Effects of intravenous injection of high-dose ascorbic acid on mitochondrial DNA copy number in chronic fatigue patients: randomized-controlled study

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Directed by Professor Duk Chul Lee

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Jin Young Shin

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ABSTRACT

Effects of intravenous injection of high-dose ascorbic acid on mitochondrial DNA copy number in chronic fatigue patients: A randomized-controlled study

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Fatigue is related with mitochondrial energy systems. Mitochondrial components can be impaired by reactive oxygen species. Vitamin C has been used as a therapeutic agent for fatigue recovery. However, the effect of that is still vague. To explore changes in fatigue scores and mtDNA copy numbers after high-dose vitamin C injection in chronic fatigue patients, double-blind randomized trial was performed. Sixty participants with moderate-severe fatigue for more than 6 months were enrolled. Each participant received a single treatment of either vitamin C 10g or same volume of normal saline. Primary outcomes are fatigue scores by brief fatigue inventory-Korean version and mitochondrial DNA (mtDNA) copy number in whole blood and buccal epithelial cell, 2 weeks later.

Mean fatigue scores were improved in both vitamin C group (n=30, 6.3±1.1 to 5.1±2.1, p=0.006) and placebo group (n=30, 6.6±0.9 to 5.6±1.6, p=0.001) (p=0.582, intergroup). In vitamin C group, mtDNA copy number was slightly increased (blood: 71.89±35.14 to 72.08±34.33, p=0.688; saliva: 398.25±326.65 to 415.56±347.49, p=0.915), while that of placebo group was decreased (blood: 74.18±29.12 to 66.47±31.74, p=0.283, saliva: 349.02±305.60 to 317.23±270.19, p=0.631). In subgroup analysis, divided into four groups according to fatigue recovery and intervention, More subjects in vitamin C group (15/15, 50%) experienced fatigue recovery than placebo group (8/22, 27%), (p=0.055).

In vitamin C and fatigue recovery group, mtDNA copy number was higher. However, the other subgroups showed lower changes. In vitamin C group, participants with lower initial mtDNA copy numbers had higher mtDNA copy number (p=0.001) and lower fatigue scores (p=0.026) than participants with higher initial copy number in peripheral blood. However, the placebo group did not show significances (p>0.05, both). In saliva sample, the change of mtDNA copy number was same (p=0.031), but we did not confirm the fatigue recovery (p=0.740). There was no important adverse event.

We cannot confirm fatigue recovery and mtDNA copy number increase by high-dose vitamin C injection in all participants. However, this treatment can reduce chronic fatigue and increase the mtDNA copy number on chronic

fatigue patient with low mtDNA copy number. Clinically, fatigue patient with low mtDNA copy numbers may receive more benefit from this treatment.

Key words: vitamin C, mitochondrial DNA, fatigue

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I. INTRODUCTION

The human mitochondrial genome is a 16.6-kb circular structure of double-stranded DNA with 100-1,000 copies per cell.[1] The number of mitochondrial DNA (mtDNA) copies has been suggested to indicate mitochondrial gene stability and biogenesis and reflect mitochondrial function.[2] Among cell types, mtDNA copy number varies.[3] Mature red blood cells are devoid of mtDNA, while oocytes contain 100,000 or more copies.[4] Depleted mtDNA and the resulting mitochondrial malfunction have been implicated in aging, diabetes, neurodegenerative disease, and cancer.[2] Decreased mtDNA copy numbers have been reported in aging,[5] Friedreich's ataxia patients and adriamycin induced injury.[6]

Fatigue is related to reduced efficiency of cellular energy systems, primarily in

mitochondria.[7] Mitochondria produce high-energy molecules such as adenosine triphosphate (ATP) through electron transportation by reducing nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH).[8] Mitochondrial components can be impaired by reactive oxygen species (ROS), resulting in oxidation of lipids, proteins, and DNA with aging and chronic illnesses.[9,10] Unlike nuclear DNA, mtDNA is not protected by histones, making it susceptible to damage from ROS.[11,12] Normally, free-radical scavenging enzymes neutralize excess ROS and repair ROS-mediated damage.[13] However, when damage accumulates and exceeds the abilities of cellular repair systems[14], fatigue and disease can occur.[13]

In fatigue patients, there is some evidence of oxidative damage to DNA, such as oxidized blood markers including methemoglobin;[15] elevated peroxynitrite levels due to excess nitric oxide; and cytokine levels that exert positive feedback on nitric oxide production,[16] urine analysis[17] and muscle biopsy samples.[18,19] However, mtDNA copy number in blood and saliva has not been measured in patients with fatigue.

Vitamin C is one of the most popular single vitamins with sales of 884 million dollars in the US in 2007. Besides its use to treat scurvy, vitamin C has been used as a therapeutic agent by physicians orally and intravenously for more than 60 years. In the US, 11,233 patients were administered intravenous vitamin C in

2006. The average dose was 28 grams every 4 days for 22 treatments.[20]. The reasons for injection were broad, mostly infection, cancer and fatigue.[18,21]

The mechanisms of vitamin C in fatigue patients are still unclear. Vitamin C enters mitochondria via glucose transporter 1 and protects mitochondrial against injury from oxidative stress by depolarizing the mitochondrial membrane.[22] Oral vitamin C supplements were reported to decrease DNA damage in smokers,[23,24] arsenic-treated rat[25], while it did not have any effect in non-smokers.[26] Predicting the effects of oral vitamin C supplements is difficult, considering bioavailability from intestinal absorption. Intravenous injection, however, reduces these variations. Moreover, injecting high-dose vitamin C can create a concentration 70 times higher than with oral intake.[27] High-dose vitamin C has been used to treat cancer and fatigue.[28-30] Since vitamin C is a potent antioxidant, it may have a role in reducing fatigue.[29]

In previous randomized controlled trial, intravenous vitamin C reduced fatigue in Korean office workers as measured by the fatigue scale,[29] however, there was no evidence that vitamin C could restore the mtDNA damage in fatigue patients. The aims of this study were to investigate mtDNA biogenesis and to examine changes in fatigue scores and mtDNA copy numbers in peripheral blood and buccal epithelial cell after high-dose vitamin C injection in patients with chronic fatigue.

