Pre-Treatment of Titanium Alloy with Platelet-Rich Plasma Enhances Human Osteoblast Responses

Ji-Hye Lee¹, Jinwoo Nam¹, Kwang Woo Nam², Hee Joong Kim^{1,3}, Jeong Joon Yoo^{1*}

¹Department of Orthopaedic Surgery, Seoul National University College of Medicine, Seoul, Korea

²Department of Orthopaedic Surgery, School of Medicine, Jeju National University, Jeju, Korea

³Medical Research Center, Seoul National University, Seoul, Korea

Osseointegration, the histological direct bone-to-implant contact, is the ultimate goal of implant healing and the first prerequisite for long-term success of endosseous implants. It is well-known that metal implants with rough surfaces achieve better osseointegration than those with smooth surfaces *in vivo*. The implantation of metal materials into bone is always accompanied by bleeding. The implant surface is initially coated with blood and these initial events could determine subsequent osseointegration. However, there is little concordance between *in vitro* results and *in vivo* findings regarding the effect of surface roughness on osseointegration. Here, we show that the osteoblast response to metal surfaces pre-treated with platelets and plasma proteins elucidates the superior osseointegration of rough surfaced implants *in vivo*. We found that osteoblast attachment, proliferation, and osteoblastic differentiation were significantly higher on a rough titanium surface pre-treated with platelet-rich plasma (PRP) than on the same surface without pretreatment. Furthermore, we found that the three-dimensional fibrillar network formed on the rough surface of the titanium by PRP pre-treatment might enhance osteoblast responses. Our results demonstrate why osseointegration is found to be most active on metal implants with a rough surface *in vivo*. We anticipate that our assay would be a useful tool for mimicking the *in vivo* model of osseointegration. Because cellular responses to the titanium implant that are pre-treated with platelet and plasma proteins on their surfaces after the biomimetic process *in vitro*, may be more similar to the events that occur *in vivo*.

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INTRODUCTION

Osseointegration is the structural and functional direct contact between natural bone and artificial implants. It is the ultimate goal of implant healing and the precondition for long term success of orthopedic or dental implants [1,2]. After insertion of an artificial implant, osteoblasts adhere around the implant and produce osteoid. The osteoid remodels into new trabecular bone and finally becomes lamellar bone. These biological processes have been defined as osseointegration and are caused by the bone surrounding implants [3,4]. However, the exact mechanism of osseointegration is not yet fully understood.

The bonding of implant and bone was considered as concerted actions of mechanical interlocking and biochemical bonding

[5]. It is well known that rough titanium implants induce better osseointegration than those with smooth surfaces in clinical applications. Especially, almost all of the implants used for artificial joint replacements or dental implants possess porous and rough surfaces that can biologically secure new bone growth. Therefore, surface roughness is a key factor in the design of metal implants such as titanium and its alloys [6]. Many techniques have been developed to increase the surface roughness of metal implants, including acid etching and grit blasting [7-10].

Many researchers have attempted to explain the effect of the surface roughness of titanium on *in vivo* and *in vitro* peri-prosthetic bone formation [7,11-15]. They reported that surface roughness modulates the attachment, proliferation, differentiation, and bone matrix synthesis of osteoblasts [16]. In animal models, titanium implants with rough surfaces induced more extensive bone formation than smooth titanium surfaces. These *in vivo* results are fully concordant with the results in clinical applications [8,17-19]. In contrast, *in vitro* studies have not shown definitive evidence of enhanced osseointegration due to increased surface roughness. Particularly, initial results on cell at-

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***Corresponding author:** Jeong Joon Yoo, Department of Orthopaedic Surgery, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 03080, Korea.

Tel: 82-2-2072-1994, Fax: 82-2-764-2718, E-mail: jjyos@snu.ac.kr

tachment modulated by surface roughness were controversial in various studies [20]. Recent literature reported that rough metal surfaces decreased cell proliferation, but enhanced osteogenic differentiation and extracellular matrix synthesis [21,22]. These observations have low reproducibility and a large spectrum of different results. The disparity in the results *in vitro*, *in vivo* and in clinical applications regarding the effects of surface roughness has not yet been addressed.

