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ORIGINAL ARTICLE

The Beneficial Effects of Chitosan Dressing in Third-Degree Swine Burn Model

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Background: Chitosan is a kind of chitin derivative, which has unique biological properties, including biocompatibility, biodegradability, and extremely low toxicity, considered as a suitable material for promoting wound healing. **Aim:** We aimed to investigate the effectiveness of chitosan dressing for burn wound healing in swine model. **Methods:** In this study, we evaluated the wound healing in a swine model of full thickness cutaneous burn to assess the benefits of chitosan dressing treatment compared to commercial gauze. We also investigated the effect of chitosan dressing in inhibiting the growth of bacteria as well as the survival ability of 3T3-L1 cells in contact with the dressings. **Results:** After wounding swine treated with chitosan dressing, it showed an increased wound closer compared to commercial gauze, suggesting that the chitosan dressing treatment results in better accelerated healing. In the antibacterial test, the results showed that antibacterial effect of chitosan dressing was better than commercial gauze. **Conclusion:** The presence of chitosan dressing was characterized by their high antibacterial activity, and it was confirmed against *Staphylococcus aureus* in the burn wound of swine. The superior wound healing effect on deep dermal burns of presented wound dressing was demonstrated in a swine model. Our finding suggests that the chitosan wound dressing has a great potential application in severe wound care.

Key words: Chitosan, antibacterial, cell viability burn wound, swine model

INTRODUCTION

Chitosan, a chitin derivative, consists of glucosamine and N-acetyl-D-glucosamine (chitin).[1] In the 1980s, chitosan and its derivatives were first used for skin and wound healing,[2,3] and they have been proven in animal and human studies to promote wound repair.[4,5] Chitosan is a cationic polymer with free acetamide groups and hydroxyl functional groups connected to the glucopyranose ring that is easily reacted by nucleophilic attack.[6] In clinical field, chitosan has unique biological properties, including biocompatibility, biodegradability, nontoxicity, antibacterial, hemostatic, and mucoadhesive properties, implying its beneficial as biomaterial for wound healing accelerator.[7-9] A study has reported the application of collagen–chitosan-glycosaminoglycans as artificial dermis for wound repair in a rat model.[10] The results showed that the dressing had relatively poor vascularization and some fibrosis of the wound. Further, another study has investigated the effect of collagen–chitosan-glycosaminoglycans dressing on human full-thickness wounds.[11] The autograft was cultured after early resection in 4 patients. After 10 days of transplantation, the histological results showed that the wounds were colonized by fibroblasts, and after 21 days, complete wound closure was developed. The results demonstrated that the use of collagen–chitosan-glycosaminoglycans as a dermal matrix could improve vascularization and promote wound healing. Numerous studies have shown that chitosan is a remarkable biocompatible material that combines a unique

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wound healing property. However, the source of chitosan must be carefully selected because impurities such as animal protein or other contaminants may affect the effectiveness of chitosan.[12]

Wound healing involved a series of events, including the formation of initial clots by platelets, inflammation, epithelial cell proliferation, and wound restoration. An early inflammatory response is a critical period in the process of wound healing, which is essential for removing bacteria and creating a suitable environment for wound healing. Excessive inflammation can cause multiple tissue and organ destruction, systemic inflammatory response syndrome, multiple organ dysfunction syndrome, and even death.[13-15]

The activation of leukocytes is an early inflammation reaction, from the microcirculation to the main white blood cells in the injured area. The number of neutrophils infiltrating the wound site is naturally proportional to the severity of the wound injury. Neutrophils help remove bacteria and necrotic tissue, and at the same time, neutrophils secrete inflammatory cytokines, such as interleukin 1α and β (IL-1α and 1 β) and tumor necrosis factor α (TNF-α), which activate a series of immune responses and stimulate epidermal cell proliferation.[16-18] IL-6 is an important inflammatory factor in the early inflammatory response of burns, and the amount of IL-6 reaches its peak on the 3–4 days after burns.[19] In skin burn wounds, it is mainly keratinocytes that produce IL-6.[20] In the rat burn model experiment, it was found that the early increase of IL-6 prolonged the inflammatory response of full-thickness burn wounds, and IL-6 further enhanced the levels of TNF and IL-1. The combination of the two amplifies the inflammatory response after burns.[21] Chitosan inhibits the production of active reactive oxygen species, degranulation, and adhesion of the neutrophils, which proves that chitosan has the function of inhibiting the activation of the neutrophils.[22] In this study, we investigated the effectiveness of chitosan dressing for burn wound healing in pig models.

