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Effects of *Urtica dioica* agglutinin on glycotargeting of the vasculature: an *in ovo* study on chicken embryo

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Abstract

The angiogenesis process is a pivotal cellular process involved in both developmental and pathological circumstances. In this study we investigated effect of *Urtica dioica* agglutinin (UDA), as an unusual phyto-lectin from the chitin-binding protein family, on the angiogenesis of chicken embryos. The UDA was extracted from plant rhizomes and purified by affinity chromatography column. The activity of this lectin was assayed by hemagglutination test on the human RBCs. Anti-angiogenic effect of UDA on the extra-embryonic layer of the chick egg was studied in the different concentrations. Our results showed that the minimum concentration of UDA for agglutination were 48.00 and 15.00 µg mL^{-1} in macro- and microscopic studies, respectively. Because the number and length of the vessels were dramatically decreased at 100 µg kg^{-1} of UDA, the lectin had an inhibitory effect on angiogenesis of the embryonic vasculature of the chick. We concluded that UDA might target the vascularization events through binding to GlcNAc-conjugates. More investigations are needed to clarify the angiogenesis-related therapeutic roles of this interesting biomolecule.

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Introduction

The *Urtica dioica* (stinging nettle) agglutinin (UDA) is a unique phyto-lectin with a high sugar tendency for N-acetyl glucosamine (GlcNAc) or chito-oligomers.[1] This chitin-binding protein contains a tandem repeat of the Hevein domain (carbohydrate-recognition site) in a single chain of nearly 89 amino acid residues. The UDA is a thermo-stable acid and the lectin capacity of this molecule has been improved by its tandem structure. This improved functional performance has proposed it to be a good option for engineering as a molecular probe. Accordingly, this bivalent agglutinin attaches to the cell surface glycans, especially TCR (T-cell receptor), enrolling as a supper-antigenic lectin simultaneously via both major histocompatibility complex (MHC) classes I and II and maximizing the T cell activation.[2-5] Additionally, a variety of biological features of this small such as lectin in immunomodulation, inhibition of microbial/viral pathogens and anti-proliferative activity on neoplastic cells suggests its surprising potencies in glycotargeting sciences.[6-9]

Angiogenesis, the organization of a new blood/ lymphatic vasculature from the pre-existing one, consists of some complex cellular events like proliferation, survival, migration, and differentiation. Although angio-genesis occurs in embryonic development and rarely in adulthood (bone repair, wound healing and reproductive system), this process is involved in the pathogenesis of several disorders such as cancer, retinopathy, rheumatoid arthritis and so on.[10,11] However, glycosylation is a fundamental regulator of cellular interactions and diverse glycosylated components of the cell membrane and extracellular matrix (ECM) including growth/death factor receptors, gangliosides, galectins, integrins, and many other factors are engaged in these events.[12] Thus, any disturbance in the glycosylated areas may prevent angiogenesis. Engineering proteins selectively towards glyco-contributors in particular GlcNAc-conjugates that mostly are Asparagine (N)-linked glycans may generate promising therapeutic clues for the introduction of chitin-binding proteins, like UDA,[4] against angiogenesis. In this context, epidermal growth factor receptor (EGFR), a prominent N-glycan,[12]

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tunes-up the growth of the stromal cells.[10,13] The stromal cells involving monocytes, fibroblasts, and platelets are key cell communities in angiogenesis.[10] Interestingly, it has been reported that UDA halted the binding of EGF to its cognate receptor (Her-1) on A431 epidermoid cancer cells.[14] Also recently, a model of programmed-cell death for the targets of plant lectins has suggested that the nettle lectin can stimulate this cascade probably through binding to EGFR.[15] Seemingly, the glycans on the vascular endothelial cells may also be putative targets for chitin-binding lectins that there is no available evidence *in vivo*, through a cell-based study has denoted that these lectins may exhibit the anti-angiogenic modes of action.[16]

For *in vivo* investigation, chick embryo chorioallantoic membrane (CAM) model has been established as a simple and low-priced technique to study angiogenesis.[17] Accessibility of this highly-vascularized tool makes it attractive and useful as an *in ovo* model. Due to challenges in present remedies, researchers have screened the impact of synthetics and natural molecules on vascularization to find an efficient pharmaceutical strategy .[17-20] In the current study, for the first time, we decided to evaluate the anti-angiogenic action of a chito-specific agglutinin (UDA) in this reproducible and reliable animal model. So, the CAM vasculature was targeted by UDA lectin.

