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Potential antiviral activity of metformin against human Adenovirus-7

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ABSTRACT

Background: Human adenovirus 7(HAdV-7) cause acute respiratory tract infections with high morbidity and mortality rates in children and immunocompromised adults. Metformin is a natural oral antihyperglycemic drug, that possesses antiviral activity. Our study aimed to investigate and compare the antiviral activity and mechanism of action of metformin and ribavirin against HAdV-7. **Methods**: The antiviral activity and cytotoxicity of each of metformin and ribavirin per se and in combination were tested using the crystal violet method. The mechanism of action of metformin against HAdV-7 was assessed during viral adsorption and replication phases. The viricidal effect and cytopathic effect inhibition of metformin was also determined. **Results**: Metformin revealed a moderate antiviral activity against HAdV-7 with a selective index = estimated CC50/estimated IC50 = 5.0 in comparison with selective index of ribavirin 1.82. Metformin demonstrated modest antiviral activity against HAdV-7 with a selective index = estimated CC50/estimated IC50 = 5.62 during the replication process, but not during the other phases of infection. The combined effect of both drugs revealed a low antiviral activity against HAdV-7 in comparison to using each drug alone; Antiviral index = 2.87, SI = 5.0. **Conclusion**: Metformin has a potential promising antiviral activity against HAdV-7.

Introduction

Human adenovirus 7(HAdV-7) belongs to the family Adenoviridae, causing acute infections of the respiratory tract, eyes, and lymph nodes in both adults and children [1-3]. They are nonenveloped double-stranded DNA viruses that persist in the environment for extended periods of time [1, 2]. They commonly inhabit and spread in crowded physical settings, with poor personal hygiene as military recruits, dormitories, nursing homes and day care centers [4,5]. They also spread in hospitals [6]. Their clinical features resemble common colds, and are found to produce latency in healthy individuals. Human adenoviruses have a tropism for the respiratory tract in adults, particularly HAdV-7. Moreover, they are mostly associated with respiratory infections in childhood where they reach high morbidity and mortality rates [7, 8]. Severe adenovirus infection in children can be complicated with acute respiratory distress syndrome, respiratory failure [9], and central nervous system dysfunction [10]. Severe systemic infections can occur in immunocompromised adults as leukemic and acquired immune deficiency syndrome (AIDS) patients [11]; stem cell and kidney or bone marrow transplantations [12].

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Metformin is a natural compound derived from Galega officinalis that is widely used as an oral antihyperglycemic drug in type-2 diabetes mellitus. It possesses microbe-modulating properties with antibacterial, antiviral, antifungal and antiparasitic activity. It has an antibacterial effect against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, [13] and *Mycobacterium tuberculosis* [14]. It improves response to treatment by altering the gut microbiota in diabetic patients. Metformin has been used as an adjuvant drug with conventional antibiotics to enhance their efficacy by improving a person's immune response. [13,14]. Moreover, **Loos *et al.*** [15] reported that the combination of antifungal agents with metformin revealed better fungicidal action than using antifungal drugs alone. On the other hand, metformin reduces the replication of hepatitis B and C viruses [16,17]. It has been suggested to be administered as an adjuvant for treating for SARS- COV 2 patients [18-20] where it reduces IL-6 levels, increases cellular pH and hence reduces viral replication.

The immunomodulatory activity of metformin reduces the production of proinflammatory cytokines, where it inhibits the expression of IL-1β, IL-6, and TNFα by activated macrophages [21,22]. Moreover, metformin inhibits the cytokine production of Th1 and Th17 cells [18].

The aim of our study was to investigate and compare the antiviral activity of metformin and ribavirin against HAdV-7 virus; as well as to determine the mechanism of action of metformin against HAdV-7.

Material and methods

This experimental study was performed by the Virology Laboratory personnel in Nawah Scientific, Almokattam, Cairo, Egypt from February to May 2022. Metformin powder (17.6 mg/ml, code no. SO8731) was obtained from Merck Ltd., Cairo, Egypt. Ribavirin IV injection ampoules were obtained from CSC Pharmaceuticals International Gamdevi, Mumbai, India. HAdV-7 and Human epithelial cells HEp-2 cells (CCL-23 ™) were provided by the National Research Center, Giza Egypt. This work was conducted according to the Operational Guidelines BSL-2 for Virology Lab that is adopted by Nawah Scientific for virology research. Cells were grown on Dulbecco's Modified Eagle medium (DMEM) that was supplemented with 10% fetal bovine serum and 0.1% antibiotic/antimycotic solution. Trypsin-EDTA, fetal bovine serum, antibiotic and antimycotic solutions and DMEM were provided by Gibco BRL, Grand Island, NY, USA.

