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Folic acid inhibits the amyloid fibrils formation of β-lactoglobulin

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Abstract: It is well known that amyloid fibrils are associated with a lot of neurodegenerative diseases. Thus, inhibition of protein aggregation and disruption of the formed amyloid fibrils have been attractive therapeutic strategies for amyloid-associated diseases. Here, we take the widely studied protein, β-lactoglobulin (β-LG), as a model protein to study the inhibition of amyloid fibrils. Using multiple biophysical and biochemical approaches, we successfully identify that folic acid can strongly inhibit amyloid fibrils formation of β-LG in 5 M urea solution. In the presence of folic acid, both the nucleation step and elongation step of β-LG fibrillation can be affected and the suppression efficiency followed a dose depended manner. Furthermore, the studies of protein intrinsic-fluorescence and anilinonaphthalene-8-sulfonic acid (ANS) fluorescence demonstrated that the folic acid decreased the hydrophobicity of the urea-denatured β-LG and changed the conformation of β-LG. Our study provides a convenient way to inhibit the amyloid fibrils formation, which has potential help to study the mechanism of amyloid-diseases.

Key words: β-lactoglobulin (β-LG); Amyloid fibrils; Lag time; Folic acid; Hydrophobic interactions.

Introduction

Proteins perform their particular biological functions mostly depending on the correct native conformations (1-3). However, under certain pathological conditions, some proteins may go through unfolding, misfolding, or forming insoluble amyloid fibrils which can be associated with many neurodegenerative diseases, termed as amyloido-sis, such as Alzheimer's, Parkinson's and Huntington's diseases (4-6). Thus, studying the biochemical properties of amyloid fibrils has been a fascinating area because of its contribution to understand the protein self-assembly and the pathogenesis of amyloid-diseases. Inhibition of protein aggregation and disruption of the formed amyloid fibrils has been an attractive therapeutic strategy for these amyloid-associated neurological diseases.

Since many disease-independent proteins can self-as-sembly into fibrils with similar structures and biochemi-cal properties under appropriate conditions, formation of amyloid fibrils has been thought to be a common charac-teristic of proteins (7, 8). Through nucleation and growth of amyloid prescursors, amyloid fibrils can be formed as long and unbranched fibrils with enhanced β-sheet struc-ture, increased surface hydrophobicity (9, 10). The kine-tics of amyloid aggregation can be described by the lag time of nucleation phase and the growth rate of elongation phase (11-13).

β-LG is one of the most abundant water soluble globu-lar proteins in bovine milk whey. It has a predominantly β-sheet structure with nine stranded antiparallel β barrels and one small helix. β-LG has been reported to bind a variety of bioactive compounds, such as fatty acids, vi-tamins and polyphenols (14). And β-LG has been exten-sively studied because it is readily available in large quan-tities and of its importance to food and dairy industry. It has been demonstrated that β-LG can form amyloid fibrils in the presence of denaturants at neutral pH (15-17).

Folic acid (schematic picture shown in Figure 1) is a synthetic and stable form of folate, which is present in a variety of foods including beans, spinach, and lentils (18), and it is an ideal choice for studying protein-ligand interactions. Since low folate levels may lead to mega-loblastic anemia, pregnancy complications, neural tube defects, and possibly increases the risk of developing cardiovascular diseases, supplementation for women in-tending to become pregnant and dietary fortification have been adopted in many counties (19,20). In addition, fo-lic acid is necessary for modulating the transfer of one carbon unites for protein and DNA synthesis, methylation and gene expression. However, folic acid can be degraded by ultraviolet radiation. This photo-degradation can be si-gnificantly reduced by binding to the β-LG surface (21). Although the interactions between folic acid and β-LG have been widely researched, studies on the effects of fo-lic acid on fibril formation of β-LG are rare.