II. MATERIALS AND METHODS

1. Study design

This double-blind, random allocated, placebo controlled trial was performed at Gangnam Severance Hospital. Participants were recruited between July and November 2013. They were over 20 years old and had moderate-severe fatigue for more than 6 months without malignancy or acute/chronic illness. We randomly assigned 30 participants to the vitamin C group and 30 to the placebo group, for a total of 60 enrolled participants (1:1 for two groups). The blinded participants received a single intravenous treatment of either vitamin C (20 ml of ascorbic acid 500 mg/ml, colorless transparent solution, Uni-C®, Unimed, Korea) with normal saline or same volume of normal saline. The Institutional Review Board of Gangnam Severance Hospital, Yonsei University College of Medicine approved this study, and informed consent was obtained from each participant. This clinical trial is registered at ClinicalTrials.gov. (NCT01926132) and was permitted by Korean Ministry of Food and Drug Safety (20130091445).

2. Participants

The sample size was estimated based on the results of a pilot study using a 2-sided 2-sample t-test: type 1 error 0.05, type 2 error 0.2, and drop-out rate 20% by a statistician. The mean difference in mtDNA copy number was 38.37±

34.75 and the mean difference in fatigue scores was -3.09± 1.11 in our pilot study. The necessary sample size was calculated to 11 participants in each arm. Using the mean difference in fatigue scores from a previous study,[29] the necessary sample size was estimated to be 30 people per group.

Sixty-two participants were screened for the following criteria: fatigue period ≥ 6 months, age ≥ 20 years, brief fatigue inventory-Korean (BFI-K) ≥ 4 (moderate to severe fatigue), and no abnormal laboratory test (glucose-6 phosphate dehydrogenase (G6PD), white blood cell count, hemoglobin, creatinine, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), thyroid stimulating hormone (TSH), or urinalysis in last 3 months.

Participants were excluded for pregnancy; lactation; history of common cold, acute gastroenteritis, uncontrolled diabetes, uncontrolled hypertension, liver disease or renal disease, gout, renal calculi; hypersensitivity to intravenous vitamin C injection, oral vitamin supplement intake within 2 days and a history of drugs that are to interaction with vitamin C, such as aspirin, appetite deprivation agents, iron supplements, phenytoin, estrogen, tetracycline, Coumadin and corticosteroids. Participants received written instructions to not take any additional vitamin supplements, but we did not limit fruit or vegetable intake.

3. Random Assignment and Interventions

After receiving a consent form, we measured height, weight, and blood pressure. All participants reported smoking status, alcohol intake, exercise, coffee consumption, depression, and sleep quality. We randomly assigned participants to the vitamin C and placebo groups. A computerized randomization list was generated by a statistician, sealed in an opaque envelope, and delivered to a pharmacist. She opened the envelope containing the randomization list in a closed room. She prepared injection materials mixing either vitamin C 10 g (20 ml) or normal saline 20 ml in normal saline bottles, according to the randomization list, then had no further involvement in the study.

A study nurse assigned consecutive numbers to participants in order of enrollment. The mixed solution of the same number was administered to each participant by the study nurse. The participants and study nurse assessing the outcomes were blinded to the group assignment until the end of trial. The vitamin C group received vitamin C 10 g in 130 ml of normal saline intravenously over 30 minutes, while the placebo group received the same volume (150 ml) of normal saline in the same manner. Each solution was concealed to protect against sunlight. Therefore, participants could not determine their study arm. After 15 minutes rest, participants return home, they were followed up 2 weeks later.[31]

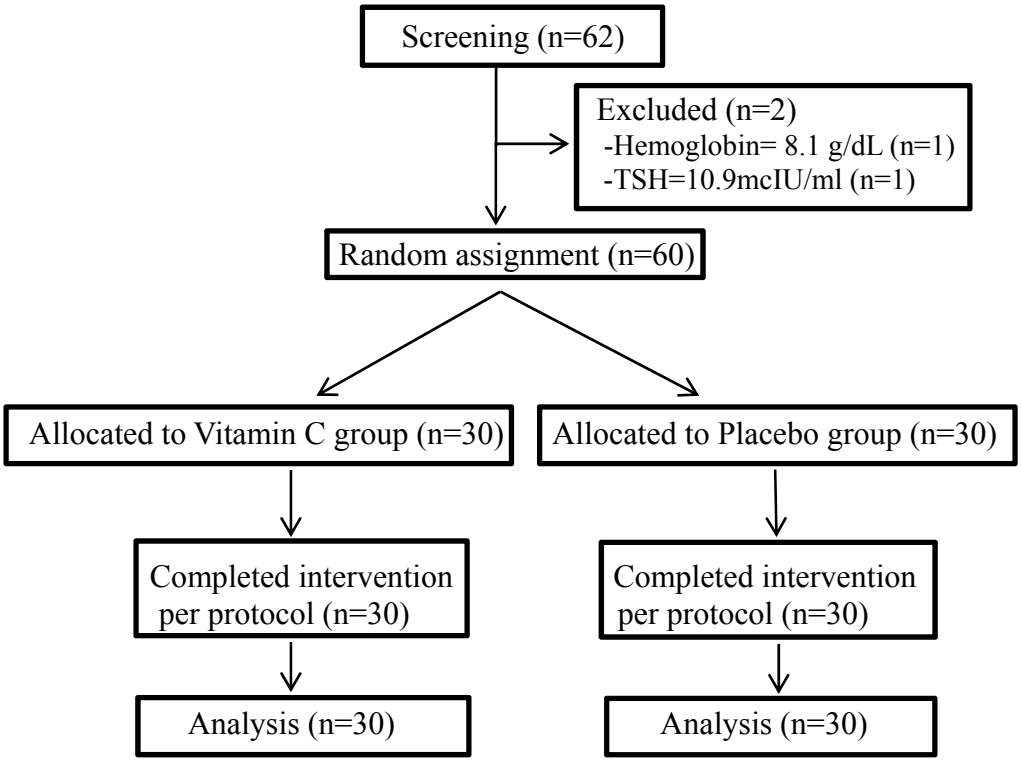

Figure 1. Study flow diagram.

