Research Article

Anti-adipogenic and antiviral effects of L-carnitine on hepatitis C virus infection[†]

Running title: Anti-HCV effect of L-carnitine

Yoko Tsukuda, Goki Suda, Seiji Tsunematsu, Jun Ito, Fumiyuki Sato, Katsumi Terashita, Masato Nakai, Takuya Sho, Osamu Maehara,Tomoe Shimazaki, Megumi Kimura, Kenichi Morikawa, Mitsuteru Natsuizaka, Koji Ogawa, Shunsuke Ohnishi, Makoto Chuma, and Naoya Sakamoto#

Department of Gastroenterology and Hepatology, Hokkaido University, Sapporo, Japan

#Address correspondence to:

Naoya Sakamoto, MD, PhD

Department of Gastroenterology and Hepatology/Graduate School of Medicine

Hokkaido University

North 15, West 7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan

Phone: +81 11-716-1161; Fax: +81 11-706-7867

Email: sakamoto@med.hokudai.ac.jp

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Abstract

Background and Aims: Hepatitis C virus (HCV) has been reported to hijack fatty acid metabolism in infected hepatocytes, taking advantage of lipid droplets for virus assembly. In this study, we analyzed the anti-HCV activity of L-carnitine, a substance involved in the transport of fatty acids into mitochondria.

Methods: JFH-1 or HCV replicon-transfected Huh7.5.1 cells were treated with or without L-carnitine to examine its anti-HCV effects. The effects of L-carnitine on HCV entry, HCV-induced adipogenesis and lipid droplet formation, and HCV-induced oxidative stress were examined.

Results: Treatment of JFH-1-infected cells with L-carnitine inhibited HCV propagation in a concentration-dependent manner. In contrast, L-carnitine had no anti-HCV activity in the HCV replicon system, which is lacking viral assembly. In addition, L-carnitine did not affect HCV entry. However, L-carnitine treatment decreased intracellular lipid droplets, which are crucial for HCV assembly in JFH-1-infected cells. The expression level of CPT-1 was decreased in JFH-1-infected cells, and L-carnitine treatment restored this expression. HCV-infected cells exhibited increased production of reactive oxygen species and glutathione oxidation. L-Carnitine decreased oxidative stress induced by JFH-1-infection, as shown by glutathione/glutathione disulfide assays and MitoSOX staining.

Conclusions: L-Carnitine exhibited anti-HCV activity, possibly by inhibiting HCV assembly and through its anti-adipogenic activity in HCV-infected cells. Moreover, L-carnitine has antioxidant properties in HCV-infected hepatocytes. Overall, these results indicated that L-carnitine may be an effective adjunctive agent in antiviral therapies to treat chronic hepatitis C.

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Introduction

Hepatitis C virus (HCV) affects 200 million people worldwide and is one of the major pathogens causing liver-related morbidity and mortality. After the development of robust models of HCV replication [Lohmann et al., 1999], a number of novel direct-acting antiviral agents (DAAs) were developed [Sarrazin and Zeuzem, 2010]. Various clinical trials revealed that DAA-based therapies improve the sustained viral response rate to over 90% [Chayama et al., 2012; Suda et al., 2016; Sulkowski et al., 2014]. However, currently available DAAs are not well adapted to patients with decompensated liver cirrhosis, severe renal dysfunction, or multiple resistant associated variants due to failure of combination therapy with DAAs. Therefore, it is important to continue investigating optional antiviral treatment regimens for such patients while awaiting the development of next-generation therapies.

Hepatic steatosis is a characteristic histological finding in the livers of HCV-infected patients [Czaja et al., 1998]. Moriya *et al.* [Moriya et al., 1997] reported that the HCV core protein induces hepatic steatosis in core-Tg mice and that overproduction of lipid droplets is observed in JFH1-transfected cells, but not in JFH1dC3-transfected cells, which lack the coding region of the core protein [Miyanari et al., 2007]. These results indicate that HCV recruits lipid droplets, resulting in hepatic steatosis. Moreover, hepatic steatosis progresses to insulin resistance, hepatic fibrosis, and oxidative stress [Moucari et al., 2008; Vidali et al., 2008] and can cause hepatocarcinoma [Fujinaga et al., 2011; Moriishi et al., 2007]. Treatment of HCV infection with interferon has shown little benefit for hepatic steatosis [Poynard et al., 2003]. HCV takes advantage of host lipid droplets as a scaffold for viral assembly. Miyanari *et al.* [Miyanari et al., 2007] reported that the HCV core protein localizes on the surface of lipid droplets and recruits nonstructural proteins, as well as replication complexes, producing infectious viral particles.

Therefore, we hypothesize that HCV-induced hepatic steatosis is a potential therapeutic target in patients with chronic hepatitis C (C-CH).

In the present study, we focused on L-carnitine (L-b-hydroxy-g-*N*-trimethylaminobutyric acid), which plays a pivotal role in the transport of long-chain fatty acids from the cytosol to the mitochondrial matrix, where β-oxidation takes place [Flanagan et al., 2010]. Rudman *et al.* [Daniel Rudman, 1977] reported that the amount of carnitine was decreased in patients with liver cirrhosis, and Roe *et al.* [Roe et al., 2011] reported that carnitine was significantly decreased by HCV infection, resulting in increased hepatocelluar fatty acids. Recent reports have shown that administration of carnitine improved liver steatosis and fibrosis in patients with nonalcoholic steatohepatitis (NASH) [Malaguarnera et al., 2010] and prevents the progression of NASH in a mouse model of this disorder [Ishikawa et al., 2014]. Therefore, we hypothesized that L-carnitine could improve HCV-induced hepatic steatosis, leading to a decrease in the propagation of HCV. The aims of the present study were to investigate the effects of carnitine on fatty acid metabolism and the propagation of this virus in HCV-infected cells.