Many researchers have investigated biomolecular mechanisms for peri-implant bone formation with only metal specimens and bone forming cells *in vitro* [23-25]. Upon implantation of metal prosthesis into bone, the implant surface is initially coated with body fluid mainly consisting of blood. Osteoblastic cells later migrate, attach, and proliferate on the implant [23]. Hence when cells approach an implant material, it is unlikely that they will make direct contact with its surface. Rather, the rapid adsorption of proteins from blood (or serum) and establishment of the three-dimensional fibrin matrix determines the cellular response, contributing to the ultimate outcomes in both implantation and tissue culture situations [24,25].

In this study, we hypothesized that the cellular responses to titanium implants with surfaces pre-treated with platelet and plasma proteins may be more similar to the events that occur *in vivo* due to the initial biomimetic process *in vitro*. We also hypothesized that the osteoblast responses to a titanium implant may be different depending on whether the surface was pre-treated with platelet and plasma proteins or not. The goal of the present study was to document the significance of platelet and plasma proteins on the implant surface with respect to the osteoblast responses. To accomplish this goal, we investigated the amount of platelets on grit-blasted titanium alloys (TiGB) after platelet-rich plasma (PRP) pretreatment and analyzed the adhesion, proliferation, and osteogenic differentiation of human osteoblasts (hOBs) on TiGB implants with and without PRP treatment.

MATERIALS AND METHODS

Specimens preparation

Titanium alloy (Titanium-6Alumina-4Vanadium, Ti-6Al-4V) discs (rough, grit-blasted; TiGB) were supplied by an orthopaedic implant manufacturer (Corentec, Cheonan, Korea). The discs were 2 mm thick and had a diameter of 14 mm, so as to fit into the well of a 24-well tissue culture plate (TCP). The manufacturing process was briefly as follows: the raw material of Ti-6Al-4V Eli rods were machined round with a three-axial CNC lathe (PUMA 300MA, Doosan Infracore, Daegu, Korea) and were treated by grit-blasting using an inhalation sanding machine (KSSA-5FD, KumkangTech, Pohang, Korea) with #16 mesh particles.

The discs were cleaned and made hydrophilic by boiling in NH_4OH (25%): H_2O_2 (30%): H_2O (1:1:5 v/v) for 5 min, followed by thorough rinsing in distilled water. All discs were sonicated in 95% ethanol for 15 min, washed three times with Dulbecco's Phosphate Buffered Saline (PBS, GibcoBRL, Gaithersburg, MD, USA) and autoclaved for 15 min at 121°C. The morphology of the disc surface was observed with a scanning electron microscope (S-570, Hitachi, Tokyo, Japan) and the roughness of the disc surface was measured using a Surfcom 1440D (Tokyo Seimitsu, Tokyo, Japan).

Platelet-rich plasma preparation

Allogenic leukocyte-depleted PRP was obtained from our hospital blood bank. PRP was obtained from supernatants of blood centrifuged at 150 g for 10 min following removal of leukocytes. The PRP of 5 donors were mixed to achieve epidemiologically representative values of growth factors. The mean platelet count in the PRP ranged from 800/nL to 1100/nL.

Human osteoblasts culture

Human osteoblasts (human fetal osteoblastic cells, hFOB 1.19, no. CRL-11372) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell monolayers were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco, Invitrogen Corporation, Carlsbad, CA, USA) and Ham's F12 medium (HAM-F12, Cultilab, Campinas, SP, Brazil) (1:1 mixture) supplemented with 10% of fetal bovine serum (FBS, GibcoBRL, Grand Island, NY, USA), 50 $\mu\text{g}/\text{mL}$ of ascorbic acid-2-phosphate (Sigma, St. Louis, MO, USA), and 0.3 mg/mL of Geneticin[®] selective antibiotic (G418 Sulfate, Thermo Fisher Scientific Inc., IL, USA). The cells were maintained in a humidified incubator with 5% CO_2 at 34°C. When cells reached confluency, they were recovered by 0.05% trypsin and 0.53 mM Ethylene diamine tetra acetic acid (EDTA, GibcoBRL, Grand Island, NY, USA). Those used in the experiment were derived from passages 0 to 2.

