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### Part A

**Category:**
- Basic Science [x]
- Clinical Science [ ]
- Public Health/Epidemiology [ ]
- Social Science [ ]
- Programmatic Review [ ]

**Best Practice/Intervention:**
- Focus: Hepatitis C [x] Hepatitis C/HIV [ ] Other: caffeine [ ]
- Level: Group [x] Individual [ ] Other: [ ]
- Target Population: patients infected with HCV
- Setting: Health care setting/Clinic [ ] Home [ ] Other: Laboratory: in vitro [ ]
- Country of Origin: Brazil [ ]
- Language: English [x] French [ ] Other: [ ]

### Part B

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**WITHIN THE SURVEILLANCE SYSTEM FOR REVIEW**

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**RESEARCH REPORTS**

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Caffeine inhibits hepatitis C virus replication in vitro

Mariana N. Batista · Bruno M. Carneiro · Ana Cláudia S. Braga · Paula Rahal

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Abstract Hepatitis C is considered the major cause of cirrhosis and hepatocellular carcinoma. Conventional treatment is not effective against some hepatitis C virus (HCV) genotypes; therefore, new treatments are needed. Coffee and, more recently, caffeine, have been found to have a beneficial effect in several disorders of the liver, including those manifesting abnormal liver biochemistry, cirrhosis and hepatocellular carcinoma. Caffeine acts directly by delaying fibrosis, thereby improving the function of liver cellular pathways and interfering with pathways used by the HCV replication cycle. In the current study, the direct relationship between caffeine and viral replication was evaluated. The Huh-7.5 cell line was used for transient infections with FL-J6/JFH-5/C19Rluc2AUbi and to establish a cell line stably expressing SGR-Feo JFH-1. Caffeine efficiently inhibited HCV replication in a dose-dependent manner at non-cytotoxic concentrations and demonstrated an IC50 value of 0.7263 mM after 48 h of incubation. These data demonstrate that caffeine may be an important new agent for anti-HCV therapies due to its efficient inhibition of HCV replication at non-toxic concentrations.

Introduction

Hepatitis C is a liver infection caused by hepatitis C virus (HCV) [5]. Usually, this infection does not generate an adequate host immune response [13], and it progresses to a chronic infection in approximately 80 % of patients [19]. HCV infection is a global public health problem and has been associated with 350,000 annual deaths related to cirrhosis and hepatocellular carcinoma [4]. Pegylated interferon alpha (PEG-INF) in association with ribavirin (RBV) has been the standard treatment for patients with chronic HCV infection in the last decade [35]. The new standard treatment includes the protease inhibitors boceprevir and telaprevir in association with PEG-IFN and RBV [29]. However, severe side effects are observed, the treatment is costly, and viral resistance has been demonstrated for all classes of directly acting antivirals [17]. Therefore, new treatments are needed.

Coffee and caffeine consumption has been associated with beneficial effects in patients with abnormal liver function, cirrhosis and hepatocellular carcinoma [3, 6, 10]. The daily consumption of approximately 3 cups of coffee [10], which is approximately 408 mg of caffeine [6], may reduce the risk of chronic liver disease progression. Caffeine has also been demonstrated to have an antiproliferative effect on liver cancer cells [28]. Moreover, caffeine consumption may limit liver fibrosis, especially in patients with chronic HCV infection (14 % milder than other fibrosis groups) [24].

It has been suggested that caffeine may interact with cellular pathways to slow the progression of liver diseases. Among these pathways, a decrease in alanine aminotransferase (ALT) [30], alkaline phosphatase and aspartate aminotransferase (ASP) and an increase in serum albumin have been observed [24]. These studies indicate that
caffeine has the potential to improve liver functions in patients chronically infected with HCV. As there is no evidence of direct interference of caffeine with the HCV replication cycle, the aim of the current study was to assess the effect of caffeine on HCV replication.

Materials and methods

HCV replicons

The replicons used in the study were as follows: FL-J6/JFH-5'C19Rluc2AUbi, a monocistronic full-length HCV genome that expresses Renilla luciferase (RLuc) [33], derived from the previously described infectious genotype 2a of the HCV genome, J6/JFH1 [22]; and SGR-Feo JFH-1, a bicistronic subgenomic replicon based on the JFH-1 sequence, which possesses the firefly luciferase gene fused to a neomycin resistance gene [36]. RNA was transcribed from the plasmids (pFL-J6/JFH-5'C19Rluc2AUbi J6-JFH1 RLUC and pSGR-Feo JFH-1) after linearization with the XbaI enzyme and mung bean nuclease treatment followed by phenol/chloroform extraction [39]. The purified product was used as a template for transcription with the T7 Ribomax™ Express Large Scale RNA Production System (Promega, Madison, WI, USA).

