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카르니틴이 비알코올성 지방간 질환에서 말초혈액 미토콘드리아 DNA 단위 반복수와 간기능에 미치는 영향

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Effects of Carnitine on Peripheral Blood Mitochondrial DNA Copy Number and Liver Function in Non-Alcoholic Fatty Liver Disease

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Background/Aims: Functional and anatomical abnormalities of mitochondria play an important role in developing steatohepatitis. Carnitine is essential for enhanced mitochondrial beta oxidation through the transfer of long-chain fatty acids into the mitochondria. We examined the impact of carnitine complex on liver function and peripheral blood mitochondria copy number in NAFLD patients. **Methods:** Forty-five NAFLD patients were enrolled. Patients were categorized into the carnitine complex-administered group and control group. Before and 3 months after drug administration, a liver function test and peripheral blood mitochondrial DNA and 8-oxo-dG quantitive analysis were conducted. **Results:** In carnitine treatment group, ALT, AST, and total bilirubin were reduced after medication. There was no difference in AST, ALT, and total bilirubin between before and after treatment in control group. In carnitine group, peripheral mitochondrial DNA copy number was significantly increased from 158.8±69.5 copy to 241.6±180.6 copy (p=0.025). While in control group the mitochondrial copy number was slightly reduced from 205.5±142.3 to 150.0±109.7. 8-oxo-dG level was also tended to decrease in carnitine group (p=0.23) and tended to increase in control group (p=0.07). **Conclusions:** In NAFLD, the carnitine improved liver profile and peripheral blood mitochondrial DNA copy number. This results suggest that carnitine activate the mitochondria, thereby contributing to the improvement of NAFLD. **(Korean J Gastroenterol 2010;55:384-389)**

Key Words: Nonalcholic fatty liver disease; Mitochondria; Carnitine

Introduction

Non-alcoholic fatty liver disease (NAFLD) is reported to be closely associated with obesity, insulin resistance. Also, NAFLD is related with various systemic complications such as cardiovascular, renal, and metabolic disease regardless of obesity and insulin resistance.[1] However a distinctive pathogenesis has not yet been reported. The development of NAFLD is known

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to be associated with mitochondrial disorders.[2] Mitochondrial disorders associated with NAFLD include structural lesions, mitochondrial DNA copy number reduction, reduced mitochondria respiratory chain (MRC), and mitochondria β-oxidation disorders. In certain researches, when administered with the drug 4,4'-diethylaminoethoxyhexestrol aimed at inhibiting the mitochondria respiratory chain (MRC) activity and mitochondria β-oxidation, it resulted in increased mitochondrial formation of reactive oxygen species and caused lipid peroxidation in rats.[3] Also, according to electron microscope findings of liver tissues in mouse model for β-oxidation defect, mitochondria grew large and swollen, the mitochondrial matrix density decreased, and paracrystalline inclusion was observed, showing structural changes of mitochondria.[4] In NAFLD patients, reduced MRC complexes activity in liver tissues were observed. This dysfunction is associated with serum tumor necrosis factor alpha, body mass index, and insulin resistance.[5] In diabetes similar to NAFLD, peripheral blood mitochondrial DNA copy number was decreased before the occurrence of diabetes.[6,7]

Wu et al.[8] reported glycyrrhizin reduced hepatic lipotoxicity by stabilizing the integrity of lysosomes and mitochondria. This suggests the restoration or protection of mitochondrial function is one of therapeutic option for NAFLD. Several anti-oxidative scavengers such as Coenzyme Q_{10}, carnitine, vitamins, and n-3-polyunsaturated fatty acid suggested modulate mitochondrial beta oxidation and function.[9] L-carnitine is a cofactor of carnitine palmitoyltransferase 1 (CPT1), and L-carnitine in hepatocytes modulate hepatic CPT1A activity. Recent data suggested that the overexpression of CPT1 maintain mitochondrial function and ameliorate lipid induced insulin resistance through acceleration of β-oxidation. Carnitine was involved in several physiological roles: Carnitine acts as free radical scavenger and essential for the transfer of long-chain fatty acids into the mitochondria.[10]

This study aimed to define the effects of carnitine on liver functions and peripheral blood mitochondrial DNA copy number in NAFLD patients.

