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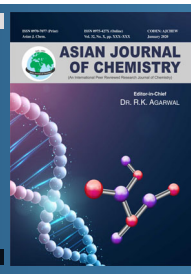

Poly Sulfoxyamine Grafted Chitosan as Bactericidal Dressing for Wound Healing

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In this study, sulfoxyamine derivative of chitosan was carried out by reaction with thionyl chloride and further treated with ammonia. FT-IR, ^{1}H NMR, elemental analysis and DSC methods are used for confirmation of modification. The results revealed that the modified chitosan exhibits better water solubility than chitosan. The evaluation of the applicability of sulfoxyamine modified chitosan in the treatment of dermal wound in rats was performed by induction of transcutaneous wound. The antibacterial activity was tested on Gram-negative and Gram-positive strains. It was found that the modified chitosan showed grater activity against Gram-negative stains as compared to Gram-positive strains. The superior wound healing and antibacterial activity might be due to the grafting of additional cationic group on the polymeric backbone and their ionic interaction with anionic cell wall of skin or bacteria. Modified chitosan also showed significant physical properties like mucoadhesion and film forming or coating properties. The modified chitosan forms film with good adhesion on wound which will protect the wound and also allows gas exchange. These properties are beneficial for treatment of wounds. Similar to chitosan, modified chitosan showed non-toxicity in skin irritation, oral acute toxicity and cytotoxicity.

Keywords: Chitosan, Wound healing, Antimicrobial, Mucoadhesion, Film forming agent.

INTRODUCTION

Chitosan, (1-4)-2-amino-2-deoxy-β-D-glucan [1,2] is a cationic polysaccharide obtained from alkaline hydrolysis or deacetylation of chitin. Chitosan has received considerable attention during the last decades due to its favourable properties including biodegradation, biocompatibility and non-toxicity. The term chitosan is generally used to copolymers having greater than 65 % 2-deoxy-2-aminoglucose monomeric units and the remainder monomeric units being 2-deoxy-2-acetamidoglucose units [3]. Chitosan is neither dissolved in water nor in organic solvents, but due to the presence of free amino groups, it is soluble in a dilute aqueous acid solution of an organic acid such as citric acid, acetic acid or lactic acid. Generally chitosan is soluble in acidic medium in a pH range from 1 to 5; this may impose formulation and its bioactivity restrictions [4]. In literature, various chitosan derivatives are prepared either by reactions involving -NH_2 group at C-2 position or non-specific reactions of -OH groups at the C-3 and C-6 positions [5-7].

To improve the mucoadhesion, aqueous solubility as well as enzymatic inhibitory and tight junction (TJ) opening abilities of chitosan through various chemical modifications, like thiolation, quaternization, halogenations, carboxylation, acylation, alkylation, PEGylation and graft copolymerization have been conducted [8,9]. Polymeric halo derivatives of chitosan were prepared by reacting chitosan with a halogenating agent [4]. Aqueous solubility of chitosan is significantly increasing by quaternization of amino group [10]. By covalent coupling with sulfhydryl bearing agents such as cysteine, thioglycolic acid and glutathione, various thiolated derivatives of chitosan are prepared [11,12]. Carboxymethyl derivatives of chitosan can be prepared by introducing -CH_2COOH groups onto 2-N and 6-O atoms [13-15]. By grafting hydrophobic compounds such as aliphatic acids (C_6-C_{16}) *via N*-acylation, various amphiphilic derivatives of chitosan can be prepared [16,17]. Derivatives of chitosan are also prepared by interaction of various chelating agents, including nitrilotriacetic acid (NTA), ethylenediamine tetraacetic acid (EDTA) and diethylenetriamine pentaacetic acid (DTPA) [18], PEGylated chitosan (CS-PEG) can be synthesi-

zed by using PEG-succinimidyl succinate or activated esters of PEG carboxylic acids to form stable amides [19].