In this study, intrinsic fluorescence, anilinonaph-thalene-8-sulfonic acid (ANS) fluorescence, thioflavin T (ThT) fluorescence, Congo Red (CR) binding assay and transmission electron microscopy were employed to in-vestigate the effects of folic acid on the conformation

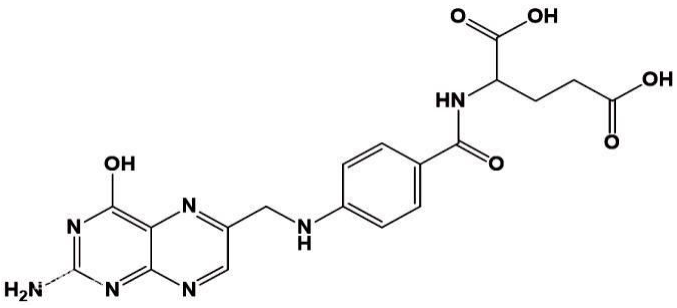

Figure 1. The chemical structure of folic acid.

change and the amyloid aggregation of β-LG. The results indicated that folic acid decreased the hydrophobic inte-ractions between urea-denatured β-LG and inhibited the amyloid aggregation of β-LG. To our knowledge, this is the first report to study the effect of folic acid on amyloid fibrils formation.

Materials and Methods

Chemicals

Bovine β-lactoglobulin (β-LG) was obtained from Sigma (Sigma-Aldrich Co, St. Louis, Mo) and used wi-thout purification. Folic acid, Thioflavin T, Sodium azide (NaN3) and Congo Red were purchased from Sigma. All the other chemical regents used were of analytical grade and were made in China.

Fibrils formation

A stock solution of β-LG was prepared in 10 mM sodium phosphate buffer at pH 7.0. Dilution with 8 M urea in 10 mM sodium phosphate buffer resulted in solutions of 1.2 mg/mL β-LG with 5 M urea. To study the effect of folic acid on the fibril formation, the desired concentra-tions of folic acid were added into the solutions containing 1.2 mg/mL β-LG and 5 M urea with 0.01% (w/v) sodium azide (NaN3). All samples were first well mixed via vor-texing and then incubated at 37oC during the course of experiments.

ThT assay

To monitor the degree of fibril formation, 100 μL ali-quots of proteins were taken at each time point and diluted 20 times with 400 μM of ThT solution in 10 mM sodium phosphate buffer (pH 7.0, final ThT concentration 20 μM). ThT fluorescence intensity measurements were performed by exciting samples at 450 nm with slit-width of 5 nm and recording emission intensity at 485 nm with slit-width of 5 nm using a Hitachi F-4600 Florescence Spectrophoto-meter. All measurements were taken in triplicate. The data from ThT fluorescence measurements were fitted against sigmoidal curves described by the following equation.

$$I = I_0 + m_i t + \frac{I_f + m_f t}{1 + e^{-[(t - t_0)/\tau]}} \quad (1)$$

Where I is the fluorescence intensity at time t, t represents incubation time and t0 represents the time reach 50% of maximal fluorescence, and I0+mit and If+mft represent the initial baselines and the final plateau line, respectively. Thus, the apparent rate constant, kapp, for the growth of fi-brils is given by $1/\tau$ and the lag time is given by $t0 - 2\tau$ (17).

Congo Red binding assay

A stock solution of 200 μM CR was prepared in 10 mM sodium phosphate solution (pH 7.0). CR absorbance of β-LG samples and the free dye control were determined by adding CR to a final concentration of 20 μM and acqui-ring spectral measurements from 400 to 700 nm at 37oC on an ultraviolet-visible spectrometer (Shimadzu UV-1800, Japan). The β-LG solutions in the absence and pre-sence of folic acid and the control solutions were allowed to interact with CR for at least 30 min in the dark before recording the absorbance spectra. All measurements were taken in triplicate.

Transmission electron microscopy

A 5 μL aliquot of samples prepared as described above was placed on formvar-coated copper grids and left at room temperature for 5 min. The grids were stained with 2% (w/v) phosphotungstic acid solution for another 1min before examination using an H-7650 transmission elec-tron microscopy (Hitachi, Tokyo, Japan) operating at ac-celerating voltages of 80 kV.

Intrinsic fluorescence spectroscopy

A total of 40 μL of 5 mg/ml β-LG samples with va-rious concentrations of folic acid was mixed with 960 μL sodium phosphate and the intrinsic fluorescence spectra were monitored with F-4600 Fluorescence Specrtopho-tometer (Hitachi, Japan) using a quartz cuvette with a path length of 1cm. The emission spectra were recorded between 300 and 450 nm by exciting the samples at 280 nm.

