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**Abbreviations:** core, viral core protein; dpi, days post-infection; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVpp, hepatitis C virus pseudoparticles; moi, multiplicity of infection; NS3, HCV nonstructural serine protease with a helicase activity; p, the p value of Student’s t-test
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Abstract

Based on multipurpose cohort studies, coffee consumption reduces the risk of hepatocellular carcinoma, one of the main causes of which is hepatitis C virus (HCV) infection. Here, we focused on the effect of caffeic acid, a major organic acid derived from coffee, on the propagation of HCV using an *in vitro* naïve HCV particle-infection and production system within human hepatoma-derived Huh7.5.1-8 cells. When cells were treated with 1% coffee extract or 0.1% caffeic acid for 1-h after HCV infection, the amount of HCV particles released into the medium at 3 and 4 days post-infection were considerably decreased. HCV-infected cells were cultured with 0.001% caffeic acid for 4 days, which was sufficient to decrease the amount of HCV particles released into the medium. Caffeic acid treatment inhibited the initial stage of HCV infection, i.e., between virion entry and the translation of the RNA genome. This inhibitory effect was observed against both HCV genotypes 1b and 2a. These results suggested that treatment of cells with caffeic acid inhibited HCV propagation.
Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Chronic hepatitis C is a persistent infection by hepatitis C virus (HCV) and is the cause of 27% of cirrhosis cases and 25% of HCC worldwide (1). In 1989, HCV was first identified as the agent responsible for blood-borne non-A and non-B viral hepatitis (2). HCV is spread by direct blood-to-blood contact associated with intravenous drug use, transfusions, and insufficiently sterilized medical equipment. Approximately 150–200 million people in the world are infected with HCV (3-5) and although numbers of new HCV infections have been dramatically reduced since its initial discovery, there is currently no vaccine to prevent this infection. HCV is an enveloped, positive-strand RNA virus with a genome size of approximately 9.6 kb and belongs to the *Flaviviridae* family. The HCV single open reading frame is translated to produce a polyprotein, which is further processed to produce HCV nucleocapsid (core), two envelope glycoproteins (E1 and E2), and nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). In 2005, an *in vitro* naïve HCV particle-infection and production system was established in cultured cells using a cloned viral genome (JFH-1 strain,
genotype 2a) (6-8) thus accelerating studies on the HCV life cycle.

Coffee consumption reportedly decreases the risk of liver diseases, including HCC (9-13). In addition, several cohort and case-control studies and meta-analyses have reported that coffee consumption is inversely associated with liver cancer (12-25). Human studies that evaluated the coffee intake of HCC and HCV patients suggested that a specific substance(s) in coffee can inhibit HCV infection and/or propagation (11, 26-28).

Coffee and green tea are both rich in caffeine (1%–2% and 2.5%–5%, respectively) and polyphenols. Particularly, coffee contains chlorogenic acids (5%–10%), whereas green tea predominantly contains catechins (12%–20%). A cohort study conducted with both healthy people and HCV patients reported that coffee consumption had an inverse association with liver cancer, whereas green tea consumption demonstrated no such correlation (29). Therefore, it is possible that the specific polyphenols present in coffee function to inhibit HCV propagation. Chlorogenic acid (an ester of caffeic and quinic acids) is one of the major polyphenols in coffee; it is heat-unstable and, following its absorption into the body, it metabolically
decomposes to caffeic and quinic acids. Coffee consumption increases caffeic acid concentration in human total plasma, whereas chlorogenic acid is undetectable (30). Therefore, we hypothesized that caffeic acid may inhibit HCV propagation. Thus, we utilized an in vitro naïve HCV particle-infection and production system with cultured cells to experimentally determine whether coffee-related organic acids affect HCV propagation.

