Physicochemical properties and in vitro osteocompatibility of different titanium surfaces stored in a saline solution

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Abstract

Objective. The study aimed to investigate the physicochemical properties of titanium surfaces with different morphologies stored in a saline solution and their effects on osteoblast behavior. Methods. Freshly prepared commercial pure titanium (cp-Ti), acid-etching titanium (SLA-Ti) and nanowire titanium (NW-Ti) were stored in 0.9% and 10% NaCl solutions, and exposure to air and double-distilled water were used as controls. After storage for two weeks, scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS), optical contact angle instrument, and optical profilometer were used to analyze the micro-morphology, elemental composition of the samples, contact angle and surface roughness. BCA protein kit was used to detect the protein adsorption capacity of the three titanium surfaces under the different storage conditions. MC3T3-E1 osteoblast-like cells were cultured on the titanium surfaces. The proliferation, adhesion, alkaline phosphatase activity, and osteogenic differentiation of MC3T3-E1 cells were assessed using CCK-8, laser confocal microscope (CLSM), alkaline phosphatase (ALP) assay, and western blotting. Results. SEM results indicated that the storage conditions did not affect the micromorphology of the titanium surfaces. The XPS and contact angle determination results suggested that cp-Ti, SLA-Ti, and NW-Ti stored in NaCl solutions showed less carbon contamination, higher hydrophilicity. The roughness results showed that the air groups and 10% NaCl had higher roughness. The protein adsorption capacity of the three titanium surfaces under the different storage conditions. The proliferation activity of osteoblasts on the three titanium surfaces was not different from the control groups after storage in 0.9% NaCl solution. However, the results of the in vitro study suggested that the cell adhesion capacity and the expression of ALP and the osteogenic-related proteins Runx2, Osterix, and Osteocalcin improved after storage in 0.9% NaCl solution. Conclusions. The storage of the different types of titanium surfaces in 0.9% NaCl solution could effectively reduce carbon contamination, maintain good hydrophilicity, improve the roughness and make the environment conducive to the differentiation of osteoblasts.

1. Introduction

The vigorous development in dental implantology has changed the traditional way of restoration of missing teeth. Dental implants have been widely accepted worldwide as the third set of teeth for humans. The long-lasting stability of the dental implants depends on the tight integration of the implants with the alveolar bone [1].

At present, titanium and titanium alloys have advantages as implant materials. Titanium is light in weight and stable, and a corrosion-resistant metal due to the presence of surface oxide layers. Moreover, titanium is biocompatible enough to form a direct interface between the implant and the bone, termed as osseointegration,
without the need of the intervening soft tissue. A dental implant made of pure titanium or titanium alloy integrates with the surrounding bone and, thus, supports the dental prosthesis. However, the total implant area covered by the bone (bone-titanium contact percentage) has been reported to be 45 ± 16%, or 50%–65%, which may be related to the aging of titanium [2] manifested by the loss of biological activity over time, with the most significant change observed in hydrophilicity [3]. Highly hydrophilic biomaterials possess better adhesion potential and cell proliferation, making them conducive to early osseointegration [4, 5].

This change in hydrophilicity over time correlates with the contamination of hydrocarbons in the surrounding environment [6]. Exposure of the newly prepared titanium surfaces to air for 4 w increased the carbon atoms on the titanium surface from 20% to 60% [7]. And in 6 months, the carbon contamination gradually worsened, affecting the time taken for protein adsorption and cell adhesion [8]. Aging inevitably occurs on the different titanium surfaces [9–11].

Before its clinical applications, newly prepared titanium implants need to go through processes such as cleaning, disinfection, packaging, distribution, and storage. The implants are stored throughout these processes up to their application and it is important to slow down the aging of titanium during this period. It was observed that storing titanium implants in isotonic saline, distilled water, or using a gaseous barrier packaging can effectively maintain hydrophilicity immediately after manufacturing [12–14].

