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Hylan G-F20 and galactomannan joint flares are associated to acute synovitis and release of inflammatory cytokines

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Abstract

Background: Injection of Hylan G-F20 (HY) into joints may provoke local flares, which mechanisms may involve reaction to protein contaminants. We have previously developed a protein-free saline-soluble galactomannan derived from guar gum (GM) that displays both analgesia and chondroprotection in experimental osteoarthritis (OA). We now demonstrate that both GM and Hylan G-F20 (HY) promote mild synovitis with cytokine release after intra-articular injection.

Methods: Mice received 100 μg/25 μL GM or HY or saline into the knees. Joint pain was evaluated using von Frey test; cell influx, interleukin (IL)-1, IL-6, and CXCL-1 (pg/mL) levels were assessed in joint lavage at 6 h. Synovia were excised for histopathology.

Results: Neither GM nor HY after being given into mice knee joints induced pain albeit promoting mild cell influx into joint washings as well as mild synovitis at histology, with no damage to the underlying cartilage. HY but not GM promoted IL-1 release into mice joints. Both compounds induced IL-6 and CXCL-1 release.

Conclusion: Intra-articular injection of HY or GM promote acute transient synovitis whilst not provoking detectable significant joint damage. Local administration of these polysaccharides induces acute intra-articular release of inflammatory cytokines, which may account for joint flares following viscosupplementation.

Keywords: Galactomannans, Hyaluronic acid, Hylan G-F20, Osteoarthritis, Polysaccharides, Viscosupplementation

Background

Viscosupplementation is a commercially available safe alternative to treat osteoarthritis (OA). Such non-surgical treatment aims to restore viscoelastic properties in diarthrodial joint cavities by injecting viscous hyaluronic acid formulations intra-articularly [1–4].

There are claims that high molecular weight viscosupplements provide better results, but definitive data to prove this assumption are yet to be obtained [5, 6]. Actually, high molar mass hylans (around 10^6 g/mol) had similar efficacy, as compared to low molar mass compounds (5–7.5 × 10^5 g/mol) in providing pain relief in OA [7]. The intra-articular half-life of viscosupplements is assumed to be around 24 h but patients may experience up to 6 months of pain relief following a single injection, suggesting that rheological properties may not be the only explanation of the therapeutic benefit [1, 7, 8]. Serious adverse reactions following viscosupplementation are uncommon. Reports of local pain following injection vary from 1 to 33% of the patients and up to 3% experience a pseudoseptic reaction, with joint swelling and intense pain [9, 10]. High molar mass compounds were more frequently associated to local flares. Reasons for this pseudoseptic reaction are ascribed to immune sensitization since they usually occur after a first injection but their occurrence following a single

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injection questions this assumption [1, 10]. Due to ethical reasons, it is hard to demonstrate whether viscosupplementation compounds promote synovitis after injection into human healthy joints. In naïve rabbits subjected to an air pouch model, viscosupplements induced a moderate inflammatory reaction and a mild chronic granulomatous reaction following injection into the knee joint [11].

Viscosupplements are primarily made of polysaccharides, but Hylan G-F20 (HY), the most commonly used compound that is isolated from rooster combs, also contains avian protein contaminants [1]. We have previously hypothesized that the polysaccharide structure rather than its rheology could explain the pain relief provided by viscosupplementation agents. Using a commercially available guar gum, a polysaccharide extracted from *Cyamopsis tetragonolobus*, composed of a galactomannan (GM) with a long central chain of mannose residues, we isolated a protein-free GM, which we use as a saline-soluble polysaccharide that is 40 times less viscous than HY [12]. Injection of this GM into the knee of rats subjected to experimental OA provided analgesia similar to that of HY regardless of using a viscous or a saline-soluble GM formulation, showing that viscosupplementation benefits may not depend on the viscoelastic properties [13]. We also showed that chronic GM administration prevented joint damage in an OA model and structure modifications of GM abrogate the therapeutic benefit [14].

Although unproven, protein contaminants and the viscous nature of viscosupplements may favor the development of local reactions, particularly when made blindly into joints. Spillage of injected compounds into perisynovial tissues could at least partially be responsible for local reactions [9]. Easy-to-handle, saline soluble, protein-free formulations could thus be a better alternative to current viscosupplements.