Materials and Methods

Cells, Media, Materials, and Antibodies Human hepatoma Huh-7.5.1 cells (7) were subcloned by limiting dilution and a highly HCV-JFH1-permissive subclonal cell line, Huh-7.5.1-8 (31), was used for this study. Huh-7.5.1-8 cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM; Wako, 045-30285) containing 10% fetal calf serum (JRH biosciences/ Sigma-Aldrich, 12603C), 100-units/mL penicillin G, 100-μg/mL streptomycin sulfate, and 1% nonessential amino acids (Invitrogen, 11140050). Coffee extracts (CE, Caffenol P-100 raw coffee bean extracts) were provided by Fuji Chemical Industry Co., Ltd. (Toyama, Japan). Caffeic acid was
purchased from WAKO (048-20983), \( p \)-coumaric acid from MP Biomedicals (102576), D-\((-\)\)-quinic acid from Alfa Aesar (L15238), and nicotinic acid from Sigma–Aldrich (N4126). Monoclonal antibodies against the HCV core protein was purchased from Anogen (MO-I40015B) and against GAPDH (ab8245) and HCV NS3 (ab18664) from Abcam. Protein concentrations were determined using the bicinchoninic acid protein assay reagent (Pierce, 23225). Luciferase activity was monitored using the PG MelioraStar-LT luciferase assay system (WAKO).

**Infection of Huh7.5.1-8 cells with HCV** Infectious HCV (JFH1 strain) particles were produced in Huh7.5.1-8 cells as described previously (32). Culture supernatant containing infectious HCV particles was collected and stored at \(-80^\circ\text{C}\) until analysis. Subconfluent cells in a 24-well plate were exposed to normal culture medium containing HCV particles (8 fmoles of core protein/well, corresponding to a multiplicity of infection (moi) of 0.1) at \(37^\circ\text{C}\) for 3 h.

For a one-shot treatment with coffee extract and caffeic acid, cells were incubated in the normal culture medium containing coffee extract or caffeic acid at \(37^\circ\text{C}\) for 1 h. After washing, cells were maintained in 500 \(\mu\)l of normal culture medium.
at 37°C for 3 and 4 days. For continuous treatment with these compounds, cells were cultured in the normal culture medium in the presence of the compounds at the indicated concentration.

To determine the amount of HCV particles released into the culture medium, amounts of the HCV core protein in the culture medium was quantified by an enzyme-linked immunosorbent assay (ELISA; Ortho® HCV antigen ELISA test, Ortho-Clinical Diagnostics, 601002).

**Immunoblotting analyses** Cells were washed twice in phosphate-buffered saline, lysed in lysis buffer (10-mM sodium phosphate [pH 7.2], 150-mM NaCl, and 1% sodium dodecyl sulfate) containing a Complete® protease-inhibitor cocktail (Roche Diagnostics, 1697498), and boiled for 10 min. Total proteins (10 µg) in the lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4%–12% Bis-Tris, Invitrogen, NP0322BOX). After transferring the proteins to a polyvinylidene difluoride membrane using a Trans-Blot SD transfer cell apparatus (Bio-Rad, 170-3940), the membranes were probed with antibodies specific to the HCV core and NS3 proteins. Immunoblots were imaged using chemiluminescence using
SuperSignal West Dura Extended Duration Substrate (Pierce, 34075) or SuperSignal West Pico Chemiluminescent Substrate (Pierce, 34077) according to standard protocols.

**Preparation of HCV pseudoparticles (HCVpp)**

HCVpp were produced as previously described (33). Briefly, 293T cells were cotransfected with a Gag-Pol packaging vector (Gag-Pol 5349), reporter vector (Luc 126), and glycoprotein (HCV E1 and E2)-expressing vector [genotype 2a, the JFH1 strain; genotype 1b, the TH strain (34)]. The medium was collected from the transfected cell cultures and used as the HCVpp source.

**Results**

**One-shot treatment with caffeic acid to the HCV-infected cells inhibits the propagation of HCV.**

To investigate whether the coffee extract inhibited HCV propagation, we examined the amount of HCV particles released into the medium after the treatment of HCV-infected cells (Figure 1A). Huh7.5.1-8 cells were infected with HCV particles for 3 h at an moi of 0.1. After washing to remove free HCV particles, the HCV-infected
cells were incubated in the normal culture medium containing 1% coffee extract at 37°C for 1 h. Thereafter, the cells were cultured in the normal culture medium without the coffee extract for 3 and 4 days. HCV particles released into the medium were investigated at 3 and 4 days post-infection (dpi) (Figure 1A). There were minimal abnormalities in cell morphology and viability under these conditions. The 1-h coffee extract treatment considerably decreased the amount of HCV particles released into the medium (p<0.004 at 3 dpi and p<0.003 at 4 dpi) (Figures 1B & C).