According to previous reports, there is no significant difference in protein adsorption and the apatite forming ability between the fresh alkali-heated and the alkali-heated titanium surfaces stored in vacuum for 52 w [15]. In another study, the smooth titanium was stored in either double-distilled water (ddH2O) or low vacuum after UV treatment. The protein adsorption and cell spreading ability of the smooth titanium surface stored in ddH2O were better than that stored in low vacuum [16]. Also, vacuum packaging requires more stringent packaging material parameters like tightness and strength compared to liquid packaging. Damage during vacuum packaging may be ignored easily. Therefore, compared to other storage methods, storing the implants in liquid is a simple, effective, and economical anti-aging strategy.

Early protein adsorption to the titanium implant interface underlies the subsequent adhesion and proliferation of osteoblasts [17]. The titanium surface stored in NaCl or CaCl2 solution has been proved to possess greater protein adsorption potential, which is due to the liquid environment separating the contamination of the titanium surface by the hydrocarbons in the air [2, 18]. In addition, the surface of TiO2 is charged under a physiological pH environment and Na+, Ca2+, and Cl− in the solution can be adsorbed on the titanium surface in different binding forms. The ions adsorbed on the titanium surfaces have positive implications for early protein adsorption in addition to eliminating hydrocarbon contamination [2, 18]. The composition, number, and binding form of the ions adsorbed to the titanium surface are closely related to the physical and chemical properties of the material surface [19]. Although the modified titanium is made from titanium metal, the biological activity and physicochemical properties of the modified titanium surface, such as surface morphology, hydrophilicity, surface charge, roughness, and chemical composition, are different [20].

Up to now, the effects of liquid storage solutions on the physicochemical properties and biological activities of the different titanium surfaces and the internal mechanisms affecting these properties have not been studied and elucidated.

In this study, commercial pure titanium (cp-Ti), acid-etched titanium (SLA-Ti), and titanium nanowires (NW-Ti) were treated with two different concentrations of saline storage solutions to study their effects on the physicochemical properties and the biological activity of the different titanium surfaces.

2. Materials and methods

2.1. Materials and equipment

Commercially available pure titanium (TAl, 99.5%, Baoji Titanium, China), SiC sandpaper (Tianjin Nanjing Abrasive Grinding Co., Ltd), 10% NaCl solution (Chinese medicine, China), fetal bovine serum, alpha-MEM medium, penicillin/streptomycin double-antibody solution (Gibco, USA), trypsin (Sigma, USA), DAPI and CCK-8 (Beyotime, China), BCA protein quantification kit (KeyGEN BioTECH, China), mouse MC3T3-E1 cell line (Cell Bank of Chinese Academy of Sciences, Shanghai, China), scanning electron microscope (1530VP, LEO, Germany), standard optical contact angle instrument (SL200B, Kono, USA), X-ray photoelectron spectrometer PHI 5000 (VersaProbe, Ulvac-Phi, Japan), optical profilometer (MicroXamTM, Phase-Shift, UP, Rtec co, USA), cell incubator (Thermo, USA), laser confocal microscope (LSM710, Zeiss, Germany), chemiluminescent gel-imaging system (Tianneg 5200, China), microplate spectrophotometer (MD, USA), AKP assay kit (Jiancheng Bioengineering Institute, China) were obtained.
2.2. Preparation of specimen and storage solution
Preparation of saline storage solution: Double-distilled water was used as ddH2O storage solution after high-pressure steam sterilization. The 10% NaCl solution was purchased and diluted 10 times with ddH2O to prepare 0.9% NaCl solution. In the current study, three kinds of titanium surfaces stored in air and ddH2O were used as controls and those stored in 0.9% and 10% NaCl solutions as the experimental groups.

Two types of pure titanium discs (φ5 mm × 1 mm and φ30 mm × 1 mm) were used.

Commercial pure titanium group (cp-Ti): Titanium discs were ground and polished step by step with SIC metallographic sandpaper (320 mesh, 600 mesh, 800 mesh, and 1200 mesh).

Acid-etching titanium surface (SLA-Ti): Titanium discs were sandblasted with Al2O3 particles at a pressure of 0.4 MPa. The SLA titanium surfaces were etched using HF/ HNO3 (at room temperature for 10 min) and H2SO4/HCl (at 80 °C in a water bath for 30 min).