MATERIALS AND METHODS

The chitosan dressing

The chitosan dressing (10 cm × 10 cm × 0.15 cm) (Coreleader Biotech Corporation, ROC) test pieces and pouched dressings were sterilized by gamma irradiation dose between 11 and 19 kGy. A carboxymethylated cellulosic fiber wound dressing, AQUACEL® Hydrofiber, was used as a control. The primary chemical difference between dressings is the presence of glucosamine functionality in the chitosan dressing which is absent in the AQUACEL® dressing. An adhesive Tegaderm™ polyurethane film covering was used to attach dressings to the burn wounds and also as a control wound covering.

Scanning electron microscope

Scanning electron microscope (SEM) images of chitosan dressing were obtained using a Hitachi S–3000N (Hitachi High Technologies, Krefeld, Germany) operated at 1.5 kV. The particle size and thickness were determined using Image-Pro® Plus software (version 5.1 from Media Cybernetics, Rockville, MD, USA) and taking the average value of 1000 particles.

Elemental analysis

The qualitative determination of chitosan dressing composition was performed through energy dispersive X-ray spectroscopy (EDS) using a Hitachi S–3000N (Hitachi High Technologies, Krefeld, Germany) operating at 20 kV.

In vitro antibacterial test

The *Staphylococcus aureus*-ATCC 9027 strain cultured in a plastic cell dish and placed commercial gauze and chitosan dressing in plastic cell dishes. When dressing the burn wound, the wounds were scraped with a cotton swab for 5 times and the exudate was collected as a sample put on the agar plate. After incubation, we followed up the growth of bacterial at day 1 and day 7.

Cell viability

Cell viability was assessed using the MTT assay. In test of cytotoxicity, we used 3T3-L1 cell line purchased from the Food Industry Research and Development Instituted, Taiwan. The condition incubation, at 37°C ± 1°C in air with 5% CO_2, with saturated moisture. Reagent control: Dulbecco's Modified Eagle Medium with 10% fetal bovine serum. Negative control: The 120 cm^2 of nitrocellulose paper was extracted in DMEM contain with FBS for 24 ± 1 h at 37°C ± 1°C. Positive control: DMEM contain with FBS and phenol. Preparation of test sample extracts: The test sample were extracted in DMEM with FBS for 24 ± 1 h at 37°C ± 1°C. Qualitative analysis: The 1.0×10^5 cells in 5 ml DMEM with 10% FBS were placed in a 6 cm sterile plastic cell dish and then incubated overnight at 37°C ± 1°C in a 5% CO_2 incubator. When the cells reached 70%–80% confluence, the culture medium was removed and replaced with 5 ml of DMEM contain with 10% FBS and 20% DMSO (positive control), extract of NC paper with 10% FBS (negative control), and extract of the chitosan dressing with 10% FBS. The cells were incubated at 37°C ± 1°C in a 5% CO_2 incubator for 24 ± 1 and 48 ± 1 h and then examined. All samples were tested in triplicate.

Burn procedure

This study was conducted in the Laboratory Animal Center at our institution. Three female domestic pigs (20–25 kg) were