Furthermore, we investigated the effect of coffee extract treatment on the intracellular levels of HCV core and NS3 proteins in the infected cells 4 dpi (Figure 1D). When cells were treated with 1% coffee extract for 1 h after HCV infection, levels of HCV core and NS3 proteins were found to be low, whereas these were more abundant in the HCV-infected cells without the treatment. The levels of total proteins in the coffee extract-treated cells was similar to that of the untreated cells (Figure 1E), suggesting that coffee extract had little effect on the cell viability and proliferation. These results indicated that HCV propagation was markedly inhibited by the 1-h coffee extract treatment.
Chlorogenic acid is a major component of coffee (approximately 5%–10%), and it is metabolically decomposed to caffeic acid and D(−)-quinic acid after absorption. As caffeic acid is present in human plasma after the consumption of coffee (30), we focused our study on this compound. And to clarify the effect of caffeic acid on HCV propagation, we investigated the abundance of HCV particles released from infected cells at 3 and 4 dpi, following a 1-h treatment with 0.1% caffeic acid and found that the number of HCV particles released was substantially decreased at 3 and 4 dpi (p<0.006 at 3 dpi and p<0.006 at 4 dpi), which was similar to the results observed after treatment with the coffee extract (Figure 1B & C). The intracellular levels of HCV core and NS3 proteins in the HCV-infected cells at 4 dpi were also a markedly decreased by the caffeic acid treatment (Figure 1D). Caffeic acid took little effect on the level of total proteins of the cells (Figure 1E), suggesting that caffeic acid had little effect on the cell viability and proliferation under these conditions. There were minimal abnormalities in cell morphology and viability under these conditions. These results indicate that, in addition to the 1% coffee extract, the one-shot 0.1% caffeic acid treatment also inhibited HCV propagation.
HCV propagation is inhibited by continuous treatment with a lower caffeic acid concentration

Next, we investigated whether HCV particle release was decreased by a continuous treatment with lower concentrations of coffee extract and caffeic acid compared with the initial concentrations (Figure 2). Huh7.5.1-8 cells were infected with naïve HCV particles for 3 h (moi = 0.1). After infection, cells were cultured in the presence of 0.01% coffee extract or 0.001% caffeic acid for 3 and 4 days (Figure 2A), after which the level of HCV particles released into the medium was estimated at both time points. As before, minimal abnormalities in cell morphology and viability under these conditions were observed. At 4 dpi, the abundance of HCV particles released into the medium was drastically decreased in the presence of both 0.01% of coffee extract and 0.001% of caffeic acid (p<0.006 and p<0.005, respectively) (Figure 2B), with a corresponding decrease in the intracellular level of HCV core and NS3 proteins (Figure 2C). Little difference of total proteins in the cell lysate was observed between the compound-treated and the untreated HCV-infected cells (Figure 2D), suggesting the
little effect of these compounds on the cell viability and proliferation. These results were similar to those of the one-shot treatment experiments, as they indicate that continuous caffeic acid treatment also inhibited HCV propagation.

**Caffeic acid inhibits the initial stage of HCV infection**

The treatment of HCV-infected cells with caffeic acid resulted in a decreased release of HCV particles. This inhibitory effect was observed even with the one-shot 1-h caffeic acid treatment. Considering these results, it is possible that caffeic acid can inhibit the initial stage of HCV infection (from viral entry to viral genome translation). To investigate this possibility, we employed infectious HCVpp (35). The entry of HCV into target cells depends on the envelope glycoproteins. HCVpp is assembled by displaying unmodified and functional HCV glycoproteins on retroviral core particles to mimic the initial stage of HCV infection. Because a firefly luciferase gene was introduced into the HCVpp viral genome, HCVpp entry into target cells can be estimated by firefly luciferase measurement. Therefore, cells will express active luciferase only when it is translated from the viral genome following HCVpp infection.
of target cells.