Antiviral activity of metformin and ribavirin

The Crystal violet method was used to test the antiviral activity and cytotoxicity [23]. This cytopathic effect (CPE)-inhibition assay was conducted to investigate the potential antiviral activity of Metformin against HAdV-7. The dose-response assay was designed to determine the range of efficacy; the 50% inhibitory concentration (IC50), and the range of cytotoxicity (CC50) for both metformin and ribavirin.

HEp-2 cells were seeded into a 96-well culture plate at a density of $2x10^4$cells/well one day prior to inducing infection. The next day, the culture medium containing serially diluted samples of metformin and ribavirin were added to the cells and incubated for 72 hours before being removed. Cells were then washed with phosphate buffer saline (PBS).

The Crystal violet method was applied to determine the infectivity of HAdV-7; in which the CPE was monitored and the percentage of cell viability was calculated. A 0.1 mL of diluted viral suspension of HAdV-7 containing the cell culture infectious dose by 50% (CCID50, $1.0 * 10^4$) of the virus stock was added to mammalian cells to produce the desired CPEs two days after infection. The antiviral activity was determined using a two-fold diluted concentration range of 0.1-1000 µg/ml. The virus controls (virus-infected, nondrug-treated cells) and cell controls (non-infected, nondrug treated cells) were included in the test. Culture plates were incubated at 37 °C with 5% CO2 for 3 days.

The development of CPE was monitored by light microscopy. Cell monolayers were washed with phosphate buffered saline (PBS), then fixed and stained with a 0.03% crystal violet solution in 2% ethanol and 10% formalin. After washing and drying; the optical density of the individual wells was quantified spectrophotometrically at 570/630 nm. The percentage of antiviral activity was calculated according to **Pauwels *et al***. [24], using the following equation:

Antiviral activity= [(mean optical density of cell controls−mean optical density of virus controls)/ (optical density of test−mean optical density of virus controls)] ×100%.

Based on these results, the 50% CPE inhibitory dose (ID50) was calculated. The results of the 50% cytotoxic concentrations (CC50) and the 50% inhibitory concentration (IC50) were determined using GraphPad PRISM software (Graph-Pad Software, San Diego, USA).

Mechanism of action of metformin on HAdV-7

The mechanism of action of metformin against HAdV-7 was assessed during viral adsorption and replication phases. The viricidal effect and CPE inhibition of metformin were also determined.

Viral adsorption mechanism

The viral adsorption mechanism was assayed using **Zhang *et al.*** protocol [25], with few modifications. Cells were cultivated in a 96-well plate (10^4cells/ml) for 24 hours at 37 °C. Metformin was added to 100 µl of DMEM without any supplements and co-incubated with the cells for 2 hours 5 at 4 °C. The inoculum containing the non-absorbed drug was removed by washing the cells three successive times with the supplement-free medium. The infectivity of virus was determined using the Crystal violet method [23], which monitored CPE and allowed the percentage of cell viability to be calculated. A 0.1 ml of the diluted HAdV-7 suspension that contained CCID50 of the virus stock was added to the mammalian cells. This was selected to produce the desired CPE. Each test sample's antiviral activity was determined using a two-fold diluted concentration range of 0.1-1000 µg/ml. The virus controls (virus-infected, nondrug-treated cells) and cell controls (non-infected, nondrug treated cells). For 4 days, culture plates were incubated at 37ºC in 5% CO2. The development of cytopathic effect was monitored by light microscopy. Following a PBS wash, the cell monolayers were fixed and stained with a 0.03% crystal violet solution in 2% ethanol and 10% formalin. After washing and drying the optical density of individual wells was quantified spectrophotometrically at 570/630 nm. The percentage of antiviral activities of the test's compounds were calculated according to the equation by **Pauwels *et al.*** [24].