Materials and Methods

Reagents and antibodies

Recombinant human interferon (IFN) alpha-2b was purchased from MSD (Tokyo, Japan). L-Carnitine was purchased from Wako Pure Chemical Industries (Osaka, Japan). The anti-core protein antibody and anti-L-carnitine palmitoyltransferase 1 (CPT-1) antibody were purchased from Abcam (Cambridge, MA, USA). The monoclonal antibodies JS-81 and M-L13 against CD81 and CD9, respectively, were from BD Biosciences (San Jose, CA, USA).

HCV replicon cell culture

The HCV subgenomic replicon plasmid, pRep-Feo (genotype 1b and 2a), expresses a fusion protein of firefly luciferase and neomycin phosphotransferase [Kato et al.; Yokota et al.,

2003]. A cell line that stably expressed the Feo replicon was established by transfecting Huh7 cells with Rep-Feo RNA and culturing the cells in the presence of 500 μg/mL G418 (Wako, Osaka, Japan).

Cells and cell culture

Huh7.5.1 cells, provided by C. M. Rice (Rockefeller University, New York, NY, USA) [Moradpour et al., 2003], were maintained in Dulbecco's modified minimal essential medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. To maintain cell lines carrying the HCV replicon (Huh7/Rep cells), G418 was added to the culture medium to a final concentration of 500 μg/mL.

HCV cell culture system

A full-length pJFH1 HCV expression plasmid was used [Wakita et al., 2005]. The plasmid was linearized at the 3′ end and applied as the template for HCV RNA synthesis using a T7 RiboMax Express Large Scale RNA Production System (Promega, Madison, WI, USA). After treatment with DNase I (RQ-1, RNase-free DNase; Promega), the HCV RNA was purified using ISOGEN (Nippon Gene, Tokyo, Japan). For transfection, Huh7.5.1 cells were washed twice with PBS, and 5×10^6 cells were suspended in Opti-MEM I (Invitrogen, Carlsbad, CA, USA) containing 10 μg of HCV RNA. Cells were transferred into a 4-mm electroporation cuvette and subjected to an electric pulse (925 μF and 275 V) using a Gene Pulser II (Bio-Rad, Hercules, CA, USA). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated in a cell culture dish under normal culture conditions.

HCV pseudoparticle (HCVpp) production and infection assays

HCVpps were produced as described previously [Bartosch et al., 2003]. HCVpps were inoculated into Huh7.5 cells with or without 500 μM carnitine. Luciferase activity was measured

72 hours after inoculation [Morikawa et al., 2014].

Luciferase reporter assay

Luciferase activity was quantified with a luminometer (GloMax-Multi+ Detection System; Promega) using the Bright-Glo Luciferase Assay System (Promega). Typically, 5×10^4 cells/cm^2, plated onto 96-well plates, were lysed using 100 μL of 1× Glo lysis buffer. The luciferase activity in 20 μL of the lysate was measured by adding an equal volume of Bright-Glo Luciferase Assay Reagent (Promega).

MTS assay

MTS assays were performed using the CellTiter 96 Aqueous One Solution Proliferation Assay (Promega). Typically, 5×10^4 cells/cm^2 were plated in 96-well plates and treated with 0, 5, 50, 500, or 5000 μM carnitine. After 96 h, 20 μL CellTiter 96 Aqueous One Solution Reagent (Promega) was added to each well, and cells were then incubated for 2 h in a humidified 5% CO_2 atmosphere. Absorbance at 490 nm was recorded using an absorbance spectrometer (GloMax-Multi+ Detection System; Promega).

Quantification of HCV core antigen in culture supernatants

Culture supernatants from Huh7.5.1 cells transfected with HCV RNA were collected and stored at -80°C. The concentrations of core antigen in the culture supernatants were measured using a chemiluminescence enzyme immunoassay according to the manufacturer's protocol (SRL, Tokyo, Japan).

Oil Red O assay

Quantification of intracellular neutral lipids was performed with a Lipid Assay Kit (Primary Cell Co., Ltd, Tokyo, Japan). Huh7.5.1 cells and HCV-RNA transfected cells were cultured in 24-well plates. Cells were fixed with 10% neutral buffered formalin (Wako, Tokyo,

Japan) overnight at room temperature. After washing with purified water, cells were stained with Oil Red O. Stained cells were washed with purified water and dried. The extraction agent (500 μL; Primary Cell Co., Ltd, Japan) was added to each well, and extracts were collected. Intracellular neutral lipids were quantified by measuring the absorbance at 560 nm.

Immunofluorescence staining for the HCV core protein

As described previously [Suda et al., 2010], HCV-JFH1 transfected or infected Huh7.5.1 cells were cultured on 25-mm round micro cover slips (Matsunami, Tokyo, Japan). For detection of the HCV core protein, cells were fixed with cold acetone for 15 min. The cells were incubated with the primary antibodies for 1 h at 37°C, then with Alexa Fluor 594 goat anti-mouse IgG antibody (Invitrogen, Eugene, OR, USA) and BODIPY 493/503 (Invitrogen) for 1 h at room temperature. Cells were mounted with DAPI Fluoromount-G (Southern Biotech, Birmingham, AL, USA) and visualized by fluorescence microscopy (BZ-8000, KEYENCE, Osaka, Japan).

