

Original Article

Inhibitory Effects of Caffeic Acid, a Coffee-Related Organic Acid, on the Propagation of Hepatitis C Virus

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SUMMARY: Multipurpose cohort studies have demonstrated that coffee consumption reduces the risk of hepatocellular carcinoma (HCC). Given that one of the main causes of HCC is hepatitis C virus (HCV) infection, we examined 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 Huh-7.5.1-8 cells. When cells were treated with 1% coffee extract or 0.1% caffeic acid for 1-h post HCV infection, the amount of HCV particles released into the medium at 3 and 4 days post-infection considerably decreased. In addition, HCV-infected cells cultured with 0.001% caffeic acid for 4 days, also released less HCV particles 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) in both HCV genotypes 1b and 2a. These results suggest that the treatment of cells with caffeic acid may inhibit HCV propagation.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Chronic hepatitis C is a persistent infection caused by the hepatitis C virus (HCV) and is the cause of 27% of cirrhosis and 25% of HCC cases worldwide (1). HCV was first identified as the agent responsible for blood-borne non-A and non-B viral hepatitis in 1989 (2). HCV is spread by direct blood-to-blood contact associated with intravenous drug use, transfusions, and insufficiently sterilized medical equipment. Globally, approximately 150–200 million people are infected with HCV (3–5), and although the number of new HCV infections has dramatically decreased since its initial discovery, there are currently no vaccines to prevent this infection. HCV belongs to the *Flaviviridae* family and is an enveloped, positive-strand RNA virus with a genome size of approximately 9.6 kb. The HCV single open reading frame is translated to produce a polyprotein, which is further processed to produce the HCV nucleocapsid (core), two envelope glycoproteins (E1 and E2), and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). In 2005, studies on the HCV life cycle were accelerated when 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).

Coffee consumption is associated with a decrease in

the risk of liver diseases, including HCC (9–13). In addition, several studies (meta-analyses, cohort, and case-control) have reported that coffee consumption is inversely associated with liver cancer (12–25). Furthermore, studies that evaluated the coffee intake of HCC and HCV patients have suggested that a specific substance(s) in coffee could 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. However, 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. For example, 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, metabolically decomposes to caffeic and quinic acids. After coffee consumption, the caffeic acid concentration in human total plasma increases; however, chlorogenic acid remains undetectable (30). Therefore, we hypothesized that caffeic acid may inhibit HCV propagation. To test this hypothesis, we utilized an in vitro naïve HCV particle-infection and production system with cultured cells to 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 a limiting dilution, and a highly HCV-JFH1-permissive sub-

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clonal cell line (Huh-7.5.1-8) (31) was used for this study. The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Wako, Osaka, Japan; 045-30285) containing 10% fetal calf serum (JRH biosciences/Sigma-Aldrich, St. Louis, MO, USA; 12603C), 100 units/mL penicillin G, 100 μ g/mL streptomycin sulfate, and 1% nonessential amino acids (Invitrogen Co., Carlsbad, CA, USA; 1140050). 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 Inc. (Solon, OH, USA; 102576), D-(–)-quinic acid from Alfa Aesar (Lancashire, UK; L15238), and nicotinic acid from Sigma-Aldrich (N4126). Monoclonal antibodies against the HCV core protein were purchased from Anogen (Orlando, FL, USA; MO-I40015B), while monoclonal antibodies against GAPDH (ab8245) and HCV NS3 (ab18664) were purchased from Abcam (Cambridge, UK). Protein concentrations were determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA; 23225). Luciferase activity was monitored using the PG MelioraStar-LT luciferase assay system (Wako).

Infection of Huh-7.5.1-8 cells with HCV: Infectious HCV (JFH1 strain) particles were produced in Huh-7.5.1-8 cells as described in a previous study (32). Culture supernatant containing infectious HCV particles was collected and stored at -80°C until analysis. Subconfluent cells in a 24-well plate were exposed to a normal culture medium containing HCV particles (8 fmoles of core protein/well, corresponding to a multiplicity of infection [moi] of 0.1) at 37°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°C for 1 h. After washing, the cells were maintained in 500 μ l of the normal culture medium at 37°C for 3 and 4 days. For continuous treatment, cells were cultured in the normal culture medium in the presence of the coffee extract or caffeic acid at the indicated concentration.