Materials and Methods

1. Patients

Forty-five NAFLD patients who visited the gastroenterology department with an increase in AST/ALT were enrolled. All patients had fatty liver on abdominal sonography and allocated by non-randomized and open labeled manner. The definition of NAFLD was as follows (i) subjects with an alcohol consumption $\leq$20 g/day, (ii) subjects who were not taking drugs such as any herbal medication, amiodarone, methotrexate, synthetic estrogens, nucleoside analogues and gluococorticoids within three months, (iii) negative study for viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis, drug-induced liver disease, and thyroid disease (hepatitis B surface antigen, anti-HCV antibody, anti-nuclear antibody, anti-mitochondrial antibody, thyroid function test).

The patients were categorized into the carnitine complex (Godex®, Hanseo pharm. Co.,Ltd, Seoul, Korea) administered group and the control group. Carnitine group took carnitine with a dose of 600 mg/day. Before and 3 months after drug administration, with patients diagnosed as NAFLD, liver function tests such as albumin, total bilirubin, AST, ALT, γ-GTP, and as well as mitochondrial DNA content were conducted. The institutional review boards of hospital approved the study.

2. Measurement of peripheral blood mitochondrial DNA

Before and after each drug administration, 5 mL of peripheral venous blood was taken, put in the EDTA tube, and immediately kept at -80^{o}C. At room temperatures, with the blood centrifugation, DNA was extracted using QIAmp DNA Mini Kit (QIAGEN, Co., Ltd, Düsseldorf, Germany), and peripheral blood mitochondria DNA was measured using real time PCR. Probes for GAPDH and mtDNA were made, and the 5' end and the 3' end were marked with 5-carboxyfluorescein (FAM) reporter and 6-carboxy-tetramethyl-rhodamine (TAMRA) quencher, respectively. mtDNA was made to undergo PCR, the PCR mixture, totaling 15 μL, consisted of TaqMan Universal PCR Master Mix (ABI, USA) 7.5 μL, primer forward (5 pmol/mL) 1.5 μL, primer reverse (5 pmol/mL) 1.5 μL, probe (1 pmol/ μL) 1.5 μL, distilled water 0.5 μL, and DNA 2.5 μL. PCR was conducted using the ABI 7500 Real-Time PCR System, with the amplification environment involving 2 minutes at 50^{o}C, one time 10 minutes at 95^{o}C, and 15 seconds at 95^{o} and one-minute 40-time repetition at 60^{o}. To analyze the content of mitochondrial DNA copy number, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was used. Primers and probes used are as follows: GAPDH forward primer; CCA GGT GGT CTC CTC TGA CTT C, GAPDH

reverse primer; GTG GTC GTT GAG GGC AAT G, mitochondria forward primer; CCA GCG TCT CGC AAT GCT, mitochondria reverse primer; CTC CAT GCA TTT GGT ATT TTC G, mitochondria probe; FAM - TCG CGT GCA CAC CCC CCA - TAMRA, GAPDH probe; FAM - ACA GCG ACA CCC ACT CCT CCA CCT T - TAMRA.

3. Measurement of 8-oxo-deoxyguanosine (8-oxo-dG)

In extracted DNA, the absorbance of oxidized guanosine was measured at 450 nm wave length using 8-oxo-dG ELISA kit (COSMO BIO Co., Ltd, Tokyo, Japan) which contained 8-oxo-dG antibodies, pursuant to the manufacturer's instructions.

4. Statistical analysis

Clinical findings of the medication groups were displayed using mean and standard deviation in the case of a normal distribution. Differences in the mean value between the two groups were compared using an unpaired *t*-test. In each group, to compare the change of clinical findings, liver function test values, and mitochondrial DNA copy number changes before and after medication, a paired *t*-test was conducted. Statistical significance level was set at p of under 0.05.

Results

1. Clinical characteristics before and after medication

Subjects totaled 45 with 27 males and 18 females, and mean age was 47.0 years old (range: 19-76). Of the subjects, 29 patients were placed on the carnitine complex medication, 16 patients were placed on the control group. Before medication, when clinical characteristics and biochemical variables were compared between the 2 groups, there was no significant difference in age, AST, ALT, total biliruibin, albumin, γ-GTP, free fatty acid, and weight (Table 1).

2. Liver function test and a change in clinical variables after medication

Between groups, 3 months after medication, liver function test results and clinical variables changes were compared. In the carnitine group, ALT, AST, and total bilirubin significantly decreased ($p<0.05$). In the control group, AST, ALT, and total bilirubin average were not significantly changed (Fig. 1).

