Original Article

Hepatitis C Virus Genotype 4 Replication in the Hepatocellular Carcinoma Cell Line HepG2/C3A

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ABSTRACT

Background/Aims: The lack of a reliable cell culture system allowing persistent in vitro hepatitis C virus (HCV) propagation is still restraining the search for novel antiviral strategies. HepG2 cells transfection with HCV allows for viral replication. However, the replication is weak presumably because of HepG2 lack of miRNA-122, which is essential for viral replication. Other agents such as polyethylene glycol (PEG) and dimethyl sulfoxide (DMSO) have been shown to increase the efficiency of infection with other viruses. This study included comparison of HCV genotype 4 UTR and core RNA levels and HCV core protein expression at different time intervals in the absence or presence of PEG and/or DMSO postinfection.

Materials and Methods: We used serum with native HCV particles in infecting HepG2 cells in vitro. HCV replication was assessed by reverse transcriptase polymerase chain reaction for detection of HCV RNA and immunofluorescence and flow cytometry for detection of HCV core protein. Results: HCV 5’UTR and core RNA expression was evident at different time intervals after viral infection, especially after cells were treated with PEG. HCV core protein was also evident at different time intervals using both immunofluorescence and flow cytometry. PEG, not DMSO, has increased the HCV core protein expression in the treated cells, similar to its effect on viral RNA expression. Conclusions: These expression profiles suggest that the current model of cultured HepG2 cells allows the study of HCV genotype 4 replication and different stages of the viral life cycle.

Key Words: Dimethyl sulfoxide, hepatitis C virus, hepatitis C virus core, hepatitis C virus 5’UTR, HepG2, polyethylene glycol

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Hepatitis C virus (HCV) is one of the most common viruses that infects the lives of more than 170 million people worldwide and is one of the leading causes of chronic liver disease, cirrhosis, and hepatocellular carcinoma. HCV is a member of the family Flaviviridae, genus Hepacivirus that was discovered in 1989. HCV genome is a linear, single stranded RNA of positive polarity, approximately 9.6 kb, which contains a single open reading frame (ORF) encoding a large polyprotein of about 3000 amino acids (aa). HCV is classified into at least six major genotypes that in turn are subdivided into sets of subtypes representing all the HCV isolates distributed all over the world. HCV genotype 4 has been identified as the principal genotype among infected individuals from the Middle East and North Africa, particularly Egypt. HCV replication takes place in the cytoplasm, and the encoded polyprotein is localized to the rough endoplasmic reticulum (ER), where it is cleaved into 10 structural (C, E1, E2, and P7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. These proteins play important roles in virus replication, assembly, and pathogenesis. HCV core protein is a structural protein of the nucleocapsid that can affect apoptosis, lipid metabolism, transcription, host cell transformation, and immune response of the infected host. Core protein exists in three forms; 21 kDa, 19 kDa, and 16 kDa. The genome sequence coding for the core protein is highly conserved within the different HCV genotypes.

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and Fas. These interactions influence the efficacy of the host antiviral immune responses, which play an important role in the development of chronic infection and in the changes of host cell sensitivity to apoptosis.\textsuperscript{[10,11]}

The lack of a reliable cell culture system continues to allow the persistent propagation of the \textit{in vitro} virus and hinders the screening of antiviral strategies. Some cell lines, particularly of lymphoid origin, are susceptible to HCV infection and permissive for HCV RNA replication.\textsuperscript{[12]} Although virus production has been achieved by long-term culture of primary hepatocytes of infected patients,\textsuperscript{[13]} efforts to propagate the virus by infection of adherent cells such as hepatoma cell lines have been discouraging because of poor yield and expression. Transfection of HepG2 cells with HCV stably replicate virus and promote both growth and tumor genesis.\textsuperscript{[14]} However, HepG2 lacks miR-122, an miRNA that is important for HCV RNA replication,\textsuperscript{[15]} and the cells weakly support HCV replication.\textsuperscript{[16]} Polyethylene glycol (PEG) and dimethyl sulfoxide (DMSO) have been shown to increase the efficiency of infection with other viruses such as hepatitis B virus,\textsuperscript{[17]} Sendai virus,\textsuperscript{[18]} herpes simplex virus types 1 and 2,\textsuperscript{[19]} and mouse hepatitis virus.\textsuperscript{[20]}

In this study, we examined the effect of PEG and/or DMSO on HCV gene expression and replication. The study included comparison of HCV 5'UTR and HCV core RNA levels and HCV core protein expression at different time intervals.