Materials and Methods

This study was approved by the ethics committee of the University of Mazandaran (#IR.UMZ. REC.1397.049) and conducted in accordance with Iran National Committee for Ethics in Biomedical Researches.

Plant sample and UDA crude extraction. The *Urtica dioica* (stinging nettle) samples were collected from the Bahnemir (Mazandaran, Iran) with a geographical location of Latitude: 36° 43' 371", Longitude: 52° 47' 205", Altitude: – 22.00 m. They were identified in the Herbarium of University of Mazandaran (#HUMZ7736). The water extraction procedure was done according to Peumans *et al*.[1] with some modifications. Fifty grams of fresh rhizomes of stinging nettle homogenates were prepared by grinding in a cold mortar with 300 mL of 100 mM, HCl (Merck, Darmstadt, Germany) pH 3.80 as a homogenization solution. The mixture was placed at 4.00 ˚C overnight. The cell lysate was further homogenized with a buffer for 20 sec. at 22.50 kHz by Misonix Sonicator 3000 (Cole-Parmer, Vernon Hills, USA) on ice, this step repeated for at least 20 times. The homogenate solution centrifuged at 20,000 *g* for 10 min, and supernatants filtrated with filter paper: Whatman Grade 3 (Merck), which contains 6.00 µm pore size and 185 µm thickness. The pH of the filterable solution was adjusted by 2.00 M, NaOH (Merck) up to 3.80. We identified this solution as a UDA crude extract.

UDA Lectin purification. To specifically purify, the crude extract was prepared and loaded on chitin affinity chromatography column (2.60 × 40.00 cm, 150 mL bed volume) of Sulfopropyl-Sephadex type C 50 (Pharmacia, Stockholm, Sweden) pre-equilibrated with acetate buffer [50.00 mM sodium acetate (Merck), pH 3.80, containing 0.10 M NaCl (Merck)] based on Peumans *et al*. method.[1] Here, each fraction, collected by 2110 Fraction Collector (BioRad, Hercules, USA) contains 10.00 mL solutions. The non-specificities were washed by acetate buffer containing 1.00 M NaCl. The UDA was eluted from fraction 45 - 60 by changing pH with acetic acid pH 2.80 (Fig. 1A). Then all peak fractions (#45 up to 54) were pooled and precipitated with 60.00%, NH_3SO_4 (Merck). Precipitated lectin dissolved in the general phosphate-buffered saline (PBS) [0.137 M, NaCl; 0.0027 M, KCl (Merck); 0.01 M, Na_2HPO_4 (Merck); 0.0018 M, KH_2PO_4 (Merck)]. One milliliter of this sample against 200 mL of PBS as a dialysis buffer, were dialysis with dialysis tube pore size 3.00 kDa (Sigma, St. Louis, USA). Finally, the purified lectin was prepared in PBS and visualized on SDS-PAGE.

Functional assay. Hemagglutination assay on human red blood cells (RBCs) was used to determine the lectin activity of the purified protein.[21] At first, the cells were washed in five volumes of normal saline and harvested at 5,000 rpm for 5 min. Then suspension of trypsin-treated RBCs prepared by 200 µL of 1.00% trypsin (Gibco, Grand Island, USA) and 2000 µL of 4.00% RBCs in PBS within 0.16%, $EDTANa_2$ -containing tube (Pars Peyvand, Tehran, Iran). Agglutination assays were carried out in small tubes in a final volume of 0.10 mL containing 80.00 µL of 1.00% trypsinated-RBCs suspension in normal saline, and 20.00 µL of different concentrations of UDA. Agglutination was monitored visually (with the unaided eye) after 1 hr at room temperature. However, for microscopic study of agglutination, after 2 - 3 hr at room temperature, this solution was centrifuged at 1,500 rpm for 3 min.