We now show that HY as well as a GM solution induce mild acute transient painless synovial cell influx into mice joints that is associated with cytokine release.

Methods

Polysaccharides

Guar gum was purchased from Sigma-Aldrich, São Paulo, Brazil and purified as described previously [12]. Hylan G-F20 (Synvisc®) was purchased from Novartis®, São Paulo, Brazil.

Animals

A total of 54 Swiss mice of either sex (25–30 g) were provided by the central animal house of our Institution. Animals were housed in cages (6 / cage) in temperature-controlled rooms with a 12 h light/dark cycle with free access to water and food. At the start of any experiments, mice were 2.5 months of age. All animal procedures and experimental protocols were approved by our local ethics committee on animal experimentation, that follows the recommendations of the Brazilian Council on Animal Experimentation (CONCEA) (protocol number 113/07). All efforts were made to minimize animal suffering and the number of animals used.

Intra-articular injection

Mice were anesthetized with i.m. ketamine (50 mg/kg) and xylazine (10 mg/kg). After local asepsia, animals received either sterile 100 μg/25 μL HY or GM (solution) or 25 μL saline intra-articular (i.art.) injections.

Assessment of pain behavior

Nociceptive behavior (regarded as joint pain) was assessed using the electronic pressure-meter nociception paw test by an observer blinded to group allocation [15]. Animals were placed in acrylic cages (12 × 10 × 17 cm high) with a wire grid floor, 15 min before the beginning of the tests, in a quiet room. Stimulations were performed only when animals were quiet, without exploratory, urination or defecation movements and not resting on their paws. The electronic pressure-meter consists of a hand-held force transducer fitted with a polypropylene tip (Electronic von Frey aesthesiometer, Insight Equipamentos Científicos Ltda., Brasil). The polypropylene tip was applied perpendicularly to one of the five distal footpads of the right hind paw. The intensity of the stimulus was automatically recorded when the paw was withdrawn. The test was repeated three times, until less than a 1 g difference between measurements was obtained. Results were expressed as the mean value of three withdrawal threshold measurements (g).

Assessment of cell influx and inflammatory mediators in joint aspirates

Groups of animals were sacrificed 6 h following intra-articular injection with exsanguination, under terminal anesthesia. The synovial cavity of the knee joints was washed with 0.05 mL saline containing 10 mmol/L EDTA. The synovial fluids were collected by aspiration and total cell counts were performed using a Neubauer chamber. Differential cell counts were performed using the panoptic Instant Prov™ staining kit (New ProvBrasil™). Cell influx will be used when referring to cell counts in joint exudates. After centrifuging (500 g/10 min), the supernatants, collected at 6 h following intra-articular injections, were stored at -80 °C until used for measuring the concentrations of Interleukin (IL)-1, IL-6 and CXCL1 using commercially available kits (R & D Systems, São Paulo, Brazil).

Histopathology

The same groups of animals used for cell counts (sacrificed 6 h after intra-articular injection) had their joint tissues excised for the histological study. Other groups were sacrificed with exsanguination, also under terminal anesthesia, 7 or 28 days following intra-articular injection and were also used for histological study. After fixation in 10% v/v formaldehyde solution and decalcification (5% v/v formic acid in 10% v/v formaldehyde solution), the whole joint, comprising the distal femoral and proximal tibial extremities, was processed for paraffin-embedding and staining with hematoxylin-eosin (HE) and safranin-O. Analysis was expressed as one result for each sample. Semi-quantitative histopathological evaluations was performed by an independent observer (VCCG) blinded to group allocation considering synovial proliferation and cell infiltration, meaning a semiquantitative evaluation of cells present in the synovial tissue, ranging from 0 to 3 (0, absent, 1, mild; 2, moderate; 3, severe). Results are expressed as the median (IQR) value for each group of six animals.

Statistical analysis

Results are presented as means ±95% C.I. for pain behaviour and cell counts in joint washings or medians (IQR) for histology of measurements made on at least six animals in each group. Assessment of normality of the pain behaviour data was done using the D'Agostino-Pearson Omnibus test. Differences between means and medians were compared using Student's t-test or Kruskal-Wallis test, respectively; $P < 0.05$ was considered as significant.