Western blot analysis

Western blotting was performed as described previously [Tanabe et al., 2004]. Briefly, 10 μg of total cell lysate was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene fluoride membranes. The membranes were incubated with the primary antibodies followed by a peroxidase-labeled anti-IgG antibody, and the signal was visualized by chemiluminescence using an ECL Western Blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK).

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from Huh7.5.1 cells and JFH-1-transfected Huh7.5.1 cell was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany); 1 μg of the total RNA was reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen). PCR amplification was performed using a 25-μL reaction mixture containing 1 μL cDNA and 12.5 μL Platinum SYBR

Green PCR Mix (Invitrogen, Carlsbad, CA, USA). β-Actin mRNA that was amplified from the same samples served as an internal control. After initial denaturation at 95°C for 2 min, a two-step cycle procedure was used (denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min) for 40 cycles on a 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Gene expression levels were determined using the comparative threshold cycle (ΔΔCt) method with β-actin as an endogenous control. Data were analyzed with Sequence Detection Systems software (Applied Biosystems). Primers were as follows: CPT-1, 5′-GACGGCTATGGTGTGTCGTA-3′ and 5′-ATAATCCCCGTCTCAGGGCAA-3′.

Glutathione (GSH)/glutathione disulfide (GSSG) assay

JFH-1-transfected Huh7.5.1 cells were cultured in 96-well plates, and L-carnitine was added. After 96 h, GSH/GSSG was measured using a GSH/GSSG-Glo Assay kit (Promega) according to the manufacturer's protocol.

Quantification of mitochondrial oxidants

Mitochondrial superoxide was quantified using MitoSOX Red indicator dye (Molecular Probes, Eugene, OR, USA) and fluorescence-activated cell sorting (FACS) analysis. Cells were incubated with 5 μM MitoSOX Red at 37°C for 30 min, collected, and washed in phosphate-buffered saline (PBS). Cells were resuspended in 500 μL FACS buffer and analyzed by a FACS Canto II (BD, Tokyo, Japan) with a 488-nm laser.

Statistical analyses

Data are presented as means ± standard deviations. Comparisons between two groups were performed by Student's *t*-tests. Statistical analyses were performed using the SPSS software package, version 20.0 (IBM, Chicago, IL, USA). Differences with *P* values of less than 0.05 were

considered statistically significant.

Results

Antiviral effects of carnitine in HCV replicon cells

Feo replicon cells were treated with 0, 5, 50, 500, or 5,000 μM carnitine for 96 h and then analyzed by luciferase and MTS assays. Carnitine alone had no antiviral effect on either genotype 1b or 2a replicon cells (Figure 1A). At these concentrations, carnitine exhibited no cytotoxicity (Figure 1B). These results indicated that carnitine did not inhibit HCV replication.

Next, Feo replicon cells were treated with 0, 1, 10, 100, or 1,000 IU/mL IFN for 96 h and then analyzed by luciferase and MTS assays. IFN showed antiviral effects in both genotype 1b and genotype 2a replicon cells (Figure 1C). At these concentrations, IFN exhibited no cytotoxicity (Figure 1D).

Antiviral effects of carnitine on HCVcc

Synthesized HCV-RNA (JFH-1 strain) was transfected into Huh7.5.1 cells, and cells were then treated with 0, 5, 50, 500 or 5,000 μM carnitine. After 96 h, the culture supernatants were collected for measurement of the concentration of the HCV core antigen, and western blotting was used to determine the amounts of specific intracellular proteins. The cells were also stained for immunofluorescent analyses.

As shown in Figure 2A, carnitine had no effect on the viability of JFH-1-transfected Huh7.5.1 cells. However, carnitine significantly inhibited the propagation of HCV in a concentration-dependent manner (Figure 2B). Carnitine also decreased the supernatant core levels in the context of HCV infection and 50% tissue cell infectious dose (TCID50) (Supplementary Figure 1). Western blot analyses showed that carnitine suppressed the intracellular expression of the HCV core protein (Figure 2C). Immunofluorescent staining showed that the number of

HCV-infected cells was suppressed by the administration of carnitine (Figure 2D, E).

Carnitine did not affect HCV entry

Carnitine exhibited anti-HCV activity in the HCVcc system but did not affect HCV replication (Figure 1A). This suggested that carnitine may affect HCV entry. To assess the effects of carnitine on HCV entry, we inoculated Huh7.5 cells with HCVpps with or without 500 μM carnitine. Seventy-two hours after inoculation, luciferase activity was measured. As controls for specificity, Huh7.5 cells were incubated for 1 h prior to HCVpp infection with the monoclonal antibody JS-81, which blocks the HCV entry factor CD81, or the monoclonal antibody M-L13 against CD9 as a nonrelevant control. HCV entry was decreased in cells treated with the JS-81 antibody, while M-L13 and carnitine had no effect (Figure 3).

