective increase in glutathione level.[15] Thus, the amount of L-cystine was determined as 700 mg/day in consideration of safety margin and that of L-theanine 280 mg/day. These test substances were generously provided by Ajinomoto Co., Inc. (Tokyo, Japan). The administration of test substances was double-blinded and visually confirmed by the attending staff every day. Each group was instructed to take the test substances for 14 days. All subjects underwent a medical examination and interview by a physician before and after the administration of the test substances, and vaccination. The entire protocol was performed during the period November 2005 to March 2006.

Vaccination

All subjects were given s.c. 0.5 mL of commercially available subvirion trivalent influenza vaccine (Denka Seiken, Tokyo, Japan) on the 15th day of the study. The vaccine included 30 μg/mL hemagglutinin of each of the following influenza strains: A/New Caledonia/20/99 (H1N1); A/New York/55/2004 (H3N2); and B/Shanghai/361/2002.

Laboratory examination

Blood samples were obtained before and after administration of the test substances, and 4 weeks after vaccination to evaluate the blood cell count and biochemistry. Serum influenza virus antibody titers against the vaccine strains were determined by the standard microtiter hemagglutination inhibition (HI) procedure from the samples using the same antigens as those in the vaccine. All serum specimens for evaluation of HI titer were stored at –40°C until simultaneous assay.[6]

Serum HI titer equal to or greater than 40 was considered as the level expected to be protective against influenza infection.[19–21] We thus analyzed data from subjects in the aggregate and whose HI titer was at a non-protective level (<40) before vaccination. The

Table 1 Clinical profiles of subjects

	Control ($n = 33$)	L-Cystine and L-theanine ($n = 32$)
Male : female	15:18	14:18
Age (years)	77.4 ± 8.9	76.0 ± 9.2
Body weight (kg)	45.7 ± 9.9	46.8 ± 9.8
Serum total protein (g/dL)	6.89 ± 0.54	6.96 ± 0.61
Serum albumin (g/dL)	3.85 ± 0.36	3.90 ± 0.44
Albumin/globulin	1.28 ± 0.20	1.30 ± 0.26
Blood urea nitrogen (mg/dL)	16.7 ± 6.1	15.5 ± 5.9
Serum creatinine (mg/dL)	0.8 ± 0.3	0.8 ± 0.3
Hemoglobin (g/dL)	12.2 ± 2.0	12.3 ± 1.7
Mean corpuscular volume (fL)	95.5 ± 5.2	93.9 ± 7.8
White blood cells (/mm^3)	6422 ± 1971	6710 ± 2420
History in the preceding season		
influenza vaccination (*n*)	22	25
laboratory-confirmed influenza/ influenza-like illness (*n*)	2/0	3/4

proportion of subjects in whom a protective HI titer (≥40) was achieved from a non-protective level after vaccination (seroconversion) was compared between the control and C/T groups to evaluate the effect of co-administration of C/T on influenza immunization.

Statistical analysis

Data are expressed as the mean ± standard deviation. HI titers were $\log_2$-transformed for analysis to obtain normality. Paired HI titers were analyzed with the Wilcoxon rank sum test. Differences in the proportion of seroconversion between groups were analyzed using Fisher's exact test and ANCOVA. $P < 0.05$ was considered significant. HI titer was analyzed for each influenza virus strain in the aggregate or with stratification for values related to nutritional status. The criteria of stratification for analogous data were determined by the mean value of all of the participants.

Results

Two individuals were excluded from the study because of hospitalization due to acute illness or trauma. The remaining 65 subjects completed the protocol. The profiles of these subjects are presented in Table 1. There were no significant differences in the clinical profiles between the control and C/T groups. Two subjects in each group had a history of influenza B infection, and one subject in the C/T group had influenza A (H1N1) infection in the preceding season. ILI was observed in four subjects in the C/T group, but in no subjects in the control group. All participants ingested almost all of the daily diet. No adverse effects of C/T or influenza vaccine were observed throughout the study, and the co-administration of C/T was well tolerated.