Results

Cell migration and hypernociception

Figure 1a shows that GM or HY induced a mild transient though significant cell influx at 6 h that subsided after 24 h, as compared to saline injection. Cell counts measured at 7 or 28 days did not differ from those at 24 h. Polymorphonuclear neutrophils were predominant in the cell exudates at 6 h (> 85%) in both HY and GM injected joints whereas there were only mononuclear cells in the saline group at this time-point. Joint washing collected at 7 or 28 days in all groups were composed

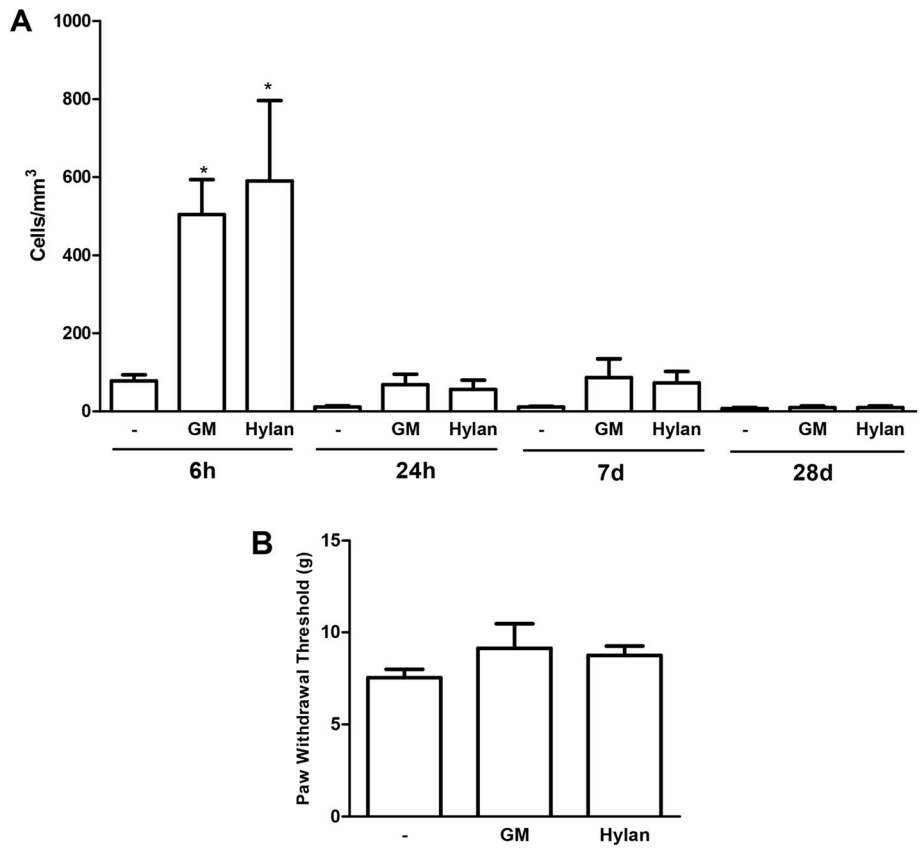

Fig. 1 Guar gum (GM) or Hylan G-F20 (HY) (100 μg/25 μL) or 25 μL saline were injected into naïve mouse joints; **a** kinetics of total leukocyte counts in joint lavage; **b** Joint pain evaluated at 5 h post-injection, using von Frey test. Data are mean ± SD of $n = 6$/group; one-way ANOVA followed by Tukey's test

solely of mononuclear cells. Despite the increase in acute cell influx, no hypernociceptive response could be demonstrated in joints that received HY or GM, as compared to control (Fig. 1b).