Effect of carnitine on HCV-induced adipogenic effects and lipid droplet formation in cultured cells

Recently, several reports revealed that compounds modulating lipid metabolism could have anti-HCV activity through suppression of viral assembly in lipid droplets (LDs) [Liefhebber et al., 2014]. Carnitine is involved in the transport of fatty acids into mitochondria and lipid metabolism. Thus, we next analyzed the effects of carnitine on HCV-induced adipogenic activity and LD formation, processes that are crucial for the assembly of HCV virions. The content of intracellular neutral lipids, with or without HCV infection, was quantified using Oil Red O. HCV infection induced significantly higher levels of intracellular lipid droplets compared with mock-transfected Huh7.5.1 cells (Supplementary Figure 2). Moreover, immunofluorescence staining showed that HCV infection induced large and abundant lipid droplets that colocalized with the HCV core protein (Supplementary Figure 3), as reported previously [Miyanari et al., 2007].

Subsequently Oil Red O was used to quantify the lipid content of HCV-RNA-transfected

cells treated with 0, 5, 50, 500 or 5,000 μM carnitine. Carnitine decreased the intracellular lipid content in a concentration-dependent manner (Figure 4A). Immunofluorescent staining of these cells showed that carnitine decreased both the number and size of the lipid droplets (Figure 4B).

Carnitine upregulated CPT-1

CPT-1 is located on the outer mitochondrial membrane and is known as a rate-limiting enzyme for β-oxidation. CPT-1 is reported to be downregulated by HCV infection [Cheng et al., 2005]. Western blotting (Figure 5A) and qRT-PCR (Figure 5B) showed that the expression of CPT-1 was downregulated by HCV infection, but was restored by carnitine.

Effects of carnitine on HCV-induced oxidative stress

The HCV core protein was reported previously to increase the production of reactive oxygen species and cause oxidative stress [Korenaga et al., 2005]. In contrast, carnitine decreases the production of reactive oxygen species [Andrieu-Abadie et al., 1999]. JFH-1 RNA was transfected into Huh7.5.1 cells. These cells were then treated with or without carnitine, and changes in GSH/GSSG and superoxide were determined 96 h post-transfection. Glutathione is a cellular antioxidant present in the reduced for (GSH) under normal conditions. In response to oxidative stress, the levels of GSH decreased while GSSG increased. The GSH/GSSG ratio decreased following HCV infection and was restored by carnitine treatment (Figure 5C). MitoSOX Red staining (Figure 5D) showed that the number of cells with elevated levels of superoxide increased in HCV-infected cells, an effect that was prevented by carnitine.

Discussion

To the best of our knowledge, the current study demonstrated the anti-HCV effects of carnitine for the first time. Carnitine could not suppress HCV replication or HCV entry. However, lipid quantification with Oil Red O and immunofluorescent images showed that carnitine

decreased the number and size of intracellular LDs. These findings indicated that carnitine may inhibit viral assembly by decreasing LDs that are crucial for HCV assembly [Miyanari et al., 2007]. Moreover, carnitine showed an antioxidant effect in HCV-infected hepatocytes. Together, these results indicated that carnitine may be useful as an adjunctive therapy for HCV-infected patients that are difficult to treat.

Carnitine is an essential substance that plays a pivotal role in the transport of long-chain fatty acids from the cytosol to the mitochondrial matrix. Carnitine binds to long-chain acyl-CoA, which is derived from fatty acids, and converts it to acylcarnitine, which can be transported to the inner mitochondrial membrane and degraded by β-oxidation [Flanagan et al., 2010]. Because carnitine is an essential nutrient involved in fatty acid oxidation, its deficiency causes health problems. A mouse model of primary carnitine deficiency, juvenile visceral steatosis, shows hepatic steatosis and hepatomegaly [Koizumi et al., 1988]. Secondary carnitine deficiency can be caused by the increased renal tubular loss of carnitine (Fanconi syndrome), hemodialysis, a poor diet [Flanagan et al., 2010], administration of pharmacological agents such as valproic acid [Verrotti et al., 2002], and liver cirrhosis [Daniel Rudman, 1977].

Hepatic steatosis is one characteristic of HCV-infected patients [Czaja et al., 1998]. Hepatic steatosis is a cause of liver fibrosis and carcinogenesis and may result in difficulties in treating these patients [Moucari et al., 2008; Vidali et al., 2008] [Fujinaga et al., 2011; Moriishi et al., 2007]. Our results (Supplementary A,B) indicated that HCV infection induced hepatic steatosis, similar to previous reports [Miyanari et al., 2007]. HCV-associated steatosis is partially caused by a decrease in mitochondrial β-oxidation [Korenaga et al., 2005]. Long-chain fatty acids, present in the cytoplasm as long chain acyl-CoA, are transesterified to L-carnitine in a reaction catalyzed by CPT-1 at the mitochondrial outer membrane. In this reaction, the acyl moiety of the

long-chain fatty acids is transferred from CoA to the hydroxyl group of carnitine. The resulting long-chain acylcarnitine esters are transported through the inner mitochondrial membrane via a specific carrier, carnitine-acylcarnitine translocase [Malaguarnera et al., 2010] and degraded by β-oxidation. Importantly CPT-1 is downregulated by HCV infection [Cheng et al., 2005] and the consumption of fatty acids is thereby decreased. Several reports have shown that HCV core proteins downregulate the expression of CPT-1 [Cheng et al., 2005], resulting in liver steatosis. Our results showed that the administration of carnitine increased the expression of CPT-1. Therefore, upregulation of CPT-1 in HCV-infected hepatocytes may mediate the anti-adipogenic effects of carnitine.