Viral replication mechanism

The effect of metformin on viral replication was identified and compared to ribavirin according to **Zhang *et al.*** protocol [25]. Cells were cultivated in a 96-well plate (10^4 cells/ml) for 24 hours at 37 °C. The virus was directly inoculated onto the cells and was incubated for 1 hour at 37 °C. The inoculum containing the non-adsorbed viral particles was removed by washing cells three successive times with supplement-free medium. Metformin was added in varying concentrations to infected cells for another contact time of 1 hour. After removing the inoculum containing metformin; DMEM supplemented with 2% agarose was added to the cell monolayer. Culture plates were also incubated at 37 °C with 5% CO2 for 72 hours. CPE was then determined [23].

Virucidal mechanism

The virucidal mechanism was assayed according to **Schuhmacher *et al***. protocol [26]. In a 96-well plate, cells were cultivated (10^4cells/ml) for 24 h at 37 °C and 100 µl of serum-free DMEM containing the virus was added to each well. After 1 hour incubation, the mixture was diluted by 10-fold, three times using serum-free medium, allowing the continued growth of viral particles on the cells. Then 100 µl of each dilution was added to the cell monolayer. After another hour contact time, DMEM was added to the cell monolayer. Plates were then incubated at 37 °C for 72 hours. The development of CPE was assessed [23].

Antiviral activity of metformin combined with ribavirin on HAdV-7

The crystal violet method was also used to evaluate the combined antiviral activity of Metformin with Ribavirin and their cytotoxicity assays using **Abou Aitah *et al.*** [27] recently reported crystal violet assay applying the same previously mentioned procedures.

Determination of the antiviral combination

Six concentrations for each of metformin and ribavirin were used in the antiviral combination test. Their concentrations, expressed as a percentage of the single-drug antiviral effective dose (ED50), were 1%, 3.125%, 6.25%, 12.5%, 25%, 50% and 100 %. Triplicate wells were used to test ribavirin in combination with metformin. The virus and cell controls were included. CPE was monitored and antiviral IC50 was calculated.

Determination of fractional inhibitory concentration

Each IC50 concentration of ribavirin in combination was re-expressed as a fractional inhibitory concentration (FIC). That is, as a fraction of the IC50 of ribavirin used alone (ribavirin FIC = ribavirin ED50 in combination/ribavirin IC50 alone). The resulting Ribavirin FIC was paired with

the FIC of metformin that was present in that combination (metformin FIC = concentration of metformin in combination/ metformin IC50 alone).

The FIC values for Metformin, in the presence of each concentration of Ribavirin, were determined (metformin FIC = metformin IC50 in combination/ metformin IC50 alone) and paired with the FIC values of ribavirin that were present in each combination (ribavirin FIC = concentration of ribavirin in combination/ribavirin IC50 alone).

Calculation of antiviral indices

Antiviral indices (AIs) were used to evaluate the specific antiviral activity of Metformin and Ribavirin and their combination; taking into consideration the cytotoxicity of these drugs and their combination. The AI of an individual drug was defined as the maximum therapeutic concentration (MTC) of the drug divided by the antiviral IC50.

The Al of the combination was calculated according to **Kirsi *et al***. [28] as follows:

Combination AI = ribavirin CC50 in combination/ribavirin IC50 in combination + metformin CC50 in combination/ metformin IC50 in combination

Statistical analysis

All data were expressed as the mean of three repeated experiments. Data analysis was performed with Two -way RM ANOVA using GraphPad PRISM software (Graph-Pad Software, San Diego, USA). *$P \leq 0.05$ was used as the limit for significance.

Results

Antiviral activity of Metformin and Ribavirin

Table 1 showed that metformin revealed a moderate antiviral activity against HAdV-7 with a selective index = estimated CC50/estimated IC50 = 5.0 in comparison with selective index of ribavirin 1.82. A significant difference was found regarding the antiviral activity between metformin and ribavirin ($p < 0.01$)

Figure 1 represented the %inhibition concentration and % viability of each drug alone, as a function of Log concentration (µg/ml) that corresponds to the average values of three repeated experiments.

Mechanism of action of metformin on Adenovirus 7

Table 2 metformin demonstrated modest antiviral activity against Adenovirus-7 with a selective index = estimated CC50/estimated IC50 = 5.62 during the replication process, but not during the other phases of infection. The difference between the three phases were found to be highly significant ($p < 0.0001$).