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

Immunoblotting analyses: Cells were washed twice in a phosphate-buffered saline, lysed in a 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, Branchburg, NJ, USA; 1697498), and boiled for 10 min. Total proteins (10 μ g) in the lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4%–12% Bis-Tris Gel, Invitrogen; NP0322BOX). The proteins were transferred to a polyvinylidene difluoride membrane using a Trans-Blot SD transfer cell apparatus (Bio-Rad, Hercules, CA, USA; 170-3940), and the membranes were probed with antibodies specific to the HCV core and NS3 proteins. Immunoblots were imaged by chemiluminescence using SuperSignal West Dura Extended Duration Sub-

strate (Pierce, 34075) or SuperSignal West Pico Chemiluminescent Substrate (Pierce, 34077) according to standard protocols.

Preparation of HCV pseudoparticles (HCVpp): HCV pseudoparticles 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 caffeic acid treatment of 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 (Fig. 1A). Huh-7.5.1-8 cells were infected with HCV particles for 3 h at a 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. The cells were then 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) (Fig. 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 value (P) < 0.004 at 3 dpi and $P < 0.003$ at 4 dpi) (Fig. 1B, 1C).

We also investigated the effect of the coffee extract treatment on the intracellular levels of HCV core and NS3 proteins in the infected cells 4 dpi (Fig. 1D). When cells were treated with 1% coffee extract for 1 h after HCV infection, the HCV core and NS3 (nonstructural serine protease with a helicase activity) protein levels were low, whereas these were more abundant in the HCV-infected cells without the treatment. The level of total proteins in the coffee extract-treated cells was similar to that of the untreated cells (Fig. 1E), suggesting that the 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 after absorption, is metabolically decomposed into caffeic acid and D-(–)-quinic acid. Given that caffeic acid is present in human plasma after the consumption of coffee (30), our study focused on this compound. In addition, 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. The results showed that the number of HCV particles released substantially decreased at 3 and 4 dpi ($P < 0.006$ for both), similar to the results observed after the coffee extract treatment (Fig. 1B, 1C). The intracellular levels of HCV core and NS3 proteins in the HCV-infected cells at 4 dpi also decreased markedly with the caffeic acid treatment (Fig. 1D). Furthermore, caffeic acid had little effect on the