***In ovo* angiogenesis assay.** The chorioallantoic membrane vascular network was used as an animal model for our anti-angiogenesis screening by Ji *et al*. method.[22] The vessels under the chorionic epithelial tissue were treated by UDA lectin at the early 8th day of incubation of the fertilized eggs, which pre-incubated at 37.00 ˚C in 60.00 - 80.00% humidity. The eggs were cleaned with a 70.00% alcohol solution, in which a hole was drilled through the pointed pole of the shell and saline injection was dropped in the eggshell membrane. Part of the CAM of the embryo was exposed by peeling a 0.50 - 1.00 cm window in the shell, which exposed the underlying blood vessels. The natural egg component (inner membrane of the shell) was selected as a carrier vehicle for local application of the treatments. Different concentrations of UDA, which filtrated by syringe filter 0.20 µm (Merck, Kenilworth, USA), were placed on the inner membrane of the shell. The window was sealed with clear adhesive tape, and the eggs were incubated for another 96 hr. At least seven eggs were used per group. On the 12th day of embryonic

development, the eggs were collected from the incubator and the CAM was cut with sterilized scalpel blade (2.00 - 3.00 cm in diameter). The implants and surrounding embryonal tissues were surgically removed and fixed in 10.00% formaldehyde. The CAM vessels for both UDA treated groups and PBS (as a negative control) group were photographed by stereomicroscope (Blue Light Industry, La Habra, California). The number and length of the vessel branches were calculated by the ImageJ Software (version 1.37; National Institutes of Health, Bethesda, USA)[23] in a blind, an independent observer analyzed and statistically analyzed by one-way ANOVA using SPSS Software (version 16.0; SPSS Inc., Chicago, USA).

Results

Purification of the UDA lectin. The UDA lectin, a mixture of different isolectins, was isolated by a carbohydrate-specific chromatography and the process is illustrated in Figure 1A.

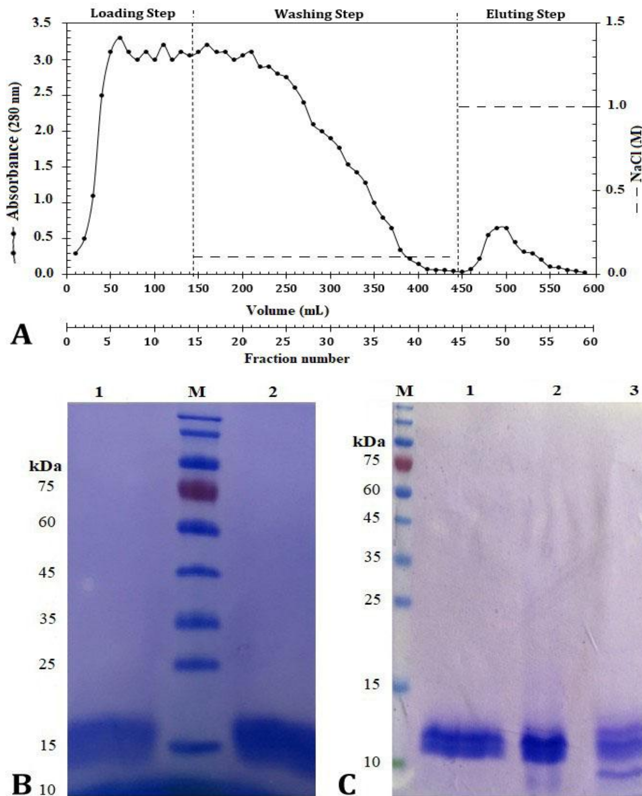

Fig. 1. Chromatogram and purified profiles result: **A)** Chromatogram profile of UDA eluted from chitin affinity column during elution, one peak (#47-54 fractions) was obtained. **B)** Purification and re-purification of the UDA lectin; Lane 1: the electrophoretic pattern of the purified lectin, Lane 2: the result of re-purification (see the text) and M: protein marker; **C)** The electrophoretic mobility of the total extract and UDA on a larger gel; Lane 1: UDA after denaturing treatment (8.00 M Urea); Lane 2: the purified lectin; Lane 3: the total protein extract; and M: protein marker of the Thermo Fisher Scientific, Bedford, USA.