4. Measurements of fatigue scale and mtDNA copy number

A. Assessment of Fatigue scale

We measured fatigue using the Brief Fatigue Inventory-Korean version (BFI-K). It consists of 9 questions. Fatigue and its interference with daily living were scored by patients on a scale from 0 to 10, which ranged from "no fatigue" to "fatigue as bad as you can imagine." The internal consistency of Korean

version was very high (Cronbach's α coefficiency; 0.96), and the validity was confirmed by factor analysis.[32] The BFI-K was evaluated for fatigue "now", "usual" fatigue, and "worst" fatigue during the previous 24 hours. These aspects included general activity, mood, walking ability, normal work (both outside of the home and daily chores) relationships with other people and enjoyment of life. Fatigue severity was then categorized into 3 groups: a global score of 1-3 was considered mild, a score of 4-6 was moderate, and a score of 7-10 was severe.[33] In the general Korean population, BFI scores for ''worst'' fatigue were 4.33±2.48 and for ''usual'' fatigue were 4.07 ± 2.27.[34] Therefore, BFI-K scores of moderate-severe fatigue patients in this study (BFI-K score ≥ 4) were similar to mean values for "usual" and "worst" fatigue in general population.

B. Quantification of mtDNA copy number

(A) DNA extraction

Blood and buccal epithelial cell samples were collected from each participant. Blood from a peripheral vein was collected in tubes containing EDTA. Buccal epithelial cells were collected from saliva rubbed with oral mucous against teeth. Saliva samples were washed by centrifuging 1,200 x gravity (g) for 3 min with phosphate buffered saline. DNA components from whole blood and saliva

was extracted using the Genomic Blood Spin Mini Kit and Genomic Cell/ Tissue Spin Mini Kit (Nucleogen, Ansan, Korea), respectively. The quantity and quality of DNA was measured with an Epoch spectrophotometer (BioTek, Winooski, VT, USA).

(B) Real-time quantitative PCR assay

The mtDNA content was measured by a real-time quantitative polymerase chain reaction (qPCR) for mitochondrial encoded NADH dehydrogenase 1 (*MT-ND1*), which was normalized to hemoglobin beta (*HBB*) (encoded by nuclear DNA). qPCR was performed using the CFX96 Real-time System (Bio-Rad, Singapore). The 15-μl reaction mixture contained 10 ng of template DNA, 1X Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan), and 7.5 pmole of primers. Thermal cycling conditions consisted of 1 cycle at 95°C for 60 sec, followed by 35 cycles of 95°C for 5 sec, 61°C for 10 sec and 72°C for 20 sec. After the PCR, a dissociation curve analysis was performed from 61°C to 95°C (in 0.5°C increments) to confirm specific amplification.

To assess the efficiency of all primer pairs, the standard curves were derived from 7 serial dilutions of reference DNA 40, 20, 10, 5, 2.5, 1.25, and 0.625 ng, using the Bio-Rad CFX Manager version 1.6 software (Bio-Rad). Primers for *ND1* were 5'-GACCCTACTTCTAACCTCCCTGT-3' and 5'-TAGGAGGTGTATGAGTTGGTCGT-3'. *HBB* primers were 5'-

ACCCAAGAGTCTTCTCTGTCTCCA-3' and 5'-TCTGCCGTTACTGCCCTGTG-3'. We also screened the UCSC database (http://genome.ucsc.edu/) to confirm unique sequence without any repeat sequences in the primers. The threshold cycle number (Ct) indicates the fractional cycle number, which is automatically determined for each primer. The ΔCt represented the relative abundance calculated as [Ct (*ND1*) – Ct (*HBB*)]. The mtDNA was quantified using $2 \times 2^{-\Delta Ct}$.[35] All experiments contained reference DNA and were repeated at least 3 times. Each experiment was normalized against serial dilutions of a control DNA sample.[36]

C. Assessment of depression and sleep quality

We assessed depression and sleep quality by self-reported questionnaire. The Beck Depression Inventory (BDI) is one of the most widely used self-report instruments for measuring the severity of depression. It is useful in screening depression in the general population, as well as measuring the severity of depression among psychiatric patients. The Pittsburgh Sleep Quality Index (PSQI) is used worldwide to evaluate sleep quality during the previous month. The Korean version of the PSQI is reliable (Cronbach's α coefficiency; 0.84), sensitive (0.943), and specific (0.844).[37]

5. Assessment of Adverse Events

During the intervention period, study nurses assessed adverse events by asking open-ended questions about duration, intensity, required treatment and outcome.

6. Statistical Analysis

All analyses were performed with SPSS for Windows (version 18.0; SPSS Inc., Chicago, IL, USA). Clinical characteristics were shown between the vitamin C and placebo groups. Data are presented as mean (standard deviation) and categorical variables are presented as frequencies (percentages). mtDNA copy numbers were normalized by logarithmic transformation. Clinical characteristics were compared using Students *t*-test or Chi-squared test. Analyses were performed with the "intention-to-treat" method. Predefined primary endpoints were the differences in mtDNA copy number and mean fatigue scores between before and 2 weeks after the intervention by paired t-test. In subgroup analyses, changes in mtDNA copy number were compared according to fatigue recovery and intervention using analysis of covariance (ANCOVA). Participants were divided in quartiles by initial mtDNA copy number. After the intervention, changes in mtDNA copy number and fatigue scores were compared using ANCOVA and p-trend. Significance was defined at the 0.05 level.