used in this study. Animals were given a standard diet ad lib several days before the investigation and be fasted overnight before any procedures. The animals were housed in individual pens upon its arrival and allowed to become acclimated for at least 7 days. They were pretreated with a transdermal fentanyl patch (50 mg/h) for pain management. On the day of burn creation, pigs were sedated with Zoletil 50 (Verbac, France) 25 mg/kg IM. These pigs were then intubated endotracheally and maintained under a surgical plane of anesthesia with isoflurane 0.5% to 2.5% in room air. Blood pressure, heart rate, and body temperature are monitored during surgery for any complications. The flank and back hair are shaved with hair clippers and the skin is scrubbed with a povidone iodine solution. Six uniform burn wounds (190°C, 30 s) were then made symmetrically on the back of each pig using a modified soldering iron with a flat contact area of approximately 20 cm^2. Two replicate burns are created for each contact time and treatment and control wounds are randomized to location, with equal distribution of wounds on either side of the dorsal midline and with respect to rostral or caudal positioning. Sixty minutes after burning, the wounds were covered with saline soaked nonadhesive gauze. The burns were covered with AQUACEL® dressing and chitosan. All wounds were then protected with nonstick cotton and acrylic fiber pad, fixed with adhesive dressing, and covered with a special garment to prevent tearing of dressings. After the burns, the animals received intravenous injections of saline (0.1 l/kg) and were placed in individual cages for recovery. Pigs from free accessed to laboratory chow and tap water. It is kept in an experimental room whose temperature was maintained between 25°C and 28°C, with natural light and dark cycles. Pig will be euthanized after these periods with lethal doses of intraperitoneal KCl.

Analysis of wound healing

Wound healing was assessed by evaluating the rates of wound re-epithelialization and contraction. The open wound area and the surrounding area of normal skin were measured using macrophotography. The healing rate was monitored every 2 days for the first 10 days and then twice a week for 6 weeks. Wound surface area and contraction were calculated as the percentage of the original wound size by image analyzer (Image J.2.0 software, NIH, USA) during healing. The wound closure % was detected as follows:

% Wound contraction = (Current wound area/wound area at the beginning) × 100.[23]

Statistical analysis

Statistical analysis between the two treatment groups was performed using the two-tailed paired *t*-test and Student's *t*-test for continuous data and the Chi-square test for noncontinuous data. Differences between groups were declared statistically significant when the $P < 0.05$.

RESULTS

Characteristic of chitosan dressing

Chitosan is a polysaccharide natural polymer obtained by the hydrolysis of chitin. Chitosan is a linear polymer with a structure similar to cellulose. As carbohydrate polymers, their protein and calcium carbonate can be removed and diluted using alkali and acid, respectively, to obtain purer chitin; after the chitin is deacetylated, it becomes chitosan, which constitutes their monomer is glucosamine as Figure 1d. The chitosan dressing was made from fiber of chitosan. The Scanning electron microscope structure of chitosan dressing analyzed the diameter of chitosan fiber and surface [Figure 1a and b]. Figure 1a showed the uniformity and consistency of chitosan dressing formation, and the polymer structure of chitosan dressing was composed of pure biological materials [Figure 1b]. We used the EDS to explore the atomic percentages of all the elements in the analyzed area of chitosan dressing [Figure 1c]; the atoms included the C, N, O, P, and Ca_2^+ in the chitosan fiber.

Cell viability of 3T3-L1 cells in control gauze and chitosan dressing

Results from MTT quantitative analysis for 24 h showed the absorbance of positive control, negative control, and chitosan dressing at an ELISA reader with 570 nm, which were 2.389 ± 0.083, 2.316 ± 0.064, 0.080 ± 0.010, and

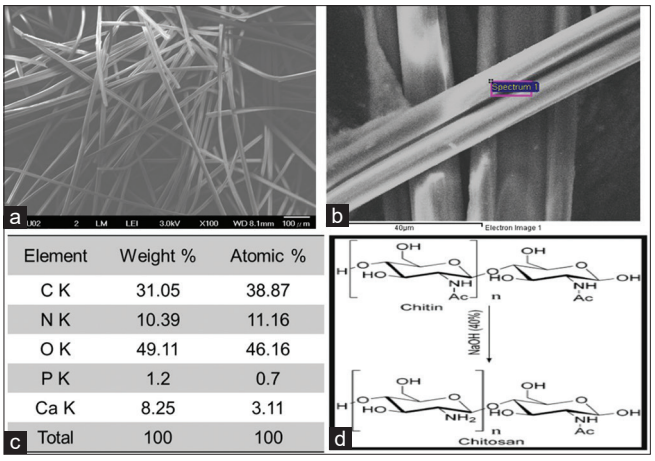

Figure 1: The morphology of chitosan dressings. (a and b) Scanning electron microscope images of chitosan dressing at ×100 and ×1000; (c) Energy-dispersive X-ray spectroscopy of chitosan dressing, showing the atomic percentages of all the elements in the analyzed area; (d) The process of deacetylation of chitin to form chitosan