Cell culture

A human hepatoma cell line (Huh-7.5) was maintained in DMEM medium supplemented with 10 % FBS (Cutilab, Campinas, SP, BR), 1 % (v/v) non-essential amino acids (Gibco Life Technologies, USA), 100 U/mL penicillin (Gibco Life Technologies, USA), 100 mg/mL streptomycin (Gibco Life Technologies, USA) and 1 % HEPES (Gibco Life Technologies, USA) in a humidified 5 % CO₂ incubator. The Huh-7.5 cell line stably expressing SGR-Feo JFH-1 was maintained in DMEM supplemented with 500 μg/mL G418 (Sigma-Aldrich, St. Louis, MO, USA).

HCV RNA transfection

Cells were transfected with transcribed HCV RNA by electroporation as described elsewhere [1]. Briefly, cells were detached by trypsin treatment and washed twice with RNase-free PBS and then resuspended in 10 mL of Cytomix buffer [34]. Ten micrograms of FL-J6/JFH-5'C19Rluc2AUbi RLUC RNA or SGR-Feo JFH-1 RNA was mixed with 4 × 10⁶ (subgenomic assay) or 8 × 10⁶ (HCVcc assay) cells in a 4-mm cuvette, and a Bio-Rad Pulser Xcell system (Bio-Rad, Amadora, PT) was used to deliver a single pulse at 0.27 kV, 950 μF and ohms. Electroporated cells were transferred to culture flasks containing complete DMEM. The stable cell line harboring SGR-Feo JFH-1 RNA was obtained after G418 selection of electroporated cells (Sigma-Aldrich, St. Louis, MO, USA) after at least three weeks of culture.

Caffeine cytotoxicity profile

The cytotoxicity of caffeine (Sigma-Aldrich, St. Louis, MO, USA) was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA). Cytotoxicity was assessed after the cells were incubated for 24 h, 48 h and 72 h with caffeine.

Cell cycle analysis by flow cytometry

Prior to the start this assay, the cells were synchronized in G0 by FBS deprivation for 24 h. To determine the caffeine-treated cell cycle pattern, synchronized Huh-7.5 cells stably expressing SGR-Feo JFH-1 were seeded on a 96-well plate (5 × 10³ cells/well) 24 h before treatment. Caffeine was then added at the following concentrations: 0, 0.250 mM, 0.5 mM, 1 mM, 2 mM and 4 mM. One set of cells was maintained without serum, which was used as positive control of cell cycle arrest in G0. Forty-eight hours post-treatment, the cells were resuspended, counted and fixed overnight with 75 % cold ethanol. On the following day, cells were stained with Guava Cell Cycle Reagent following the manufacturer’s instructions (EMD Millipore Corporation, Hayward, CA, USA). Subsequently, these cells were analyzed using easyCyte 5HT flow cytometry (Guava Easycheck, Millipore Corporation, Hayward, CA, USA) with 10,000 events acquired from each sample.

Indirect immunofluorescence assay

Cells were fixed with 4 % paraformaldehyde and permeabilized with 0.1 % Triton-X-100. The cells were then incubated with a primary polyclonal sheep antibody against the NS5A protein (kindly provided by Professor Mark Harris, University of Leeds) at a dilution of 1:4,000 followed by incubation with a 1:500 dilution of an Alexa Fluor® 594-conjugated donkey anti-sheep IgG (H+L) (Life Technologies, Carlsbad, CA, USA), both for 2 h at room temperature.

Virus titration

Cell supernatants were serially diluted 10-fold in complete DMEM and used to infect 5 × 10³ Huh-7.5 cells per well in 96-well plates. The inoculum was incubated with the cells for 48 h at 37 °C and then fixed with 4 % paraformaldehyde. HCV infection was detected by
immunofluorescent staining for the HCV NS5A protein. The viral titer was expressed as focus-forming units per milliliter of supernatant (FFU/mL), determined by calculating the average number of NS5A-positive foci present at the highest dilutions.