PRP pre-treatment

The TiGB discs were inserted into the bottom of 24 well plates and 200 μL of PRP was added to the surface of the TiGB discs. The plates were then incubated for 20 min to bring the platelets closer to the titanium metal surface at room temperature. After the 1st incubation, the plates were further incubated at 37°C on a horizontal shaker (70 rpm) for 60 min. Following the 80 min contact time, the wells were washed three times with PBS to remove residual PRP and loosely adherent platelets.

Platelet adhesion analysis

The 24 well plates were washed three times with PBS after

PRP pre-treatment and the collected lavage fluids were used to determine the degree of platelet adhesion using Lactate dehydrogenase (LDH) assay kit (ROCHE, Basel, Swiss). The LDH activity assay was conducted and the cytotoxicity (%) was calculated according to the instructions of the manufacturer.

Osteoblast culture and assays

For the cell adhesion and proliferation assay, previously cultured osteoblasts were seeded onto both TiGB pre-treated with PRP (TiGB-PRP) discs and non-PRP treated TiGB (TiGB) discs at a cell density of 3×10^4 cells/disc. Then they were incubated in growth media at 34°C in 5% CO_2 gas. The number of cells was determined at 4 h for adhesion and at 1, 3, and 5 days of culture for proliferation by using a cell counting kit (CCK-8, Dojindo, Tokyo, Japan). In brief, the wells were washed with PBS, then 10 μL of CCK-8 solution for each 100 μL of media was added to all wells and reacted at 37°C for 3 h to form formazan crystals. Absorbance was estimated using VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm after centrifugation at 1400 rpm for 5 min.

Adhered cell morphologies and fibrin networks on TiGB and TiGB-PRP discs were observed at 1 and 3 days of culture by using a field emission scanning electron microscope (FE-SEM, JSM-7401F, JEOL Ltd., Tokyo, Japan). The cells on the discs were washed three times with PBS and were fixed in 1000 μL of 2% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 2 h. After washing with PBS, the cells were dehydrated in increasing concentrations of ethyl alcohol (70 to 100%) and were critical point dried in a vacuum. Then, the samples were sputter coated with a thin gold-palladium layer on an aluminum stub for FE-SEM.

To analyze osteogenic differentiation of hOB on TiGB and TiGB-PRP discs, the cells were seeded on each disc at a density of 2×10^4 cells/disc, and cultured in growth media to allow enough cell adhesion on discs. After 24 h, the culture media was changed to osteogenic differentiation media composed of DMEM and HAM-F12 (1:1) supplemented with 10% FBS, 50 $\mu\text{g}/\text{mL}$ of ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 10^{-8} M of 1,25-dihydroxy vitamin D3 (Sigma-Aldrich, St. Louis, MO, USA), 10^{-8} M of menadione (Sigma-Aldrich, St. Louis, MO, USA), and 0.3 mg/mL of Geneticin[®] selective antibiotic at 39°C in 5% CO_2 .

Alkaline phosphatase activity was detected after 14 days of differentiation using an ALP diagnostic kit (#245; Sigma-Aldrich, St. Louis, MO, USA). Enzyme activity was normalized against the total protein content, which was determined using a BIO-RAD Protein assay kit (kit II; BIO-RAD Laboratories, Hercules, CA, USA), and expressed as μmol of p-nitrophenol/min/mg of protein. For the calcium content assay, discs with cells

were immersed in 500 μL of 1N HCl solution after 21 days of differentiation for 24 h at -70°C to dissolve calcium. Calcium content was measured using a QuantiChrom[™] calcium assay kit (DICA-500, BioAssay Systems, Hayward, CA, USA). Calcium contents were expressed as micrograms per milliliter.

To measure the amount of platelet-derived growth factor-AA (PDGF-AA) and transforming growth factor- β (TGF- β), the culture media was collected after 14 days of differentiation and analyzed by an ELISA kit (R&D systems, NE, USA).

Statistical analysis

Each experiment was repeated three times with five samples for each group, resulting in a total of fifteen samples per group. All data were reported as the mean $\pm$ standard deviation and were compared using Mann-Whitney test among PRP-treated discs or not-treated ones, separately. Differences were considered significant for p values < 0.05 .