ANS fluorescence spectra

A total of 50 μL of β-LG sample solution was mixed with 950 μL of 20 μM ANS in sodium phosphate buffer. ANS fluorescence spectra were made on an F-4600 Fluorescence Specrtophotometer using 1 cm light path quartz cuvette. A slit width of 2.5 nm was used on both excitation and emission wavelengths, respectively. The emission intensity was measured between 400 and 600 nm by exciting at 380 nm. The fluorescence of the solvent was subtracted and all measurements were taken in triplicate.

Results

Effect of folic acid on amyloid fibrillogenesis of β-LG To verify the effect of folic acid on amyloid fibrillization of β-LG, we employed the commonly used ThT fluores-cence assay. ThT as an extrinsic fluorescent dye has been widely used to study the formation kinetics of amyloid fibrils. Upon binding to amyloid fibrils, its fluorescence intensity increases (23-25). To investigate the influence of folic acid on the amyloid fibrils formation of β-LG, we added folic acid into the β-LG solutions with 5 M urea and monitored the changes of ThT fluorescence emission. As shown in Figure 2, samples containing freshly-prepared β-LG with 5 M urea displayed low ThT fluorescence at the beginning of incubation, indicating the lack of amy-loid fibrils in these samples. Upon further incubation at 37oC, a dramatic increase in ThT fluorescence emission was observed during 10-12 days, suggesting the formation of β-LG amyloid fibrils with 5 M urea in the absence of folic acid, which was consistent with the previous results.

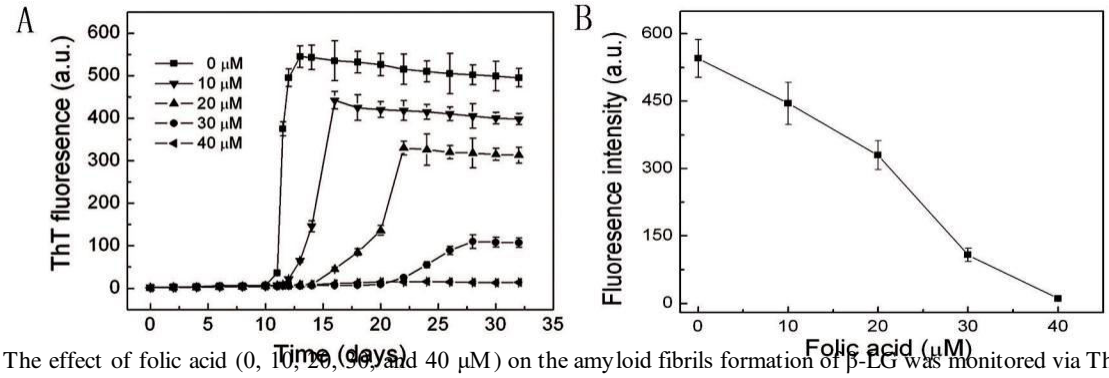

Figure 2. (A) The effect of folic acid (0, 10, 20, 30, and 40 μM) on the amyloid fibrils formation of β-LG was monitored via ThT fluorescence as a function of incubation time. **(B)** The dose-response curve indicating the plateau value of ThT fluorescence intensity of β-LG sample against folic acid concentration. Data are the average of three independent experiments.

The kinetic curves of ThT fluorescence intensity were consistent with a nucleation-dependent polymerization model in which the lag corresponded to the nucleation phase and the exponential part to elongation phase (fibril growth) (26). However, upon co-incubation with 10 μM folic acid in 5 M urea solution, the lag time of fibrillation was increased from 12 to 14 days, accompanied by a re-markable decline of the maximum ThT fluorescence in-tensity. Specifically, no enhancement of ThT fluorescence intensity was observed for β-LG solution in the presence of 40 μM folic acid at pH 7.0 even incubated for 50 days (supporting information figure S1). Control experiments showed that the reduced ThT fluorescence was not due to the interactions between ThT and folic acid (supporting information figure S2). Thus, the results demonstrate that folic acid has the inhibitory effect on amyloid fibril forma-tion of β-LG and the inhibitory effect is dependent on the concentrations of folic acid.