Luciferase reporter assay

To determine the IC50 value of caffeine against the virus, HCV subgenomic replicon protein expression in Huh-7.5 cells was analyzed during caffeine treatment. Huh-7.5 cells stably expressing SGR-Feo JFH-1 were seeded on a 96-well plate (5 × 10^3 cells/well) 24 h before treatment. Caffeine was then added at the following concentrations: 10 mM, 3.17 mM, 1 mM, 0.317 mM, 0.1 mM, 0.0317 mM, 0.01 mM, 0.00317 mM, 0.001 mM, 0.000317 mM. The cells were incubated at 37 °C in a humidified atmosphere with 5 % CO2 for 48 h. Cyclosporin A (CsA) (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control for inhibition, and Milli-Q water (the diluent of the caffeine solutions) was used as a negative control for inhibition. After treatment, the cells were disrupted with 30 μL of passive lysis buffer (Promega, Madison, WI, USA), and the luciferase substrate (Promega, Madison, WI, USA) was added. Luciferase activity was measured using a luminometer (FLUOstar Omega/BMG LABTECH, Offenburg, BW, DE), and a BCA protein assay kit (Thermo-Scientific, Rockford, IL, USA) was used to normalize protein concentrations.

qPCR analysis

Total RNA was extracted from caffeine-treated cells using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Five hundred nanograms of RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Complementary DNA was amplified in triplicate using primers to the 5' UTR region of the HCV genome (forward: 5' CGGGA-GAGCCATAGTGG; reverse, 5' AG-TACCAAACGCTCTTCG) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, CA, USA). A standard curve was generated with 10-fold dilutions of HCV JFH-1 RNA control that had been pre-quantitated by spectrophotometry.

Western blotting analysis

To analyze HCV protein expression upon caffeine treatment, a western blot was performed. In this assay, Huh-7.5 cells containing SGR-Feo JFH-1 or infected with FL-J6/JFH-5'CI9Rluc2AUbi were seeded at 2 × 10^5 cells/well on a 6-well plate. Cells were treated with the appropriate drug concentrations, and after 48 h, the cells were lysed with the CelllyticTM M (Sigma-Aldrich, St. Louis, MO, USA) containing protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA). The protein in the cell lysate was then quantified using the BCA assay (Thermo-Scientific-Pierce, Rockford, IL, USA) following the manufacturer’s instructions. Subsequently, the proteins were separated by SDS-PAGE, transferred to a PVDF membrane (Millipore, Bedford, MA, USA), blocked in TBS-T with 5 % skim milk (Bio-RAD, Amadora, PT) and incubated overnight with a polyclonal anti-NS5 mouse antibody (1:3,000) (Abcam, San Francisco, CA, USA). The membrane was then incubated with anti-mouse IgG (whole molecule) (Abcam, San Francisco, CA, USA). The membrane was incubated for 1 minute with the Pierce ECL Western Blotting Substrate (Thermo-Scientific-Pierce, Rockford, IL, USA), and luminescence intensity was captured on a Chemi-Doc System (Bio-Rad, Amadora, PT). GAPDH (Abcam, San Francisco, CA, USA) was used as the endogenous control for normalization of protein expression.

Statistical methods

The half-maximal inhibitory concentration of caffeine (IC50) was calculated using linear regression. The results of the inhibition of SGR-Feo JFH-1 were calculated as the percentage of the negative control (medium, no drug). All statistical analyses were performed by one-way ANOVA with Tukey’s post test or two-way ANOVA with Bonferroni post-tests using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

Results

MTT

Caffeine cytotoxicity against Huh-7.5 cells containing SGR-Feo JFH-1 was determined by MTT assay. The solubility of caffeine in water is 16 mg/mL at room temperature, and we used this point of reference to test different concentrations in cell culture. We observed that cell viability was affected by caffeine in a time- and dose-dependent manner (Fig. 1a). When the cells were treated with...
caffeine concentrations above 2 mM for 48 h, the cell viability was close to 80 %. After 72 h of treatment, the cells reached this arbitrary cell viability cutoff at concentrations below 1 mM. Thus, we observed maximum viral inhibition at safe concentrations after 48 h; this time point was therefore selected for all subsequent experiments. Caffeine concentrations equal to or below 1 mM were safely tolerated by the cells, but a twofold increase (2 mM) reduced cell viability to approximately 80 % after 48 h. Therefore, 2 mM was the highest concentration utilized for further experiments.

Inhibitory effect of caffeine on HCV replication

To evaluate the effect of caffeine on HCV replication, SGR-Feo JFH-1 cells were incubated with the following concentrations of caffeine: 0.5 mM, 1 mM and 2 mM. Replication efficiency was measured 24 h (Supplementary Material S1) and 48 h after treatment using luciferase assays. The half-maximal inhibitory concentration (IC_{50}) of caffeine was determined to be approximately 0.726 mM (Fig. 1b). When the cells were treated with 0.5 mM caffeine, viral replication was inhibited by 33.56 ± 7 % (n = 9) compared with the mock control. An increase in the drug concentration showed an increase in the inhibition of viral replication in a dose-dependent manner (Fig. 2a and e). The highest safely tolerated concentration (2 mM) of caffeine reduced the luciferase activity by 82 ± 2 % (n = 9) of the mock control values. Protein expression levels were also verified by western blot analysis of the NS3 virus protein (Fig. 2b). After 48 h of treatment, caffeine reduced viral protein expression by at least 70 % at the highest drug concentration.