RESULTS

The gross image of the TiGB disc showed light reflection by the roughened surface (Fig. 1A). The SEM image showed a rough surface composed of various complex grooves, crevices, pit and hills (Fig. 1B). The surface roughness was measured and the R_a , arithmetic average roughness, was 7.75 ± 0.61 μm , while the R_z , the average ten point height, was 42.02 ± 3.25 μm .

The amount of platelet adhesion was compared in the TCP and the TiGB disc after PRP treatment using the same procedures (Fig. 2). The number of adhered platelets was significantly higher on TiGB than on TCP. More than 4 times the number of platelets on TCP were observed on the TiGB discs.

Initial cell adhesion of hOBs was analyzed after 4 h of culture on TiGB and PRP-TiGB discs (Fig. 3). The cell adhesion was increased by approximately 2 folds on the TiGB-PRP discs than on the TiGB discs. The proliferation of hOBs on TiGB and TiGB-PRP discs was analyzed at 1, 3, and 5 days of culture (Fig. 4). The cells on TiGB proliferated gradually during the culture

Figure 1. Morphology of grit-blasted titanium alloy (TiGB) disc. (A) Macroscopic image of TiGB disc. (B) Scanning electron microscope image of TiGB disc ($\times 1000$ magnification).

period, but twice the numbers of cells were observed on TiGB-PRP discs after 5 days of culture.

The adhesion morphologies of hOBs were observed at 1 and 3 days of culture (Fig. 5). The cells on TCP were large and flat with lamellipodia at day 1 and the morphology was maintained until day 3. On TiGB, the cells were stretched over the ridges of the disc at day 1 and contacted each other by day 3. The cells on TiGB-PRP were observed with lots of surrounding fibrin networks at day 1 and had expanded over the ridge of discs as well as the 3-D fibrin networks.

The morphology of hOBs on TiGB-PRP was magnified to observe the interaction of cells and fibrin networks (Fig. 6). The cells were completely surrounded by dense fibrin networks at $\times 3000$ magnification and adhered to the fibrin network which covered the TiGB-PRP disc. The connection of cells and fibrin

networks were further magnified to $\times 10000$. The fibrin networks formed a 3D porous structure and individual fibrin fibers were connected on the cell surface.

The osteogenic differentiation of hOBs on TiGB and TiGB-PRP was analyzed by measuring ALP activity and calcium content. The ALP activity after 14 days of differentiation was significantly higher on TiGB-PRP than on TiGB (Fig. 7A). The calcium content after 21 days of differentiation showed a trend similar to the ALP activity, which was more than 3 folds higher on TiGB-

Figure 2. Number of adhered platelets on tissue culture plate (TCP) and TiGB disc. The data are presented as the mean $\pm$ standard deviation of 7 samples ($n=7$). * $p<0.01$, compared with TCP. TiGB: grit-blasted titanium alloys.

Figure 3. Adhesion of human osteoblasts on discs after 4 hours. The data are presented as the mean $\pm$ standard deviation of 5 samples ($n=5$). * $p<0.01$, compared with non-PRP treated TiGB disc. TiGB: grit-blasted titanium alloys, PRP: platelet-rich plasma.

Figure 4. Proliferation of human osteoblasts on discs. The data are presented as the mean $\pm$ standard deviation of 5 samples ($n=5$). * $p<0.01$, compared with 1 day after culture. TiGB: grit-blasted titanium alloys, PRP: platelet-rich plasma.

Figure 5. FE-SEM images ($\times 1000$ magnification) of adhered hOBs after 1 and 3 days of culture on TCP and discs with or without PRP pretreatment. *adhered cells. TCP: tissue culture plate, TiGB: grit-blasted titanium alloys, PRP: platelet-rich plasma, FE-SEM: field emission scanning electron microscope, hOBs: human osteoblasts.

PRP than on TiGB (Fig. 7B).

Finally, the secretion of the osteogenic growth factors, PDGF-AA and TGF- β into the culture media was measured after 14 days of differentiation (Fig. 8). The amount of PDGF-AA was significantly higher on TiGB-PRP than on TiGB. The amount of TGF- β was also significantly higher on TiGB-PRP than on TiGB.