Water soluble natural polymers are becoming increasingly important compound useful in a broad range of applications. Their importance lies, in part, in their ability to function in environmentally "friendly" ways. Only few natural or synthetic polymers are water soluble. The polysaccharides cellulose and chitin are the most abundant natural and linear polymers with poor water solubility. In neutral and basic aqueous solutions, chitosan is essentially insoluble [20,21]. Wound healing is a dynamic process, which involves various mechanisms like coagulation, matrix synthesis and deposition, fibroplasias, angiogenesis, epitheliazation, contraction and remolding [22,23]. Several studies reported that the chitosan and its derivatives can be used in all stages of wound healing. During initial healing phase, it shows its haemostatic property and promotes infiltration and migration of neutrophil and macrophase [24,25] thereby allowing fibrous tissue formation and re-epitheliazation. Chitosan is able to decreases the scar tissue and allowing for a good re-epitheliazation [26,27].

EXPERIMENTAL

All synthetic chemicals were procured from LOBA Chemie Pvt. Ltd, Mumbai, India. Melting points were uncorrected. The microwave was used for synthesis of modification of chitosan.

Ethical clearance: Protocol used in this study for the use of mice as an animal model for acute oral toxicity and skin irritation study and rats for wound healing activity were approved by the Institutional Animal Ethical Committee, Gourishankar Institute of Pharmaceutical Education and Research, Limb, Satara, India.

Synthesis: Sulfoxyamine chitosan was synthesized as per schematic presentation given in Fig 1. In brief, 10 g of chitosan was added in 100 mL of pyridine. To this, 8 mL of thionyl chloride was added slowly with shaking. The reaction mixture was irradiate in microwave for 1 min and kept overnight for digestion. The solid product was filtered and then dispersed in 50 mL ethanol containing 5 % ammonia. The flask was kept aside for 2 h then filtered and washed with 50 mL rectified sprit. Finally, the solids were collected and dried.

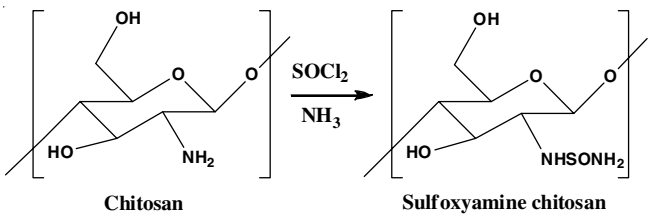

Fig. 1. Synthesis of sulfoxyamine grafted chitosan from chitosan using thionyl chloride and ammomia

Wound healing activity: The wound healing activity was evaluated as described method [28-30] by excision wound model in the adult albino rats having weight 150-200 g. The wound healing activity was performed as per protocol. The animals were numbered, weighed and then divided into three groups with six animals in each as follows: **Group I**: Control and provides no any treatment; **Group II**: chitosan powder; **Group III**: modified chitosan powder.

The anaesthetized animal was placed on the operation table in normal position. The dorsal fur of all the rats was shaved with an electric clipper. On the back of animals, anticipated area of the wound to be created was outlined. From the demarked area, full thickness skin was excised to get a wound area of diameter 2 cm. After surgery, animals were kept in separate cages. All the animals showed good general health condition throughout the study and were fed with commercial rat food and water. The animals were sacrificed after 26 days. The area wound was calculated on 0th, 5th, 9th, 14th, 17th and 22nd post wounding day. The degree of wound healing was calculated as % closure of the wound area from the original wound using a formula:

$$\text{Closure } (\%) = 1 - \frac{A_d}{A_0} \times 100$$

(A_d - Wound area on corresponding days, A_0 - Wound area on day zero).

Antimicrobial activity: Antimicrobial activity of modified chitosan and chitosan were evaluated as reported method [31] against Gram-positive (*Staphylococcus aereus*) and Gram-negative (*Escherichia coli*) bacteria. The bacterial strains were obtained from fresh cultures in the Department of Microbiology, Gourishankar Institute of pharmaceutical Education and Research, Limb, India. The study was simultaneously performed for the reference standard moxifloxacin. The chitosan solution was prepared by dissolving 100 mg of chitosan in 100 mL of 0.2 % acetic acid. Acetic acid (0.2 %) was used as blank for chitosan only. Modified chitosan solution was prepared by dissolving 100 mg of chitosan in 100 mL of distilled water. Moxifloxacin and modified chitosan solution was prepared by mixing 0.5 mL of moxifloxacin and 0.5 mL of modified chitosan solution. The measurement of antimicrobial activity of chitosan and modified chitosan were done by agar diffusion method. The bacteria were grown on agar plate. Under sterile condition, 0.1 mL of all the solutions was directly placed on the cups of agar plate. The plats were incubated at 37 ºC for 24 h.