HCVcc inhibition by caffeine

To verify whether caffeine could also interfere with full-length HCV replication, Huh-7.5 cells were infected with cell-culture-derived HCV (HCVcc) at a multiplicity of infection of 0.1 and treated with different concentrations of caffeine. Similar to what was observed in the stable cell line, caffeine reduced viral replication in a dose-dependent manner (Fig. 2c). Using the drug at 2 mM, the intracellular levels of luciferase were reduced by 79.3 ± 9 % of the mock control, whereas at 0.5 mM, luciferase levels were reduced by 40.6 ± 13 % of the mock control. Analysis of the NS3 virus protein by western blot showed that after 48 h of treatment, viral protein expression was reduced by at least 60 % at the highest drug concentration (Fig. 2d).

Physiologic state analysis

To evaluate the influence of caffeine on the cell cycle, caffeine-treated cells were submitted to cell cycle assay by flow cytometry. We observed that cells treated with or without caffeine remained in the division process, ranging
from the S to the G2/M phase. At the highest concentration used in inhibition assays (2 mM), 74.15 % of cells were undergoing division (S or G2/M), and in caffeine-free cells (negative control), 78 % of the cells were undergoing division, and thus, there was no statistical significant differences in the frequency of cell division. Cells maintained without serum were used as a positive control for cell cycle arrest, and 52.45 % of the cells were in cell cycle arrest in G0/G1, while in the negative control, 22 % of the cells were at this stage (Fig. 3).

After cell cycle analysis, we tested the capacity of caffeine to induce cell apoptosis by poly ADP ribose polymerase (PARP) expression analysis [18]. This analysis was performed using western blotting to anti-PARP. Western blotting assay was performed on cells treated with caffeine for 48 h, and doxorubicin was used as a positive control of apoptosis induction [9]. The percentage of apoptotic cells in the overall population upon caffeine treatment did not differ significantly from that of the negative control (zero caffeine) (Fig. 4).

Discussion

Viral replication is an essential step in the maintenance of virus infection, and the process represents an important target of anti-HCV therapy. New drugs against HCV are based on inhibiting viral replication [12]. In this study, we clearly demonstrated that caffeine, a phytochemical found in many plants, was able to reduce HCV replication. This
effect was observed using the HCV genotype 2a stably expressed in Huh-7.5 cells. To our knowledge, this is the first work to demonstrate the relationship between the in vitro administration of caffeine and a decrease in HCV replication.

Clinical reports have shown no change in HCV RNA levels in the serum of infected patients [24] after long-term caffeine consumption. This may be explained by the fact that the caffeine concentration tested in our study was much higher than that found in regular caffeine intake. Additionally, conventional therapies are restricted only to the inhibition of HCV replication, but caffeine, in addition to its effect on viral replication, can also reduce the liver damage caused by chronic HCV infection. Caffeine can act on detoxification pathways [32] and improve the expression of liver enzymes, such as ALT, ASP, alkaline phosphatase and albumin [6, 10, 24]. Also, it has already been reported that caffeine may alter cancer cell proliferation by inducing cell cycle arrest [28] and cellular apoptosis [23] and by inhibiting pro-inflammatory gene expression [14].

In this study, we showed that caffeine treatment resulted in dose-dependent inhibition and reduced viral replication by up to 80% of the mock control when tested at 2 mM. This concentration is the highest non-cytotoxic

![Fig. 3 Effect of caffeine on the cell cycle pattern of Huh-7.5/SGR-Feo JFH-1 cells. Huh7.5 cells stably harboring the SGR-Feo JFH-1 replicon were treated with increasing doses of caffeine. Cells were analyzed for their DNA content and the percentage of cells in the different stages of the cell cycle. Cells maintained with serum deprivation were used as a cell cycle arrest control. Stable replicon cells treated with caffeine diluent were used as a negative control. The assays were performed twice with 10,000 events. ***P < 0.0001 vs. control. a) Illustration of results showing histograms from the cell cycle assay. b) Graphic representation of the cell cycle assay.](image-url)
Caffeine inhibits hepatitis