Table 1. Baseline Characteristics of Subjects

	Control (n=16)	Carnitine group (n=29)	p-value*
Age (yr)	42.25±14.74	49.66±15.34	0.122
AST (IU/L)	43.06±18.48	51.10±26.97	0.295
ALT (IU/L)	50.69±19.08	60.00±49.20	0.375
T-Bil (mg/dL)	0.87±0.38	0.71±0.22	0.078
γ-GTP (mg/dL)	74.62±26.86	58.14±34.84	0.237
Albumin (g/dL)	4.51±0.31	4.62±0.36	0.152
FFA (mg/dL)	806.3±211.6	799.6±439.2	0.965
Bwt (kg)	74.88±11.62	72.95±12.60	0.638
mtDNA copy No.	205.5±72.1	158.8±69.5	0.145
8-oxo-dG (pg/mL)	1.37±0.32	1.35±0.34	0.823

AST, aspartic acid transaminase; ALT, alanine transaminase; Bwt, body weight; T-Bil, total bilirubin; FFA, free fatty acid; γ-GTP, r-glutamyltranspeptidase.
mean±SD.
* $p<0.05$ by one way *t*-test.

3. Change in mitochondrial DNA copy number before and after medication

Baseline mitochondrial DNA copy number of the control group showed higher tendency than carnitine group. However in carnitine group, mitochondrial DNA copy number increased from 158.8±69.5 before treatment to 241.6±80.6 (p=0.025) after treatment. Mitochondrial copy number was unchanged from 205.5±72.1 to 150.0±50.4 after treatment in the control group (p=0.232) (Fig. 2).

4. Change in 8-oxo-deoxyguanosine (8-oxo-dG) before and after medication

Comparing between two groups, there was no statistical difference in base line 8-oxo-dG level between the control and carnitine group (1.37±0.32 *vs.* 1.35±0.34, p=0.82). 8-oxo-dG level of carnitine group was lower than control group after treatment (1.50±0.26 *vs.* 1.28±0.37, p=0.048). Comparing within group, 8-oxo-dG showed decreasing tendency in carnitine group and increasing tendency in control group (Fig. 3).

Discussion

Mitochondria is an important organ that processes internal oxidative stress and simultaneously very susceptible to oxidative stress. Current drugs for mitochondrial function disorders include vitamin E, coenzyme Q-10, creatine, and N-acetylcy-