Mucoadhesion property: Mucoadhesive property was evaluated by the reported method [32]. A modified balance method was used for determining mucoadhesion strength. For bioadhesive test, sheep buccal mucosa was used. The buccal mucosa was removed immediately after slaughter from the sheep and transported to laboratory in tyrode solution and kept at 40 ºC. After removing fat and loose tissues, the mucosal strips/pieces were prepared and washed with tyrode solution. A piece of buccal mucosa was tightly fitted with glass side and glass slide fitted with lower support. The diameter of each exposed mucosal membrane was 1 cm. The 1 % w/v solution of chitosan and modified chitosan were prepared in dilute acetic acid and water, respectively. The film of above solution on another glass slide (10 mm × 10 mm) was prepared by dispensing 0.1 mL solution. Polymer content of resulting film was about 1 mg/cm^2. The two sides of balance were made equal before the study by keeping suitable weight. Both slides (upper and lower) were kept in contact with each other for 6 min so that adhesion bonding could be established. A vertical acting force was slowly increased (1 g increment) until the polymer became detached from mucosa. This detachment force gives the mucoadhesion strength.

Film forming and coating properties: Film forming and coating properties was determined as reported method [33]. Film forming property was carried out by pouring chitosan and modified solution (10 mL of 1 % w/v) on a patriplate, then drying the preparation in a oven at 40 ºC. Coating property was studied by spraying 1 % w/v solutions on fruits.

Toxicity study

Skin irritation test: It was performed by reported method [34]. In brief, hair on the backside area of mice was removed. The animals of **group I** were served as control (without treatment), without any treatment. On **group II** standard irritant was applied and in **group III** modified chitosan was applied. Standard irritant and modified chitosan were applied for 7 days. The application sites were graded according to a visual scoring and edema scale: 0 for none, 1 for slight, 2 for well defined, 3 for moderate, and 4 for severe.

Acute toxicity: OECD guideline-423 was used for performing oral acute toxicity. The toxicological effects were observed in terms of mortality and expressed as LD_{50} [35].

SRB assay: SRB assay was performed according to reported method [36]. The total growth inhibition (TGI) and LC_{50} values were calculated.

RESULTS AND DISCUSSION

The % of elements was found to be C: 35.078, H: 6.479, N: 6.421, O: 42.285. DSC curve of modified chitosan showed a broad endothermic peak at about 89.11 ºC.

In a chitosan primary amine (RNH_2) showed broad signal at 3360-3302 cm^{-1} with two sharp spikes that two sharp spikes were absent in chloro-sulfoxy modified chitosan which indicate that substitution occurred on that primary amine of chitosan. Two resonances H-1 and H-2 at 4.8 ppm occur due to 2-amino-2-deoxy- D-glucopyranose. Peak at 4.8 ppm of 2-amino-2-deoxy-D-glucopyranose and peak of internal standard overlap each other and therefore peak of (-CH) of glucosamine is observed. The (-CH-NH) proton is represented by a peak at 2.8 ppm. Chemical shifts at 3.5 and 3.7 ppm correspond to protons of $-CH_2-OH$. Chemical shifts from 4.5-4.6 ppm correspond to HOH_2C-CH-, $CH-CH_2-$ and $-CH_2-OH$ protons of glycoside ring.

Wound healing is a process by which damaged tissue is restored as closely as possible to its normal state where as wound contraction is the process of shrinkage of area of the wound. Wound healing depends on the repairing ability of tissue which may be reduced due to infections. It was measured to find the extent of reduction in wound area at different periods of treatment. Fig. 2 shows a set of healing pattern. These patterns were observed from day 0 to day14 and showed that topical application of modified chitosan improved wound healing. When compared with control group, wound area was decreased rapidly in the modified chitosan treated animal. It was observed that wound area of control animal increases during the first days, which were not observed in the animals present in the group 2 and 3. Chitosan and modified chitosan were hydrated rapidly when applied on wet wound by exudates absorption