**MATERIAL AND METHODS**

**HCV samples**

We used five serum samples that were identified as positive for anti-HCV antibodies and negative for anti-HBV and anti-HIV antibodies. Viral titer was determined by the Diagnostic Molecular Biology Unit of Pathology Department, College of Medicine, King Saud University, using real-time polymerase chain reaction technique and Cobas Taqman assay (Roche Molecular Diagnostics, California, USA). High viral titers were used in these studies ranging from 300,000 to 3,000,000 copies/mL.

**HCV genotyping and sequence logo**

All samples were genotyped using direct sequencing method. Viral RNA from HCV-positive sera was extracted using QiAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany). RNA was then amplified using QiAEn One Step RT-PCR kit for Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) (QIAGEN) on the GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). We used the primer sets listed in Table 1 for amplification of 5'UTR\textsuperscript{[21]} and core regions. PCR products were purified using EXO-SAP IT\textsuperscript{®} kit (USB Products Cleveland, Ohio, USA). Sequencing of the purified fragments was done by BigDye\textsuperscript{®} Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) for the tagging of sequencing dyes. The products were then purified by BigDye\textsuperscript{®} X Terminator v3.1 purification kit (Applied Biosystems) following the manufacturer's instructions. The purified fragments were then separated by capillary electrophoresis, collected, and detected by GA-3130 genetic analyzer (Applied Biosystems). Alignment, data analysis, and genotyping were done by using MEGA 5.05 software, Blast http://blast.ncbi.nlm.nih.gov/Blast.cgi and HCV data base http://www.hcvdb.org/, respectively. HCV sequences logo for the used HCV isolates was created by application of the resulted sequences into WebLogo 3 software http://weblogo.threeplusone.com/create.cgi according to user's manual instructions.

**Cell culture**

Human hepatocellular carcinoma cell line HepG2/CA3 (ATCC, Manassas, VA, USA) was used to establish the \textit{in vitro} HCV replication system. HepG2/CA3 were grown in EMEM growth medium (LONZA, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), 1% glutamax, and 1% penicillin/streptomycin, then they were incubated in 5% CO\textsubscript{2} incubator at 37°C. The culture medium was renewed by fresh medium every three days.

**HCV infection**

We optimized the viral dose for infection using 0.05, 0.1, and 0.15 copy/cell and found that 0.05 copy/cell gave the best replication result. Then we adjusted the viral titer of the used samples to 1,000,000 copy/mL and used 0.5 mL/10\textsuperscript{5} cells. HepG2 cells were cultured in 6 cm\textsuperscript{2} culture plates at density of 5 × 10\textsuperscript{4} cells/plate. Cells were grown to semi-confluence in complete medium, washed twice with FBS-free medium, then inoculated with a serum sample (500 μL serum and 500 μL FBS-free EMEM/1 × 10\textsuperscript{6} cells) obtained from HCV-infected patients, giving a final concentration of 0.05 copies/cell. After 90 min, EMEM containing FBS was added.\textsuperscript{[22]} Cells were maintained overnight at 37°C in 5% CO\textsubscript{2}. On the next day, adherent cells were washed three times with culture medium to get rid of the remaining infection serum and incubation was continued in complete medium containing FBS with regular medium changes. In the supplemental experiments, PEG (final concentration 4%) and DMSO (final concentration of 1.5% or 2% when combined with PEG) were added to the fresh medium.