We first purified the lectin from the total extract. Then, the purified lectin eluted in acetic acid prepared in acetate buffer by dialysis in order not to interfere with equilibrium of the column (acetate buffer was also used as the equilibrium buffer). Next, we loaded the prepared lectin on the column again by applying the similar condition of chromatography to re-purify (re-load) it. Re-purification was only conducted to check the possible effect of re-loading on the pattern of the lectin on the gel. Finally, we observed the same pattern on the gel even after re-loading or re-purification. Therefore, re-loading of the purified lectin ensured that there were not any possible undesired bands. Observing the indifferent pattern was also interesting, because we saw a UDA band in the two different weight positions at the molecular weight (MW) between 10.00 and 15.00 kDa (Figs. 1B and 1C). Moreover, we were able to continue our next experiments more confidently according to these observations.

To verify the purified lectin, we performed the reducing and denaturing 12.00% SDS-PAGE by Tris-Glycine buffer. At first, we observed different patterns of the purified lectin on the gel. As shown in Figure 1B, the protein marker with a molecular weight of 15.00 kDa represents the size of our purified lectin. Subsequently, the 10.00 kDa-size marker was overlapped with the electrophoresis loading dye resulting in some doubts for seeing the exact profile of the purification product on the gel. In order to allow the protein marker to be separated well, we carried out the electrophoresis of the lectin on the larger gel that provided a suitable distance for the clear visualization of the marker (Fig. 1C). The mini gel showed the molecular weight and electrophoretic pattern in consistent with the previous reports mentioned in the next section.

The pattern of the purified protein on the larger gel (Fig. 2) represents a different MW that also confused us to show it. Surprisingly, the discrepancy in molecular weights of our lectin seen in gel of Figures 2 were due to the biochemical properties of the UDA molecule.

A discrepancy in MW is routinely occurred in SDS-PAGE for high acidic proteins. These proteins are highly negatively charged and they do not easily interact with SDS. Therefore, the lower isoelectric point (pI) of these molecules results in the occurrence of their aberrant migration in SDS-PAGE. The UDA molecule is also highly acidic and, according to the purification method, we isolated this lectin in acidic condition (pH 3.80 for preparation of both the extract and chromatography column). In Tris-Glycine buffer, the applied ionic strength ranges from 3 to 9: pK1= 3.14 and pK2= 8.25 for glycine and pK1= 6.50 and pK2= 9.00 for Tris. Because of the high acidic nature of UDA, this protein disobeys from the logarithmic rule of mobility of a protein in Tris-glycine SDS-PAGE. As a result, UDA

displayed a different behavior on our gels. In this regard, we also calculated the relative migration of the purified lectin on both gels by using the logarithmic formula, which obtain from log of the migration each marker in the gel in mm, and log of the MW of each marker in kDa. Based on the measured relative mobility of the band in lane 1, we found that the average MW of the purified lectin was estimated 16.60 kDa and 11.70 kDa in the gel, respectively (Figs. 1B and 1C). Eventually, the agglutination assay confirmed the purified lectin. Lane 3 in Figure 1C shows the protein extract pattern before purification. Also, after applying the denaturing conditions (8.00 M, Urea; 5.00% 2-mercapto ethanol and heating at 100 ˚C for 10 min) to the lectin, the electrophoretic mobility of the lectin (lane 1) was similar to untreated UDA (lane 2) as compared in Figure 1C.

Lectin activity of UDA. Qualitative agglutination activity of the purified lectin on the trypsinated RBCs confirmed its functionality to bind to the cell surface-glycans. The macroscopic and microscopic analysis of this activity for our total UDA isolectins at concentrations from 12.00 to 96.00 µg mL^{-1} resulted in observations illustrated in Figure 3. In this case, the clear agglutination was macroscopically found at 48.00 and 96.00 µg mL^{-1} of the lectin, as compared to the lower concentrations (15.00 and 24.00 µg mL^{-1}) and PBS (negative control) after 1 hr (Fig. 2A). As shown in microscopic photographs, the UDA agglutinated more cells in 24.00 µg mL^{-1} (Fig. 2B) than in 15.00 µg mL^{-1} (Fig. 2C). The minimum concentration of UDA for agglutination was 15.00 µg mL^{-1} because no agglutinated RBCs were observed in 12.00 µg mL^{-1} of UDA (not shown) which was as same as PBS-treated cells (Fig. 2D).