HCVpp-infected cells were washed to remove free HCVpp, followed by a 1-h incubation in medium containing either 1% coffee extract or 0.1% caffeic acid. Cells were washed again and then cultured in the normal culture medium for 2 days, at which point the cellular luciferase activity was measured (Figure 3A). When the HCVpp-infected cells were treated with 1% coffee extract or 0.1% caffeic acid for 1 h, the luciferase activity was markedly decreased compared with the untreated HCVpp-infected cells (p<0.002 and p<0.001, respectively). These results suggest that coffee extract and caffeic acid severely inhibit the initial stage of HCV infection.

We then performed the similar experiment to investigate the effect of continuous caffeic acid treatment at a lower concentration on the initial stage of HCV infection (Figure 3B). Huh7.5.1-8 cells were infected with HCVpp for 3 h. After washing the cells to remove free HCVpp, they were cultured in the presence of 0.001% caffeic acid for 2 days. Thereafter the activity of cellular luciferase was measured. When the HCVpp-infected cells were treated with caffeic acid, the activity of cellular luciferase decreased (p<0.005) (Figure 3B), thus indicating that continuous caffeic acid
treatment also inhibits the initial stage of HCV infection.

Next, we focused on another of the major HCV genotypes, particularly, 1b (Figure 3C). We prepared HCVpp genotype 1b and infected Huh7.5.1-8 cells for 3 h as described above. After infection and removal of free HCVpp, the cells were treated for 1 h with either 1% coffee extract or 0.1% caffeic acid. Following treatment, the cells were washed and further cultured in normal medium for 2 days, at which point the cellular luciferase activity was measured. As was the case for HCVpp genotype 2a, the HCVpp genotype 1b-infected cells treated with 1% coffee extract or 0.1% caffeic acid had drastically decreased cellular luciferase activity ($p<0.001$ and $p<0.001$, respectively). These results suggested that the inhibitory effect of coffee extract and caffeic acid on the initial stage of HCV infection is independent of viral genotype.

**Higher concentration of $p$-coumaric acid inhibits the propagation of HCV**

We further investigated the anti-HCV effects of additional organic acids from the coffee extract, including $p$-coumaric acid, quinic acid, and nicotinic acid (Figure 4). Huh7.5.1-8 cells were infected with HCV particles for 3 h and cultured in the presence
of 0.1% $p$-coumaric acid, D(-)-quinic acid, or nicotinic acid for 3 and 4 days. The number of HCV particles released into the medium was then examined. Only $p$-coumaric acid strongly inhibited the HCV particle release ($p<0.008$ at 3 dpi and $p<0.001$ at 4 dpi), although these organic acids were used at a concentration 100-fold higher than that of caffeic acid. Little difference of total proteins in the cell lysate was observed among the compound-treated and the untreated HCV-infected cells (Figure 4), suggesting little effect of these compounds on the cell viability and proliferation.

**Discussion**

In this study, we used an *in vitro* naïve HCV particle-infection and production system and demonstrated that coffee extract and caffeic acid inhibited the propagation of HCV. The one-shot treatment of HCV-infected cells with 1% coffee extract and 0.1% caffeic acid for 1 h inhibited HCV propagation at 3 and 4 dpi. Moreover, continuous treatment of HCV-infected cells with 0.01% coffee extract and 0.001% caffeic acid demonstrated the similar inhibition. We further demonstrated that caffeic acid inhibited the initial stage of HCV infection. Our results strongly suggest that caffeic acid derived
from the coffee extract inhibits HCV propagation.