**HCV RNA RT-PCR**

Total RNA was isolated from cells using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer protocol. RT-PCR was performed using one-step RT-PCR kit (QIAGEN, Hilden, Germany). Universal primers, KY80 and KY78, were used for amplification of the 5'UTR HCV viral region,\textsuperscript{[23]} whereas we used our custom-designed primers for the HCV core region [Table 1]. Because of the diversity

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**Table 1**: Primer sets listed in Table 1 for amplification of 5'UTR and core regions.

| Primer Set | 5'UTR | Core |
|------------|------|------|
| KY80       |      |      |
| KY78       |      |      |
| KY90       |      |      |
| KY91       |      |      |
| KY92       |      |      |
| KY93       |      |      |

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This information is presented in a structured manner, focusing on the methodology and results of the study, along with necessary background information to understand the context. The text is clear and logical, ensuring that all parts are coherent and easy to follow.
between the different sequences of HCV genotype 4 subtypes, three primer sets for the core region were used. One forward primer was used in separate reactions with one of the three different reverse primers to produce 326 bp fragments. These primer sets were tested and the products were genotyped by sequencing. 500 ng of viral RNA per reaction. Amplification conditions for HCV 5’UTR amplification were as follows: 50°C for 30 min; 94°C for 15 min; 40 cycles of 94°C for 30 s, 65°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. Amplification conditions for the HCV core region were the same except for the annealing temperature (58°C for 1 min). PCR products were analyzed using 1.5% agarose gel electrophoresis, ethidium bromide, and visualized using gel documentation system, GEL DOC XR (Bio-Rad, Pennsylvania, USA). PCR band intensities were determined using Image J software version 1.47 (http://imagej.nih.gov/ij, National Institute of Mental Health, Bethesda, MD, USA) and numerical values representing each PCR band were used for Microsoft Excel graphing.

**Immunofluorescence**

Infected cells were plated at density of 8 x 10⁴ cells/well. After 2, 4, and 6 days, the medium was removed and cells were fixed and permeabilized by 2% paraformaldehyde (Santa Cruz Biotechnology, Dallas, TX, USA) and 0.1% Triton X-100 (LKB Bromma, Sweden) in PBS for 30 min. After washing with PBS, cells were blocked with 1% BSA (Santa Cruz Biotechnology) in PBST (1% Triton X-100 in PBS) for 30 min. The cells were incubated with the monoclonal primary antibody Hep C cAg (C7-50) (Santa Cruz Biotechnology) specific for HCV core detection using 1:50 dilution for 30 min. The pellet was washed three times and incubated with the FITC-conjugated secondary antibody (1:100 dilution) for 30 min, washed and suspended in 500 μL 1% paraformaldehyde. The cells were analyzed by BD FACS CALIBUR cell analyzer (BD Biosciences, San Jose, CA, USA). The BD Cell Quest™ Pro software version 6.0 (BD Biosciences), supplied with the analyzer, was used for cytometric analysis and data presentation.

**Compliance with ethical standards**

Written informed consents from participating subjects from Saudi Arabia were obtained. The project and data forms were approved by the Ethics Committee at College of Medicine and King Khalid University Hospital, King Saud University, Riyadh, Saudi Arabia, in compliance with the Helsinki declaration (http://www.wma.net/en/30publications/10policies/b3/index.html).

**RESULTS**

**HCV genotyping**

All samples were amplified and sequenced for both HCV 5’UTR region [Figure 1a] and HCV core region [Figure 1b] and only samples identified as genotype 4 were selected for in vitro infection of HepG2 cells. Genotyping of HCV samples was done using the direct sequencing method and the resulting sequences were screened using HCV blast data base for maximum identity. For 5’ UTR sequences, identities ranged from 98% to 100%, obtained by alignments of 5’UTR sequences with their consorts from HCV complete genome references with the Gene Bank accession numbers FJ462441 and FJ462439. For core sequences, the maximum identities
were 94%–99% when compared with HCV core sequences of the HCV genotype 4 complete genome references, FJ462437.1, FJ62439.1, and JX227979.1.