Figure 2 represents the % inhibition of metformin as a function of Log concentration (µg/ml) in the three phases of infection namely; the virucidal, adsorption and replication phases. The optimum antiviral activity of metformin was during the replication phase.

Antiviral activity of metformin combined with ribavirin on Adenovirus 7

Table 3 Effect of combined compounds with Antiviral index = 2.87, showed low antiviral activity against Adenovirus-7 in comparison to using each drug alone; SI = 5.0. Difference between both were highly significant ($p < 0.0001$).

Figure 3 represented the % inhibition concentration and % viability of the combination of metformin and ribavirin as a function of Log concentration (µg/ml) that corresponds to the average values of three repeated experiments.

Table 1. Antiviral activity of metformin and ribavirin against Adenovirus-7.

Drug	**CC50**	**IC50**	**SI**	***P* value**
Metformin µg/ml	58.58	11.72	5.0	0.0061
Ribavirin µg/ml	18.91	10.38	1.82	

CC : cytotoxicity IC: inhibitory concentration SI: selective index

Table 2. Mechanism of action of metformin against Adenovirus-7.

Mechanism of Action	**CC50**	**IC50**	**SI**	***P* value**
Adsorption µg/ml	58.58	31.76	1.85	$p < 0.0001$
Replication µg/ml	58.58	10.42	5.62	
Virucidal µg/ml	58.58	22.75	2.57	

Table 3. Antiviral activity of metformin combined with ribavirin on Adenovirus 7.

Mechanism of Action	CC50/FIC	IC50/FIC	AI*	*P* value
Metformin µg/ml	1.01	1.98	2.87	$p < 0.0001$
Ribavirin µg/ml	4.57	1.76		
Both combined µg/ml	86.4	20.58	-------	

*Antiviral index

Figure 1. Antiviral activity of metformin and ribavirin.

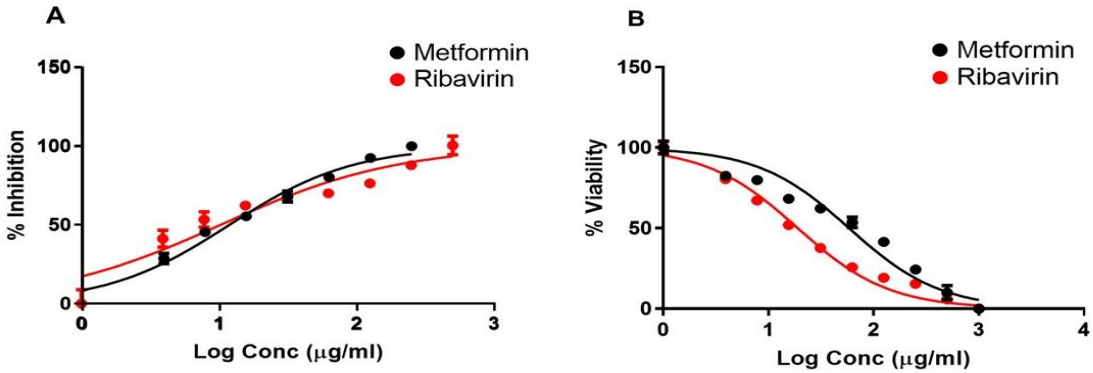


Figure 2. Mechanism of action of metformin (% inhibition) against HAdV-7.

