Toxicological and genotoxic evaluation of anacardic acid loaded-zein nanoparticles in mice

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ABSTRACT
Anacardic acid extracted from cashew nut shells of Anacardium occidentale L. has demonstrated important biological activities, such as antibacterial activity against the cariogenic specie Streptococcus mutans. Zein nanoparticles containing anacardic acid (9.375 μg/mL) were evaluated in terms of toxicity and genotoxicity in vivo. The subacute toxicity assay was used to evaluate the cumulative effects of the oral administration of nano-encapsulated anacardic acid at 2.25 and 112.5 μg/kg for 7 days in mice, simulating a mouth rinse short-term clinical course treatment. Blank zein nanoparticles and saline solution 0.9 % were used as negative controls. Peripheral blood samples were collected to evaluate the genotoxicity in polychromatic erythrocytes using the micronucleus test. The animals were anesthetized, euthanized and the target organs collected, weighed and submitted to histopathological analysis. Liver, kidney and spleen relative weights did not change. Nevertheless, stomach, lung and heart increased the relative weights in the group receiving the highest dose, in which occasional histopathological findings were also identified. Both doses maintained the micronucleus frequency within the normal range and the animals treated with the highest dose presented a discrete weight lost, which could explain the organs’ relative weight reductions. Blank and anacardic acid loaded zein nanoparticles were nontoxic when administered repeatedly for 7 days, as no relevant histopathological changes neither genotoxicity were observed. These preparations demonstrated limited toxicity under the conditions used in this study and could become an antibacterial alternative for preventing/treating oral infections in short-term treatments.

1. Introduction
Caries and periodontal disease can be prevented and controlled by mechanically removing the biofilm and using additional mouthwashes [1]. Chlorhexidine is currently considered the gold standard chemical antiseptic agent, although its prolonged use is associated to staining teeth and restorations, desquamation and pain in the mucosa, discoloration of the taste buds and taste changes [2] and the emergence of resistant species [3]. Therefore, medicinal plants and their phytochemicals are a potential source of therapeutic options for the treatment and prevention of biofilm- dependent oral diseases, considering several advantages, such as their biological properties, low cost and availability [4].

Anacardic acid is found as the major constituent extracted from cashew nut shells of Anacardium occidentale L., representing up to 70 % of its overall content [5]. It can be found as 4 distinct phenolic molecules whose difference remains in the unsaturation found in the 15-carbon aliphatic chain, correlated with the enhancement of biological properties [6-8].

This compound has gained attention in the last years in view of its biological activities, such as: anti-inflammatory [9,10], larvicide [5], antinociceptive [11], GABA A receptor-mediated anxiolytic [12],...
antioxidant [5,7] and antimicrobial [6], comprising oral bacteria [4,13]. Nonetheless, its low pH, high viscosity, hydrophobia and anti-aesthetic color discouraged its clinical use in dentistry. The incorporation in polymeric systems can overcome these disadvantages. Polymeric nanoparticles have been extensively studied as drug delivery systems, providing drug protection, increased stability, controlled release, specific targeting and decreased toxicity [14–16]. Apart from overcoming the pharmacokinetic and pharmacodynamics limitations of many potential molecules, whose otherwise could be therapeutically useless, they may also be useful for enhanced permeability and retention effect, important features for local delivery in the biofilm control in the oral cavity [17]. Zein (Zea mays L) is a polymer biocompatible, biodegradable and highly hydrophobic [18,19]. It was used in the development of polymeric nanoparticles as a drug delivery system [20–22], incorporating several active compounds such as epigallocatechin gallate (EGCG) [18], curcumin [23,24], rutin [25], thymol [26] and glimepiride [20].

Anacardic acid loaded zein nanoparticles were able to improve important properties of this molecule, such as antioxidant and antibacterial activities against the cariogenic Streptococcus mutans in vitro [13]. Although several studies have attempted to evaluate the biological activities of anacardic acid, very little is found in the literature regarding its safety patterns. In view of the improved potential found in the anacardic acid nanoencapsulation and the potential use in the composition of mouthwashes to treat oral biofilms, the subacute toxicity and genotoxicity in vivo of anacardic acid loaded-zein nanoparticles administered orally to mice were investigated.