HCV logos were built for both 5'UTR and core sequences alignments. HCV 5'UTR logo [Figure 2a] shows the alignment of 196 nucleotides (excluding primers sequences) of which 191 were similar among the different HCV samples (97.5%), indicating nucleotide conservation. Variations appeared in 5 nucleotide positions where substitution between nucleotides A and G or T and C is shown (2.5%). For HCV core logo [Figure 2b], the sequence identity among the used isolates was 81.4% (231 nucleotide out of 285, excluding primers sequences). The variations resulted from mismatches, with no gaps, in 53 positions of the 285 compared nucleotides (18.6%). Substitutions were either between 2 nucleotides (43 positions) or 3 nucleotides (9 positions) whereas the 4 nucleotides substitution in one position (position 243) indicated maximum variation.

**HCV 5'UTR and core RNA expression in HCV infected cells**

HCV RNA expression was tested by RT-PCR at different time intervals after virus infection. Gel electrophoresis results for 5'UTR PCR products of RNA that was extracted from the infected cells up to two months postinfection (data not shown) displayed weak signals. In an attempt to enhance HCV 5'UTR and core PCR signals, we decided to use larger amounts of total RNA (1 µg) extracted from cells after infection. Gel electrophoresis for both HCV 5'UTR and core regions with β-actin, as internal control, was performed at three time intervals, 2, 4, and 6 days [Figure 3a]. Bands intensities were measured to reveal slight increase in 5'UTR bands raised against the 2 days and 4 days postinfection [Figure 3b] in contrast to the core results that show a remarkable increase at day 6 while the intensity increased about three times more than the other intervals.

**HCV 5'UTR and core RNA expression in HCV-infected cells treated with supplements**

To enhance the HCV replication, we supplemented the culture media with PEG and/or DMSO. We compared cell cultures supplemented with PEG and/or DMSO with the noninfected cells as negative control. PCR products of 5'UTR at two time intervals; 2 and 4 days [Figure 4a], showed a remarkable increase in band intensity with PEG treatment when compared with nontreated cells. The addition of DMSO to the culture media induced a decrease in the HCV 5'UTR signal [Figure 4a], which was potentiated with PEG/DMSO mixture [Figure 4a]. HCV core RT-PCR results showed no difference between HCV-infected cells with no supplement and HCV-infected cells with PEG and/or DMSO except for a slight increase with PEG addition.
to the culture medium [Figure 4b], especially after 2 days postinfection.

**HCV core protein expression in HCV-infected cells using immunofluorescence**

We studied HCV core protein expression by immunofluorescence [Figure 5]. At the same culture conditions, we also supplied culture media with PEG and/or DMSO. Cells were studied at 2, 4, and 6 days postinfection. HCV core protein was expressed in cells with no supplement, especially at day 6 postinfection [Figure 5c]. Cells supplemented with PEG only showed strong core protein expression increase from day 2 up to day 6. The use of DMSO as a media supplement with or without PEG seems to have no effect on the expression levels of HCV core protein, and moreover, DMSO may have had an inhibitory effect on the enhanced expression of HCV core protein, induced by PEG alone [Figure 5b and c].

**HCV core protein expression in HCV-infected cells using flow cytometry**

Flow cytometric analysis showed that HCV core protein was detected inside the infected HepG2 cells 2, 4, and 6 days postinfection [Figure 6]. The flow cytometry histograms and dot plots indicate expression of HCV core protein in cells at 2 days postinfection [Figure 6b], 4 days post-infection [Figure 6c], and 6 days postinfection [Figure 6d] when compared with the noninfected cells [Figure 6a and d]. The overlay histogram [Figure 6d] clearly shows that HCV core protein is more expressed in a larger population of cells 6 days postinfection when compared with 2 and 4 days postinfection. Flow cytometric data analysis also indicates increased positive cell population stained for HCV core at 2 days postinfection (22.9%) and 4 days postinfection (30%) when compared with the noninfected cells.