To understand the influence of folic acid on the amy-loid formation kinetics of β-LG in detail, we quantita-tively analyzed the obtained ThT fluorescence data using a nucleation-dependent polymerization model. This mo-del is described by a sigmoidal curve with two key pa-rameters, the growth rate constant and the lag time. The values of growth rate constant and the lag time for β-LG samples containing various folic acid concentrations were determined and listed in Table 1. The growth rate of fibri-lization of β-LG was slowed down as well as the lag time was increased in the presence of folic acid at the concen-trations from 10 to 30 μM demonstrating that folic acid could affect both the nucleation step and the elongation step to prolong the amyloid fibrils formation of β-LG in urea solution. The results also demonstrated that folic acid prolongs amyloid fibrils formation. Furthermore, the final amount of amyloid fibrils in the presence folic acid was monitored by the ThT end-point fluorescence intensity. Fi-gure 2 showed that the ThT end-point fluorescence inten-sity was gradually decreased as the concentration of folic acid increased. Taken together, these results demonstrated that folic acid could delay the amyloid fibrils formation of β-LG and inhibit the production of the final amyloid species. Furthermore, IC50, defined by the concentration at which half of ThT fluorescence intensity reduced, was used to present the effectiveness of folic acid in inhibiting β-LG fibrillation. According to an analysis of a dose-de-pendent curve shown in Figure 2B, IC50 of folic acid was determined to be 22 μM.

Table 1. The estimated values of the lag time and growth rate constants for β-LG samples containing various concentration of folic acid.

Folic acid concentration (μM)	Lag time (days)	k_{app} ($days^{-1}$)
0	11.6	9.60
10	12.8	1.25
20	15.6	0.48
30	21.5	0.26

CR absorbance spectroscopy has been widely used to probe the presence of amyloid fibrils (27, 28). To further explore whether folic acid can exert an inhibitory effect on the formation of β-LG fibrils, samples were also ana-lyzed by using the CR binding assay. There's an absorp-tion maximum at 490 nm for the samples without fibrils. However, after incubated in 5 M urea without folic acid for 12 days, the β-LG solution's absorption maximum shifted higher to 504 nm (Figure 3). Simultaneously, a se-cond shoulder peak at 540 nm became visible indicating the presence of fibrils. In contrast, in the presence of 40 μM folic acid, no change of the maximum absorption of CR was observed for β-LG at pH 7.0 and the absorption spectrum of β-LG+CR was almost the same as that of CR alone, further supporting the results of ThT binding assay.

To further confirm the results obtained from previous

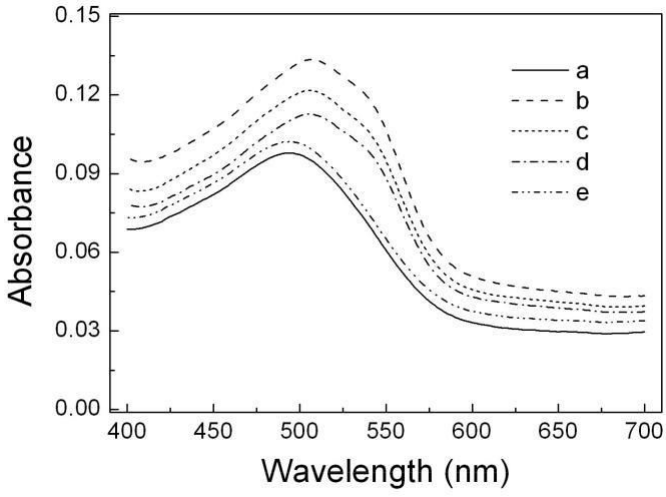

Figure 3. CR binding assays characterize the effect of folic acid on amyloid fibrils formation of β-LG with 5 M urea. The sample of CR+-buffer (a) was performed as a control and the absorbance data are shown for amyloid fibrils formation of β-LG at different conditions: CR+β-LG incubated for 60 hours in 0 (b), 10 (c), 20 (d), and 40 μM (e) folic acid, respectively.