LDs induced by HCV are involved in the production of infectious HCV particles because HCV uses LDs as a scaffold for virus assembly [Miyanari et al., 2007]. In the current study, carnitine did not show any anti-HCV effects in the HCV replicon system (Figure 1A) and did not affect HCV entry (Figure 3). In contrast, carnitine suppressed HCV propagation in the JFH-1 cell culture system (Figure 2A). This discrepancy may result from the presence or absence of HCV nonstructural proteins, particularly core proteins, and may indicate that carnitine suppresses HCV assembly but not HCV replication and entry. Quantification and immunofluorescent images of intracellular lipids (Figure 4B) showed that carnitine decreased the number and size of LDs. In addition, the expression levels of both intracellular NS5A protein and HCV core protein were decreased by carnitine administration (Supplementary Figure 4). Taken together, these results plus previous findings showing that depletion of LDs in cells would reduce the stability of the HCV core and NS5A [Liefhebber et al., 2014], we speculated that carnitine administration could reduce HCV-induced LD, resulting in instability of the HCV core and NS5A and impairing assembly of HCV.

The basic anti-adipogenic mechanism of carnitine involves facilitating the transport of long-chain fatty acids into mitochondria. Serum carnitine levels are significantly lower in patients with C-CH than in healthy individuals [Azin Nassiri, 2014]. An in vitro study showed that carnitine levels are significantly decreased by HCV infection [Roe et al., 2011]. Therefore, infection with HCV may induce carnitine deficiency, and supplementation with carnitine may normalize the transport of long-chain fatty acids into mitochondria.

An in vitro study by Gülçin *et al.* [Gulcin, 2006] revealed the antioxidant activities of carnitine. HCV infection and HCV core proteins lead to oxidative stress [Korenaga et al., 2005] in hepatocytes. Oxidative stress is involved in hepatocarcinogenesis through telomerase activation or increased neovascularization [Jo et al., 2011]. Ishikawa, using the NASH mouse model, reported that carnitine reduces oxidative stress and the subsequent development of hepatocellular carcinoma by upregulating mitochondrial β-oxidation and redox systems in the liver [Ishikawa et al., 2014]. In the current study, both the ratio of GSH/GSSG and the number of cells stained with MitoSOX were decreased after carnitine administration. These results indicated that carnitine could reduce oxidative stress in HCV-infected hepatocytes and may prevent HCV-related hepatocellular carcinoma. Further studies are required in models of HCV-related tumorigenesis, such as HCV transgenic mice, to fully understand the mechanisms underlying HCV-induced cancer.

L-Carnitine has been suggested for the treatment of various liver diseases. In patients with liver cirrhosis, carnitine levels are decreased [Daniel Rudman, 1977], and the administration of L-carnitine reduces ammonia levels. This effect is thought to reflect the ability of L-carnitine to improve hepatic mitochondrial function [Malaguarnera et al., 2011]. Malaguarnera *et al.* [Malaguarnera et al., 2010] reported that carnitine treatment improved liver steatosis and fibrosis in patients with NASH. In patients with chronic hepatitis B, carnitine treatment decreases alanine

aminotransferase levels [Jun et al., 2013].

The relationship between HCV and carnitine is supported by the finding that serum carnitine levels are decreased in patients with C-CH [Azin Nassiri, 2014] and in vitro analyses showing that HCV infection decreases carnitine, resulting in increased hepatocellular fatty acids [Roe et al., 2011]. The current study was the first to reveal that L-carnitine inhibited HCV replication and had anti-HCV-induced adipogenic effects in hepatocytes. L-Carnitine administration in patients with chronic hepatitis C may be an effective adjunctive therapy, as with other liver diseases. DAA-based therapies show significant antiviral effects. However, DAA therapy has not been studied in patients with decompensated cirrhosis [Carrion et al., 2009; Forns et al., 2003]. Therefore, L-carnitine may be an effective adjunctive therapy in these patients.

There were several limitations to this study. First, we did not determine the changes in endogenous carnitine levels after HCV infection or exogenous carnitine administration. Additionally, the concentration of carnitine (500 μM) used for our primary analyses was higher than the serum carnitine concentration in patients with liver cirrhosis. Indeed, Shiraki et al. [2016] showed that the serum carnitine level in patients with liver cirrhosis was 61.8 μM (mean value). However, after administration of 1800 mg carnitine orally, serum carnitine levels were upregulated to 122.9 μM (mean value), and one patient's serum carnitine level reached more than 250 μM [Shiraki et al., 2016]. Therefore, we think the discrepancy between the in vitro and in vivo concentration may not be critical.

In conclusion, we confirmed that L-carnitine had anti-HCV and anti-adipogenic activity in HCV-infected cells. Moreover, we found that L-carnitine had antioxidant properties in HCV-infected hepatocytes. Taken together, these findings demonstrated that L-carnitine has potential as an adjunctive agent in antiviral therapies of C-CH.

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Figure Legends

Figure 1. Analysis of the anti-HCV effects and cytotoxicity of carnitine on HCV replicon cells

A total of 5×10^4 /cm^2 Feo replicon cells (genotypes 1b and 2a) were plated in 96-well plates and treated with carnitine or IFN-alpha 2b. Ninety-six hours later, luciferase (A, C) and MTS (B, D) assays were conducted. Assays were performed in triplicate, and the data are shown as means ± SDs.