III. RESULTS

Of the 62 participants initially recruited, 60 (30 in the vitamin C group and 30 in the placebo group) were enrolled (**Figure 1**). No participants were dropped; all completed the trial. **Table 1** summarizes the baseline characteristics. There was no G6PD deficient subject. Two participants were excluded for anemia and hypothyroidism. The mean age was 41.4 years, female gender was dominant (81.6%). There were differences between groups in comorbidity, medications, and worst fatigue in the previous 24 hrs.

Table 1. Baseline characteristics of study population

	Vitamin C group (n=30)	Placebo group (n=30)
Age, years (SD)	41.5 (8.6)	41.3 (13.4)
Sex, n		
Male (%)	4 (23.3)	7 (23.3)
Female (%)	26 (86.7)	23 (76.7)
BMI, kg/m^2 (SD)	22.2 (3.3)	22.6 (3.0)
Smoking status, n (%)		
Current smoker	2 (6.7)	1 (3.3)
Former smoker	3 (10.0)	1 (3.3)
Non smoker	25 (83.3)	28 (93.3)
Drinking status, n (%)		
No drinking	15 (50.0)	16 (53.3)
Drinking(more than one day/week)	15 (50.0)	14 (46.7)

Exercise, n (%)		
No exercise	17 (56.7)	12 (40.0)
One day/week	10 (33.3)	10 (33.3)
More than 2 day/week	3 (10.0)	8 (26.7)
Comorbidity*, n (%)		
None	29 (96.7)	26 (86.7)
Hypertension	0	2 (6.7)
Depression	0	0
Sleep problem	0	0
Osteoporosis	0	2 (6.7)
BPH	1 (3.3)	0
Medication*, n (%)		
None	29 (96.7)	25 (83.3)
Anti-hypertension	0	2 (6.7)
NSAIDs	0	1 (3.3)
5a-reductase inhibitor	1 (3.3)	0
bisphosphonate	0	2 (6.7)
Coffee intake (cup/week)	5.6	5.6
Brief Fatigue Inventory, mean (SD)		
Mean value	6.3 (1.1)	6.6 (0.9)
Right now	6.6 (1.6)	7.0 (1.1)
Usual fatigue during 24hrs	6.7 (1.6)	7.1 (1.3)
Worst fatigue during 24hrs*	7.6 (1.4)	8.5 (1.1)
General activity	6.6 (1.7)	6.3 (1.1)
Mood	6.2 (2.0)	6.4 (1.3)
Walking ability	5.2 (2.3)	5.0 (2.6)
Normal work	6.9 (1.7)	6.9 (1.3)

Relation with other people	6.0 (1.7)	6.2 (1.9)
Enjoyment of life	5.2 (2.4)	5.5 (2.5)
mtDNA copy number, mean (SD)		
peripheral blood	71.89 (35.14)	74.18 (29.12)
saliva	398.3 (326.7)	349.0 (305.6)
Beck Depression Inventory, mean (SD)	14.3 (7.7)	16.7 (5.6)
Pittsburgh Sleep Quality Index, mean (SD)	6.3 (2.4)	6.2 (2.2)

***** <0.05

1. Effect of Vitamin C on Fatigue scale

Both the vitamin C and placebo groups had lower fatigue scores (p=0.006, p=0.001, respectively). The score of fatigue and general activity were lower. But there was no difference between groups (p=0.582, **Table 2**).

Table 2. The changes of fatigue score between vitamin C group and placebo group.

	Vitamin C			Placebo			p-value
	pre	post	p-value	pre	post	p-value	
Mean	6.3±1.1	5.1±2.1	0.006	6.6±0.9	5.6±1.6	0.001	0.582
Right now	6.6±1.6	5.6±2.1	0.019	7.0±1.1	6.1±1.8	0.010	0.808
Usual fatigue	6.7±1.6	5.4±1.8	0.001	7.1±1.3	5.8±1.7	<0.001	0.999
Worst fatigue	7.6±1.4	6.3±2.3	0.002	8.5±1.1	6.8±1.8	<0.001	0.553
General activity	6.6±1.7	4.9±2.3	0.001	6.3±1.1	5.4±2.0	0.015	0.159

Mood	6.2±2.0	5.0±2.4	0.033	6.4±1.3	5.7±2.2	0.091	0.533
Walking ability	5.2±2.3	4.5±2.8	0.212	5.0±2.6	4.7±2.2	0.596	0.572
Normal work	6.9±1.7	5.1±2.3	0.001	6.9±1.3	5.5±2.2	0.001	0.499
Relation with other people	6.0±1.7	5.1±2.6	0.112	6.2±1.9	5.1±2.1	0.002	0.569
Enjoyment of life	5.2±2.4	4.5±2.7	0.308	5.5±2.5	5.5±2.0	0.928	0.389

2. Effect of Vitamin C on mtDNA copy number

In the vitamin C group, mean mtDNA copy number of blood and saliva was sustained or increased, while that of placebo group was decreased. However, there were no statistically differences (**Table 3**).