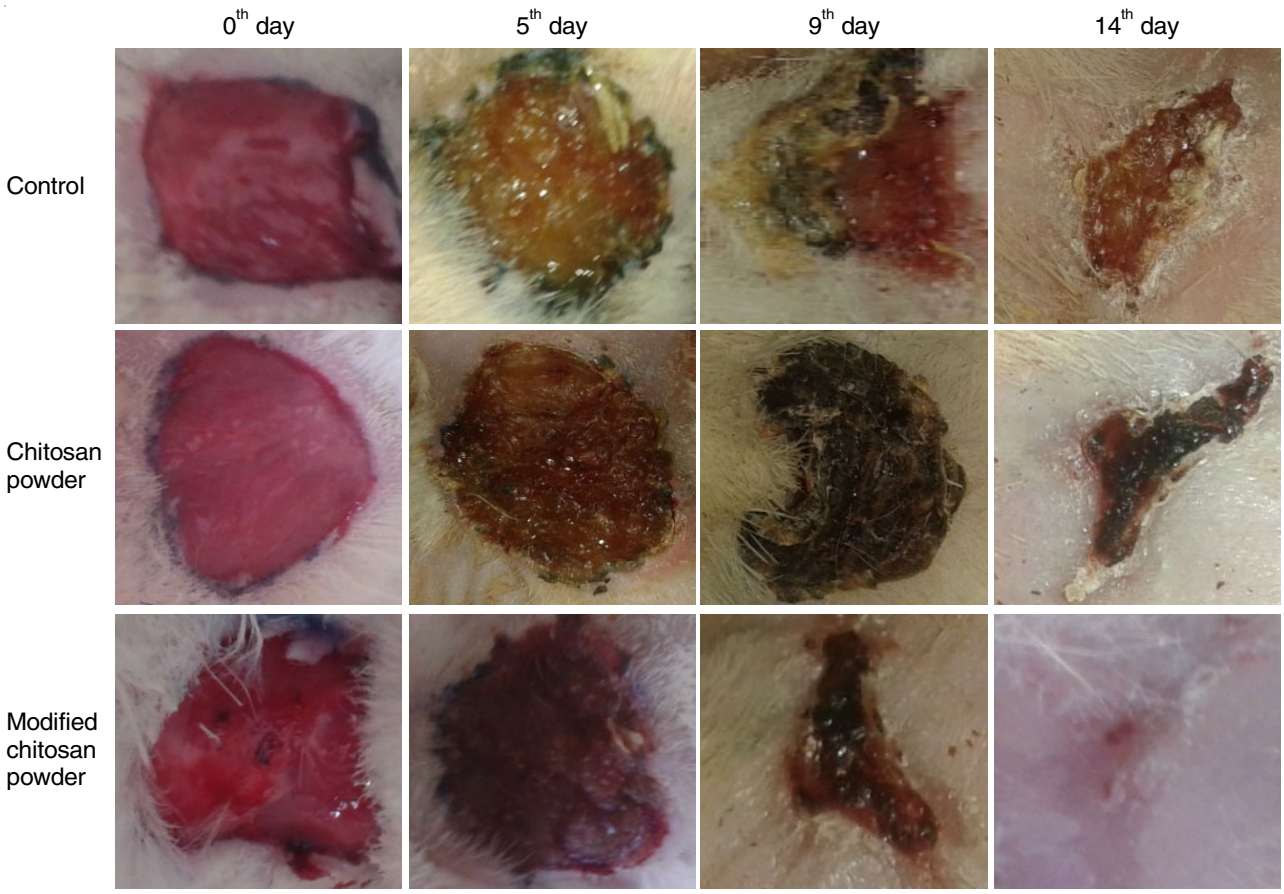

Fig. 2. Photographs of wound healing panorama with different treatment over 14 days. 2 cm diameter wound at the dorsal skin of rat treated with chitosan and modified chitosan

forming hydrogel or film at the wound surface and the wound was dried within few hours. In addition, the absence of local irritation on topical application was observed. This indicates the promoting role of chitosan and modified chitosan in wound healing. The wound closer time was lesser, as well as the percentage of wound contraction was more with the modified chitosan powder treated group. In the modified chitosan treated rats the wounds were completely healed in 14 ± 2 days whereas in the control animals, it took more than 23 ± 2 days. Chitosan required 18 ± 2 days to heal the wound. The epithelization of wound with modified chitosan powder treated group was found to be earlier as compared to chitosan.