Figure 2. Concentration-dependent inhibition of HCV replication by L-carnitine in the JFH-1 HCV cell culture system

(A) Huh7.5.1 cells were transfected with 10 μg of JFH-1 RNA or subjected to mock transfection. One hour later, cells were cultured with 0, 5, 50, 500, or 5000 μM carnitine. Ninety-six hours after transfection, MTS assays were conducted. Assays were performed in triplicate, and the data are shown as means ± SDs. (B) Huh7.5.1 cells were transfected with 10 μg of JFH-1 RNA or subjected to mock transfection. One hour later, cells were cultured with 0, 5, 50, 500, or 5000 μM carnitine. Ninety-six hours after transfection, culture supernatants were isolated. The concentrations of core antigen in the culture supernatants were measured using a chemiluminescence enzyme immunoassay. Assays were performed in triplicate, and the data are shown as means ± SDs. Asterisks indicate *p*-values of less than 0.05. (C) Huh7.5.1 cells were transfected with 10 μg of JFH-1 RNA or subjected to mock transfection. One hour later, cells were cultured with 0, 5, 50, 500, or 5000 μM carnitine. Ninety-six hours after transfection, intracellular proteins were isolated. Western blotting was conducted using an anti-core protein antibody. The numbers show quantification of the densitometry (CPT-1/β-actin). (D) Huh7.5.1

cells (5×10^6) transfected with 10 μg of JFH-1 RNA were fixed at 96 h post-transfection. Immunofluorescent staining was performed using a mouse anti-core antibodies (*red*) and DAPI (*blue*). Representative images of several independent experiments are shown. (E) Numbers of HCV core-positive cells were counted in three fields for each sample. Error bars indicate means ± SDs. Asterisks indicate *p*-values of less than 0.05.

Figure 3. Carnitine did not affect HCV entry

Huh7.5 cells were inoculated with HCVpps with or without 500 μM carnitine. Seventy-two hours later, luciferase activity was measured. As controls for specificity, Huh7.5 cells were incubated for 1 h prior to HCVpp infection either with the monoclonal antibody JS-81, which blocks the HCV entry factor CD81, or with the monoclonal antibody M-L13 against CD9 as a nonrelevant control, with or without L-carnitine. Assays were performed in triplicate, and the data are shown as means ± SDs. Asterisks indicate *p*-values of less than 0.05. HCVpp: HCV pseudoparticle, NS: not significant.

Figure 4. Carnitine decreased intracellular lipid droplets

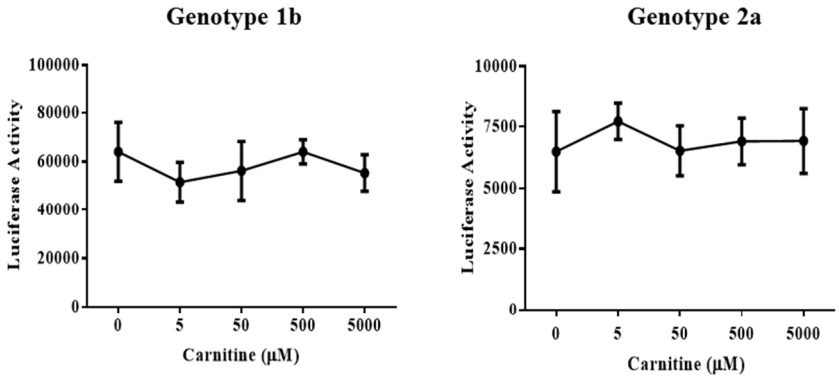
(A) Huh7.5.1 cells (5×10^6) were transfected with 10 μg JFH-1 RNA and treated with 0, 5, 50, 500, or 5,000 μM carnitine. After 96 hours, Oil Red O was used to quantify lipids in HCV-infected cells treated with or without carnitine. Assays were performed in triplicate, and the data are shown as means ± SDs. Asterisks indicate *p*-values of less than 0.05. (B) Carnitine decreased the amount and size of intracellular lipid droplets. Huh7.5.1 cells were transfected with JFH-1 with or without carnitine treatment and were fixed at 96 h post-transfection. Immunofluorescent staining was performed using mouse anti-core antibodies (*red*), BODIPY (*green*), or DAPI (*blue*).

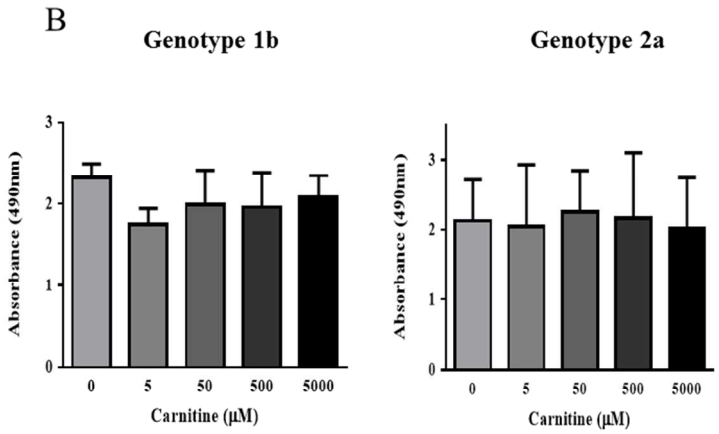
Figure 5. Effects of carnitine on HCV-induced oxidative stress