2.278 ± 0.093, respectively [Figure 2a]. In 48 h, the OD of values were 2.876 ± 0.037, 2.748 ± 0.020, 2.746 ± 0.032, and 2.671 ± 0.082, respectively [Figure 2b]. Those values were not significantly different statistically ($P \leq 0.05$). However, the absorbance of positive control was 0.062 ± 0.010, the value was significantly different from that of positive and negative control ($P \leq 0.05$). Comparing the biocompatibility of chitosan dressing with surgically sterilized gauze (as control group), chitosan dressing showed higher cell growth efficiency at either 24 or 48 h significantly ($P \leq 0.001$). The OD570 ratio values: Chitosan dressing versus control gauze = 1 ± 0.05 versus 0.72 ± 0.07 for 24 h; 1.23 ± 0.18 versus 1.20 ± 0.08 for 48 h [Figure 2c]. The data showed that chitosan dressing has good cell viability.

Burn wound observation

As shown in dressing procedure of burn wound [Figure 3], pigs were sedated with Zoletil 50 (Verbac, France) 25 mg/kg IM. These pigs were then intubated endotracheally and maintained under a surgical plane of anesthesia with isoflurane 0.5% to 2.5% in room air. The wound surface samples were collected at time points as a follow-up dressing antimicrobial test. The outer layer of the wound was then covered with gauze. Figure 4 shows a set of typical wound beds shortly after the surgical procedure and application of the chitosan dressing. The healing patterns were observed after 12, 23, and 41 days, and showed that topical application of chitosan dressing improved wound healing. The wound area decreased in the presence of chitosan dressing when compared with the control AQUACEL® [Figure 4a and b] until day 41. The results obtained were statistically significant at day 12 ($P \leq 0.01$). Although the wound size of AQUACEL® presented smaller then chitosan dressing on day 12, the wound size of chitosan dressing was 0.86 folds smaller than that of AQUACEL® until day 41, and the appearance of wound was smoother and less red and swollen.

Antibacterial test of dressings from burn wounds

As shown in Figure 5, chitosan dressing and control gauze were collected from burn wound, and the exudate sampling from the dressing surface were collected by swab scrolled five times, then placed onto the inoculated agar plates along with the *S. aureus* at 37°C for 24 h. Afterward, followed up the colony-forming unit (CFU) of bacterial in 1 and 7 days by photos were performed. The results showed that the CFU levels were less in chitosan gauze than control gauze during the experimental.

DISCUSSION

During tissue injury, the inflammatory response is the host's defense, which will respond quickly to harmful substances in the injured area with four major changes: (1) vasodilation in the injured area, increasing local blood flow; (2) changes in microvascular structure, causing white blood cells and inflammatory cells to leave the blood circulation; (3) transfer of white blood cells from the microcirculation to the injured area; (4) stimulation white blood cells to remove

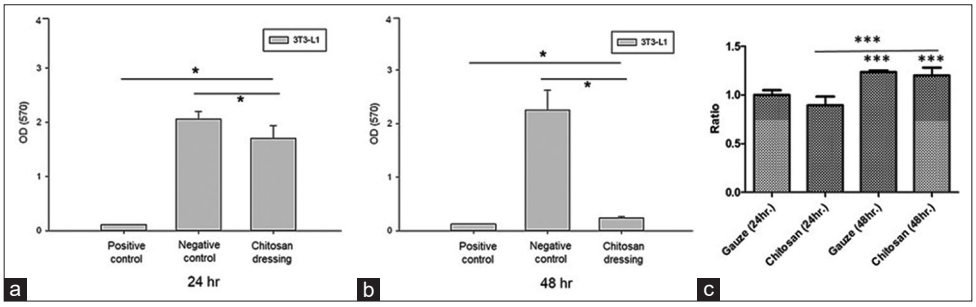

Figure 2: Cell viability assay of chitosan dressing. (a) OD 570 nm detection in 3T3-L1 cells incubate with chitosan dressing for 24 h and (b) 48 h. (c) Comparison of cell survival ratio between control gauze and chitosan dressing for 24 and 48 h. Quantitation of the cell viability ratio in triplicates is represented as mean ± standard deviation. * $P < 0.05$, *** $P < 0.001$