The one-shot treatment with 0.1% caffeic acid for 1 h effectively inhibited HCV propagation *in vitro*. Interestingly, the continuous treatment with 0.001% caffeic acid had a similar effect. Coffee consumption results in increased caffeic acid in human total plasma (30, 36). The caffeic acid concentration in human total plasma at approximately 1-h after coffee consumption is approximately 80–116 nM, which gradually decreases (approximately 50 nM at 8 h) and reduces to almost zero at 12 h. The caffeic acid concentration used for the continuous treatment (0.001%) was approximately 55 nM. Thus, the increased caffeic acid concentration in human plasma after coffee intake may inhibit HCV infection and/or propagation in humans. According to human studies, the amount of coffee consumed per day correlates with a decreased risk of chronic liver diseases (11, 15, 28). Furthermore, coffee consumption is associated with a response to peginterferon and ribavirin therapy in patients with chronic hepatitis C (27). The results presented in this study suggest that the plasma concentration of caffeic acid is important for the inhibition of HCV infection and HCV propagation.
We found that the one-shot treatment with the coffee extract and caffeic acid severely inhibited the initial stage of HCV infection, an effect that is independent of viral genotype. Therefore, this may partly contribute to the decrease in HCV propagation. Considering that continuous treatment with lower concentrations of both substances also inhibited HCV propagation, they may affect intracellular processes essential for HCV propagation, such as polyprotein processing; RNA replication; and virion assembly, transport, or release, in addition to viral entry and viral polyprotein translation. It is possible that caffeic acid inhibits in part RNA-replication in HCV-infected cells, since it has been reported that caffeic acid inhibits HCV-replication in HCV subgenomic replicon cells at higher concentration (EC50, about 37 µM) (37). Further studies are warranted to clarify the step(s) that is inhibited by these treatments.

The 1-h treatment with coffee extract or caffeic acid following HCV infection was sufficient to severely inhibit HCV propagation. HCV cellular entry is mediated via many receptors, including (at least) human CD81, low-density lipoprotein receptor, occludin, scavenger receptor class B type I, claudin-1, and the Niemann–Pick C1-like 1 cholesterol absorption receptor. The time course of HCV entry and the mechanism by
which these receptors contribute to HCV entry remain unclear. Further studies are required to clarify these questions.

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Conflict of interest: None to declare.


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Figure Legends.

Figure 1. HCV propagation is inhibited by one-shot treatment of HCV-infected cells with coffee extract and caffeic acid for 1 h. (A) The schedule for exposing Huh7.5.1-8 cells to naïve HCV particles and compounds is schematically represented. Huh7.5.1-8 cells were infected with naïve HCV particles (JFH-1, genotype 2a) for 3 h. After washing free HCV particles, HCV-infected cells were incubated in the culture medium containing 1% coffee extract or 0.1% caffeic acid at 37°C for 1 h. The medium containing HCV-infected cells incubated in the culture medium for 1 h was used as the positive control. After washing the cells, they were incubated at 37°C in the culture
medium for the indicated time points. Black and dotted bars indicate the intervals with and without treatments, respectively. (B) The effect of 1% coffee extract and 0.1% caffeic acid on HCV particle released into the medium. After HCV infection, cells were treated with 1% coffee extract (open squares) or 0.1% caffeic acid (open triangles) at 37°C for 1 h. The medium containing cells incubated the culture medium for 1 h was used as the positive control (closed squares). The relative amounts of HCV core proteins with that of HCV particles released from nontreated HCV-infected cells at 3 dpi set to 100% is shown. Error bars indicated the standard error of the mean (S.E.M., n = 4). (C) The relative amount of HCV core proteins in the medium at 3 dpi. (D) The intracellular level of HCV-related proteins in HCV-infected cells under the same conditions at 4 dpi. HCV core and NS3 proteins in the cell lysates were visualized by immunoblotting using the appropriate antibodies. GAPDH was employed as the loading control. (E) Little effect of 1% coffee extract and 0.1% caffeic acid on total proteins in HCV-infected cells. The schedule for exposing Huh7.5.1-8 cells to HCV particles and compounds was the same as that shown in (A). Error bars indicated the standard error of the mean (S.E.M., n = 4).
Figure 2. HCV propagation is inhibited by continuous treatment of HCV-infected cells with coffee extract and caffeic acid. (A) The schedule for exposing Huh7.5.1-8 cells to compounds and naïve HCV particles is schematically represented. Huh7.5.1-8 cells were infected with naïve HCV particles (JFH-1, genotype 2a) for 3 h. After washing free HCV particles from the medium, HCV-infected cells were cultured in medium containing 0.01% coffee extract or 0.001% caffeic acid at 37°C. Black and dotted bars indicate the intervals with and without treatments, respectively. (B) The amounts of HCV particles released into the medium in the presence of 0.01% coffee extract and 0.001% caffeic acid is shown. After HCV infection, cells were cultured in the normal culture medium in the presence of 0.01% coffee extract (open squares) or 0.001% caffeic acid (open triangles) at 37°C. The medium containing HCV-infected cells incubated the culture medium for 1 h was used as the positive control (control, closed squares). The relative amount of HCV core proteins with that of HCV particles released from nontreated HCV-infected cells at 3 dpi set as 100% is shown. Error bars indicated the standard error of the mean (S.E.M., n = 4). (C) The intracellular levels of...
HCV-related proteins in infected cells under the same conditions at 4 dpi. HCV core and NS3 proteins were visualized by immunoblotting with the appropriate antibodies. GAPDH was employed as the loading control. (D) Little effect of continuous treatment of HCV-infected cells with coffee extract and caffeic acid on total proteins in HCV-infected cells. The schedule for exposing Huh7.5.1-8 cells to HCV particles and compounds was the same as that shown in (A). Error bars indicated the standard error of the mean (S.E.M., n = 4).