(A) Carnitine upregulated the expression of CPT-1. Huh7.5.1 cells (5×10^6) were transfected with 10 μg JFH-1 RNA and treated with 0 or 500 μM carnitine. After 96 h, total protein was isolated, and western blotting was performed using an anti-CPT1 or anti-β-actin antibodies. Band intensities were quantified by densitometry. Values are expressed relative to the intensity of β-actin. (B) qRT-PCR analysis. Huh7.5.1 cells (5×10^6) were transfected with 10 μg JFH-1 RNA and treated with 0 or 500 μM carnitine. After 96 h, total RNA was extracted. Relative expression levels of CPT-1 were measured by qRT-PCR. Assays were performed in triplicate. (C) GSH/GSSG assays. Huh7.5.1 cells (5×10^6) were transfected with 10 μg JFH-1 RNA and treated with 500 μM carnitine. Ninety-six hours after transfection, cells were harvested, and oxidative stress was assessed by measuring GSH/GSSG. Assays were performed in triplicate, and the data are shown as means ± SDs. Asterisks indicate *p*-values of less than 0.05. (D) MitoSOX staining. Huh7.5.1 cells (5×10^6) were transfected with 10 μg JFH-1 RNA and treated with 500 μM carnitine. Ninety-six hours after transfection, cells were harvested, and oxidative stress was assessed by staining with MitoSOX. HCV-RNA-transfected and mock-transfected cells were incubated with MitoSOX Red at 37°C for 10 min, collected, and washed with PBS. Cells were resuspended in FACS buffer and analyzed by FACS Canto II (BD, Tokyo, Japan) with a 488 nm laser.

Figure 1

A





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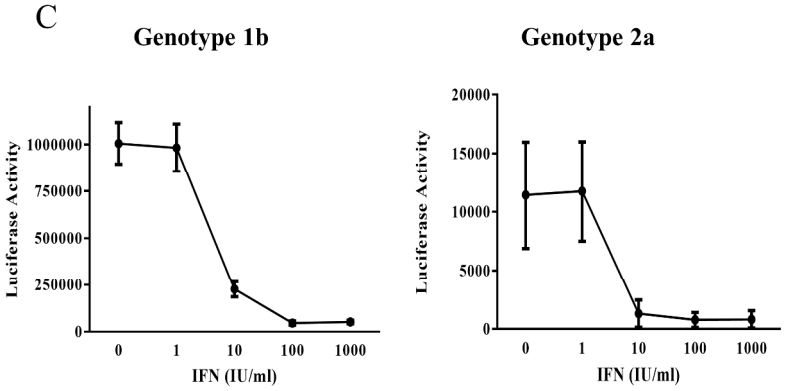

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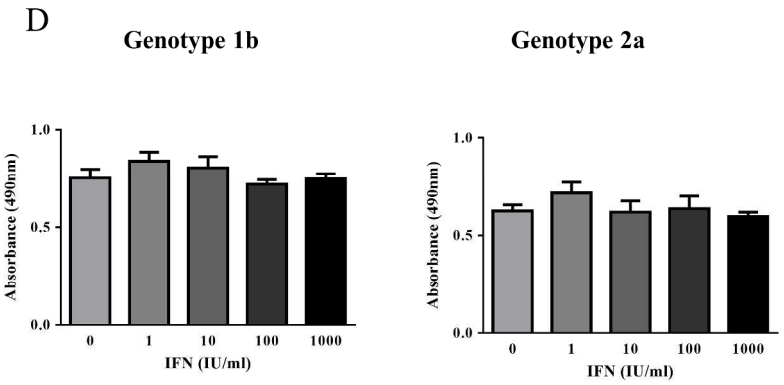

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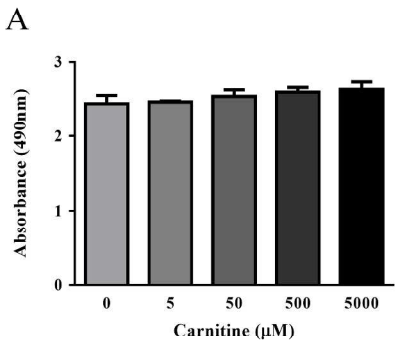

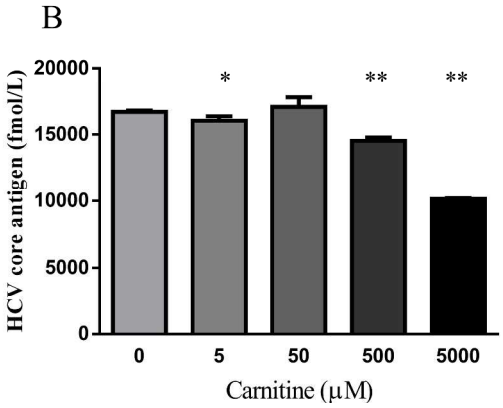

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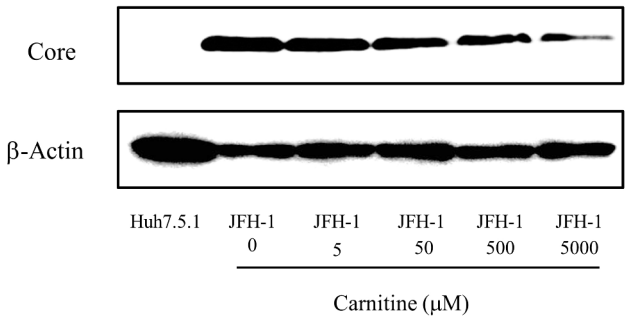

