Radiation suppresses neointimal hyperplasia through affecting proliferation and apoptosis of vascular smooth muscle cells

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ABSTRACT

Purpose: To study the effect of x-ray radiotherapy on vascular smooth muscle cells (VSMCs) and elucidate the mechanisms in preventing neointimal hyperplasia of prosthetic vascular grafts.

Materials and methods: In model I, twelve mongrel dogs underwent revascularization with prosthetic grafts and half the dogs underwent irradiation of the graft at 28 Gy. In model II, human VSMCs (hVSMCs) were maintained and divided into six groups to which external radiation was applied at six different doses: 0 Gy, 2 Gy, 8 Gy, 16 Gy, 24 Gy and 30 Gy. In both models, specimens were harvested and examined by using morphological, immunological, cellular and molecular methods.

Results: After irradiation, the neointima thickness was significantly lower in irradiated groups (p≤0.01). The radiotherapy could up-regulate p27kip1, and down-regulate proliferating cell nuclear antigen (PCNA) and S phase kinase associated protein 2 (Skp2). X-ray irradiation inhibits the proliferation of hVSMCs via acting on G1/S phase of cell cycle. The apoptosis of hVSMCs increased significantly with dose and time. The expression of PCNA and Skp2 were decreased after a first increasing trend with dose, but had a significant negative correlation with time. The expression of p27kip1 had a significant positive correlation with dose and time.

Conclusions: Postoperative external fractionated irradiation after prosthetic vessel replacement of the abdominal aorta suppressed the development of hyperplasia in the graft neointima in the short term. There was a prominent time- and dose-dependent inhibition of VSMC proliferation by radiation when it was administered.

Keywords: Apoptosis, Neointimal hyperplasia, Proliferation, Radiation, Vascular smooth muscle cells

Introduction

Replacement of a target vessel by a prosthetic vascular graft is one of the most important surgical treatments for diseases involving major vessels, and how to maintain patency of the prosthetic graft is a major clinical problem. The most frequent cause of long-term graft failure is neointimal hyperplasia, in which the vascular smooth muscle cell (VSMC) activation, proliferation, and migration play critical roles (1). Advances in understanding the mechanisms of neointimal formation have given us many potential targets for prevention of neointimal hyperplasia in humans.

Radiation therapy remains an important treatment in clinical practice. Our previous preliminary experimental study demonstrated that perioperative radiotherapy could significantly reduce the neointima thickness of the prosthetic grafts (2). The mechanisms by which radiotherapy inhibits the VSMC proliferation and migration in prosthetic grafts are not yet fully understood, and the cell cycle control system seems to play an important role. An understanding of VSMC cycle regulation may consequently lead to more effective therapeutic strategies. S phase kinase associated protein 2 (Skp2) gene is considered to be highly associated with proliferation of VSMCs and could affect the cell cycle via p27kip1 regulation (3, 4). Therefore, we hypothesize that radiotherapy could inhibit the duplication of Skp2-DNA and down-regulate the expression of Skp2, thus, break the cell cycle in G1/S phase, as well as attenuate the VSMC migration from anastomoses to central area and its phenotype transition.
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The purpose of this study was to determine if x-ray radiation therapy delivered superficially from an external source could reduce VSMC proliferation in two models – the first involving experimentally induced neointimal hyperplasia in vivo and the second involving cell culture in vitro. We hope to explore its possible mechanisms in the prevention of neointimal hyperplasia of prosthetic vascular grafts. By using an aortic interposition model, our study also shed some light on the feasibility and mechanisms of preventing dialysis access stenosis by radiation, as VSMC proliferation also plays a critical role in the pathophysiology of dialysis vascular access dysfunction.

Methods

Animals

Twelve adult mongrel dogs, weighing between 8-12 kg (mean 9.1 ± 0.52 kg), were used in the study. The experimental design and all animal procedures were approved by our University Institutional Animal Care and Use Committee. Animals were cared for according to National Research Council’s Guide for the Care and Use of Laboratory Animals (2013). A minimum of 7 days before surgery were allowed for the dogs to acclimatize to the environment of the laboratory.