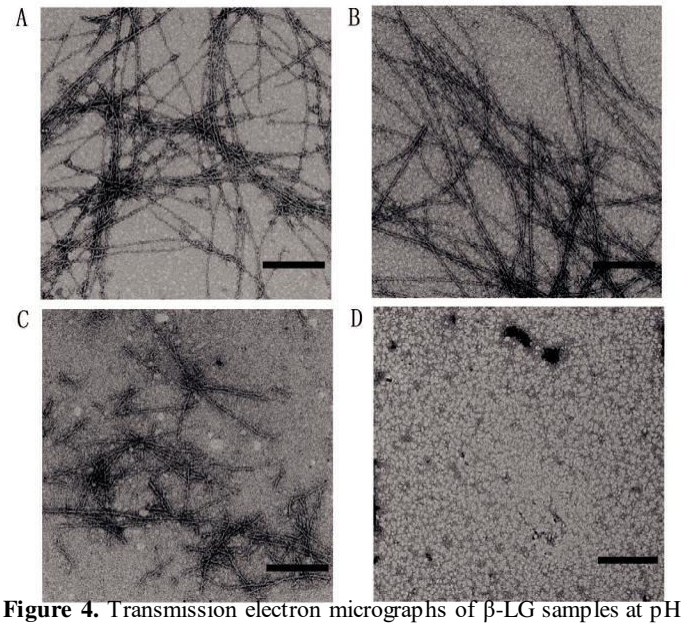

Figure 4. Transmission electron micrographs of β-LG samples at pH 7.0 and 37°C after incubation for 30 days in the presence of 0 μM(A), 10 μM(B), 20 μM(C), and 40 μM (D) folic acid. All of the scale bars represent 200 nm.

sections, the morphological studies of the end-point pro-ducts of different β-LG samples in urea solution were measured by TEM. As shown in Figure 4A, in the ab-sence of folic acid the mature β-LG fibrils showed typical amyloid morphology, characterized by long, smooth and partly bundled fibrils with diameters ranging from 5 to 20 nm and several micrometers in length. However, incuba-tion of β-LG with 10 and 20 μM folic acid led to marked attenuation of β-LG fibrillogenesis as shown in Figure 4B and Figure 4C, respectively. And no amyloid fibrils were observed when the concentration of folic acid up to 40 μM (Figure 4D) indicating that amyloid fibrillation of β-LG was completely inhibited by folic acid, which was consistent with ThT data.

Our TEM findings allowed us to conclude that folic acid would serve as a potential molecule with inhibitory properties against β-LG fibrillation. Combining with our ThT fluorescence results, it was found that the suppressing effect of folic acid on β-LG fibril formation at 37oC fol-lowed a dose-dependent manner.

Effect of folic acid on tertiary structure of β-LG

Owing to the high sensitivity to the conformational state of proteins, intrinsic fluorescence is a very use-ful tool for obtaining conformational information about proteins (29, 30). To characterize the effect of folic acid on the conformation of β-LG, intrinsic fluorescence emission spectra were used to monitor the conformational changes of β-LG in the absence and presence of folic acid. Figure 5A showed fluorescence emission spectra of the protein samples with different concentrations of folic acid. We ob-served that intrinsic fluorescence of partly unfolded β-LG decreased along with a red shift as the concentrations of folic acid increased. This indicated an increase change of polar environment around tryptophan residues. It demons-trated that folic acid changed the tertiary structure of part-ly unfolded β-LG, which may further inhibit the formation of amyloid fibrils.

Alteration in the hydrophobic exposure of β-LG in the presence of folic acid

Hydrophobic interactions have been thought to act very important roles for amyloid fibrils formation. ANS is a hy-drophobic marker that exhibits unique fluorescence along with a notable blue shift upon binding to the hydrophobic regions of proteins (31-33). Thus, ANS has been widely used for the detection of hydrophobic regions of proteins. Figure 5B showed the ANS fluorescence spectra of de-naturant-induced hydrophobic exposure of β-LG. In the absence of folic acid, the denaturant-induced hydrophobic exposure of β-LG showed very high ANS fluorescence in-tensity at 485 nm. The increase of fluorescence intensity may be attributed to more access of ANS to the hydro-phobic portion presenting in protein upon denaturation. The increasing interactions between protein molecules induced by these hydrophobic patches led to the amyloid aggregation of β-LG. But in the presence of folic acid, the observation of significant decrease of ANS fluorescence intensity indicated the loss of ANS binding sites. Gradual decrease of emission maximum of ANS fluorescence in-tensity has a marginal red shifting in the presence of folic acid, which suggested that the interactions between β-LG and folic acid decreased the solvent-exposed hydrophobic regions of partially unfolded β-LG.