Table 3. Pre- and post-mean mtDNA copy number of blood and saliva samples

	Vitamin C group			Placebo group		
	pre	post	*p*-value	pre	post	*p*-value
Blood	71.89±35.14	72.08±34.33	0.688	74.18±29.12	66.47±31.74	0.283
Saliva	398.3±326.7	415.6±347.5	0.915	349.0±305.6	317.2±270.2	0.631

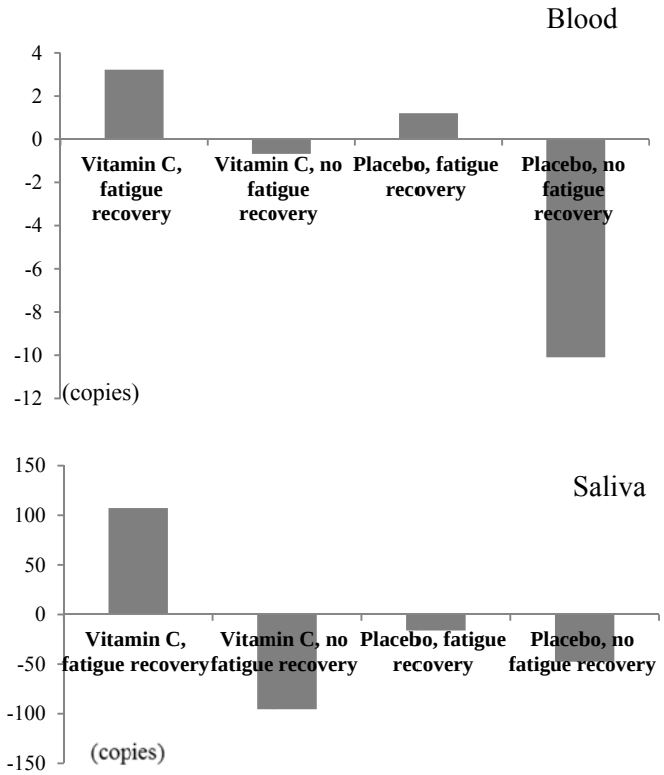

Figure 2. The changes of mtDNA copy number according to fatigue recovery and intervention

Subjects were divided into 4 groups according to fatigue recovery and intervention. This fatigue recovery was defined as decreasing score from severe to moderate or mild; moderate to mild categories after intervention. The vitamin C and fatigue recovery group increased the mean mtDNA copy number in blood and saliva samples. The non-fatigue recovery subjects decreased the

mtDNA copy numbers, although they had injection of vitamin C. In spite of placebo group, subjects experiencing fatigue recovery increased the mtDNA copy number in peripheral blood (**Figure 2**). However, there were no significances (**Table 4**). More subjects in the vitamin C group (15/15, 50%) had fatigue recovery than in the placebo group (8/22, 27%) by Chi-squared test (*p*-value=0.055).

Table 4. Mean mtDNA copy number according to intervention and fatigue recovery

	mtDNA copy number of blood		mtDNA copy number of saliva	
	pre	post	pre	post
Vitamin C, fatigue recovery (n=15)	68.94±39.40	72.16±38.30	386.5±289.1	493.7±383.9
Vitamin C, no fatigue recovery (n=15)	67.71±28.16	67.03±31.08	482.4±473.8	386.9±330.2
Placebo, fatigue recovery (n=8)	59.09±29.80	60.29±30.33	342.8±224.3	326.6±301.0
Placebo, no fatigue recovery (n=22)	76.66±26.68	66.56±31.73	416.3±368.7	368.7±314.3
P trend	0.232	0.686	0.856	0.751

Adjusted for age, smoking status, comorbidity and medication history by ANCOVA test.

3. Effect of Vitamin C on mtDNA copy number and BFI-K score

Changes in mtDNA copy number and BFI-K are shown for quartiles by initial mtDNA copy number in **Table 5, 6 and Figure 3**. Q_1 and Q_2 of both the vitamin C and placebo groups had increased mtDNA copy numbers. Q_3 and Q_4 of both groups had decreased mtDNA copy numbers. The vitamin C group was significantly different (blood: p=0.001, saliva: p=0.031). Participants with lower initial mtDNA copy numbers had larger increases in mtDNA copy number and greater fatigue recovery than did participants with higher initial mtDNA copy numbers. The vitamin C group had significant changes than did placebo group. Differences of mean fatigue score and in the categories of usual fatigue, general activity, mood, and relation were significant (**Table 5**).

Table 5. The changes of mtDNA copy number and BFI-K score according to quartiles of initial mtDNA copy number in blood sample

			Q_1	Q_2	Q_3	Q_4	*P*-trend
ΔmtDNA copy number		Intervention	35.98±38.77	12.76±39.90	-18.09±43.65	-44.51±22.98	0.001
		placebo	23.71±25.85	14.29±42.05	-23.95±25.65	-24.93±32.01	0.059
BFI-K	ΔMean	Intervention	-2.5±2.7	-0.19±0.59	0.26±1.4	-0.39±1.3	0.026
		placebo	-1.2±1.7	-0.87±0.62	-0.99±1.1	-0.96±1.0	0.727
	ΔRight now	Intervention	-2.2±3.2	-0.86±1.3	0.33±1.0	-1.0±2.5	0.139
		placebo	-2.2±2.3	-0.57±1.7	-0.56±1.1	-0.33±2.1	0.071
	ΔUsual fatigue	Intervention	-2.8±1.9	-0.43±1.3	-0.33±1.6	-1.0±2.4	0.014