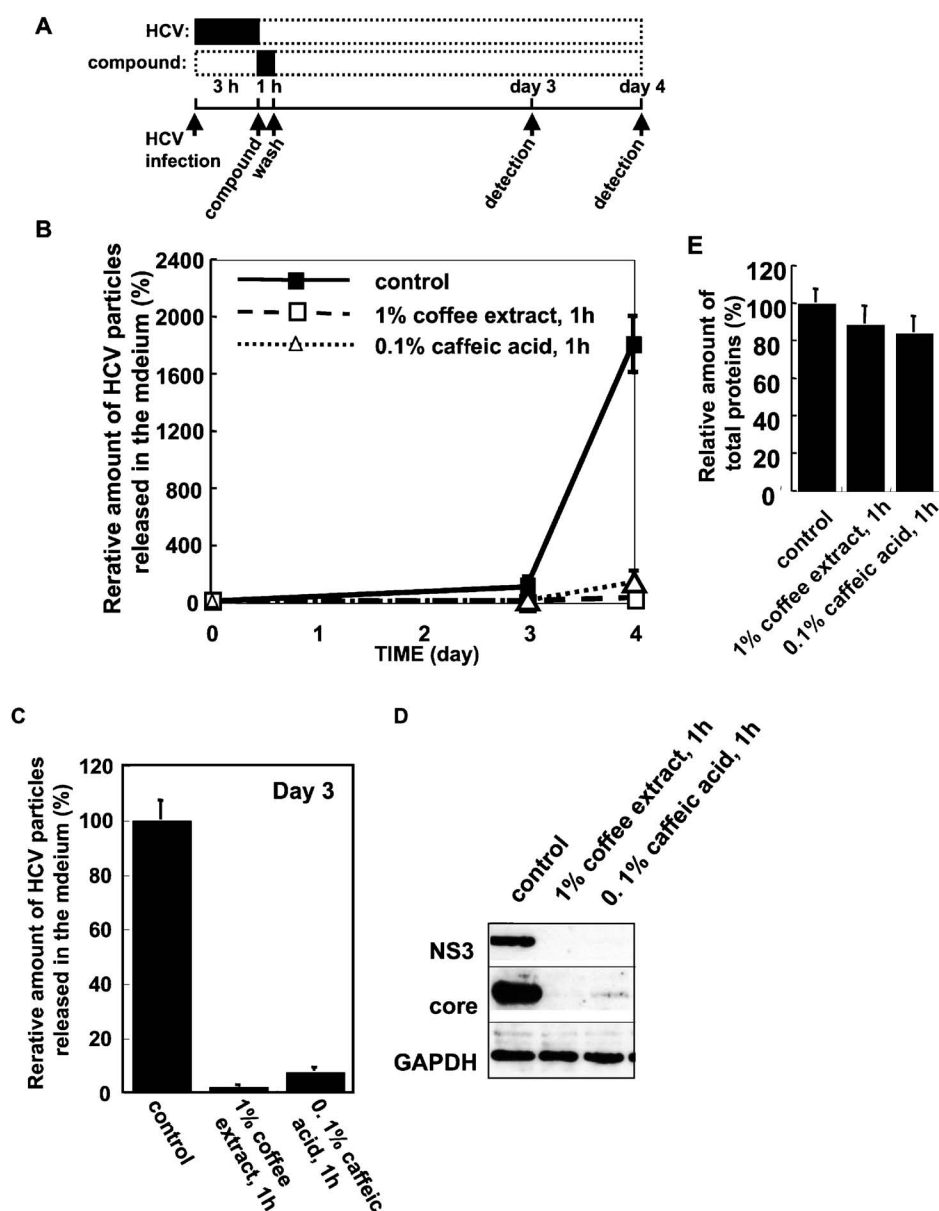


Fig. 1. HCV propagation is inhibited by one-shot treatment of HCV-infected cells with coffee extract and caffeic acid for 1 h. (A) The schedule for exposing Huh-7.5.1-8 cells to naïve HCV particles and compounds is schematically represented. The cells were infected with naïve HCV particles (JFH-1, genotype 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. After washing the cells, they were incubated at 37°C in the culture medium. Black and dotted bars indicate the periods with and without treatments, respectively. The medium containing HCV-infected cells incubated in the culture medium for 1 h was used as the positive control. (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 in the normal culture medium without these compounds 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 non-treated 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 relative amount of HCV core proteins in the medium at 3 dpi. (D) The intracellular level of HCV-related proteins in HCV-infected cells 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 or 0.1% caffeic acid on total proteins in HCV-infected cells. The schedule for exposing Huh-7.5.1-8 cells to HCV particles and compounds was the same as that shown in (A). Error bars indicate the standard error of the mean (S.E.M., $n = 4$).

level of total proteins of the cells (Fig. 1E), suggesting that caffeic acid did not impede cell viability or proliferation under these conditions. Abnormalities in cell morphology and viability under these conditions were negligible. These results indicated 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: We also investigated whether HCV particle release decreased with a continuous treatment with lower concentrations of coffee extract and caffeic acid (Fig. 2). Huh-7.5.1-8