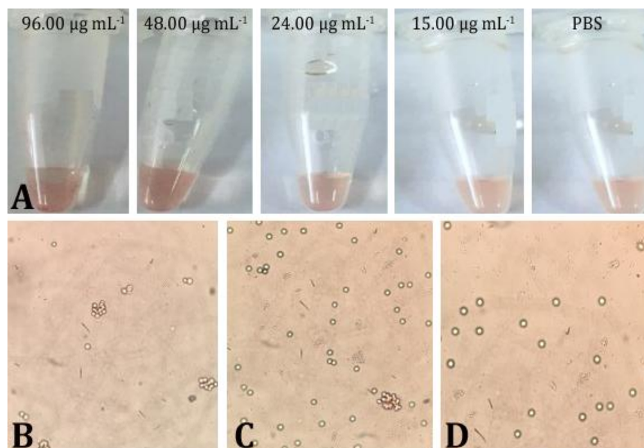

Fig. 2. Human red blood cells (RBCs) hemagglutination test of different *Urtica dioica* agglutinin (UDA) concentrations. **A)** in macroscopic agglutination, clear agglutination was not found in 15.00 and 24.00 µg mL^{-1} of UDA and phosphate-buffered saline (PBS)-treated RBCs; **B-D)** in microscopic agglutination, number of agglutinated cells in 24.00 µg mL^{-1} (B) was higher than 15.00 µg mL^{-1} of UDA (C) and PBS (D) treatments (400×).

Anti-angiogenesis activity of UDA. A total of 21, 8-day old embryos (seven eggs in each group) with an average weight of 56.00 ± 4.00 enrolled in this assay. The stereo-microscope of the CAMs showed the status of the vessel density in our treatments (Fig. 3). The CAM vessels were exposed to different concentrations of 50.00 and 100 µg kg^{-1} UDA. As shown in Figures 3 and 4, the number and length of the vessel branches in 100 µg UDA kg^{-1}-treated CAM were significantly lower than in other treatment groups in comparison to PBS-treated CAM ($p < 0.05$). Moreover, the number and length of the branches were not different between 50.00 µg UDA kg^{-1} treated and untreated (negative control) groups, with $p > 0.05$ (Fig. 4). The embryos in all groups were alive at the end of the test.

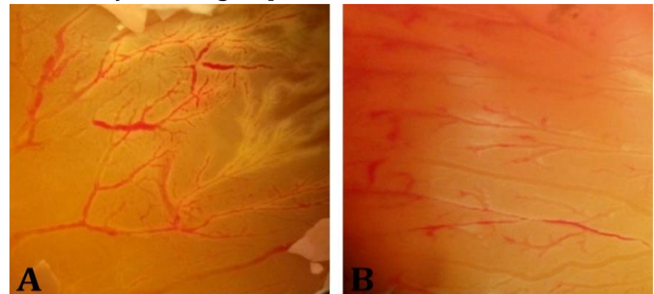

Fig. 3. Stereophotography of the chorioallantoic membrane (CAM) vascular networks in **A)** control group and **B)** UDA (100 µg kg^{-1})-treatment group. The treatments were directly applied to the inner membrane of the shell and then areas of about 1000 × 1000 peak cells of the photographed CAM at the 12th embryonic day were analyzed (A and B × 4 × 10 × 0.62).

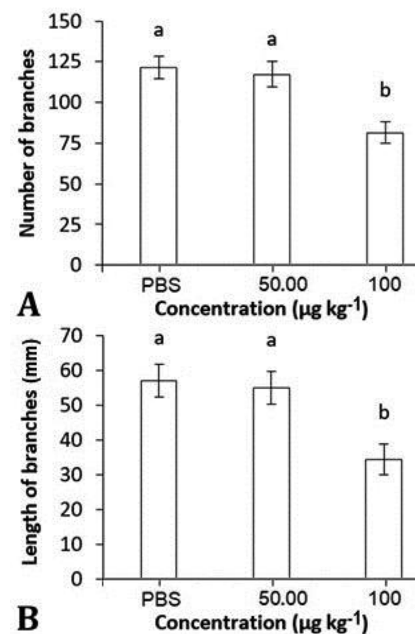

Fig. 4. Bar charts of the number and length of the treated vasculatures: **A)** the number and **B)** the length of the vessel branches in UDA (50.00 and 100 µg kg^{-1}) and PBS-treated CAMs. Differences in both the number (mean ± SD) and length (mm; mean ± SD) of branches between 100 µg kg^{-1} of UDA and control (PBS) were significant ($p < 0.05$).