	placebo	-1.8±1.3	-0.86±1.8	-1.1±2.0	-1.3±2.3	0.637
ΔWorst fatigue	Intervention	-2.2±2.2	-0.71±1.4	-0.50±1.9	-1.1±2.7	0.135
	placebo	-2.2±2.4	-1.1±1.7	-1.9±1.6	-1.7±1.0	0.729
ΔGeneral	Intervention	-3.1±2.7	-0.57±1.6	-0.83±1.7	-1.4±2.7	0.011
activity	placebo	-0.83±3.5	-1.0±0.82	-1.1±2.0	-0.5±1.0	0.797
ΔMood	Intervention	-3.0±2.9	0.0±1.3	0.17±2.8	-1.4±3.7	0.018
	placebo	-1.3±3.8	-0.43±1.3	-0.33±2.0	-1.3±2.9	0.925
ΔWalking ability	Intervention	-0.78±4.8	-0.57±2.0	1.2±2.3	-2.6±3.0	0.604
	placebo	0.33±1.0	0.86±4.6	-0.56±3.5	-1.7±1.9	0.235
ΔNormal work	Intervention	-2.8±3.9	-1.4±1.1	-0.83±1.9	-1.4±3.1	0.063
	placebo	-1.7±2.3	-1.0±2.6	-1.4±2.5	-1.2±1.0	0.692
ΔRelation	Intervention	-2.8±3.0	0.0±1.8	0.67±2.6	-0.57±3.3	0.009
	placebo	-1.5±1.9	-0.43±1.3	-1.6±2.7	-1.5±1.9	0.830
ΔEnjoyment of	Intervention	-2.6±3.8	-0.29±2.4	2.5±4.3	-1.6±3.0	0.254
life	placebo	0.0±1.5	0.0±1.3	-0.33±2.9	0.83±1.9	0.589

Adjusted for age, smoking status, comorbidity and medication history.

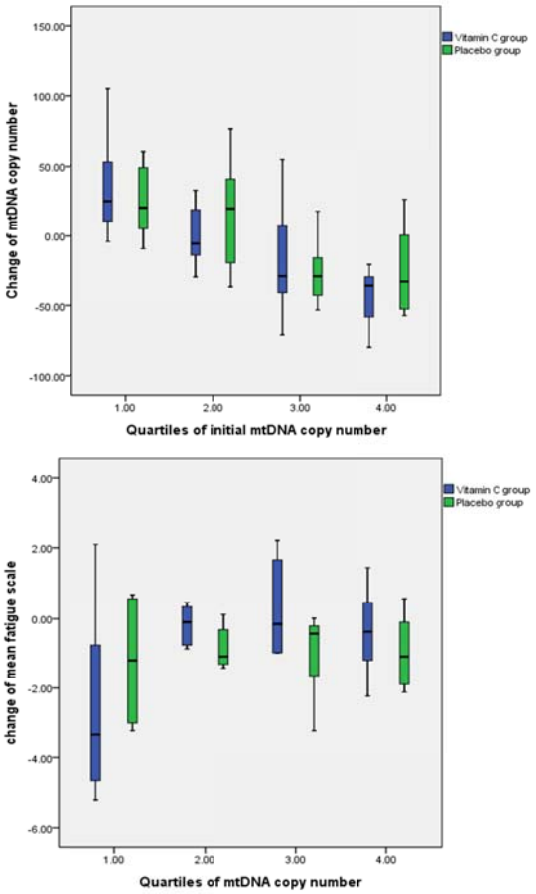

Figure 3. The changes of mtDNA copy number and mean fatigue scale according to initial blood quartile mtDNA copy number

In saliva sample analysis, the mtDNA copy number showed the same feature among the quartiles, and vitamin C group had a significant difference. However, BFI-K scale did not show the trend (**Table 6**).

Table 6. The change of mtDNA copy number and BFI-K score according to quartiles of initial mtDNA copy number in saliva sample

			Q_1 (<165.2copies)	Q_2 (165.2-343.2)	Q_3 (343.2-530.8)	Q_4 (>530.8)	*P*-trend
ΔmtDNA copy number		Intervention	129.5±177.8	139.5±408.4	-86.9±246.1	-439.1±608.0	0.031
		placebo	167.1±305.5	259.3±413.0	36.9±182.0	-341.0±657.7	0.099
BFI-K	ΔMean	Intervention	-0.89±2.4	-0.90±2.1	-2.0±2.5	-1.0±2.1	0.740
		placebo	-0.67±1.0	-0.71±9.4	-1.3±1.8	-1.0±0.7	0.639
	ΔRight now	Intervention	-1.3±2.0	-0.29±1.89	-1.1±2.4	-1.0±2.3	0.801
		placebo	0.16±1.5	-1.5±1.8	-1.25±2.1	-1.2±1.9	0.309
	ΔUsual fatigue	Intervention	-0.86±1.3	-1.4±2.2	-1.9±2.5	-1.1±2.1	0.824
		placebo	-0.75±1.3	-1.5±2.0	-2.1±2.1	-0.7±1.5	0.358
	ΔWorst fatigue	Intervention	-0.86±1.7	-0.43±2.8	-2.9±2.4	-1.3±2.2	0.184
		placebo	-0.86±0.8	-1.7±1.0	-2.4±2.5	-1.7±1.5	0.355
	ΔGeneral activity	Intervention	-1.1±2.8	-1.9±2.6	-2.1±2.9	-1.7±1.9	0.898
		placebo	0.16±2.4	-0.88±1.4	-2.0±2.1	-0.83±0.9	0.169
	ΔMood	Intervention	-1.0±4.0	-1.3±2.3	-2.0±2.8	-0.67±2.9	0.850
		placebo	-1.0±3.2	-1.0±2.4	-1.1±2.2	0.33±1.8	0.684
	ΔWalking ability	Intervention	-1.1±3.8	-0.86±3.8	-1.3±3.1	0.0±3.0	0.872
		placebo	-1.3±1.7	1.1±3.1	0.75±3.9	-2.2±2.0	0.112
	ΔNormal work	Intervention	-1.1±3.0	-1.3±3.3	-2.4±2.4	-2.2±2.8	0.776
		placebo	-0.88±1.5	-1.1±1.9	-1.6±2.6	-2.0±2.6	0.768
	ΔRelation	Intervention	-0.57±3.2	0.14±2.8	-2.4±2.5	-0.67±3.0	0.409
		placebo	-1.4±1.8	-0.63±0.7	-2.0±3.3	-0.83±0.8	0.545
	ΔEnjoyment of life	Intervention	0.0±5.1	-0.86±3.1	-2.1±3.9	0.0±2.9	0.670
		placebo	0.0±2.6	0.88±1.8	-0.38±1.8	-0.33±2.0	0.438

Adjusted for age, smoking status, comorbidity and medication history.