Nanowire titanium surface (NW-Ti): Titanium discs were etched using 0.4% HF at room temperature for 30 min and then ultrasonically washed using distilled water. Then, the discs were soaked in a 10% NaOH solution (at 70 °C in a water bath for 15 min). The three kinds of titanium surfaces were ultrasonically cleaned using deionized water, 75% ethanol, and deionized water again. Then, the specimens were dried at 60 °C, and placed in the culture plates and irradiated by ultraviolet light overnight. Finally, these titanium discs were stored in the different media (air, ddH2O, 0.9% NaCl solution, and 10% NaCl solution) under ambient temperatures for 2 w since the wettability and cytocompatibility of the titanium surfaces decreases after 2 w [21].

2.3. Surface characterization
The three titanium surfaces were stored under different conditions for 2 w and the surface micro-topography of the specimens was observed using a scanning electron microscope (SEM). The surface elemental compositions of the samples were analyzed using X-ray photoelectron spectroscopy (XPS). The surface wettability of the specimens was evaluated in terms of contact angle. Briefly, 2 μl of water was dropped onto the surface at a randomly selected point and the contact angles were measured using a standard optical contact angle meter. An optical profilometer (MicroXamTM, Phase-Shift, UP, Rtec co, USA) was used to evaluate the roughness of each sample over a 100 μm × 100 μm area. For the surface characterization test, three points were randomly selected and measured from three different samples (n = 3) per group.

2.4. Protein adsorption
Samples were placed in 96-well plates and stored under the four storage conditions for 2 w. Then, the storage solution was discarded and the samples were rinsed with PBS three times except for those stored exposed to air. 2 ml α-MEM was added to the samples and incubated for 24 h. The samples were rinsed with PBS three times again. The proteins were extracted from the titanium surfaces using the RIPA lysis buffer and the protein concentrations were measured using the BCA protein assay kit. The experiment was repeated three times.

2.5. Cell culture
MC3T3-E1 osteoblast-like cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). MC3T3-E1 cells were resuspended in vitro and cultured in α-MEM medium containing 10% fetal bovine serum and 0.9% penicillin-streptomycin at 37 °C in a cell incubator (95% relative humidity, 5% CO2). The medium was changed every 3 d and the cells were passaged at a ratio of 1:4 after reaching 80% cell confluence.

2.6. Cell adhesion
Samples were placed in 96-well plates, and after storage for 2 w under the different storage conditions, MC3T3-E1 cells (5 × 10^3 cells per well) were seeded on the samples (titanium surfaces). After incubation for 8 h, the cells were fixed in 4% paraformaldehyde for 30 min and rinsed three times in PBS. Then the cells on the sample were stained with 100 nM rhodamine phalloidin (Cytoskeleton, USA) for 30 min, and stained with DAPI (Beyotime, Shanghai, China) for 90 s. The cell adhesion morphology of each group was observed in 3 randomly selected fields using a laser confocal microscope (CLSM).

2.7. Cell proliferation
MC3T3-E1 cells (5 × 10^3 cells per well) were seeded on the surface of the samples stored under the different storage conditions in 96-well plates. After culturing the cells for 1, 3, and 6 d, a fresh medium containing 10% CCK-8 reagent was used to replace the culture medium and incubated for 2 h. The absorbance of the incubated media was measured using a microplate reader (Spectramax190, MD, USA) at 450 nm wavelength. The experiment was repeated three times.
2.8. Alkaline phosphatase (ALP) activity

MC3T3-E1 cells (8 × 10⁵ cells per well) were seeded on the surface of the samples and subjected to osteogenic induction. After 7 d of culture, cells were lysed using the RIPA lysis solution and crushed ultrasonically. The lysed cells were centrifuged at 4 °C for 10 min (12000 R/min), and the supernatant was collected. The protein concentration and AKP values were determined using the BCA protein assay and AKP kits, respectively, following the manufacturer’s instructions. The ALP activity of each titanium disc was measured according to the instructions of AKP assay kit.