Fig. 3 shows a set of wound beds after surgical procedure and application of chitosan and sulfoxyamine modified chitosan. The healing patterns were observed for 22 days. The wound area decreases rapidly without producing any bacterial growth in the presence of modified chitosan when compared with the control.

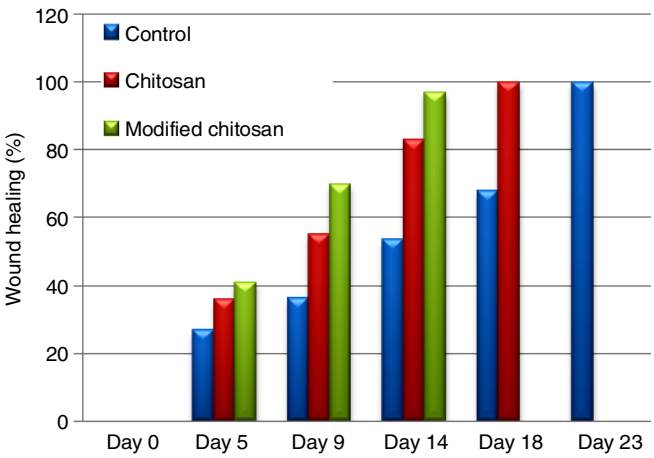

Fig. 3. Effect of control, chitosan and modified chitosan on rat wound. Each result is a mean of six independent experiments

Antibacterial efficiency of chitosan and modified chitosan against Gram +ve and Gram -ve strain is somewhat controversial and was found to be concentration dependence. The results are presented in Fig. 4. Concerning the efficiency against the *S. aereus* strain, both chitosan and modified chitosan showed interior activity when compared to the effect measured on the *E. coli*. The highest zones of inhibition of modified chitosan with chitosan (7 and 5 mm) indicate the significant antimicrobial activity against both microbes. In the present study, superior antibacterial activity was observed than chitosan. This is because of additional positive charge of amino group and presence of thio group. This creates a polycationic structure, which can be expected to interact predominantly with anionic components like lipopolysaccharide and protein of the bacterial cell wall [37-39].

In case of mucoadhesion study, the detachment force *i.e.* the force required for separating the polymer from the tissue surface was determined. In the present study, mucoadhesive strength of modified chitosan is more than chitosan (Fig. 5). The higher bioadhesion may be due to the formation of additional ionic interaction of cationic functionality of polymer with negatively charged residue present in mucosa.

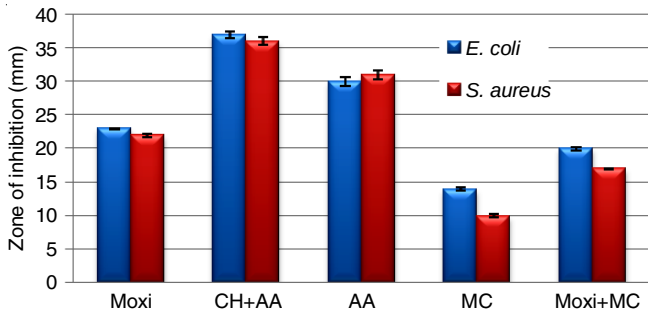

Fig. 4. Bactericidal activity of moxifloxacine (std drugs), chitosan with acetic acid (AA), acetic acid blank, sulfoxyamine modified chitosan (MC) and moxifloxacine + MC combination against *E. coli* and *S. aereus* strains. Each result is a mean ± S.D. of three independent experiments

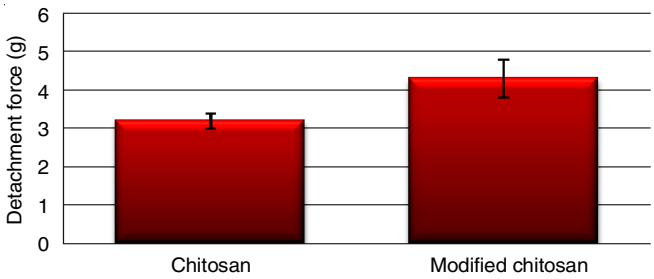

Fig. 5. Force of detachment of chitosan and modified chitosan. Each result is a mean ± S.D. of three independent experiments