**DISCUSSION**

HCV is one of the most manifold viruses, where it is divided into six major genotypes which, in turn, are subdivided into many subtypes. The multiple HCV genotypes are characterized by variable geographic distribution and different modes of transmission. The most common HCV infections all over the world to worldwide are caused by subtypes 1a, 1b, 2a, 2b, and 3a; however, infection in some restricted geographic areas is caused by specific HCV strains, including HCV-4a in

**Figure 3:** RT-PCR results for HCV infected cells. (a) Gel electrophoresis of HCV 5'UTR (upper), HCV core (middle) and β-actin (lower) at 2, 4, and 6 days postinfection with uninfected cells (negative). (b) Bar chart of band intensity calculated for HCV 5’UTR, core and β-actin at 2, 4, and 6 days postinfection

**Figure 4:** Effect of PEG and DMSO on HCV replication. (a) The 244 bp fragment of the amplified 5’UTR region using the different additives on different time intervals. (b) The 326 bp fragment of the amplified HCV core region using the different additives at different time intervals
Egypt, 5a in South Africa, and 6a in Southeast Asia.\textsuperscript{[24]} Most HCV researches have been conducted on strains or genomes of genotypes 1 or 2 in Europe, Americas, and Far East.\textsuperscript{[25]} In this study, we used serum samples obtained from Saudi patients with chronic HCV to examine the infectivity and replication of HCV-4a in hepatocytes \textit{in vitro}.
Comparison of nucleotide sequence of HCV genotypes has revealed significant genetic heterogeneity of the HCV genome. HCV 5’UTR region, which consists of 341 bp, is known to be the most conserved region of HCV RNA in terms of primary sequence and secondary structures. HCV core region contains 573 bp and is very useful for the differentiation of subtypes because it is more variable than 5’UTR region. A meta-analysis study for HCV genotyping, based on 5’UTR and core regions alignment, revealed that the rate of conservation in 5’UTR region is the highest compared with other regions; 99.2% in genotype 1, 98.9% in genotype 2, 98.6% in genotypes 3 and 4, and 99.5% in genotypes 5 and 6. The HCV core region showed conservation rate of 98.7% in genotype 1, 98.1% genotype 2, 99.7% genotype 3, 98.5% genotype 5, and 99.4% genotype 6. HCV genotype 4 has the lowest conservation rate of 93.2%.

This is in agreement with our results with 2% nucleotide variation in the 5’UTR region and the higher variation rate of 16.5% in the HCV core regions.

Although HCV molecular biology has progressed rapidly, our understanding of viral replication and pathogenicity is still hindered by the lack of efficient cell culture systems. To achieve a reliable in vitro system, we need to obtain a biological status wherein the virus–host interactions mimic exactly what happens naturally in vivo. Previous studies showed that primary hepatocytes are the most suitable in vitro model for biotransformation in the human liver as well as their ability to identify compounds that are potentially toxic to humans. The difficulty of obtaining human liver material and the absence of the proliferation process shortens the usage of primary hepatocytes for the long-term expression of viral hepatitis. HepG2 cell line, which is derived from a human hepatoblastoma, was considered to be a suitable model for in vitro studies. HepG2 cells express liver-specific metabolic proteins such as the canalicular marker MRP2 (multidrug-resistant protein-2) and Bsep (bile salt export protein). These cells form polarized cell membranes over time in culture consistent with the development of apical lumens that constitute the apical bile canaliculi. There is also a great similarity in biosynthetic pathways between primary hepatocytes and HepG2 cells.

HCV infects hepatocytes through four cellular receptor molecules. These are CD81, scavenger receptor class B member 1 (SR-B1), and the tight junction proteins Claudin-1 and Occludin. Naive HepG2 cells do not express CD81; however, complementation with exogenous CD81 induces susceptibility to HCV infection. In the current study, the use of infectious viral particles containing intact RNA genome could guarantee the presence of the necessary elements involved in translation of polyprotein precursor and viral replication. We utilized infectious serum with native viral particles presumably containing the full-length viral RNA genome in infecting HepG2 cells in vitro.

Both 5’UTR and 3’UTR untranslated regions of HCV RNA genome play an essential role in translation of viral proteins via interaction with cellular factors including eukaryotic initiation factor 3 eIF3, 40S ribosomal subunit, poly pyrimidine tract binding (PTB) protein, and mitochondrial-associated membranes. The same importance holds true for HCV core protein as it has pleiotropic functions. It is a structural protein of HCV nucleocapsid that has the capability of influencing the apoptosis, lipid metabolism, transcription, host cell transformation, and immune response of the infected host. Also the genome sequence coding for this protein is highly conserved even within the different HCV genotypes. The core protein predominantly localizes within the cytoplasm of infected hepatocytes and often shows a punctuated granular distribution within the cells. It has been observed that the majority of the core is located at the ER membrane, on the surface of lipid droplets, and on mitochondrial and mitochondrial-associated membranes.