Discussion

The transformation of soluble protein into insoluble fi-brillar structure, commonly known as amyloid fibrils has been investigated for many globular proteins. These stu-dies showed that many globular proteins can self-assemble into amyloid fibrils under harsh conditions, such as high temperature, low pH, or the presence of denaturants (34). Such amyloid aggregation was tied to many fatal diseases, such as Alzheimer's and Parkinson's diseases. Due to the

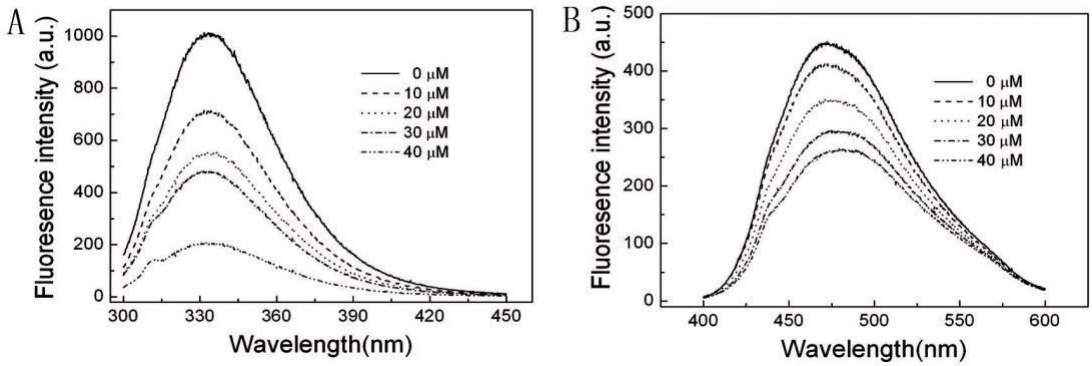

Figure 5. Intrinsic fluorescence (A) and ANS fluorescence (B) spectra of β-LG incubated with 5 M urea in the presence of 0, 10, 20, 30, and 40 μM folic acid.

increasing serious impacts of amyloid diseases on the aging population of the world, a wide range of strategies for the prevention or treatment of these diseases has been actively explored. One of the most attractive therapeutics options is to search small molecules which can prevent the accumulation-prone species by inhibiting the amyloid formation. Thus, studying the effect of small molecule inhibitors on the amyloid formation is of significance for understanding the mechanism of aggregation and preventing the amyloid-disease (35-38).

The binding between folci acid and β-LG has been wi-dely studied. Remarkablly, Liang et al. concluded that folic acid binds to the surface hydrophobic pocket in the groove between the helix and the β-barrel of β-LG by comparing the binding between the rentiol and β-LG in phosphate buffer. The binding between folic acid and β-LG increased folic acid photostability and prevented folic acid photodegradation (21). In this work, we studied the effect of folic acid on the amyloid formation of β-LG. The results of current study clearly showed that the binding between folic acid and β-LG increased the lag time and reduced the amounts of β-LG fibrils. Thus, the folic acid had the inhibi-tory effect on the amyloid formation of β-LG. In addition, we also investigated the effect of folic acid on the conformation and hydrophobicity of urea-denatured β-LG. The results showed that folic acid significantly quenched the tryptophan fluorescence and decreased the hydrophobility of β-LG in the presence of 5M urea. Since the influence of folic acid on β-LG is similar, we speculated that the binding sites between folic acid and β-LG are identical in the absence and presence of 5M urea. It has been demons-trated that hydrophobic interactions play an important role during the amyloid fibrils formation (39). Based on these results, we proposed that the decreasing proportion of the partially folded β-LG and the intermolecular association between such partially folded species was the major factor for inhibiting the formation of amyloid fibrils.

In conclusion, via multiple biophysical and bioche-mical approaches, we demonstrated that folic acid had an inhibitory effect on β-LG fibrillation. The kinetics of fibril nuclear and extension varied in the presence folic acid in a dose-dependent manner. The inhibitory effect of the amyloid aggregation was due to the decreasing of the hydrophobic interactions between partially folded species of β-LG when incubated with folic acid. This study is of significance for understanding the molecular mechanism of protein aggregation and amyloid-diseases.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interests.

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