2.9. Western blotting

The samples were stored in 6-well plates under the four conditions for 2 w. MC3T3-E1 cells (2 × 10⁵ cells per well) were seeded on the samples. After 7 d of culture, protein samples were harvested using the RIPA lysis buffer. The concentration of the protein samples was measured using the BCA protein assay kit. The protein samples (20 mg) were separated using electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). 5% skimmed milk was used to block the membranes at room temperature for 1 h, and then the membranes were incubated with different primary antibodies against Runx2 (12556; CST, Beverly, MA, USA), Osterix (22552, Abcam, UK), OCN (ab93876, Abcam, USA), and GAPDH (BM0627, Boster, China) overnight at 4 °C. These membranes were then incubated for 2 h with secondary antibodies (ZB-2301, Goat anti-Rabbit IgG, ZSGB-BIO, China; AP124P, Goat anti-Mouse IgG, Millipore, USA). GAPDH was used as an internal control. The protein levels were measured using a fluorescence chemiluminescence gel-imaging system (Millipore, USA). The experiments were performed in triplicates.

2.10. Statistical analysis

One-way analysis of variance (ANOVA) and Student Newman Keuls (SNK) post hoc test were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) to compare the differences between the groups. A P-value of less than 0.05 was considered as the threshold for significance.

3. Results

3.1. Surface characteristics

The surface micro-topographies of cp-Ti, SLA-Ti, and NW-Ti after storage under the four storage conditions for 2 w are shown in figure 1. At low magnification, there were no obvious differences within the groups stored exposed to air and in ddH₂O. NaCl crystals were observed on the surfaces of cp-Ti, SLA-Ti, and NW-Ti after storage under 0.9% and 10% NaCl solutions. Compared to the 0.9% NaCl group, more crystals were adsorbed to the three titanium surfaces in the 10% NaCl group. At high magnification, the differences across cp-Ti, SLA-Ti, and NW-Ti were observed, such as the neat mechanical scratch on cp-Ti, the uniform micro-scale dimples on SLA-Ti, and the nano-scale protrusions on NW-Ti. After storage under different conditions, the characteristic micro-topographies of the three titanium surfaces did not change.

The surface elemental composition of the three surfaces stored under the different conditions was studied using XPS and the results are shown in figure 2. On the same titanium surface, the carbon (C) content in the NaCl groups was relatively low, while those of titanium (Ti) and oxygen (O) were relatively high.

As shown in figure 3, under the same storage conditions, NW-Ti showed greater hydrophilicity, followed by SLA, and then cp-Ti. Also, the hydrophilicity of the titanium surfaces stored in liquid was significantly higher than that of the titanium surfaces stored exposed to air. Moreover, the hydrophilicity of the titanium surfaces stored in NaCl solutions was significantly higher than that of the control groups. Specifically, the titanium surfaces stored in 10% NaCl exhibited the highest hydrophilicity. The roughness of SLA-Ti and NW-Ti was significantly higher than that of cp-Ti, as shown in figure 3. In the three groups of specimens, the roughness of air groups increased relative to ddH₂O groups, and the roughness of titanium surface increased with the increase of salt concentration.

3.2. Protein adsorption

The protein adsorption potential was evaluated by measuring the amount of protein adsorbed by the samples immersed in the α-MEM medium. As shown in figure 4, under the same storage conditions, NW-Ti adsorbed more protein in the culture medium, followed by SLA-Ti, and then cp-Ti. For the same titanium surface, storage in 0.9% NaCl solution provided higher protein adsorption capacity. However, the high concentration of NaCl solution (10% NaCl) did not further promote the protein adsorption capacity of the three surfaces.
3.3. Cell adhesion

The adhesion morphology of the MC3T3-E1 cells seeded for 8 h on the surfaces of the samples stored under the different storage conditions is shown in figure 5. Compared to the storage when exposed to air and ddH2O, the cells in the 0.9% NaCl group exhibited better morphology with a clear skeleton and more pseudopodia. However, the cell adhesion of those in the 10% NaCl group was relatively poor since the cells cultured on the surfaces shrunk into clusters.
3.4. Cell proliferation

The results of cell proliferation, as measured using the CCK-8 assay, are shown in Figure 6. The proliferation of osteoblasts on all surfaces gradually increased with time. By days 1, 3, and 6, there were no significant differences in the proliferation in the air, ddH2O, and 0.9% NaCl solution groups. However, the proliferation ability of the cells was significantly inhibited by 10% NaCl solution.