2. Materials and methods

2.1. Materials

Zein was purchased from Sigma-Aldrich® (St. Louis, MO, USA). Anacardic acid was extracted from cashew nut shell liquid of Anacardium occidentale. Deuterated solvents D2O 99.9% and CD3OD 99.8% were purchased from Eurisotop (Saint-Aubin, France). Giemsa solution was purchased from Laborclin (Pinhas, Parana, Brazil). The anesthetics ketamine 10% and xylazine 2% were purchased from Venco (Londrina, Parana, Brazil). All the other reagents were pure grade and used as received.

2.2. Purification and characterization of anacardic acid

Anacardic acid was isolated from cashew nut shell liquid extracted and purified according to the method of Trevisan et al. [8], through the formation of precipitated calcium anacardate, acidification and extraction with hexane, followed by evaporation. The extracted compound (~400 mM) was dissolved in 0.6 mL of CD3OD:D2O 90:10 (v/v) and submitted to Nuclear Magnetic Resonance (1H NMR) appreciation. 1H NMR spectrum was measured at 25 °C using a 17.6 T Bruker NEO-750 NMR spectrometer (750 MHz proton frequency) and processed with MestraNova® software version 12.0.

2.3. Preparation and characterization of nanoparticles

The formulations were obtained by nanoprecipitation of the protein according to our previously described method [13], using zein protein as a carrier and anacardic acid at 9.375 μg/mL (ZAa), the maximum loading reached. This concentration demonstrated the antibiofilm activity of ZAa against S. mutans in vitro [27]. Blank nanoparticles (ZB) were prepared in the same manner, except for the absence of drug, and used as control. The blank and loaded nanoparticles were appreciated morphologically by transmission electron microscopy (TEM) (JEOL JEM-2010, Electron Microscope) and characterized in terms of size (nm), polydispersity index (pdi), zeta potential (ζ) (mV) using a dynamic light scattering (DLS) analyzer (Zetasizer® Nano-ZS90, Malvern Instruments) before and after dilution.

Anacardic acid-loaded nanoparticles (ZAa) were administered in a volume of 0.3 mL. To obtain the lowest dose 2.25 μg/kg, freshly prepared nanoparticles (112.5 μg/kg) were diluted 1:50 in purified water. This dilution was chosen based on the possibility of ingestion of the residual amount left in the oral cavity after a daily administration. The blank nanoparticles were administered in a volume equivalent to the highest loaded dose.

2.4. Animals

Twenty-three female Swiss mice (Mus musculus), nulliparous, non-pregnant, with 25–30 g, were obtained from the bioterium of the Christus University Center, Fortaleza, Brazil. All animals were acclimated and kept under controlled temperature (20–25 °C) and relative humidity (50–60 %), following a 12 -h light-dark cycle. The animals were housed in cages with 4 or 5 animals and provided standard commercial diet and ad libitum drinking water. This study was based the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and was approved by the Animal Use Ethics Committee (CEUA) of Christus University Center under the protocol number 020/18.

2.5. Subacute toxicity

The cumulative effect of a short-term course treatment was assessed by a subacute toxicity assay for 7 consecutive days, according to the OECD 407 protocol [28] with some modifications, justified by the low doses administered, compatible to its effective dose [27] and the potential use as mouthwash in oral biofilm diseases prevention and control. ZAa nanoparticles were administered by oral gavage daily in two doses (ZAd1: 2.25 μg/kg and ZAad2: 112.5 μg/kg) (5 animals per group), while blank zein nanoparticles (ZB) and saline solution 0.9 % were administered in a volume equivalent to the highest dose to groups of four animals, each. The treatments are compiled in Table 1. The number of animals was based in another in vivo study focused in the antiproliferative activity of AA in experimental model of mice bearing human prostate tumor xenografts [29], which needed four animals by experimental group to reject the null hypothesis, similarly to the protocol used in this work.