4. Adverse events

Adverse events after intervention are shown in **Table 7**. No participants withdrew from the trial due to an adverse event. Adverse events did not differ between groups, and the occurrence was lower than previously reported.[29]

Table 7. Adverse events after intervention

	Vitamin C group (n=30)	Placebo group (n=30)
Withdrawal from trial for adverse event, n	0	0
Total adverse events, n (%)	2 (6.7)	2 (6.7)
Itching sense/pain at injection site	1	1
Dry mouth	1	0
headache	0	1
nausea	0	0
myalgia	0	0
dizziness	0	0
Chest discomfort	0	0
palpitation	0	0

IV. DISCUSSION

In this study, we cannot confirm the fatigue recovery by high-dose vitamin C intravenous injection. And this injection did not change the mtDNA copy number in chronic fatigue patients. Although more subjects had fatigue recovery in the vitamin C group than in the placebo group, we cannot get what we wanted to know, and the effect of vitamin C was not uniform for these participants. However, the vitamin C and fatigue recovery group had higher mean mtDNA copy numbers in subgroup analyses. Though the vitamin C group, participants without fatigue recovery did not show any increase in mean mtDNA copy number. The placebo group with fatigue recovery from any causes showed an increase of mtDNA copy number. From this study, we cannot insist that fatigue was recovered by increasing the mtDNA copy number. However, considering the effect of vitamin C was not clinically uniform, patient with fatigue and low mtDNA copy numbers may receive more benefit from this treatment than might patients with high mtDNA copy numbers.

MtDNA copy number was regulated by tissue type and smoking pattern.

The effect of vitamin C may be different in tissue. Vitamin C enters mitochondria through the facilitative glucose transporter 1 (Glut 1) where the

vitamin C is oxidized and protects mitochondria against oxidative injury.[22] Vitamin C in its oxidized form, dehydroascorbic acid, is transported into mitochondria and reduced to mitochondrial ascorbic acid, which quenches ROS and inhibits mitochondrial membrane depolarization.[22] The distribution of transporters differs by cell type. Although, the regulation mechanism of mtDNA copy number is unclear, in a previous study, mtDNA copy number of blood and buccal mucosa showed the same variations from intervention.[38] Moreover, mtDNA mutation in non-malignant patients was detected more efficiently in saliva samples than in blood samples.[39] However, in this study, mtDNA variations of blood and saliva did not match. These discrepancies can be explained by age, mtDNA turnover, and metabolic activity of cells in the participant.[2] The absolute mtDNA copy numbers differ in liver, brain, and muscle tissue (**Figure 4**).[40] These tissue-specific mtDNA copy number indicates the metabolic activity of cells.[2] In this study, vitamin C injection changed the metabolism of blood mtDNA in fatigue patients 2 weeks later. However, mtDNA turnover and metabolism of salivary mtDNA may differ. In buccal epithelial cells, mtDNA may be turned over slowly, because the metabolic activity is lower than in other tissues.[38] Mutation of some mtDNA copies is called heteroplasmy. Heteroplasmy can be different in a single person and in time. These variations have been associated with disease severity. [41]

Another, ROS from smoking can affect cellular vitamin C levels and mtDNA damage.[26,42]

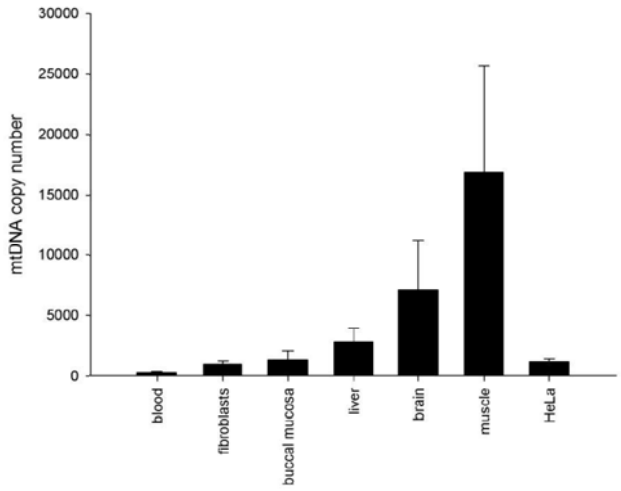

Figure 4. Comparison of the absolute mtDNA copy number in different tissues.[40]

Possible mechanisms of changing mtDNA copy number

In a previous study, mtDNA copy number was negative correlated with its oxidative stress.[43] Vitamin C may affect cellular metabolism by increasing the expression of antioxidant enzymes. Participants with lower initial mtDNA copy numbers had larger increases in mtDNA copy number and fatigue recovery.