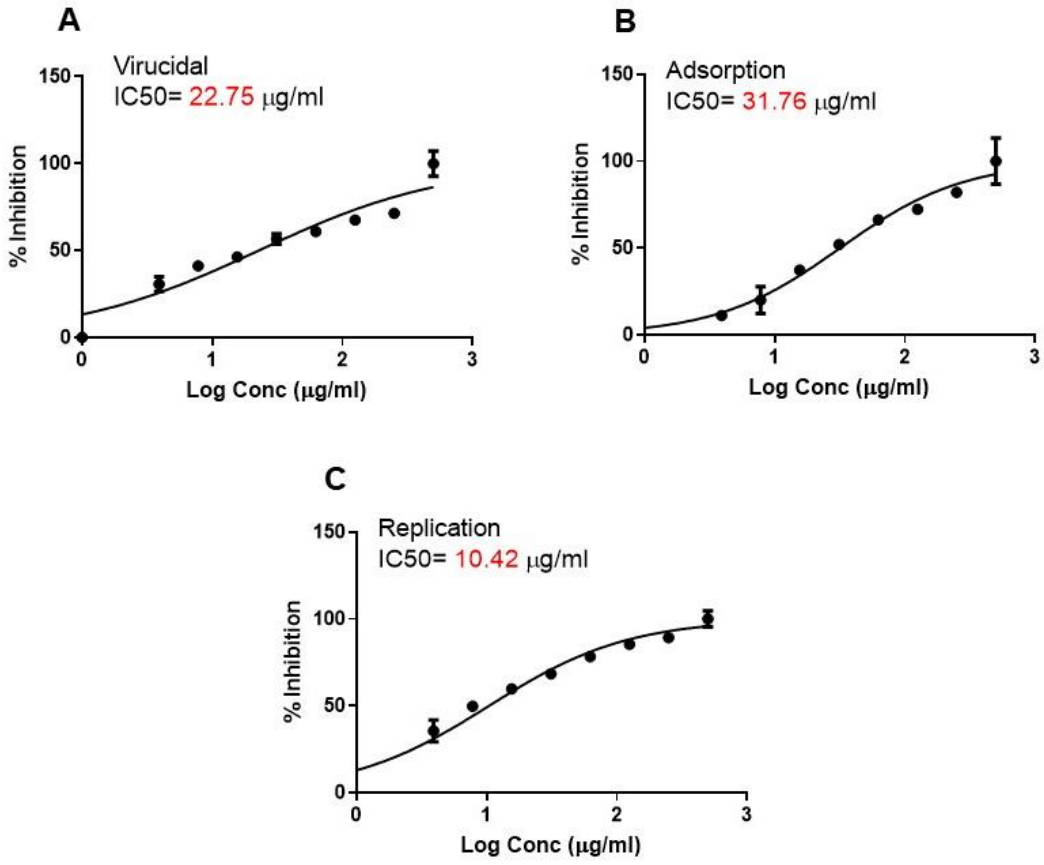
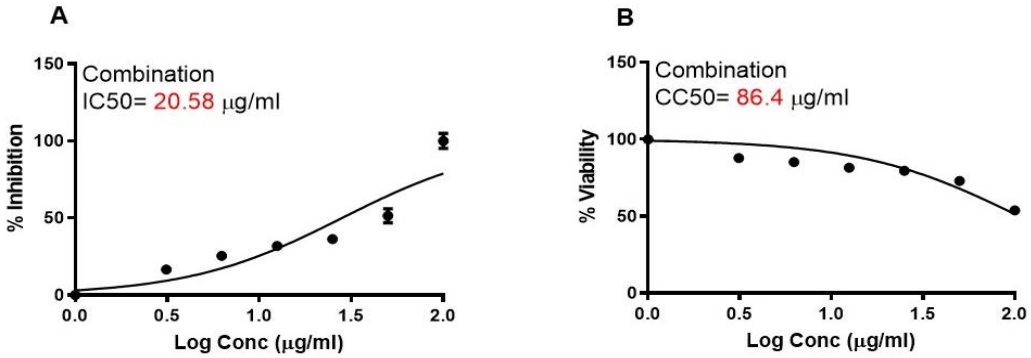


Figure 3. Antiviral activity of metformin combined with ribavirin.



Discussion

Metformin is the first line, most favoured antidiabetic drug in patients with type 2 diabetes mellitus (T2DM), that is safe and with minimal cost of treatment. Metformin has immunomodulatory effects in different pathological conditions as cancer, hyper-inflammatory and infectious diseases through direct or indirect regulation of the host innate and adaptive immune response [18]. There is a growing interest in research regarding the antiviral activity of metformin and its repurposing as an antiviral agent. To our best knowledge no studies have addressed the antiviral activity of metformin against adenoviruses, particularly HAdV-7 .

We attempted to investigate the in vitro antiviral activity of metformin against HAdV-7 and compare it with the conventional antiviral drug used to treat HAdV-7 infections. Metformin showed a moderate antiviral activity against HAdV-7 that was significantly better than ribavirin.