3.5. Osteogenic differentiation

As shown in Figure 7, under the same storage condition, the ALP activity on the surface of NW-Ti with nano-morphology was significantly higher. Also, the method of storage of the titanium surfaces affected the ALP activity of the osteoblasts. Compared to the surfaces exposed to air and ddH2O, those exposed to 0.9% NaCl showed relatively better alkaline phosphatase activity, while the alkaline phosphatase activity of the titanium surface stored in 10% NaCl decreased.
Figure 5. The adhesion morphology of the MC3T3-E1 cells (at 200× magnification) cultured on the surfaces of cp-Ti, SLA-Ti, and NW-Ti for 8 h after storage under the four storage conditions.

Figure 6. The results of the CCK-8 assay showing the proliferation of MC3T3-E1 cells cultured on the surfaces of cp-Ti, SLA-Ti, and NW-Ti after storage under the four storage conditions. Data are presented means ± SD. *, P < 0.05.

Figure 7. ALP activity of the MC3T3-E1 cells cultured on the surfaces of cp-Ti, SLA-Ti, and NW-Ti after storage under the four storage conditions. Data are presented as means ± SD. *, P < 0.05; a, P < 0.05 versus air; b, P < 0.05 versus ddH₂O; c, P < 0.05 versus 0.9% NaCl; d, P < 0.05 versus 10% NaCl.
Figure 8 shows the levels of expression of osteogenesis-related proteins Runx2, Osterix, and OCN that were used to evaluate the osteogenic differentiation capacity of the cells cultured on the surfaces of cp-Ti, SLA-Ti, and NW-Ti. Cells cultured on the surfaces of cp-Ti, SLA-Ti, and NW-Ti stored in 0.9% NaCl solution showed better osteogenic differentiation capacity since the expression of Runx2, Osterix, and OCN was higher in that group. However, storage under the high-concentration NaCl solution (10% NaCl) did not promote the differentiation of cells on the titanium surfaces but inhibited it to a certain extent.

4. Discussion

The aging phenomenon of titanium implants is getting more and more attention. Although various modifications have improved the biological activity of titanium, the surface of titanium exposed to the atmosphere ages over time, resulting in a decrease in its biological activity. Therefore, it is important to develop a universal implant storage solution. However, the different modification methods of the titanium surface lead to huge differences in the physical and chemical properties of these surfaces. We tried to elucidate the differences in the anti-aging performance in the same storage liquid across different types of surfaces.

In our study, the freshly prepared cp-Ti, SLA-Ti, and NW-Ti were stored exposed to air and in ddH₂O as controls. The three kinds of titanium surfaces stored in 0.9% and 10% NaCl solutions were used as the experimental groups. The electron microscopy results indicated that after 2 w of storage, the microstructure of cp-Ti, SLA-Ti, and NW-Ti did not change significantly apart from the fact that different numbers of sodium chloride crystals were observed on the surfaces of titanium stored in saline solutions (figure 1). These results suggested that different storage conditions and the concentration of the saline storage solution do not affect the inherent morphology of the titanium surface, which is consistent with previous reports [18, 21]. However, Shen et al found that the sandblasted and acid-etched titanium surfaces appeared nanostructured after storage in an aqueous solution, while the titanium surface that was stored in air-tight containers did not [22]. The effect of aqueous solution on the surface morphology of the bio-materials needs to be further elucidated but the findings suggested that aqueous solution seems to be better than storage using a gas barrier.

Hydrophilicity is an important feature that affects the biocompatibility of the titanium surface and is conducive to the early osseointegration of the implants [23]. It was found that all the three titanium surfaces stored in saline solutions showed eminent hydrophilicity after 2 w of storage (figure 2). Moreover, XPS analysis of the elemental composition of the titanium surfaces showed that the carbon content of the titanium surface stored in the liquid condition was relatively low, and that of the titanium surfaces stored in the saline solutions was the lowest (figure 3). Besides hydrophilicity, surface roughness is another important characteristic that affects the cell response and early protein adsorption [24, 25]. The surface roughness of the three titanium surfaces exposed to air the roughness of the titanium surfaces increased relative to that stored in the ddH₂O group, which may cause by carbon contamination on the titanium surfaces. In addition, the roughness of the three titanium surfaces increased with the increase of salt concentration, which may be related to the NaCl crystals adsorbed onto the titanium surfaces. However, the roughness of the titanium surfaces stored in 10% NaCl solution increased probably due to the large amount of NaCl crystals adsorbed onto the titanium surfaces. These results indicated that storage in the saline solution could effectively reduce the carbon deposition on titanium surfaces with different morphologies and improve the hydrophilicity and roughness. This may be due to the competitive adsorption of the storage solution solutes and hydrocarbons to the titanium surfaces. A high