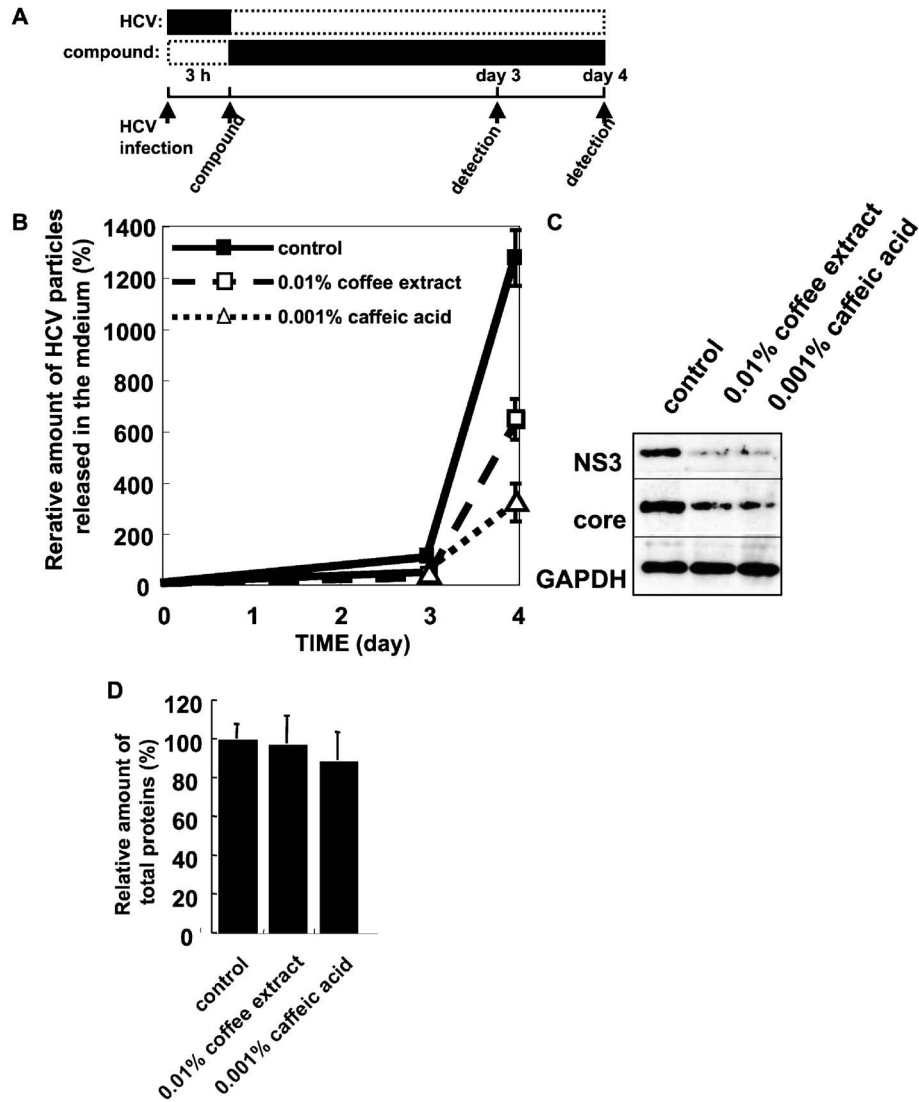


Fig. 2. HCV propagation is inhibited by continuous treatment of HCV-infected cells with coffee extract and caffeic acid. (A) The schedule for exposing Huh-7.5.1-8 cells to compounds and naïve HCV particles is schematically represented. The cells were infected with naïve HCV particles (JFH-1, genotype 2a) for 3 h. After washing to remove free HCV particles, 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 or 0.001% caffeic acid are 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 in the normal culture medium without these compounds 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 non-treated HCV-infected cells at 3 dpi set as 100% is shown. Error bars indicate 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 or caffeic acid on total proteins in HCV-infected cells. The schedule for exposing Huh-7.5.1-8 cells to HCV particles and compounds was the same as that shown in (A). Error bars indicate the standard error of the mean (S.E.M., $n = 4$).