Figure 3. Caffeic acid inhibits the initial stage of HCV infection. (A) The one-shot treatment of caffeic acid inhibits the initial stage of HCV infection. The schedule for exposing Huh7.5.1-8 cells to compounds and HCV pseudoparticles (HCVpp) (genotype 2a) was the same as that shown in Figure 1A. The medium containing HCVpp-infected cells incubated in the normal culture medium without these compounds was used as the positive control. Relative activities of luciferase with the activity of the nontreated HCVpp-infected cells (control) was set to 100% are shown. The data from three independent experiments are shown. Error bars indicated the standard error of the mean.
(S.E.M.). (B) Continuous caffeic acid treatment also inhibits the initial stage of HCV infection. The schedule for exposing Huh7.5.1-8 cells to compounds and HCVpp (genotype 2a) is the same as that shown in Figure 2A. The medium containing HCVpp-infected cells incubated in the normal culture medium was used as the positive control. Relative activities of luciferase with the activity of the nontreated HCVpp-infected cells (control) set to 100% are shown. (C) Caffeic acid inhibits the initial stage of HCVpp genotype 1b infection. The experiments were performed as described in (A) using HCVpp genotype 1b instead of HCVpp genotype 2a.

Figure 4. High concentration of p-coumaric acid inhibits the HCV propagation. (A) The schedule for exposing Huh7.5.1-8 cells to compounds and naïve HCV particles is schematically represented. (B) The effects of D-(−)-quinic acid, nicotinic acid, and p-coumaric acid on the HCV propagation were investigated. The relative amount of the HCV core protein in the medium with its amount in the medium without compounds at 3 dpi set to 100% is shown. The data from three independent experiments are shown. Error bars indicated the standard error of the mean (S.E.M.). (C) Little effect of these
compounds on total proteins in HCV-infected cells. The schedule for exposing Huh7.5.1-8 cells to HCV particles and compounds was the same as that shown in (A). The data from two independent experiments are shown. Error bars indicated the standard error of the mean (S.E.M., n = 4).
Figure 2

A

HCV:  
compound:  

3 h  
day 3  
day 4  

HCV infection  
compound  
detection  
detection  

B

Relative amount of HCV particles released in the medium (%)  

0  
200  
400  
600  
800  
1000  
1200  
1400  

TIME (day)  
0  
1  
2  
3  
4  

control  
0.01% coffee extract  
0.001% caffeic acid  

C


D

Relative amount of total proteins (%)  

0  
20  
40  
60  
80  
100  
120  

control  
0.01% coffee extract  
0.001% caffeic acid
Figure 4

A

HCV: 
compound: 
3 h 
day 3 
day 4

B

Relative amount of HCV particles released in the medium (%)

Control
0.001% caffeic acid
0.1% nicotinic acid
0.1% D(-)-quinic acid
0.1% p-coumaric acid

TIME (day)

C

Relative amount of total proteins (%)

control
0.01% coffee extract
0.001% caffeic acid
0.1% nicotinic acid
0.1% D(-)-quinic acid
0.1% p-coumaric acid