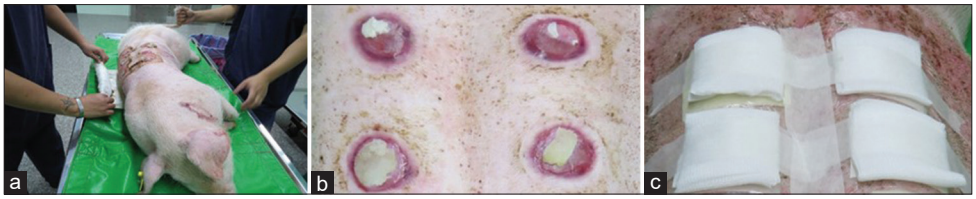

Figure 3: (a) Anesthetized the pig during dressing changes. (b) Opening the wound dressing and taking photos for every 2 days. (c) Replacing the wound dressing and covering with gauze

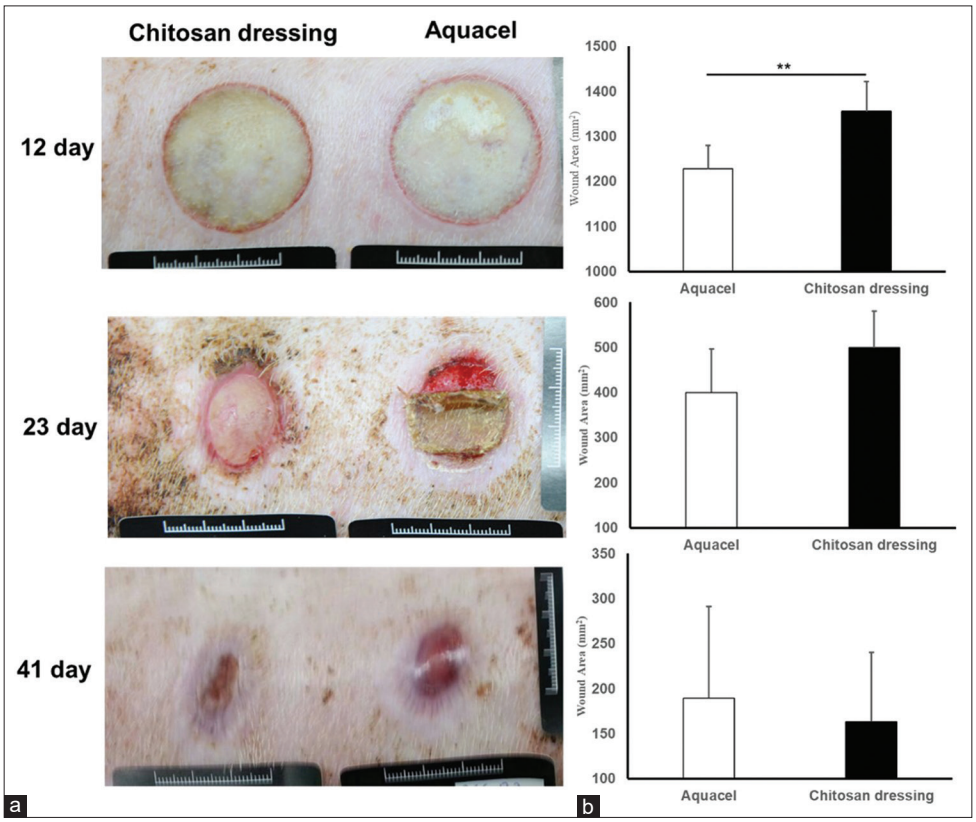

Figure 4: Burn wound observation and calculation of wound closure area. (a) Skin burn wound progression in pigs. The images of the mid-row wounds from one of representative pigs (n = 3) are shown. Burn wound (horizontal) progression is indicated in two rows of wounds and healing at day 23. (b) Quantitation of the wound size in triplicates is represented as mean ± standard deviation. The results showed that wound healing effect of chitosan gauze was better than that of commercial gauze until 41 days. ** P<0.01