Synovitis

The histopathological appearance of the synovia of mice subjected to GM or HY i.art. Injection is illustrated in Fig. 2 showing synovial proliferation and cell infiltration starting at 24 h that could also be seen at 7 and 28 days. Synovia excised at 6 h following injection of either HY or GM to joints had no demonstrable changes, as compared to control. Detailed histological analysis of the samples in the various time points (24 h, 7 and 28 days) shown in Fig. 2 also reveal the mild character of the synovitis particularly in the long term. Cell infiltration into the synovia, meaning presence of polymorphonuclear neutrophils, lymphocytes, and macrophages was mild and did not differ between HY and GM groups. Similarly, synovial proliferation did not differ between HY and GM groups, although being significantly higher as compared to saline-injected joints. There were no samples with areas of granuloma or giant cells formation revealing the mild synovitis seen following intra-articular injection of either HY or GM. There were no alterations in the cartilage or subchondral bone that could be demonstrated in the hematoxylin-eosin and safranin-O stained material (data not shown). Therefore, at least under light microscopy, no structural damage, despite the above-mentioned synovitis, could be associated to the compounds.

Levels of inflammatory mediators in joint lavage

Table 1 shows an almost three-fold increase in IL-1 level in HY-injected joints whereas IL-1 levels did not increase in GM-injected joints, as compared to control. Remarkably, there was an over 20-fold increase in IL-6 levels in the joints of mice that received either GM or HY, as compared to control. Levels of the chemokine CXCL-1 displayed a 6-fold or 12-fold increase in joints that received HY or GM, respectively, as compared to saline-injected joints.

Discussion

We have previously shown that there is no detectable nitrogen in our isolated GM leading us to consider it a protein-free compound [12] bearing an average molar mass (Mw) of 3.9×10^6 g/mol [14]. HY is a mixture of hylan A and hylan B with an average molar mass of approximately 6.0×10^6 g/mol, being a hydrated gel that contains small amounts of avian proteins (http://products.sanofi.ca/en/synvisc-information-for-use.pdf).

We have recently shown that our protein-free GM formulation does also provide chondroprotection in experimental OA. In that study, rats received 9 weekly injections of GM and did not appear to develop systemic adverse effects [14]. In the present report, the cell influx into the washings of mice knee joints following GM or HY injection subsides at 24 h being no longer detected at 14 or 21 days (data not shown).

Previous reports have shown that viscosupplementation agents may induce severe joint pain, sometimes pseudoseptic, but the mechanistic is yet to be described. Inadvertent injection into the surrounding joint tissues rather than exclusively inside the joint space has been suggested to provoke those reactions [9]. Thus, guided imaging is currently recommended when performing joint injections in humans, but into the knee, in order to avoid those pseudoseptic flares [1]. There can be a mild, painful flare in the joint that disappears within 48 h following injection, but sometimes severe reactions take place [16].

We cannot completely rule out some spillage into perisynovial tissue in our experiments. However, the mild and transient cell influx seen in the present study, despite the small joint size, likely results from the careful joint injection, trying to be sure that compounds were solely, or at least predominantly, injected inside the joint. Despite the acute cell influx into joint cavities, neither GM nor HY elicited joint pain to mouse joints, which is similar to what we reported with both compounds in a rat study [13]. We did not evaluate the effect of repeated administrations in the present study since the size of mice joints represents a limitation. It is worth mentioning though that HY is marketed to be used as a single injection of a 6 mL gel formulation and in our hands GM and HY dosages were comparable regarding analgesia in experimental OA [13].

Using an air-pouch model, it was previously shown that HY induced granuloma formation and fibrosis. On the other hand, when injected into rabbit knees, those changes were milder [11]. Although the air-pouch model is a classic tool to evaluate inflammation, singularities of the synovial cavity might explain the more pronounced inflammation in the former. The synovial tissue has a rich blood supply. Vascular permeability and blood flow increase during inflammation thus increasing the already fast clearance rate in this tissue. In the present study, species differences as well as different dosing might have influenced the apparently less severe synovitis seen. As expected, there were no alterations in cartilage or in the subchondral bone. We are not aware of previous studies that have shown such a result, but it is worth mentioning since it adds to the safety profile of viscosupplementation agents, demonstrating absence of relevant chronic synovitis and joint damage provoked by the compounds.