Fig. 4 Western blot analysis of apoptosis induction after treatment with different concentrations of caffeine. After treatment with caffeine at different concentrations for 48 h, Huh7.5/SGR-Feo JFH-1 cells were subjected to western blot analysis. Doxorubicin (10 \( \mu \)g/mL) was used as a positive control for apoptosis. Relative band intensities were measured by densitometry analysis with ImageJ software. The values were normalized to GAPDH, and the intensity of the doxorubicin band was set as 1.

concentration for this cell line. Similar to what has been described previously by Okano et al. [28] for some hepatocellular carcinoma cells (HCC) and by Lu et al. [23] for osteoblasts, the caffeine toxicity profile on Huh-7.5 cells increases in a time- and dose-dependent manner. In those previous studies, the cause of the loss of viability was cell cycle arrest [28] or induction of apoptosis [23]. To demonstrate that the effect of caffeine is directly related to the capacity of the drug to inhibit virus replication, rather than to interfere with normal cellular functions, some physiological pathways were analyzed in our study. We demonstrated that the cell cycle progression pattern in caffeine-treated cells remained unchanged at the concentrations used for virus inhibition (0.5 mM, 1 mM and 2 mM) compared to negative control. Analysis of PARP expression indicated no changes in the level of apoptosis in caffeine-treated cells in relation to untreated ones. This result is consistent with those of Kawano et al. [16], who showed that caffeine was unable to induce apoptosis in HCC cells. Other studies have demonstrated changes in cell cycle parameters. When Huh-7 cells were treated with caffeine at concentrations higher than 2.5 mM and for longer incubation periods (72 h), these HCC cells show a tendency to undergo cell cycle arrest in G0/G1 phase [28].

Another mechanism related to cell death, autophagy, was also investigated in our study. As described previously by other authors using liver cells [31], we observed that caffeine treatment increases the proportion of cells in an autophagic state, but this difference was not statistically significant. That process was first described as a mechanism of cellular maintenance and survival as a response to stressful stimulus. However, in some situations, autophagy may be responsible for destruction of cells as a result of protracted atrophy of the cytoplasm. In this case, autophagy acts as a non-apoptotic programed cell death [7]. In this study, it was not determined whether this process led to cell death, but it is possible that the observed result may explain the reduction in viability that was observed in the MTT assay. An increase in the autophagy rate has been correlated with an improvement in HCV replication [8, 25]. Caffeine has the ability to inhibit proliferation of liver cancer cells and induce their death [16, 28], and therefore, cell viability might increase if caffeine were tested on non-cancer cell lines, such as primary human hepatocytes.

Although a caffeine-mediated inhibition of HCV replication was clearly demonstrated in this study, the inhibitory mechanism remains unknown. Caffeine reduces the expression of proteins in cell pathways used by HCV replication, including the following: HSP90, which interacts directly with NS5A [2, 26, 27]; Ras-ERK, which interacts directly with NS5B and the 5' end of the negative RNA strand of HCV [28, 37, 38]; and COX-2 [14, 15]. However, there is not much evidence supporting these candidate pathways.

The enzyme cyclooxygenase-2 (COX-2) is strongly associated with HCV replication, and many studies have shown an interaction [11, 20, 21]. For example, COX-2 can increase virus expression and can restore the replication capacity of HCV after HCV inhibition by catechins from green tea [21]. Kang et al. [14] demonstrated the ability of caffeine to inhibit COX-2 in microglial cells. Caffeine may also inhibit some ERK pathway proteins that are responsible for regulating COX-2 expression [14, 28]. Therefore, we hypothesize that caffeine may regulate HCV replication by altering COX-2 expression in treated cells, although more studies are needed to test this hypothesis. Nevertheless, COX-2 is a good candidate that may be involved in the inhibitory effect of caffeine on HCV; however, we cannot rule out the possibility that caffeine may interact directly with a viral protein.

Conclusion

The data presented in this study indicate that pure caffeine significantly inhibited genotype 2a HCV replication in vitro. However, a definitive mechanism of the inhibitory effect of caffeine was not established. Further studies on the cell pathways and viral proteins expressed upon caffeine exposure as well as an efficient in vivo caffeine delivery method are needed.

Acknowledgments The authors are grateful to Professor Mark Harris (University of Leeds, UK) for providing the polyclonal anti-NS5A antibody, and to CAPES (Coordination for the Improvement of Higher Education Personnel) and FAPESP (grant 2012/19074-1 São Paulo Research Foundation) for financial support. The authors would also like to thank all members of the Laboratory of Genomics Studies (IBILCE-UNESP, BR) for their helpful suggestions and discussions.

Conflict of interest The authors declare that they have no conflict of interest.
References