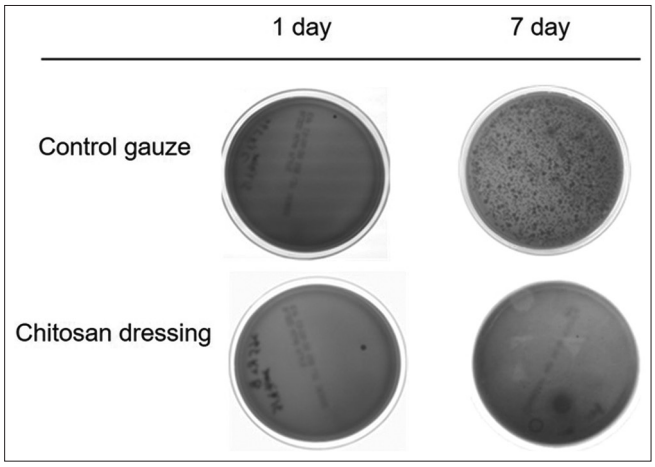

Figure 5: Antibacterial test in burn wound dressing. Pictures show the photographic documentation of representative dressing results

harmful substances. This response involves various types of immune and nonimmune cells and triggers the production of inflammatory factors, recruits and activates immune cells, and increases the production of free radicals.[24] Hence, the control of inflammation at an early stage with a proper dressing is thus crucial for managing the healing process. In burn wound, a proper dressing needs to be used immediately to prevent wound infection and excessive inflammation. Our results demonstrated that chitosan dressing showed significantly low toxicity compared to commercial gauze [Figure 2]. These results are similar to a previous study.[12] In our pig burn animal model, there were fewer bacteria on wound treated with chitosan dressing compared to commercial gauze. It proves some degree of early inflammation, as well as antibacterial property, was better with chitosan dressing treatment for burn wounds. The wound area of the control animals increased during the 23 days [Figure 4], which was not observed in the animals treated with chitosan dressing. These results corroborate what has been reported previously in the literature.[5,8] Therefore, this supports the promoting role of chitosan in wound healing.

Furthermore, the antibacterial effect of chitosan dressing was also better than that of control gauze [Figure 5]. Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria, which binds to toll-like receptor 4 and induces inflammation through inhibitory kappa B (IκB) or activated protein kinase (MAPK)-dependent pathways.

IκB triggers the inflammatory response by displacing nuclear factor-κB (NF-κB) into the nucleus, which in turn induces the transcriptional genes of the inflammatory response.[25,26] Chitosan has been extensively demonstrated the abilities to inhibit the inflammatory response induced by LPS or other stimuli in various types of cell and animal model experiments.[27] Chitosan has been also shown to inhibit the inflammatory response induced by LPS in macrophages, including the expression and release of pro-inflammatory mediators, including TNF-α, IL-6, inducible nitric oxide synthase, cyclooxygenase-2, prostaglandin E2, and nitric oxide. The mechanism by which chitosan inhibits the inflammatory response induced by LPS in macrophages involves the suppression of JNK1/2 and IκB degradation as well as preventing the translocation of NF-κB into the nucleus. Chitosan has also been demonstrated to prevent death. In a mouse model of LPS-induced sepsis, chitosan treatment significantly reduced LPS-induced neutrophil infiltration and lipid peroxidation in the damaged organs. Chitosan inhibited the phosphorylation of JNK1/2 and p38 in the liver of mice injected with LPS. These studies show that chitosan inhibits NF-κB and MAPK signals to inhibit LPS-induced inflammation.[28-30]

In the future, we may use more animals to investigate the function of chitosan dressing in burn wounds, including inflammation indexes and exploring pain signaling pathways. The investigators suggested that, based on the test results together with the biodegradable characteristic of chitosan including antibiotic and pig burn wound healing, this polymer has an advantage over other materials in treating burn wound injury.

CONCLUSION

According to the *in vitro* antibacterial test and the burn wound results, compared with the commercially available gauze, the chitosan dressing has a better antibacterial effect and burn wound closure. We recommend the use of chitosan dressings to prevent wound infections and improve burn wound healing.

Ethical approval

The animal experiments in this study were approved by and conducted in accordance with the National Defense Medical Center Institutional Animal Care and Use Committee (NDMC_IACUC-14-219).

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Data availability statement

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

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Conflicts of interest

There are no conflicts of interest.

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