Following the daily administration, the animals were carefully observed in individual cages for 1 h, regarding their general behavior, signs or symptoms of toxicity such as tremors, convulsions, changes in posture and activity or death, according to Carvalho et al. [30]. The body mass of each animal (in g) was checked in the beginning and end of the experiment using a 2 digits electronic scale to appreciate the variation between groups. Finally, the animals were anesthetized with ketamine 150 mg/kg and xylazine 50 mg/kg anesthetic mixture, euthanized and the target organs were removed to determine their relative weight (organ weight/animal body weight) and submitted to macroscopic and microscopic examination.

| Formulation | Size (nm)  | pdI    | ζeta potential (mV) |
|-------------|-----------|--------|----------------------|
| ZAd1        | 381.6 ± 2.12 | 0.215 ± 0.02 | -15.9 ± 0.60 |
| ZAad1 (5:10) | 228.4 ± 1.609 | 0.245 ± 0.019 | -34.7 ± 1.61 |
| ZB          | 376.5 ± 3.95 | 0.137 ± 0.01 | -6.56 ± 0.22 |
| ZAad1 (5:10) | 785.1 ± 143.0 | 0.231 ± 0.022 | -14.1 ± 3.65 |

ZAad1, ZAad2 and ZB: anacardic acid loaded zein and blank nanoparticles, respectively. (5:10) means after 1:50 dilution in ultrapure water. Results are expressed as mean ± SD.
2.6. Morphological and histopathological evaluation

For the histological evaluation, the organs (liver, spleen, heart, lung and kidneys) were carefully analyzed in terms of mass, size, color changes and suggestive lesions such as organ structure, degenerative, necrosis and signs of inflammation [31]. Subsequently, they were stored in 10 % buffered formaldehyde for 48 h and subjected to sample preparation for histopathological purposes: dehydrated in crescent alcohol series, embedded in paraffin, sectioned in 4 μm thick slices, stained with hematoxylin-eosin [32] and assembled in Canada balsam for conventional microscopic analysis.

2.7. Genotoxicity evaluation

The genotoxic effects of ZAad1, ZAad2 and ZB were evaluated using the micronucleus test in polychromatic erythrocytes according to the method used by Carvalho et al. [30], which evaluated anacardic acid isolated, in agreement with OECD 474 protocol [33]. The negative and positive control groups received 0.9 % saline solution by oral gavage and doxorubicin (DXR) 15 mg/kg intraperitoneally, respectively. A blood drop was collected from each animal’s tail tip and dragged over a clean microscopic blade to obtain the blood smear, dried at room temperature, followed by fixation in absolute methanol for 5 min and a second drying at room temperature. The blades were stained with diluted Giemsa dye [34] for 20 min, washed to remove excess of dye, dried at room temperature and finally analyzed in an optical microscope (Opton TIM-2008) on the immersion objective (100x) [35].

Micronucleated polychromatic erythrocytes (PCEMNs) in peripheral blood samples were calculated from 2000 cells [35] and the micronucleus prevalence (in %) determined.

3. Results and discussion

3.1. Nanoparticles characterisation

The nanoparticles showed adequate physical-chemical parameters: particle size bellow 500 nm, monodispersed (pdl <0.3) [21,22] and positive zeta potential for ZB and negative for ZAa, attributed to the salicylic acid groups [7]. The characteristics of the formulations obtained and after their dilution are compiled in Table 1.

Although blank nanoparticles doubled its size after dilution, it did not collapse (pdf = 0.231). This phenomenon can be attributed to a change in the nanoparticles conformation (confirmed by the zeta potential inversion: +6.56 to -14.1 mV), as the protein depends of a certain proportion of ethanol to maintain its stability and nanoparticles reduced size. In the anacardic acid loaded nanoparticles size was reduced at almost half and remained stable after dilution (pdf 0.245 ± 0.019), while zeta potential reduced its value to -34.7 ± 1.61 mV and did not change their charge, indicating a more stable system. The appreciation of the nanoparticles by TEM, confirms the particle size values obtained by DLS, ranging from 300–490 nm for ZAad1 and ZB (Fig. 1). Both systems showed spherical and non-aggregated characteristics.

The 1H NMR spectrum of AA, naturally found in the cashew shells of *Anacardium occidentale* is shown in Fig. 2. The signal assignment confirms the solely presence of this compound in the purified supply used to prepare the nanoparticles. The monoene and diene aliphatic chain molecules were predominant and no impurities or their degradation products (such as cardol or cardanol) were evident.

3.2. Subacute toxicity

During the subacute toxicity experiment no behavioral changes, death or severe toxicity signs were observed. Apart from the negative control group (NC), all the other groups presented weighting loss, though they did not differ from each other (p > 0.05) (Table 2).