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 (Fig. 2A). The level of HCV particles released into the medium was estimated at both time points. With the one-shot treatment, abnormalities in cell morphology and viability were minimal, and at 4 dpi, the abundance of HCV particles released into the medium drastically decreased in the presence of both the 0.01% coffee extract and 0.001% caffeic acid treat-

ments ($P < 0.006$ and $P < 0.005$, respectively) (Fig. 2B). A corresponding decrease was also demonstrated in the intracellular level of HCV core and NS3 proteins (Fig. 2C). Little difference of total proteins in the cell lysate was observed between the compound-treated and the untreated HCV-infected cells (Fig. 2D), suggesting that these compounds had little effect on cell viability and proliferation. These results were similar to those of the one-shot treatment experiments, thereby indicating that the continuous caffeic acid treatment also inhibited

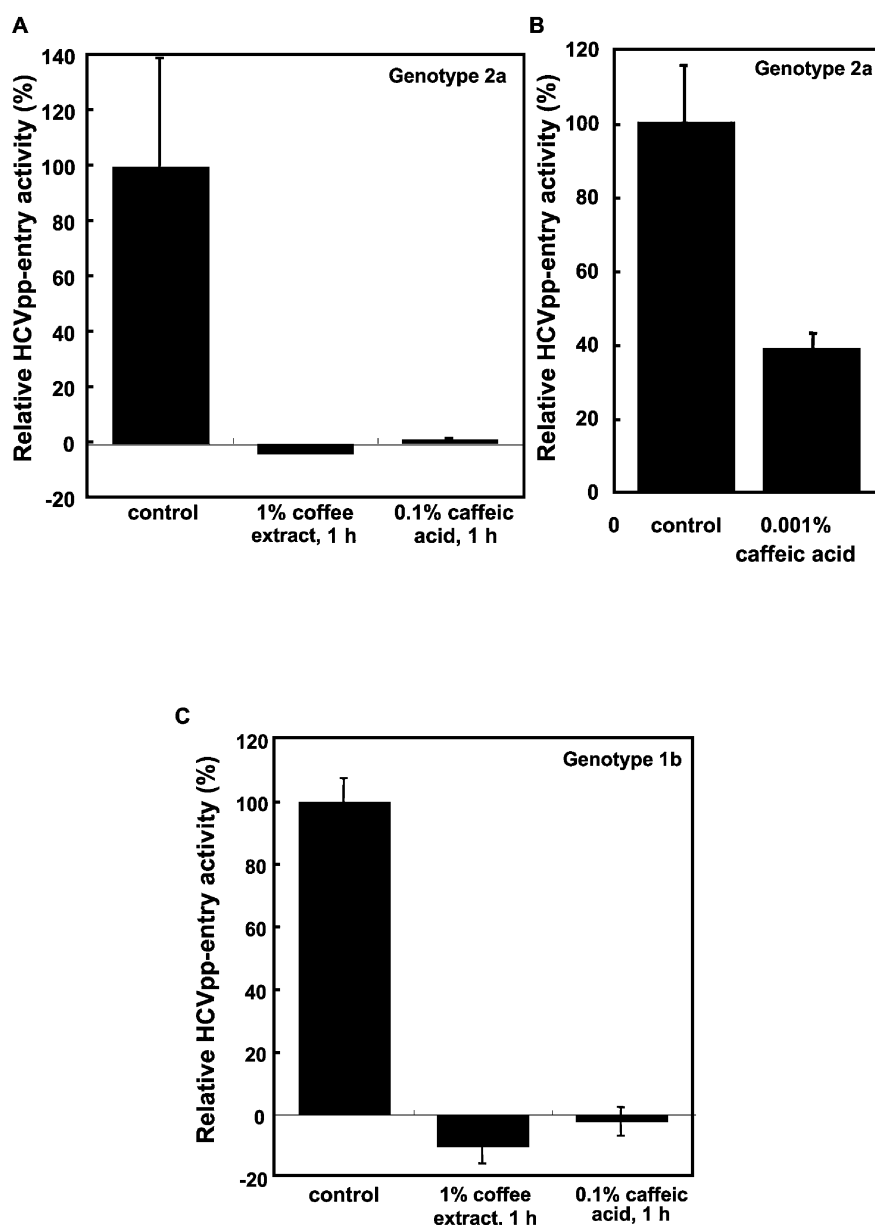


Fig. 3. Caffeic acid inhibits the initial stage of HCV infection. (A) The one-shot treatment of caffeic acid inhibits the initial stage of HCV infection. The schedule for exposing Huh-7.5.1-8 cells to compounds and HCV pseudoparticles (HCVpp) (genotype 2a) was the same as that shown in Fig. 1A. The medium 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 as 100% are shown. The data from three independent experiments are shown. Error bars indicate the standard error of the mean (S.E.M.). (B) Continuous caffeic acid treatment also inhibited the initial stage of HCV infection. The schedule for exposing Huh-7.5.1-8 cells to compounds and HCVpp (genotype 2a) is the same as that shown in Fig. 2(A). 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 non-treated HCVpp-infected cells (control) set as 100% are shown. (C) Caffeic acid inhibited the initial stage of HCVpp genotype 1b infection. The experiments were performed as described in Fig. 3A using HCVpp genotype 1b instead of HCVpp genotype 2a.

HCV propagation.

Caffeic acid inhibits the initial stage of HCV infection: The treatment of HCV-infected cells with caffeic acid resulted in the release of fewer 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 measurements. Therefore, cells will express active luciferase only when it is translated from the viral genome following HCVpp infection of the target cells.