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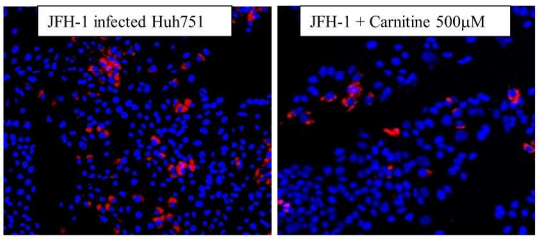

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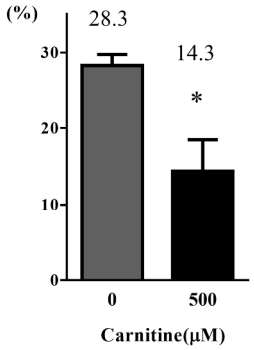

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Figure 3

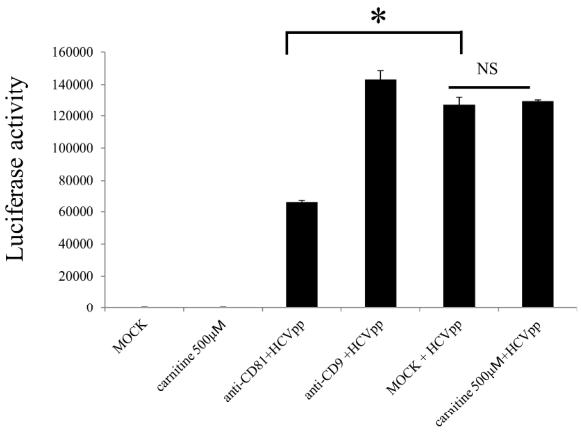

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Figure 4

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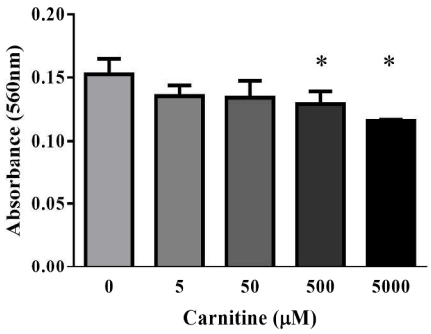

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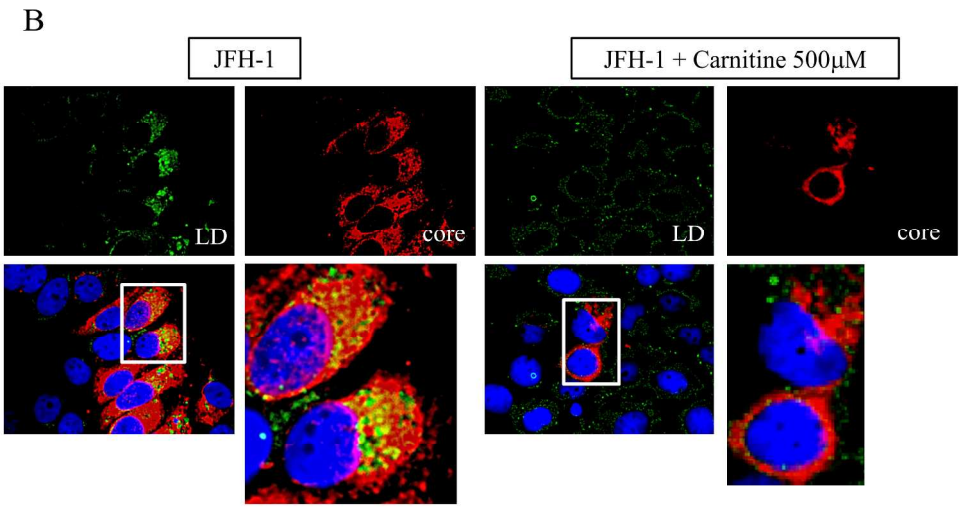
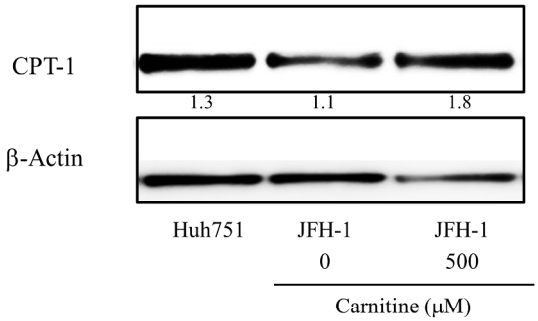


Figure 5

A



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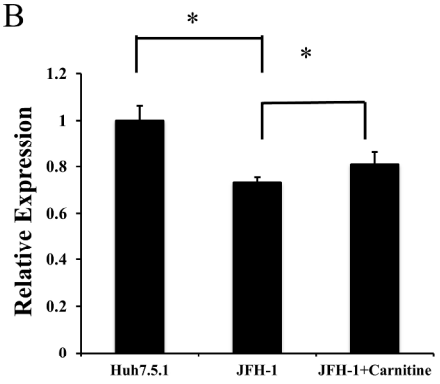

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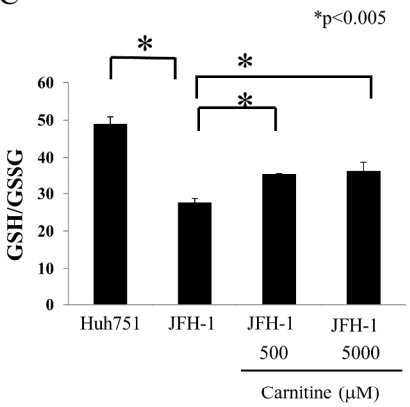

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