Carvalho et al. [30] found the lethal dose of anacardic acid and cashew nut oil to be above 2000 mg/kg in an acute toxicity study. In the same study the oral administration of anacardic acid at 300, 600 and 1000 mg/kg during 30 days did not incur in biochemical and/or hematological or body weight change, similarly to the results of Konan et al. [36] who studied the toxicity of *A. occidentale* leaves extract. The reduction in the body weight was found in the highest dose (ZAad2) (Table 2), although it has been unspecific and did not differ statically (p > 0.05) from the other groups.

In the relative weight analysis, no significant alterations were found in spleen (Fig. 3A), liver (Fig. 3B) and kidneys (Fig. 3C) in comparison with the negative control group (p > 0.05). However, an increment in the stomach (Fig. 3D), lung (Fig. 3E) and heart (Fig. 3F) (p < 0.05) relative weights of the ZAad2 group were found. This finding can be partly explained by the body weight reduction in this group; as the absolute organ weights (heart and lung) did not differ significantly from the negative control groups.

Carvalho et al. [30] reported an increment in the relative weight of spleen in female mice treated with anacardic acid (600 mg/kg) for 30 days. Nonetheless, when a single dose of anacardic acid (2000 mg/kg) was administered, this aspect was not observed. A reduction in the relative lung weight was also observed in treated male mice. Contrariwise, doses below 300 mg/kg have not produced signs of chronic toxicity. Even so, the doses used in that study are much superior than those administered (2.25 and 112.5 μg/kg) in this experimental protocol. Therefore, the changes observed are more likely to be related to the body weight reduction observed in the group treated with 112.5 μg/kg ZAad2 nanoparticles, as aforementioned.

![Fig. 1. TEM images of the nanoparticle formulations before dilutions. a) ZAad2, Anacardic acid-loaded zein nanoparticles and b) ZB, Blank zein nanoparticles.](image-url)
ZAad and ZB presented signs of sinusoidal hemorrhage and in the groups NC and hepatocyte necrosis in any group. One animal from the groups NC and noticeable in the parameters color, texture, size or anatomical aspects.

Body weight variation in mice submitted to subacute toxicity assay. Weight assessment was performed at the beginning and after 7 days (results are expressed as mean ± SD).

| Day | ZAad1 | ZAad2 | ZB | NC |
|-----|-------|-------|----|----|
| 1   | 27.60 ± 2.30 | 26.60 ± 2.07 | 26.50 ± 2.38 | 25.50 ± 3.10 |
| 7   | 27.20 ± 2.28 | 22.40 ± 3.50 | 24.00 ± 2.44 | 25.75 ± 2.06 |

ZAad1: Anacardic acid loaded zein nanoparticles administered at 2.25 μg/kg; ZAad2: Anacardic acid loaded zein nanoparticles administered at 112.5 μg/kg; ZB: Blank zein nanoparticles.

NC: Negative control group, treated with sterile saline solution 0.9 %.

**3.3. Histopathological evaluation**

In the macroscopic analysis of the organs, no alterations were noticeable in the parameters color, texture, size or anatomical aspects. The spleen preserved follicles and the white and red pulps were intact in their constitution. No vascular congestion or inflammatory cells were found, only the common presence of megakaryocytes in all groups (Fig. 4A), demonstrating no relevant histopathological changes.

Liver samples were constituted by hepatocyte cords without swelling, with portal and central lobular vein congestion, Kupffer cell hyperplasia, absence of micro or macro vesicular steatosis neither focal hepatocyte necrosis in any group. One animal from the groups NC and ZB presented signs of sinusoidal hemorrhage and in the groups NC and ZAad2 occasional focus of inflammation. In the groups ZAad1, ZAad2 and NC, mild to moderate hydropic degeneration was observed (Fig. 4B).

Tedong et al. [37] reported the hepatotoxicity of *A. occidentale* L. leaves’ hexane extracts, causing vascular congestion, degeneration and necrosis in the dose of 14 g/kg. Nevertheless, the therapeutic doses associated with its biological properties: anti-inflammatory [9], anti-bacterial [38,39], anti-tumor [40], anti-ulcerative [31] and antioxidant [42] are considerably lower than those used in that study.