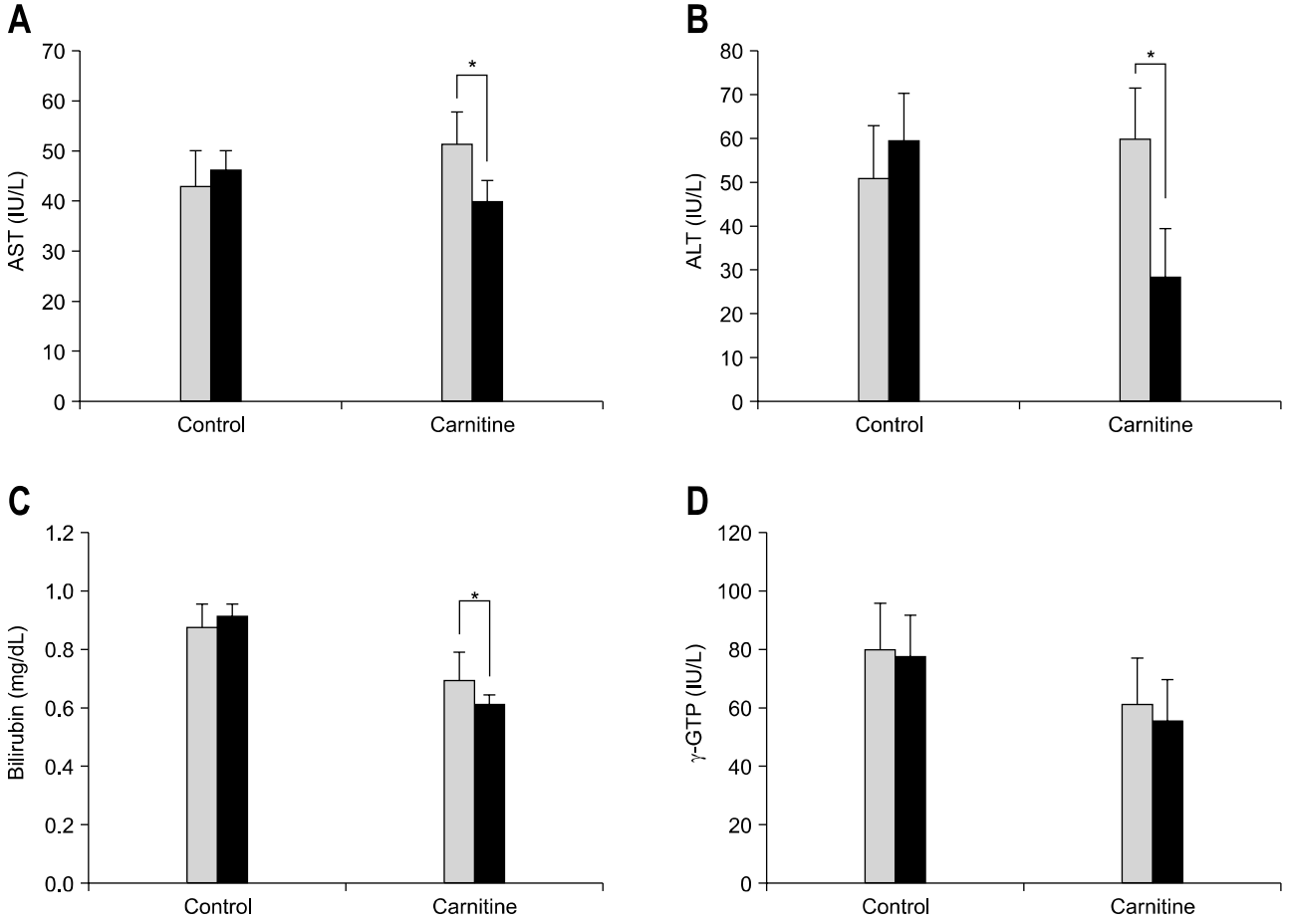

Fig. 1. Changes of liver chemistry between values at baseline and 3 month in the same group. Grey bar, before medication; Black bar, after medication. (A) Aspartic acid transaminase, (B) Alanine transaminase, (C) Bilirubin, (D) γ-GTP.
* $p<0.05$ by paired *t*-test.

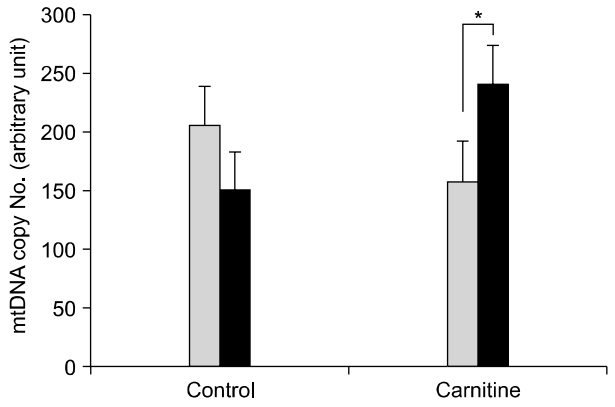

Fig. 2. Changes of mitochondrial DNA copy number before and after medication in both groups. Grey bar, before medication; Black bar, after medication.
* $p<0.05$ by paired *t*-test.

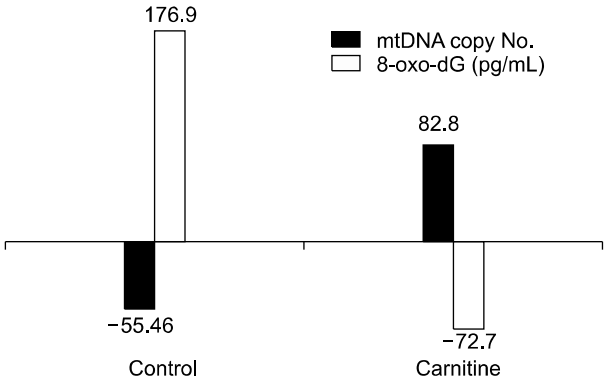

Fig. 3. Difference of 8-oxo-dG before and after medication in both groups.

steine. However, further research is needed to study such drugs' clinical effects.[9] Carnitine is a substance necessary for letting long-chain fatty acids pass the mitochondria internal membrane for inducing β-oxidation.[10] Some researchers report that a lack of carnitine may be associated with fatty deposition in the liver and drug-induced steatohepatitis.[11,12] Other reports say that after carnitine administration in patients with chronic hepatitis C treated with interferon and ribavirin, fatty liver improved.[13] In

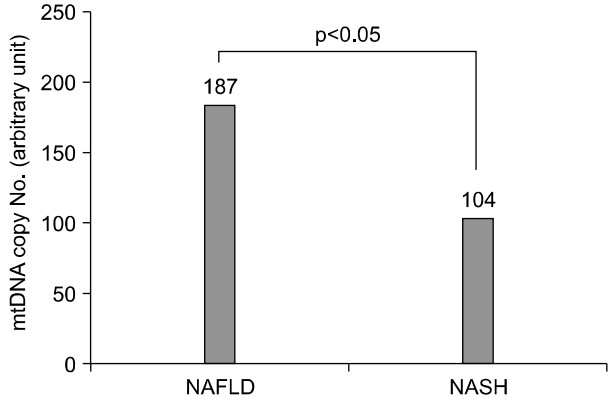

Fig. 4. Peripheral blood mitochondria DNA copy number between biopsy proven steatosis and steatohepatitis.