This study revealed that the antiviral activity of Metformin against HAdV-7 was during the replication stage. Metformin inhibits the respiratory-chain complex I of the mitochondrial electron transport chain, directly affecting reactions requiring ATP and indirectly activating AMP-activated protein kinase (AMPK)[29]. The activation of AMPK inhibits viral replication by altering cellular metabolism and energy utilization by reducing ATP production and increasing AMP levels. This, in turn, results in the inhibition of fatty acid synthesis and the interference with signaling pathways in host cells in which viruses exploit for their replication. Hence the switch off of mTOR signaling [30], results in reduced cellular energy consumption, inhibiting viral replication and propagation. Moreover, it was reported that pre-treating cells with metformin prior to viral infection can inhibit viral replication and protein synthesis, thus significantly reducing viral titers [31,32]

Metformin has been reported to have to inhibit other viral infections [33-36] as dengue virus infection by restoring AMPK activity [33] as well as decreasing viral replication of Coxsackievirus B3[35]. It also inhibits in vitro, the viral gene expression and virion production of Kaposi sarcoma herpesvirus [34] and the replication of hepatitis B virus (HBV) in primary human hepatocytes by repressing viral transcription-related genes. The combination of metformin and entecavir inhibits HBV replication more significantly than either alone [36]. Moreover, metformin may also inhibit SARS-CoV-2 infection by interfering with its interaction with ACE2 via the activation of AMPK and increases ACE2 expression by enhancing its stability [18-20].

Previous studies reported that the combination of metformin along with pegylated interferon- alpha and ribavirin increased the viral response rate and improved insulin sensitivity in chronic hepatitis C patients. Viral infections induce transient insulin resistance, and hence the addition of metformin promotes the viral response to therapy [37].

Ribavirin is a broad spectrum antiviral nucleoside analogue that is used to treat various viral

infections including adenovirus infections. As a purine analogue, it can function in multiple cellular and viral processes. It has the ability to act simultaneously through multiple mechanisms where it possesses five mechanisms of action; both direct and indirect. The direct mechanisms include the interference with RNA capping, polymerase inhibition and lethal mutagenesis, while the indirect mechanisms include inosine monophosphate dehydrogenase inhibition and immunomodulation [38]. Like ribavirin, cidofovir displays a similar mode of action; but with higher toxicity and side effects in patients compared to ribavirin. Conflicting data regarding the efficacy of ribavirin treatment was reported in studies. Small scale studies revealed the successful treatment with ribavirin in immunosuppressed patients with adenovirus infection, while other large scale studies reported that therapeutic efficacy was insignificant. Such conflicting results may be attributed to the selective efficacy in adenovirus serotypes [39].

We also assessed the antiviral activity of metformin and ribavirin combined together; to test whether or not this combination posed an additive value. Studies have reported that combination of antiviral drugs with other non- antiviral drugs may yield an expected additive or synergistic effect. In our work, the combined antiviral activity of both drugs was lower than using metformin alone, however higher than ribavirin used per se. This may be explained by the fact that Metformin acts on the replication of the virus, while Ribavirin interferes with viral RNA synthesis. Another suggestion may be that ribavirin may counteract with metformin producing an antagonistic effect. Moreover, HAdV-7 may have acquired resistance against one or both drugs thus reducing the combined efficacy.

Conclusion

Consequently, we conclude that metformin has a potential promising antiviral activity against HAdV-7. Metformin is a good candidate for further extensive studies and experiments as an anti-Adenovirus drug.

Limitation

Our findings suggested that the best selective index of metformin was during the replication phase. However further investigations are required for verify the exact mechanism of metformin against viral HAdV-7.

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Author's contribution

Gihan A. ELbatouti and Ibrahim A. Abdelwahab contributed to the conception and design of the study. Ibrahim A. Abdelwahab contributed in the laboratory work. Gihan A. ELbatouti performed the results and interpretation of the data. Gihan A. ELbatouti and Ibrahim A. Abdelwahab contributed in writing and drafting of the paper for publication. Both authors revised the paper critically for intellectual content and approved the final version to be published. Both authors agreed to be accountable for all aspects of the work.

Conflict of interest

The authors report no conflict of interest

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