Kupffer cells have phagocytic purposes and their hyperplasia shows that all animals were exposed to hepatic oxidative stress [43]. Hydric degeneration is a vacuolization in which the amount of intracellular water increases, caused by disturbance of ionic and fluid balance, commonly reversible and nonlethal process, but can lead to cellular degeneration (necrosis) in more severe cases [43,44], suggesting that anacardic acid may have contributed to some level of ionic unbalance.

The kidneys of all groups presented normal distribution of preserved glomerular structures in the cortex and renal medulla, without tubular epithelial swelling or vacuolar degeneration. No inflammatory cells and necrosis were observed. In ZAad1 and ZAad2 groups, mild tubular and interstitial hemorrhage was found, while the presence of hyaline cylinders could be observed solely in the highest dose (ZAad2) (Fig. 4C).

Alterations such as inflammation and mild hemorrhage in the renal tissues may occur and are considered occasional, but it can progress in severity to renal insufficiency and tubular necrosis [45–47]. Hyaline cylinders are formed from the accumulation of mucoproteins secreted by epithelial cells in the renal tubules and may be related to acute tubular irritation, nephritis, hemorrhages or changes in the renal flow [48–50].

Carvalho et al. [30] did not found hepatic or renal morphological alterations after acute and subacute anacardic acid administration in doses between 300 and 2000 mg/kg. Anacardic acid 0.1 % (p/p) has also been evaluated in rat supplementation diet for 29 days and no changes in the hepatic and renal markers were found [51]. Mice fed with a high-fat and high-sucrose diet treated orally with 500 μg/kg of AA slowed down the lipid accumulation rates in the liver and mitigated insulin resistance, demonstrating its protective effect in metabolic disorders [52].

The animals of all groups showed heart tissues predominantly represented by cardiac striated muscle, longitudinal and transverse fibers preserved with absence of hemosiderin pigments or inflammatory cells. Discrete areas of hemorrhage were noticed in three animals of ZAad2 from whom one presented vacuolation in the myocytes (Fig. 5A). This vacuolation in myocytes is considered a non-lethal degeneration process, characterized by clear spaces, ranging from 0.1–5 μm [53,54]. Nonetheless this aspect is more likely be related to cardiotoxicity when associated with other changes such as infiltration of inflammatory cells, necrosis and fibrosis [55–57], hemorrhagic spots accompanied by necrosis and the presence of macrophages and hemosiderin [58,59]. All these factors were absent, demonstrating only focal damage, not related to cardiotoxicity, and limited to the highest dose (ZAad2) treated group.

Lung samples were represented in all groups by alveolar spaces with bronchioles, bronchi of preserved morphology and intact pleura. Occasional areas of edema or hemorrhage were observed in one individual animal of the groups treated with ZB, ZAad1 (lowest dose) and NC group, all without inflammatory cells, not configuring a relevant and treatment-related sign of toxicity. ZAad2 group showed moderate hemorrhagic points and occasional inflammatory cells (Fig. 5B). Despite its rare occurrence, it may result from side effects of drugs that alter hemostatic mechanisms or disruption of capillaries [60,61]. Despite that, anacardic acid was able to decrease induced inflammatory and oxidative processes in mice treated orally in the doses of 50 a 250 mg/kg for 30 days, suggesting its possible protective activity in the lung [62].

Some histological changes in the stomach were found and its relative weight increased in the groups ZB, ZAad1 and ZAad2, corroborating with the histological changes observed, such as mucosal thickening of the fundus and disorganization (Fig. 6A and 6B). Moreover, no inflammatory infiltrate was observed in these groups (Fig. 4B), which could possibly be associated with the gastroprotective effect of anacardic acid [63] and zein nanoparticles, hence the blank formulations were consonant with this behavior.