Our data showed that mouse joints that received HY had a remarkable increase in IL-1 β levels. We are not

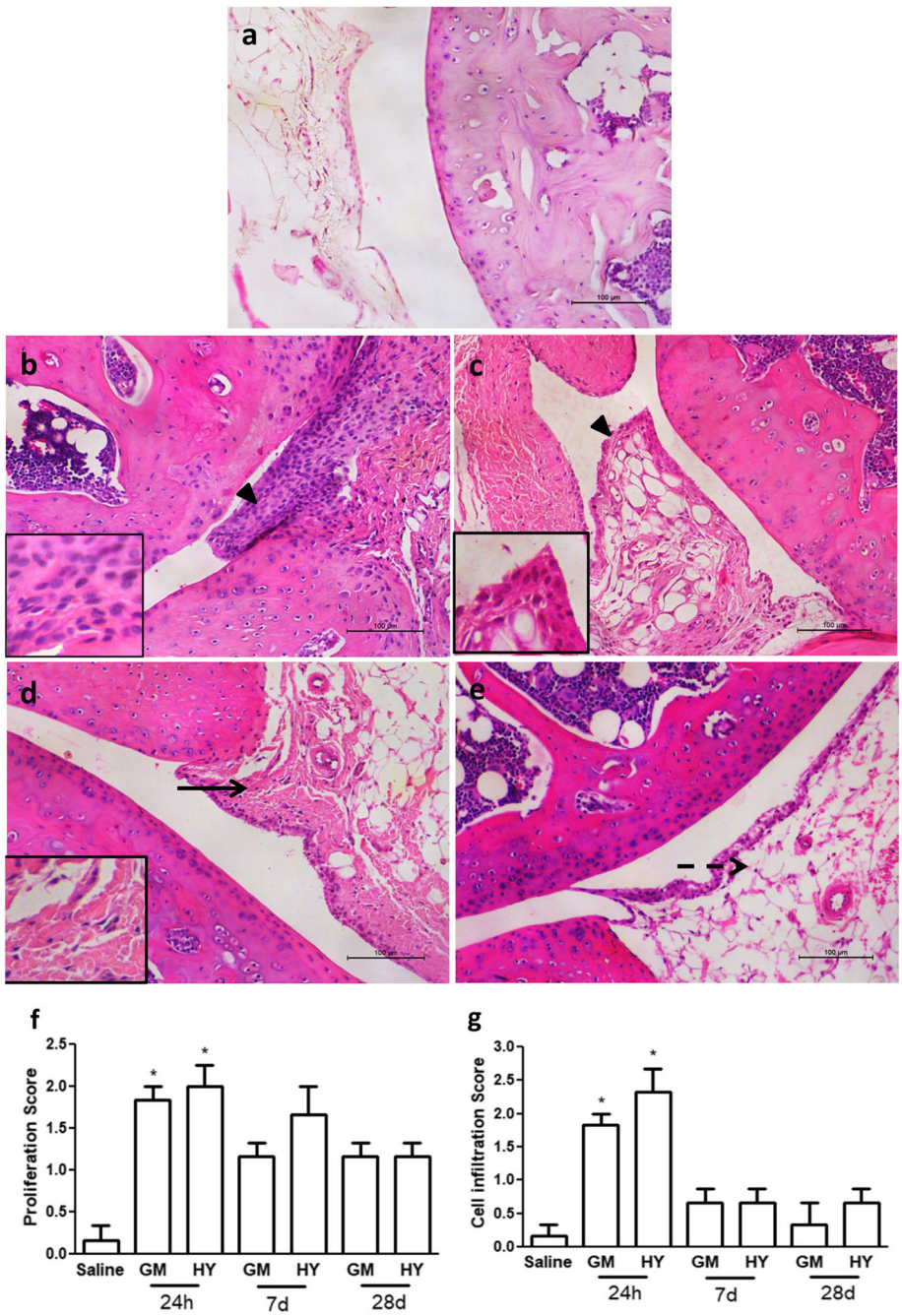

Fig. 2 Representative illustration of the histopathology of mice knees after injection of guar gum (GM) or Hylan G-F20 (HY) (100 μg/25 μL) or saline into naïve joints and analysis of histopathology scores. Knee joints were excised after 7 or 28 days and processed for hematoxylin–eosin staining. Saline-injected joints appear normal (**a**); there is mild synovial hyperplasia (arrow-heads) in GM and HY-injected joints at 7 days (**b** and **c**, respectively; insets); at 28 days, there is no long synovial hyperplasia with mild fibrosis in HY (**d**, arrow; inset) with apparently normal fat subsynovial tissue in a GM-injected joint (**e**, dashed arrow) (Original magnification × 200). Proliferation of synovial cells (**f**) and cell influx (**g**) into the synovia were evaluated under optical microscopy using HE and Safranin-O staining. Data are medians (IQR) of $n = 6$/group; *$p < 0.05$ using Kruskal-Wallys test