HCVpp-infected cells were washed to remove free

Caffeic Acid Inhibits HCV Propagation

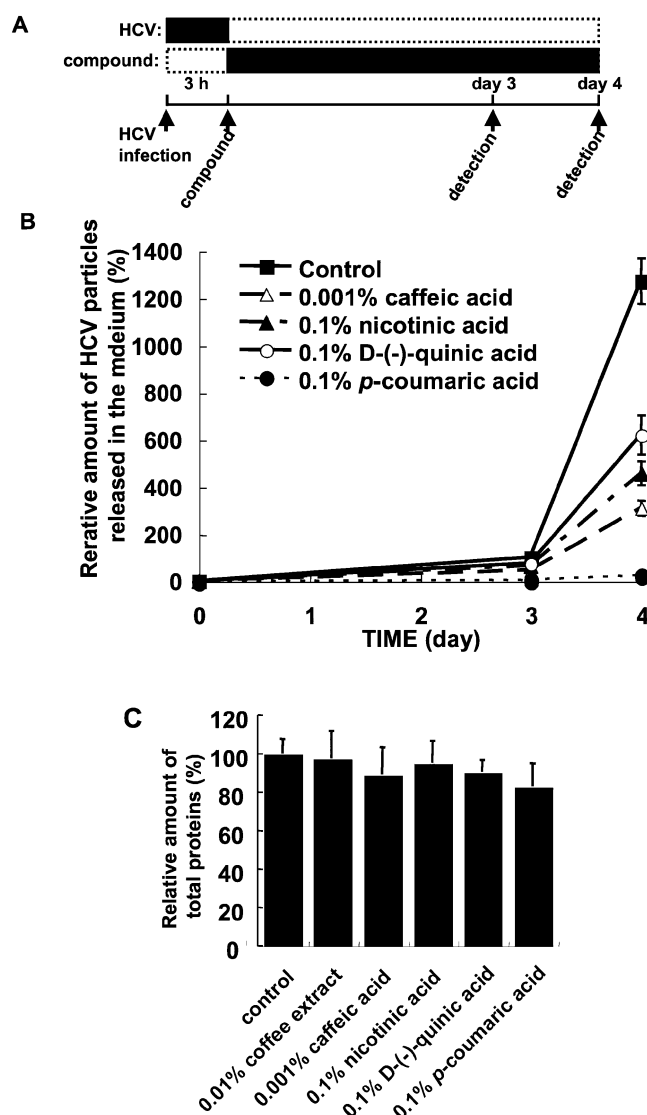


Fig. 4. High concentration of *p*-coumaric acid inhibited the HCV propagation. (A) The schedule for exposing Huh-7.5.1-8 cells to compounds and naïve HCV particles is schematically represented. (B) The effects of D-(-)-quinic acid, nicotinic acid, and *p*-coumaric acid on the HCV propagation were investigated. The relative amount of the HCV core protein in the medium with its amount in the medium without compounds at 3 dpi set as 100% is shown. The data from three independent experiments are shown. Error bars indicate 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 Huh-7.5.1-8 cells to HCV particles and compounds was the same as that shown in (A). The data from two independent experiments are shown. Error bars indicate the standard error of the mean (S.E.M., $n = 4$).

HCVpp, followed by a 1-h incubation in a 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 (Fig. 3A). When the HCVpp-infected cells were treated with 1% coffee extract or 0.1% caffeic acid for 1 h, their luciferase activity 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 a similar experiment to investigate the effect of continuous caffeic acid treatment at a lower concentration on the initial stage of HCV infection (Fig. 3B). Huh-7.5.1-8 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 cellular luciferase activity decreased ($P < 0.005$) (Fig. 3B), indicating that continuous caffeic acid treatment also inhibits the initial stage of HCV infection.

We also focused on HCV genotype 1b, another major HCV genotype (Fig. 3C). We prepared HCVpp genotype 1b and infected Huh-7.5.1-8 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 cultured in normal medium for 2 days. The cellular luciferase activity was measured at 2 dpi. As was the case for HCVpp genotype 2a, the HCVpp genotype 1b-infected cells treated with

1% coffee extract or 0.1% caffeic acid showed a drastically decreased cellular luciferase activity ($P < 0.001$ for both). 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: In addition, we investigated the anti-HCV effects of additional organic acids (*p*-coumaric acid, quinic acid, and nicotinic acid) found in coffee extract (Fig. 4A, 4B). Huh-7.5.1-8 cells were infected with HCV particles for 3 h and then 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 measured at 3 and 4 dpi. Although the