Unlike these aforementioned findings, where heart, lung and stomach organs showed relative weight increment and few toxicity signs in the higher dose tested (ZAad2), similar studies with anacardic acid and *A. occidentale* extracts did not observe apparent macroscopic or microscopic changes in the target organs analyzed in higher doses [30,36]. This fact could be attributed to the nanoencapsulation of anacardic acid, which may have contributed to enhance its bioavailability and thus exacerbate the side effects in the highest dose assayed. Further studies exploring the pharmacokinetics of different doses and also comparing the non-encapsulated to the nanoencapsulated anacardic acid are mandatory to confirm this hypothesis and could provide further insight for novel therapeutic approaches.
3.4. Genotoxicity assay

Clastogenesis or aneugensis can occur during the process of erythrocyte maturation, resulting in micronucleus [31]. These changes can be identified in the analysis of immature (polychromatic) erythrocytes in peripheral blood or bone marrow samples [42].

No relevant difference on the micronucleus frequency was observed in polychromatic erythrocytes between treated and negative control groups. A very low frequency of micronucleus was found in immature (polychromatic) erythrocytes of both treated and control groups, which implies in non-genotoxic characteristics [36] (Table 3). The lowest dose (ZAad₁) triggered a lower frequency of PCEMNs than the negative control (p > 0.05), blank zein nanoparticles (p > 0.05), ZAad₂ (p > 0.05) and DXR (p < 0.001). DXR acts through the topoisomerase II inhibition,

Fig. 3. Target organs weight variation (organ weight/body weight x 100) in mice orally treated for 7-days with ZAad₁, ZAad₂, ZB nanoparticles and NC. (A) Spleen, (B) Liver, (C) Kidneys, (D) Stomach, (E) Lung and (F) Heart. (results are express as Mean ± SD).

**p < 0.01 ZAad₂ versus NC, ZB and ZAad₁, *p < 0.05 ZAad₂ versus CN, ZB and ZAad₁. ZAad₁: Anacardic acid loaded zein nanoparticles administered at 2.25 μg/kg. ZAad₂: Anacardic acid loaded zein nanoparticles administered at 112.5 μg/kg; ZB: Blank zein nanoparticles. NC: Negative control group, treated with sterile saline solution 0.9 %.

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causing evident genotoxicity by damaging the DNA, used as positive control in micronucleus assays [64–66].

Moreover, no difference between ZB and negative control group (p > 0.05) was found. Despite the absence of genotoxicity, the largest number of PCEMNs were found in the ZAad group, attributed to the highest concentration of nanoencapsulated anacardic acid and its transient toxicity. Despite this fact, the frequency of micronuclei was much lower compared to the DXR treated group, demonstrating its lack of genotoxicity.

Different doses of anacardic acid were evaluated up to 300 mg/kg, and no difference in the micronucleus frequency was observed in comparison to their controls [30,35]. A micronuclei frequency of 0.26 % was found in rats treated with 2000 mg/kg of hydroethanolic extract of leaves *Anacardium occidentale*, while the negative control had 0.11 % and over 1 % was found in the positive control group (cyclophosphamide – CYP 50 mg/kg) [36], these data are in agreement with those obtained in this study (Table 3). The highest frequency of micronucleus was found in the DXR-treated group (1.75 %), followed by the highest dose of anacardic acid loaded-zein nanoparticles (ZAad 2 – 0.31 %), which is considered non-genotoxic under the treatment course used.

4. Conclusions

Novel blank and anacardic acid-loaded zein nanoparticles, designed
findings were correlated with high toxicity in these organs. In the gen-
be related to the weight loss observed in the nanoparticles treated
the lung, heart and stomach relative weight was observed, which could
Although, they did not show relevant signs of toxicity, an increment in
for treating oral biofilms, were administered orally for 7 days in mice.
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Table 3

| Groups   | PCE (n°) | PCEMN (n°) | MN (%)   |
|----------|----------|------------|----------|
| NC       | 8000     | 21         | 0.26 ± 0.013a |
| ZB       | 8000     | 20         | 0.25 ± 0.009a |
| ZAad₁    | 8000     | 17         | 0.21 ± 0.012a |
| ZAad₂    | 8000     | 25         | 0.31 ± 0.012a |
| DXR      | 8000     | 140        | 1.75 ± 0.712a |

PCE, polychromatic erythrocytes, MN, frequencies of micronucleus, DXR, doxorubicin (15 mg/kg). One-way ANOVA followed by Tukey test. Different uppercase letters indicate statistical difference (p < 0.001).

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**Declaration of Competing Interest**

The authors report no declarations of interest.

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**CRediT authorship contribution statement**

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