The chitosan and modified chitosan form a film which is transparent and colourless. The average thickness of both films was 10 ± 2 µm. The tensile strength of chitosan and modified chitosan were found to be 2.90 ± 0.4 and 3± 0.2 N/m^2, respectively. In the film forming property, no significant difference was observed. The coating/film forming property were further studied by spraying 1 % solution of chitosan and modified chitosan on apples and bananas. It was observed that bananas and apples treated with chitosan and modified chitosan still looked fresh after 4 days of storage at ambient temperature, while untreated fruits were not fresh and infected with either fungus or bacteria. This indicate that similar to chitosan, modified chitosan are also permeable to air/oxygen when coated on fruits and act as preservative. Keeping in mind the wound dressing application, the porosity of modified chitosan promotes air/oxygen exchanges which help in early healing process.

Most of the studies describe chitosan is a safe material, inducing low or minimal toxic effect; therefore, it is generally recognized as a safe for food application. Upon oral administration, lethal dose of chitosan was (LD_{50}) more than 16 g/kg in mice [40]. The toxicity study indicate that modified chitosan does not having any skin irritation, LD_{50} value more than 2000 mg/kg indicates less acute toxicity and LC_{50}, TGI and GI_{50} values more than 80 indicates the non-cytotoxicity (Fig. 6).

Skin irritation studies were performed to investigate the potential of polymer to cause irritant or allergic reactions. The results depicted in Table-1 indicate that polymer does not produce any erythema and edema. On the other hand, the standard irritant, *viz.* formalin was found to produce severe erythema and edema effects (Table-2).

Conclusion

Polymeric sulfoxyamine modification of chitosan was done by reacting chitosan with thionyl chloride in the presence of

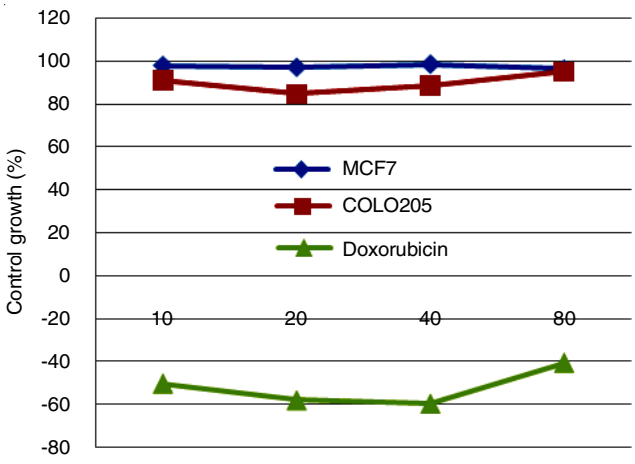

Fig. 6. % Control growth of MCF7 & COLO205 cell line by modified chitosan with references to doxorubicine. Each result is a mean of three independent experiments

TABLE-1
SKIN IRRITATION STUDY

Compounds	Erythemia	Edemia
Normal	00	00
Modified chitosan	00	00
Formaline (0.8 % v/v)	3	3.12

TABLE-2
ACUTE TOXICITY OF MODIFIED CHITOSAN*

Dose level (mg/ kg)	Number of mortality
300	Nil
2000	Nil

*Determined as per OECD guideline 423

pyridine and further treatment with ammonia. The modified chitosan exhibits better water solubility than chitosan. The less wound closer time and more wound contraction as compared to chitosan indicated the applicability of modified chitosan in wound dressings. The addition of sulfoxyamine group to this natural polymer may produce superior antibacterial activity and aid the remolding wound and their prefect healing. Modified chitosan may provide additional cationic character for mucoadhesion, hence showing grater property than chitosan. The film of modified chitosan has been extensively studied for applications as films or coating material. The films have selective permeability for various gases, therefore it is used as preservative film for fruits, vegetables and eggs also helpful for wound healing. No skin irritations were observed. The LD_{50} value of modified chitosan was more than 2 g/kg indicated less acute toxicity. The LC_{50}, TGI and GI_{50} values more than 80 µg/mL indicated non-cytotoxicity of modified chitosan. In short, introduction of sulfoxyamino group on the backbone of chitosan has succeeded in increasing its activities like wound healing, antibacterial, mucoadhesion and film forming or coating.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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