concentration of all 3 organic acid was 100-times greater than that of caffeic acid, only *p*-coumaric acid strongly inhibited the HCV particle release ($P < 0.008$ at 3 dpi and $P < 0.001$ at 4 dpi). Little difference of the total proteins in the cell lysate was observed among the compound-treated and the untreated HCV-infected cells (Fig. 4C), suggesting negligible effects of these compounds on cell viability and proliferation.

DISCUSSION

In this study, we used an in vitro naïve HCV particle-infection and production system to demonstrate that coffee extract and caffeic acid inhibits 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 similar results. Caffeic acid was also shown to inhibit the initial stage of HCV infection. Our results strongly suggest that caffeic acid derived from 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 post-coffee consumption is approximately 80–116 nM, it gradually diminishes (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. Human studies have shown that the amount of coffee consumed per day is correlated 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, regardless of the viral genotype. Therefore, one-shot treatment may partly contrib-

ute to the decrease in HCV propagation. Considering that continuous treatment with lower concentrations of both substances also inhibited HCV propagation, it is possible that coffee extract and caffeic acid may affect intracellular processes essential for HCV propagation (e.g., polyprotein processing, RNA replication, virion assembly, transport, or release, in addition to viral entry and viral polyprotein translation). It is possible that caffeic acid partially inhibit RNA-replication in HCV-infected cells, since it has been reported that caffeic acid inhibits HCV-replication in HCV subgenomic replicon cells at higher concentrations (EC_{50} , about 37 μ M) (37). However, further studies are warranted to clarify the mechanisms through which these treatments inhibit HCV infection.

The 1-h treatment with coffee extract or caffeic acid following HCV infection was sufficient to severely inhibit HCV propagation. Although HCV cellular entry is mediated via many receptors, (including 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 remains unclear. Further studies are necessary to clarify these issues.

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Conflict of interest None to declare.

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