![Figure 8. The expression of the osteoblast-related proteins in the MC3T3-E1 cells cultured on the surfaces of cp-Ti, SLA-Ti, and NW-Ti after storage under the four storage conditions.](image-url)
concentration of saline solution could compete for more adsorption sites, thereby reducing the adsorption of hydrocarbons to the titanium surface. In addition, since sodium chloride is water-soluble, the adsorbed sodium chloride on the titanium surface might also help to improve the hydrophilicity of the material.

Once the biomaterial is implanted in the body, the active protein in the blood quickly adsorbs to the surface of the material, and then the cells adsorb. Cells are attached to the protein layers instead of directly to the chloride on the titanium surface. In addition, sodium chloride is water-soluble, the adsorbed sodium concentration of saline solution could compete for more adsorption sites, thereby reducing the adsorption of related genes that low concentrations of Na\(^+\) solution. In addition, sodium ions may also be involved in the regulation of osteoblast behavior. Lu et al found that low concentrations of Na\(^+\) can directly promote the differentiation of osteoblasts and the expression of the related genes [33]. Another study found that the biological activity of the titanium surfaces was improved after sodium ion implantation [34]. In the 10% NaCl group, although the high concentration of salt improves the physicochemical properties of the three titanium surfaces, the adsorption of excessive sodium chloride crystals on the surface and the formation of a local hypertonic environment may damage cell function.

The results from the current study suggested that the use of a saline solution to store the different forms of titanium surfaces effectively reduces carbon deposition on the titanium surfaces, maintains hydrophilicity, promotes protein adsorption, and induces osteoblast differentiation. However, further in vivo research using a longer storage period is required to elucidate the anti-aging effects of the saline solution. Titanium surfaces stored at a high concentration of saline exhibited excellent hydrophilicity but lacked biocompatibility. It is suggested that both the physicochemical properties and biocompatibility of the biomaterials should be taken into account in the development of the storage solution. The addition of bioactive ions such as Ca, Mg, Sr, Ag, and Zn to the storage solution is expected to resist the aging of the implant while further improving the biological activity of the titanium surface, which has broader applications. The appropriate concentration of the implant storage solution may be needed to be determined urgently. The role of hydrocarbon contamination in implant aging has been confirmed [2, 6]. Therefore, before storage, it is necessary to treat the implants using, for example, ultraviolet radiation [35], to reduce hydrocarbon contamination. In addition, studies have found that certain steps can be taken to reverse the aging of titanium before implant placement. Henningsen et al found that non-thermal plasma (NTP) treatment could activate the titanium surface and improve the survival of mouse osteoblasts [36]. Another study treated aged titanium surfaces with NaOCl for 24 h and found that it essentially changed the titanium surface from a hydrophobic to a super-hydrophilic state, resulting in an increased cell adhesion ability of the titanium surfaces [37]. It is foreseeable that storing implants with different surface modifications in a storage solution of the appropriate concentration of salt combined with some anti-aging treatments before the clinical application is an effective anti-aging strategy.

5. Conclusions

The use of the saline storage solution to store the different titanium surfaces could effectively reduce carbon deposition on the titanium surfaces, maintain good hydrophilicity, promote protein adsorption, and induce...
osteoblast differentiation. Storing implants with different surface modifications in a storage solution of the appropriate concentration of salt combined with certain other anti-aging treatments before the clinical application is an effective anti-aging strategy.

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Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

Author contributions

KMT and WSZ contributed to the design of the study, data acquisition and analysis, and drafted the manuscript. YL contributed to data acquisition. WQZ contributed to the design of the study and data analysis. The corresponding author JQ contributed to the conception and the design of the study and data interpretation, and critically revised the manuscript. All authors provided consent and agreed to be accountable for the work.

Conflict of interest

All authors declare that they have no conflicts of interest with the contents of this article.

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