experiments with animals, after carnitine administration, mitochondrial enzyme activity was found to rise. This is probably because carnitine may increase mitochondrial enzyme activity, boost electron transport within mitochondria.[14] In our study as well, after carnitine complex administration, some liver function test findings indicated improvement, and the mitochondrial DNA copy number also increased. Interestingly, our study found that the carnitine complex medication group increased DNA copy number without changing weight (72.9 kg *vs.* 72.4 kg, p=0.314). Since it was reported that carnitine has a mechanism of inhibiting the development of reactive oxygen species (ROS) which may be a cause for hurting mitochondria, a rise in mitochondrial DNA copy number may be associated with oxidative stress reduction.[15]

Since the DNA copy number varies from one organ to another, the DNA copy number of liver tissue should be measured in order to determine the relation between NAFLD and mitochondrial disease. There are somewhat differences between liver and peripheral blood. It was reported that, in diabetes patients associated with insulin resistance and oxidative stress elevation, the mitochondrial DNA copy number in peripheral blood declined before the occurrence of diabetes. The peripheral blood DNA copy number increased due to reduced weight following physical activity therapies.[7] Although peripheral blood mitochondrial DNA does not perfectly represent the mitochondrial DNA of a particular organ, its correlation with disease can be estimated to a certain degree. An easy securing of specimens is another advantage. Before this study, we investigated the differences of mitochondria DNA copy number between biopsy proven simple steatosis and steatohepatitis patients in peripheral blood. Of fourteen patients who had a liver biopsy, 6 and 8 patients had steatohepatitis and eight were simple steatosis, respectively. In steatohepatitis patients, peripheral blood mitochondrial DNA copy number was lower than simple steatosis patients (104.2±43.1 *vs.* 187.0±92.1, p=0.025) (Fig. 4). Also, since we did not study other diverse functions of and structural changes in mitochondria, we could not identify the direct impact on mitochondria in the hepatocytes, which caused a limitation to our study.

In conclusion, in NAFLD patients, carnitine complex increased peripheral mitochondrial DNA copy number, and showed a reducing tendency of internal oxidative stress. Further research will be necessary to elucidate the role of hepatic mitochondrial function in the development of NAFLD.

요 약

목적: 미토콘드리아의 기능, 구조 이상은 비알코올성 지방간염의 발생에 중요한 역할을 한다. 카르니틴은 장쇄 지방산을 미토콘드리아로 이동시켜 베타 산화를 유발하여 미토콘드리아 기능 유지에 중요한 역할을 한다. 이번 연구의 목적은 비알코올성 지방간 환자에서 카르니틴 투여가 말초혈액 미토콘드리아 수와 간기능에 미치는 영향을 알아보고자 하였다. **대상 및 방법**: 45명의 비알코올성 지방간 환자를 대상으로 카르니틴 복합체 투여군과 대조군으로 나누었다. 약물 투여 3개월 전과 후의 간기능 검사, 산화자극 및 말초혈액에서의 미토콘드리아 수의 변화를 측정하였다. **결과**: 3개월 후, 대조군에서는 ALT, AST 및 빌리루빈 수치에 변화가 없었으나 카르니틴을 투여한 군에서는 의미있게 감소하였다. 카르니틴 투여군에서 말초혈액 미토콘드리아 수는 증가(158.8±69.5 copy *vs.* 241.6±180.6 copy, p=0.025)하였으나 대조군의 경우 말초혈액 미토콘드리아 수는 감소(205.5±142.3 copy *vs.* 150.0±109.7 copy)하였다. 혈청 8-oxo-dG 농도는 미토콘드리아 수와 반대로 카르니틴 군에서는 감소, 대조군에서는 증가하는 경향을 보였다(p=0.23, p=0.07). **결론**: 비알코올성 지방간 환자에서 카르니틴 투여는 간기능 호전 및 말초혈액의 사립체 수를 증가시켰다. 이러한 결과를 볼 때 카르니틴은 미토콘드리아를 활성화시킴으로써 지방간의 호전에 기여를 할 수 있다는 사실을 시사한다.

색인단어: 비알코올성 지방간, 미토콘드리아, 카르니틴

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