Analysis of HI titers in the aggregate

Vaccination significantly elevated HI titers for all three strains of influenza viruses (A/New Caledonia [H1N1], A/New York [H3N2] and B/Shanghai) in both groups (Fig. 1). HI titers after vaccination were not significantly different between the two groups for either strain. However, HI titer for influenza B in the C/T group before vaccination was significantly greater than that in the control group. The percentage of subjects who had a protective level of antibody (HI, ≥40) was greatest for A (H1N1), smallest for A (H3N2), and intermediate for the B strain before vaccination, and significantly increased after vaccination for A (H3N2) in both groups. The C/T group had a relatively greater percentage of subjects whose HI titer was equal to or greater than 40 than the control group both before and after vaccination (Table 2). The seroconversion rate was compared between the control and C/T groups. A greater response, though non-significant, was observed in the C/T group for all three strains (Fig. 2).

Sub-analysis of HI titers

Seroconversion rates were compared between the C/T and control groups after stratification for serum total protein, albumin, A/G ratio, cholesterol hemoglobin and white blood cell count. The analysis revealed a clear contrast between the two groups when stratified for serum total protein or hemoglobin. The seroconversion rate was significantly greater for influenza A (H1N1) in

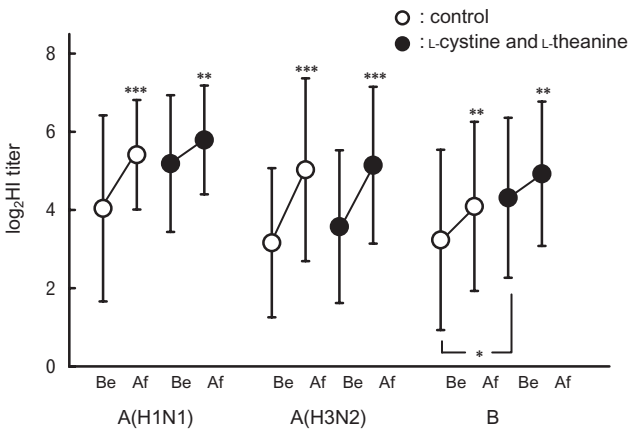

Figure 1 Hemagglutination inhibition (HI) titer before and after influenza vaccination. Influenza vaccination significantly elevated HI titer for all strains in both the control ($n = 33$) and the L-cystine and L-theanine ($n = 32$) groups. Note that the pre-vaccination titer for influenza B in the L-cystine and L-theanine group was significantly greater than that in control group. Data are shown as the mean ± standard deviation in $\log_2$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Af, after vaccination; Be, before vaccination.

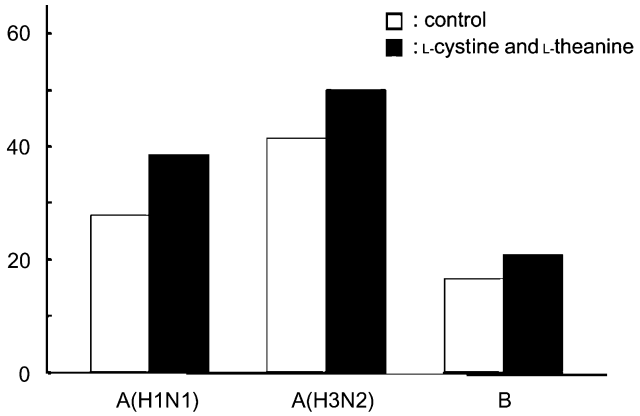

Figure 2 Rate of seroconversion in the aggregate. Seroconversion was defined as the change of hemagglutination inhibition titer from a non-protective level (<40) to a protective level (≥40). The seroconversion rate in the aggregate was greater, though non-significant, in the L-cystine and L-theanine group than in the control group.