Figure 6. Magnified FE-SEM images of adhered hOBs after 1 day on PRP pretreated TiGB disc. (A) $\times 3000$ magnification, (B) $\times 10000$ magnification. FE-SEM: field emission scanning electron microscope, hOBs: human osteoblasts, PRP: platelet-rich plasma, TiGB: grit-blasted titanium alloys.

Figure 7. Quantitative results of osteogenic differentiation of hOBs on discs. (A) ALP activities of hOBs after 14 days of culture. (B) Calcium content of hOBs after 21 days of culture. The data are presented as the mean $\pm$ standard deviation of 5 samples ($n=5$). * $p<0.01$, compared with non-PRP treated TiGB disc. ALP: alkaline phosphatase, TiGB: grit-blasted titanium alloys, PRP: platelet-rich plasma, hOBs: human osteoblasts.

DISCUSSION

The surface roughness of implants for orthopedics and dentistry has been widely investigated, with roughened implants showing positive results for osseointegration in animal and clinical studies. However, the results of *in vitro* studies are not consistent with those *in vivo* or with clinical results. Most studies examined the direct interaction between metal surfaces and osteogenic cells but excluded the effect of body fluid, which is the first material to come in contact with the implants after *in vivo* implantation. In this study, we hypothesized that pre-treatment with PRP could mimic the *in vivo* environment and that the cell behaviors on metal discs may be influenced by the presence or absence of PRP treatment.

Ti alloys, including Ti-6Al-4V, are metal materials widely used for orthopedic and dental implants due to their biocompatibility and superior mechanical strength compared to pure titanium [26]. Ti alloys demonstrated greater bone regeneration

Figure 8. Quantitative results of growth factor secretion. (A) PDGF-AA secretion after 14 days of culture. (B) TGF- β secretion after 14 days of culture. The data are presented as the mean $\pm$ standard deviation of 5 samples ($n=5$). * $p<0.01$, compared with non-PRP treated TiGB disc. PDGF-AA: platelet-derived growth factor-AA, TiGB: grit-blasted titanium alloys, PRP: platelet-rich plasma, TGF- β : transforming growth factor- β .

and bone integration effects in many studies when they possessed rough surfaces rather than smooth surfaces [4,6]. Therefore, several methods were developed to increase the surface roughness of metal materials including acid etching, grit-blasting and plasma spray [8,27]. We used Ti-6Al-4V that underwent grit blasting and soaking in NH_4OH solution to make the surface hydrophilic. Grit blasting is a method for creating grooves on the surface of a material by using various types of particles and the degree of surface roughness can be modulated by regulating the particle size or pressure. Many investigators have used grit-blasting to increase surface roughness [28,29]. Grit blasting on metals improves bone integration and enhances the adhesion of hydroxyapatite on the metal substrate [28,29]. Surface roughness can be presented in R_a (average roughness value) and grit blasted Ti alloys shows R_a between 0.32–11 μm , which is similar to the R_a for TiGB in our study (7.8 μm). The surface morphology of TiGB was also similar to those in previous reports using rough surfaces composed of complex ridges and grooves [28,29].

After implantation, the implant comes into contact with blood prior to contact with osteogenic or immune cells. Among the components of blood, platelets play an important role in thrombogenesis and help with regeneration of various types of tissues by secreting various growth factors. Therefore, we pretreated the Ti alloy with PRP containing plenty of platelets and plasma proteins to mimic the *in vivo* environment after implantation. We observed large amounts of platelets on the TiGB rather than on the TCP. Nygren et al. [30] reported increased platelet adhesion on hydrofluoric acid-etched surfaces compared to pure titanium surfaces. Platelet adhesion and activity were higher on the surface of the dual acid etched Ti and 320 grit abraded Ti than on the machined and polished surface [24]. This may be due to the increase in surface area from the increased surface roughness.