To mimic the conditions under which HCV replicates in vivo, HCV should infect highly differentiated undivided human hepatocytes. To fulfill these conditions, DMSO and PEG were used as enhancement factors of HCV replication in different cell lines. DMSO has been shown to affect cell membrane integrity, alter intracellular signaling processes (eg, protein kinase C activity and integrin expression), and affect cellular alternative splicing. All of which may contribute to its potential to promote cell differentiation and alter cell proliferation. Previous studies showed that the human hepatoblastoma cell line, HUH-7 cells, undergo cytological differentiation when treated with 1% DMSO. DMSO-treated Huh7 cell culture system has the capacity to maintain individual cultures for extended periods of time without splitting. PEG is known to be a membrane-fusing agent and increases the efficiency of infections in a number of virus systems and cell lines by fusing viral and cellular membranes and therefore increasing penetration rates. It was also found to favor virus–liposome fusion. Thus the general mechanisms of virus–cell membrane fusion as well as cell–cell communication during the initiation of infection could be the cause of enhanced virus propagation during HCV in vitro infections supplemented with PEG. Addition of PEG to the primary hepatocyte cultures maintained in the presence of 20 g/L DMSO markedly increases the infection of HBV but not HCV.

In the present study, we tested the susceptibility of HepG2 cell line to HCV infection and established a cell model that could support HCV long-term replication in vitro. The expression of viral RNA (5’UTR and core) and viral
protein (core) in infected cells suggests that this cellular model allows study of HCV life cycle. Our results indicate that expression of HCV genotype 4 viral RNA and protein was possible in HepG2 cells with or without treatment with DMSO and PEG. HCV 5’UTR and core RNA were expressed at different time intervals, but RNA expression was enhanced specifically when the cells were treated with PEG. Using immune fluorescence and flow cytometry the HCV core protein expression was also established in this in vitro cell culture model. Treating HepG2 cells with DMSO and/or PEG showed similar effects such as RNA expression where PEG gave better HCV core protein expression than other settings.

HCV replication is determined by RT-PCR to detect HCV RNA levels that are indicative of virus replication. This method proved useful in detecting low levels of HCV RNA; however, it also presented new challenges including the potential for random priming by cellular nucleic acids, contamination of RNA samples, and the lack of strand specificity due to RNA self-priming. HCV replication was reported in nontransformed human fetal hepatocytes, which maintained and secreted HCV particles for 2 months after transfection. As the previous study showed that DMSO had no effect on short-term expression of viral genes in infected HepG2 cells, where HCV RNA was detected only at the 9th day postinfection, in agreement with our results as HCV RNA amplification showed remarkable increase in HCV replication when cells were treated with PEG, in contrast to those treated with DMSO alone or combined with PEG.

From previous studies, flow cytometric analysis showed that HCV core protein was detected in infected HepG2 cells after 24 h (5.7%) and protein expression increased after 3 days (13.5%). Our findings indicated higher expression levels of HCV core protein in HepG2 cells with 22.9% of the cells at 2 days postinfection and 30% at 4 days were positive for HCV core protein. Since detectable HCV structural proteins in cells after infection may represent the residue of the inoculated virus after releasing the viral genome to cytoplasm, it is necessary to demonstrate that HCV structural proteins detected in the infected cultures are newly synthesized rather than residuals of viral inoculum. The observed increase in core expression reflects part of de novo synthesized structural viral proteins. Similarly, in our study, flow cytometric results of HCV core protein became evident at day 4 postinfection.

CONCLUSION

We report an in vitro system of cultured HepG2 cells infected with HCV genotype 4. These cells support viral replication and consistent expression of viral genes, which make this model optimum for studying HCV life cycle, screening for anti-HCV drugs and testing the efficacy of therapeutic antibodies.

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Conflicts of interest

There are no conflicts of interest.

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