Table 2 Percentage of subjects with a hemagglutination inhibition titer of ≥40

Type of influenza virus	Control ($n = 33$)		L-Cystine and L-theanine ($n = 32$)	
	Before	After	Before	After
A (H1N1) (%)	45.5	60.6	59.4	75
A (H3N2) (%)	12.1	48.5*	18.8	59.4*
B (%)	27.3	39.4	37.5	53.1

$^*P < 0.01$ vs before.

the C/T group with low serum total protein (<7.0 g/dL) or low hemoglobin (<12.0 g/dL) (Fig. 3). The result remained unchanged even after adjustment for confounding factors such as total protein or hemoglobin. A similar tendency indicating the efficacy of co-administration of C/T, though statistically non-significant, was observed in subjects with relatively low albumin or cholesterol, or a low white blood cell count (Table 3).

Discussion

This study suggested the effectiveness of co-administration of C/T on the immune response to influenza vaccination. C/T increased the seroconversion rate after influenza vaccination in elderly subjects with suspected low serum total protein or hemoglobin. Frail elderly persons have a poor antibody response to immunization. Serological response could not be predicted by body mass index or Barthel index.[22] Some age-related changes of nutritional or oxidative stress are suggested to be responsible for the impaired immune response in elderly people.[23,24] Poor nutritional status is a commonly observed phenomenon in the elderly and limits the efficacy of vaccination.[25–27] Dietary supplements including antioxidants improves the immune response to influenza vaccination.[23,28,29] Accumulation of free radicals is recognized as a major constituent of aging and age-associated degenerative diseases, which lead to decreased absorptive and metabolic capacity and could result in impaired nutritional status.[30,31] Thus, the immune response in the elderly seems dependent on nutritional status, in which oxidative stress plays a major role. We therefore investigated substances that have an antioxidant property. Among several dietary constituent, amino acids that are categorized as food would be safe and favorable for supplemental usage. L-Cystine is a precursor of glutathione, a potent antioxidant, and L-theanine collaborates in the absorption of L-cystine and stimulates to release cytokines.[32]

Levels of glutathione in leukocytes and plasma are decreased in subjects with malnutrition.[33] Deficiency of intracellular glutathione is relevant to oxidative imbalance, and results in impaired macrophage and dendritic cell function with reduced production of cytokines.[34–36] A defect of interleukin-2 production is responsible for decreased antibody production.[9] These facts suggest that antibody production after vaccination might be depressed markedly in elderly persons with poor nutritional status,[37] and that supplementation with amino acids that increase both intracellular glutathione and plasma interleukin-2 levels is reasonable. L-Cystine requires glutamate to be taken up into the cytosol for conversion to glutathione,[16] while L-theanine is split