Cell adhesion is the first occurrence in cell-materials interaction. Therefore, many researchers have examined the relationship between surface roughness and cell adhesion. They reported decreased cell adhesion on rough titanium surfaces compared to TCP [8,13,31]. These results may be because rough surfaces provide only a few points for cells to form focal adhesion and integrin binding. In this study, poor cell adhesion was observed on TiGB, which is consistent with the results from previous studies, but the adhesion was higher on the TiGB-PRP disc. In our previous study, we demonstrated that PRP coated Poly(Lactide-co-Glycolide) (PLGA) scaffolds had a higher amount of adhered cells than non-coated PLGA scaffolds [32]. In our current study, cell adhesion was affected by increased platelet adhesion on TiGB resulting from increased surface roughness which exposed a larger surface area.

In this study, the number of cells observed on TiGB-PRP was

twice the number on TiGB at 5 days after culture. However, the proliferation rate was not affected by PRP treatment because the initial adhesion on TiGB-PRP was already twice that on TiGB. Theoretically, there are two ways to enhance bone formation. One is proliferation of osteogenic cells and the other is induction of a large amount of bone formation by increasing the osteogenic biosynthesis of individual cells [18]. In this context, a greater number of cells on TiGB-PRP may be favorable for bone formation in several ways. One possible reason why PRP treatment did not affect cell proliferation may be the strong effect of surface roughness on determining cell shapes, which impedes cell proliferation.

Cell-materials interactions can be analyzed by observing cell morphologies on the materials. In this study, we found that the morphology of cells were larger and flatter on TCP whereas the cells were stretched spindle shapes on the TiGB discs. Especially, dense fibrin networks were distributed extensively on the TiGB-PRP disc with no empty spaces. Moreover, these complex fibrin networks were tightly connected with the osteoblasts and trapped the cells on the rough surface of TiGB. The results correspond with a previous study which suggested that a more extensive and three dimensional complicated fibrin network is formed as surface roughness increases [14]. These 3D porous fibrin networks may offer appropriate conditions for the adhesion, migration, and differentiation of osteoblasts [25,33]. These close interactions between cells and fibrin networks on rough surfaces may play an important role in cell adhesion and differentiation.

The osteogenic differentiation of osteoblasts was enhanced on TiGB-PRP compared to TiGB, as we expected. There have already been extensive investigations on whether PRP facilitates the osteogenic differentiation of osteoblasts. In our previous study, we showed enhanced osteogenic differentiation of human bone marrow stromal cells (BMSCs) on PRP treated TCPs. PRP was applied using the same procedures as in the current study and the result was a fully covered surface with abundant fibrin networks connected to the cells [34]. van den Dolder et al. [35] demonstrated results corresponding to ours which showed that PRP treated TCP facilitated the growth and differentiation of rat BMSCs in addition to enhancing ALP activity and calcium synthesis.

We speculate that PRP treatment induces the adhesion of platelets and the adhered platelets subsequently modulate the behavior of osteoblasts by growth factor secretion. The most important growth factors involved in osteogenic differentiation of osteoblasts are known as PDGF-AA and TGF- β . We monitored the secretion of growth factors and both growth factors were higher on TiGB-PRP than on TiGB, as we expected. These results showed that the PRP treatment provided an appropriate environment for osteogenic differentiation by stimulating

growth factor secretion. Yang et al. [36] suggested that PDGF-AA was secreted by the osteoblasts and stimulated by the autocrine loop which is considered a self-imposed factor. Moreover, secretion of PDGF-AA from osteoblasts was up-regulated by TGF- β signaling. Therefore, our results could be explained by the stimulation of increased TGF- β secretion on TiGB-PRP, which subsequently stimulated the secretion of PDGF-AA by osteoblasts.

In this study, we selected PRP as a representative component of blood, with an abundance of platelets and plasma proteins, for the treatment of metal surfaces [25]. However, we did not take into consideration the effect of inflammatory reaction or cytokine secretion by leukocytes and macrophages during *in vivo* bone formation around implants [37]. Therefore, we agree that PRP pre-treatment on metal implants cannot completely reproduce the *in vivo* situation and may possibly exaggerate or underestimate the state of the surrounding environment after implantation.

In conclusion, our results showed that PRP pre-treatment on titanium alloy enhances adhesion, proliferation, and differentiation of osteoblasts. These results revealed that the presence of body fluids might be considered when examining the *in vitro* interactions between cells and biomaterials, especially with regards to peri-implant bone formation.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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