Discussion

The UDA is a blend of about 11 structurally similar isolectins with various lengths and types of amino acid residues.[24] Besides, quantitative analysis of several *U. dioica* rhizome samples by capillary electrophoresis and mass spectrometry demonstrated a remarkable variation in UDA isolectin pattern so that isolectins I, II and VI are the most dominant forms.[25] Based on these evidence, even though the electrophoretic migration of the protein on the denaturing gel (Fig. 1B) was similar to the previous report,[26] the mobility of the purified UDA with molecular sizes around 13.00 to 17.00 kDa might reveal the presence of multiple isolectins in our work. In contrast, our observations (in Fig. 2B) have confused us for the mysterious mobility of UDA with sizes of 8.00- 9.00 kDa. Surprisingly, the UDA isoforms have functionally closely related activities in chitin-binding and agglutination tests.[24,26] Hence, we used the total UDA isolectins rather than an individual isolectin for our aims of the study. Our result showed that the minimum agglutination concentration of UDA lectin for agglutination is 15.00 µg mL^{-1} (Fig. 2). This concentration has been reported by the previous researcher, too.[1] Also, the lectin activity of UDA was comprehensively assessed to improve our insights toward its uses for human disease treatments and rapid detection systems. In the current study, UDA was locally administered to the CAM vascular networks to assess the inhibitory function of this chitin-binding protein on vascularization in an animal condition.

The results suggested that this GlcNAc oligomer-specific agglutinin could impress the angiogenesis progression. We found that UDA at 100 µg kg^{-1} was able to prevent the vascularization events in our animal model. Given that the anti-angiogenic activity of this lectin was not found in concentrations lower than 100 µg kg^{-1} (our highest treatment), a possible dose-dependent manner in higher concentrations must be tested in future attempts. The present observation provides important evidence suggesting that the CAM introduces different glyco-forms bearing GlcNAc-oligomeric side chains because interaction of UDA with the CAM potentially reflects the abundance of these carbohydrate structures on the surface of this membrane. Thus, the vascular system on this membrane may be sensitive to chitin-binding proteins and the CAM model can also be applicable to assess the potential anti-angiogenic characteristics of these molecules in line with carbohydrate-mediated targeting of angiogenesis as a promising therapeutic approach. From a mechanistic point of view, UDA (as a representative of chitin-binding lectins) may disrupt the CAM integrity through targeting specific glycans present in the glycomic profile of the CAM developing a novel selective glyco-science towards angio-genesis. Among the most established angiogenic mediators, EGFR (a well-known N-glycan) signaling is critical for the survival and proliferation of epithelial and stromal cells.[13,27] Also, to our best knowledge EGFR is a putative target for UDA and this lectin may probably affect the angiogenesis via this receptor. Importantly, the chorioallantois is a multi-layer tissue full of ECM (glyco) proteins such as fibronectin, laminin, collagen type-I and integrin alpha(v)-beta (III).[28] On the other hand, several other GlcNAc-conjugates participate in angiogenesis-related signaling pathways, particularly vascular endothelial growth factor receptors.[12] But, there is no -clue that these receptors can be targeted by UDA or other chitin-binding proteins. The sample treatment in CAM assay may trigger secondary angiogenesis as a result of non-specific inflammatory responses, tissue granulation, epithelial hyperplastic and hyperosmotic lesion. We believed that all of these induced by the influences of carrier and salt crystal, though the CAM has an immature immune system and these side effects may also take place in other *in vivo* models.[29-34] Also, the localized physical abnormality of the vessels may affect the obvious angiogenesis induction in a focal application due to insufficient liquid sample to fulfill all area of the membrane.[35-37] In contrary to these limitations, CAM assay remains the most widely used method to identify a novel angiogenic stimulator/inhibitor. Nevertheless, UDA did not threaten the life of our embryos and this might also suggest the potential safety of UDA, a property that should not be forgotten in animal studies and in clinical trials.

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Conflict of interest

The authors have no conflicts of interest to declare.

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