The mechanism remains uncertain. In the threshold hypothesis of mtDNA copy number, a low mtDNA copy number triggers replication by up-regulating transcription-related proteins such as DNA polymerase γ (POLG)[44], mitochondrial single-stranded DNA-binding protein (mtSSBP)[45], Twinkle (the mitochondrial helicase)[46] and mitochondrial transcription factor A (TFAM)[45,47]. A higher mtDNA copy number triggers degradation. These changes push the mtDNA copy number toward a middle range and regulate the amount of mtDNA in each cell.[2] This study is consistent with the threshold hypothesis. In fatigue patients who have decreased metabolic activity, vitamin C may modify metabolism rate through the regulation of TFAM expression.[31,48]

Several mechanisms have been proposed for TFAM to regulate mtDNA copy number.[49] First, a higher frequency of TFAM binding at a light strand promoter increases transcription-mediated priming of replication. In another, genome-wide binding by TFAM stabilizes steady-state levels of mtDNA, perhaps by reducing the rate of DNA turnover. Lastly, the regulation of TFAM activity

contributes to copy number control.[49] Oral vitamin C administration has been reported to prevent ROS from exercise from accumulating by regulating TAFM expression in rat.[48]

High-dose vitamin C has been assumed to act as pro-oxidant promoting DNA single-strand breakage in vitro.[50] Removing an electron from ascorbic acid generates ascorbic free radical, which is generally considered a relatively stable radical.[51] In vivo, ascorbic free radical was rapidly eliminated, as evidences by a pro-oxidant biomarker, and there was no evidence of a pro-oxidative effect at plasma concentrations of less than 100 μmol/L.[52]

There is little evidence to report changes in of mtDNA copy number after intervention. In HIV infected patients, high-dose coenzyme Q supplements, which have oxidative capacity, for 90 days did not change mtDNA levels in fat cells or peripheral blood mononuclear cells.[53] This study is the first study to suggest that intravenous high-dose vitamin C injection can restrictively change the mtDNA copy number.

Our study has some limitations. Participants could not show the same outcomes from vitamin C injection, because chronic fatigue was originated from various etiologies. The range of mtDNA copy numbers was larger than in our pilot test. Because the sample size was estimated from our pilot test and similar trials, it was hard to obtain clear statistical significance in subgroup analysis with these

small participants. Inevitable error in handling and degradation in long post-excision time, autolytic process and freeze-thaw cycles were considered.[54,55] In addition, we did not show a correlation between fatigue scores and mtDNA copy number. Though the questionnaire was well design, it is difficult to relate subjective questions and biologic measurements. However, as the theoretical background was certain, further studies are needed to confirm the correlation between fatigue and mtDNA copy number. Finally, the study was designed with a single injection and measurement 2 weeks later. We planned the follow-up period considering mtDNA turnover from a few studies and clinical application. Further studies are needed to find the effective duration and optimal treatment method.

V. CONCLUSION

High-dose ascorbic acid intravenous injection can reduce chronic fatigue and increase the mtDNA copy number on chronic fatigue patient with low mtDNA copy number. Clinically, fatigue patient with a low mtDNA copy number may receive more benefit from an intravenous injection of ascorbic acid than might patients with a high mtDNA copy number. Further research is needed to confirm the effect of high-dose ascorbic acid on mitochondrial DNA copy number in chronic fatigue patients, considering the optimal treatment method and assessment tool.

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ABSTRACT (IN KOREAN)

만성피로 환자에서 미토콘드리아 DNA 개체 수에 대한 고 용량 비타민 C 정맥 주사의 효과: 무작위배정 임상연구

<지도교수 이 덕 철>

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신 진 영

피로는 활성 산화물질에 의한 미토콘드리아 손상으로 에너지 생성의 효율이 감소되어 나타나는 것으로 생각된다. 항산화 물질인 비타민 C는 피로 회복을 위해 오랜 시간 투여되어 왔지만 아직 그 효과는 불분명하다. 따라서 본 연구는 고농도 비타민 C 정맥 주사 이후 피로도의 감소와 미토콘드리아 DNA 개체 수의 증가를 확인하기 위해 이중 맹검, 무작위 임상시험을 실시하였다. 6개월 이상, 중등도 이상 강도로 피로한 20세 이상 성인 60명을 대상으로 무작위 배정을 통해 30명에게는 비타민 C 10g을, 대조군 30명에게는 동량의

생리식염수를 주사한 후 2주 후, 혈액과 타액에서의 미토콘드리아 DNA 개체 수 측정 및 피로도 설문을 시행하였다.

피로도는 비타민 C 투약군과 대조군 모두에서 호전되었으며, 두 군간의 통계적인 차이는 없었다. (각각 p=0.006, p=0.001; 군간의 차이 p=0.582). 미토콘드리아 DNA 개체 수는 비타민 투약군에서 증가하였으나 대조군에서는 감소하였다. 비타민 투약군에서 피로감 호전을 경험한 대상이 더 많았으며 (p=0.055), 비타민 투여 후 피로도가 호전된 군은 미토콘드리아 DNA 개체수가 증가하였으나, 이외의 군에서는 그 변화가 미미하였다. 초기 미토콘드리아 DNA 개체 수에 따라 사분위로 나누었을 때, 비타민 군에서는 초기 미토콘드리아 DNA 개체수가 적을수록 유의하게 피로도가 호전되고 (p=0.026), 그 개체수가 증가하였다 (p=0.001). 그러나 대조군에서는 뚜렷한 변화가 없었다 (p>0.05). 타액 검체를 통한 초기 미토콘드리아 DNA 개체수에 따른 사분위 군에서는 미토콘드리아 DNA 개체 수는 유의한 변화를 보였으나(p=0.031), 피로도 호전은 확인하지 못하였다(p=0.740). 본 실험에서 중대한 이상반응은 관찰되지 않았다.

본 연구를 통해 모든 대상군에서 고농도 비타민 C 주사를 통해 피로도와 미토콘드리아 DNA 개체수의 의미 있는 변화는 확인하지 못하였다. 그러나 초기 미토콘드리아 DNA 개체수가 적은 피로 환자에서 비타민 C 투약에 따라 피로도 호전과 미토콘드리아 DNA 개체수의 증가를 확인하였으므로, 임상적으로, 고농도 비타민 C 정맥 내 주사는 적절한 대상군에서 효과적일 수 있음을 시사한다. 이에 대한 후속 연구가 필요하다.

핵심 단어: 비타민 C, 미토콘드리아 DNA, 피로