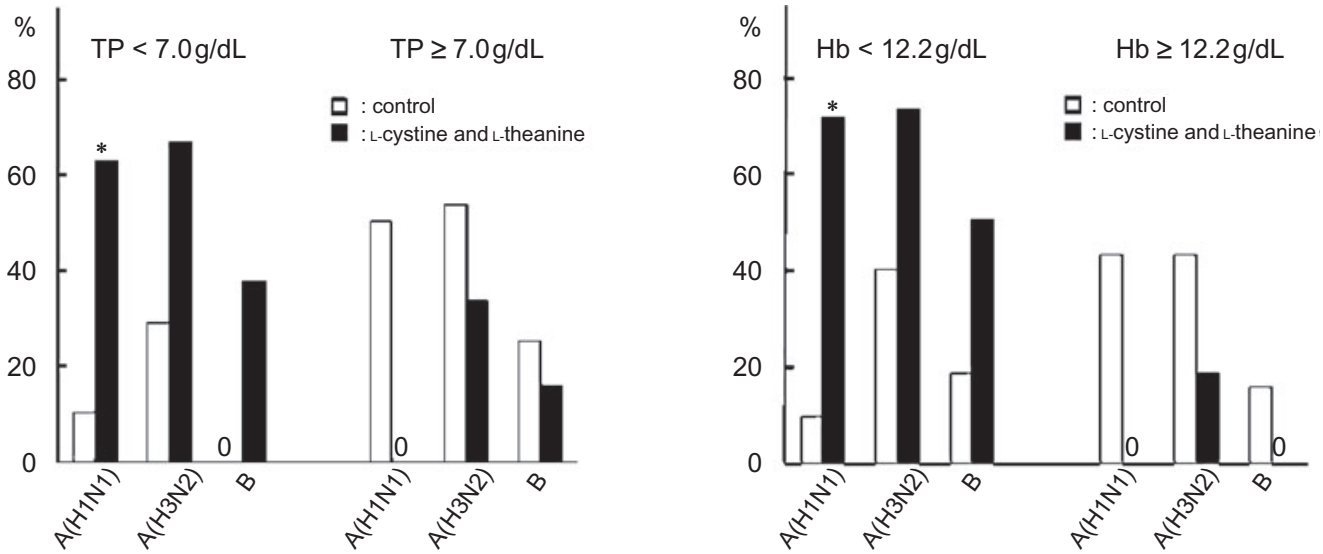

Figure 3 Rate of seroconversion after stratification for serum total protein or hemoglobin. The seroconversion rates stratified for serum total protein (left panel) and hemoglobin (right panel) were significantly greater in the L-cystine and L-theanine group than in the control group for the hemagglutination inhibition titer of influenza A (H1N1). A similar tendency, though non-significant, was observed for influenza A (H3N2) and B. Hb, hemoglobin; TP, serum total protein.

Table 3 Stratified analysis of seroconversion rate

Type of influenza virus	Total cholesterol <192 mg/dL Control (%)	C/T (%)	Total cholesterol ≥192 mg/dL Control (%)	C/T (%)
A (H1N1)	17	57	22	20
A (H3N2)	33	69	36	30
B	25	30	10	25
	WBC < 6564/mm^3 Control (%)	C/T (%)	WBC ≥ 6564/mm^3 Control (%)	C/T (%)
A (H1N1)	8	44	67	20
A (H3N2)	24	57	67	38
B	8	20	27	27
	Albumin < 3.9 g/dL Control (%)	C/T (%)	Albumin ≥ 3.9 g/dL Control (%)	C/T (%)
A (H1N1)	25	71	30	0
A (H3N2)	46	62	44	38
B	22	33	13	18

C/T, L-cystine and L-theanine; *n*, number of subjects; WBC, white blood cells.

into glutamate and ethylamine *in vivo*. Glutamate is coupled with the conversion of L-cystine to glutathione and the latter stimulates γδ T cells to release interleukin-2.[32] The combination of C/T therefore might, at least in part, restore impaired immune competence.

Stratified analysis might reveal a significant effect of C/T on the immune response after influenza vaccination in subjects with relatively low serum total protein or hemoglobin. Differences of ingested amino acids were negligible because the daily diet and tea served in the nursing home were the same among all participants before and throughout the study period. An increased incidence of anemia is associated with aging, and leads to a greater risk of infectious diseases.[38,39] Deficiency of iron, vitamin B_{12} or folate is a common cause of anemia in the elderly. Mean corpuscular volume was within the normal range in both groups. Because most elderly subjects with anemia have a normocytic profile even with deficiency of these trace elements,[40] latent deficiencies of the elements might be responsible for the difference

in the immune response in subjects with relative anemia. Low serum total protein is often accompanied by deficiencies of serum micronutrients due to age-related chronic diseases or disabilities.[24,28,41] Of the micronutrients, Hara *et al.*[24] emphasize a deficiency of vitamin E, a free radical scavenger, where an increased oxidative stress results in impaired immune response. Thus, administration of C/T could improve immune response via increased synthesis of glutathione, another potent free radical scavenger, in elderly subjects with low serum total protein and suspected deficiencies of micronutrients that have antioxidant properties.