aware of previous data showing that HY or any other viscosupplementation agent induces in vivo IL-1β release. This major inflammatory cytokine has been associated to catabolic effects in joints affected by OA as well as in other inflammatory arthropathies [17]. Actually, intra-articular hyaluronic acid treatment was shown to decrease IL-1 β levels both in experimental models and human OA [18]. As opposed to HY, GM injection did

Table 1 Levels of inflammatory mediators following injection of a galactomannan (GM) or Hylan G-F20 (HY) into mouse joints

Group	IL-1β	IL-6	CXCL-1
Control	16.30 ± 1.694	25.39 ± 11.56	23.88 ± 1.125
HY	47.57 ± 12.60*	542.9 ± 303.4*	144.0 ± 33.35*
GM	11.43 ± 4.154	617.9 ± 262.6*	288.0 ± 58.65*

Mice received GM, HY or saline into the knee. Interleukin (IL)-1, IL-6 and CXCL-1 (pg/mL) levels were measured in joint washings after 6 h. Data are mean ± SD of $n = 6$/group; *$p < 0.05$ using one-way ANOVA followed by Tukey's test

not induce IL-1 β release whereas IL-6 and CXCL1 levels were greatly increased following injection of either of the compounds. IL-6 induces the production of C-reactive protein, an inflammatory marker that is associated to a systemic inflammatory state leading to morning stiffness and fever [19]. Humans subjected to viscosupplementation that developed flares with pseudoseptic reactions were shown to have increase in erythrocyte sedimentation rate as well as in C-reactive protein levels [9, 10]. Cytokine release in our hands was detected as early as 6 h following injection of the compounds. Neutrophils, which are not relevant sources of cytokine production, represent the major cell component in joint exudate at this time. Hence, we speculate that resident cells, possibly synoviocytes, stimulated by the injection of GM or HY, are producing those mediators.

We are not aware of any previous demonstration of IL-6 or CXCL1 chemokine (a neutrophil chemoattractant) release following the administration of viscosupplements [20]. Indeed, although increase in IL-1 levels following HY injection was comparably mild, those of IL-6 and CXCL1 were very prominent. This unexpected release could be linked to the local inflammatory flares mentioned in some patients after receiving joint injection of such compounds. Increase in joint CXCL1 levels may also at least partially explain the acute migration of neutrophils. Discussing the inflammatory role of these cytokines in chronic arthropathies is beyond our present scope, but we should emphasize that, to the best of our knowledge, this is the first demonstration that a viscosupplementation agent and a galactomannan, shown to be analgesic and chondroprotective in an OA model [14], paradoxically induce a mild local inflammatory synovitis that is associated with cytokine release. Guar gum has proven to be a safe compound over a wide dose range [21] and HY safety has not been an issue of major concern [1]. However, though unlikely, the release of cytokines could be associated to systemic rare adverse effects given the rich blood supply and fast clearance characteristic of the synovial membrane thus driving compounds into the systemic circulation.

Conclusion

Our data demonstrate that HY and GM induce mild synovitis when injected into naïve mice joints that is associated with the release of inflammatory cytokines. We believe this may account for local flares following injection of viscosupplements into human joints.

Abbreviations

HY: Hylan G-F20; IL: Interleukin; GM: Galactomannan; OA: Osteoarthritis

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Authors' contributions

RML, VCCG, JPAF, FACR contributed in the conception of the protocol; RML, ACMDP, VCCG, FACR performed animal studies, including histology reading (VCCG); RML, VCCG, PLRC, JPAF, FACR wrote the manuscript; all authors revised and approved final version of the manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval

All animal procedures and experimental protocols were approved by our local ethics committee on animal experimentation (protocol number 113/07). All efforts were made to minimize animal suffering and the number of animals used.

Consent for publication

Not applicable.

Competing interests

The author and co-authors declare that there are no conflicts of interest to disclose concerning the publication of this article.

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