The HI titer measured 1 month after vaccination was similar between the two groups. This result can be explained by two possibilities. One is that the peak production of antibody is delayed in elderly people.[9] Measurement later than 1 month after vaccination might have revealed a different response. The other possibility is that the elevation of antibody is limited by a ceiling phenomenon whereby the higher the level of antibody before vaccination, the smaller the elevation after vaccination.[42] The pre-vaccination HI titer was greater in the C/T group despite random allocation. The percentage of subjects whose HI titer was equal to or greater than 40 was greater in the C/T group than in the control group, and this elevated pre-vaccination HI titer may have masked the difference between the two groups. Although the number of cases of laboratory-confirmed influenza was similar between the two groups, that of ILI in the preceding season was greater in the C/T group. Some of the cases of ILI might have been found to be influenza if laboratory examination had been performed, which might explain the difference of pre-vaccination HI titer. The difference of the pre-vaccination HI titer between the two groups has made the changes of HI titer by vaccination difficult to compare. Thus care must be taken in the interpretation of the results.

We analyzed data by the rate of seroconversion, which was defined as a change of HI titer from less than 40 to equal to or greater than 40. Some investigators have defined the efficacy of influenza vaccination as a four-fold rise of HI titer, but the fold rise of antibody is an underestimation due to the pre-vaccination titer, which is negatively associated with antibody induction.[42] The post-vaccination HI titer could therefore be reduced, negating the difference. People who are immunized on an annual basis have better protection than first-time vaccinees. Our record showed that approximately 70% of the subjects had received influenza vaccine in the preceding season. The purpose of vaccination is to cause subjects without a protective level of immunity against influenza infection to produce a protective level of antibody. The absolute value of post-vaccination titer seems a better surrogate for protection than the fold increase, which may count an insufficient immune response such as a change of HI titer from less than 10 to 20. The definition of seroconversion used in this study is therefore rational for the evaluation of immune response in the elderly.[41]

Other investigators have reported attempts to achieve higher levels of antibody in elderly subjects using statins or greater amounts of antigen in the vaccine.[21,43] Although these trials were successful, the pharmacological methods used to enhance the efficacy of vaccination may potentially have systemic or local adverse effects.[21,44] The amino acids used in our investigation showed no adverse effects, possibly because they are constituents of food and the amounts used in this study were within the range of allowed daily intake. Co-administration of C/T in humans would therefore be a safe and effective strategy to enhance the immune response to vaccination.

The subjects enrolled in this study were residents of a nursing home. Because they do not necessarily represent the general population of the elderly, the results obtained in this study are not applicable universally. For the generalization of the data, the study should be designed to sample subjects from a target population by simple random, systematic, multistage or stratified sampling. Further study with appropriate sampling of subjects is required to exclude the bias in the clinical background and might provide more accurate data on the efficacy of C/T.

Limitations of this study

Subjects with ILI in the preceding influenza season were included in C/T group. Since they might have affected the HI titer before vaccination, care must be taken in the interpretation of the data.

The ratio of L-cystine to L-theanine, and the period of administration and the amounts given were extrapolated from data obtained from experimental studies. Levels of cytokines such as interleukin-2 produced in response to C/T were not measured in this study. Therefore, the relationship between C/T and the immune response in humans was not confirmed by laboratory data.

Measurement of cytokine levels would provide important and informative data which may have suggested the role of co-administration of C/T on immunological response after vaccination. However, we had to refrain from drawing sufficient blood repeatedly to allow measuring parameters other than HI titer, biochemistry and hematology from elderly subjects because their build were rather small. Information from this study is not necessarily applicable to elderly persons in the community because of the method to sample subjects. Further studies are required to clarify these points.

In conclusion, co-administration of C/T may be a safe strategy to enhance the immune response to influenza

vaccination. The effect is notable in elderly subjects with low serum total protein or hemoglobin.

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