Surgical procedure

The dogs were anesthetized using intramuscular injection of ketamine (5 mg/kg) and intravenous thiamylal sodium (15 mg/kg). They were then intubated and mechanically ventilated. Median laparotomy and open retroperitoneal were performed to expose the infra-renal abdominal aorta. Heparin was given intravenously (100 U/kg) before clamping. The subrenal abdominal aorta was then clamped, transected, and replaced with an expanded polytetrafluoroethylene (ePTFE) graft that was 5-6 mm in internal diameter and 2.5-3.0 cm in length. Both proximal and distal anastomotic stomas were performed in an end-to-end fashion using continuous 6/0 monofilament polypropylene sutures.

The silver clip was marked at each anastomotic stoma. Then, the exposed abdomen was carefully closed. Antibiotics were intravenously given during the operation and administered intramuscularly for a total of three days. Each animal was under surveillance after anesthesia until alert and then returned to the animal cage after surgery. They were allowed free access to food and water. No anti-platelet medication or heparin therapy was postoperatively administered.

Irradiation procedure

At two weeks after surgery, six dogs were selected at random and anesthetized again. Using an external beam from a linear accelerator (Elekta Precise 5745, Elekta, Sweden), a total radiation dose of 28 Gy was delivered in four daily fractions of 7 Gy. Radiation portals were designed to keep the spinal cord outside of the irradiated field. A vascular surgeon assisted the radiologist in accurately localizing the radiation field by the guidance of the clips, which were approximately 3×4 cm², encompassing the two anastomotic stomas.

Specimen harvest

The specimens were retrieved under terminal anesthesia after five weeks by an overdose of sodium pentobarbital (control group, n = 6; radiotherapy group, n = 6). Immediately after vital signs had ceased, the aorta was perfusion-fixed in 10% phosphate-buffered formalin at a pressure of 80-100 mmHg for 1 hour. Then, the anastomoses, including a 0.5 cm adjacent section of the host vessels, were retrieved, excised and trimmed.

Histopathology and morphometry study

Specimens were cut into three sections: the proximal anastomosis, the distal anastomosis and the middle section of the graft. The proximal and the distal anastomosis were immersion-fixed in 10% phosphate-buffered formalin solution for 24 hours. The two anastomoses were longitudinally cut and then embedded in paraffin and sectioned at 4- to 5-μm intervals. Sections were stained with hematoxylin-eosin and examined by an experienced observer blinded to the treatment received. The presence of neointima and anastomatic healing was evaluated using histopathologic techniques. Sections were imaged using an Olympus microscope (Olympus CX21, Tokyo, Japan) equipped with a Sony camera (HX1, Shanghai, China). The intim thickness of the graft neointima was measured by hematoxylin-eosin staining test in the proximal and distal regions of the graft using image-analysis software (Image Pro Plus 7.0, Media Cybernetics, Inc, Bethesda, MD, USA). Immunohistochemistry tests were undertaken to analyze the anastomotic healing and neointima formation. The neointima in the middle section of the graft was separated and stored in liquid nitrogen for detecting quantitative polymerase chain reaction (qRT-PCR) and Western blot analysis.

Western blot and qRT-PCR analysis in vivo

Western-blot and qRT-PCR were applied to detect the protein and mRNA expression of proliferating cell nuclear antigen (PCNA), Skp2, and p27kip1. β-actin was used as an internal control. Western-blot analysis was performed as described elsewhere (5). Primary antibodies used in the present study were as follows: anti-p27 (mouse. #sc-1641, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Skp2 (Rabbit. #sc-7164, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-PCNA (Rabbit. #sc-7907, Zymed, San Francisco, CA, USA).

RNA extraction and cDNA synthesis was performed as described elsewhere. The sequences for the gene-specific primers were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>PCNA</td>
<td>5’-GTGTTGAGGCCCACCTCAAGGA-3’</td>
<td>5’-GGTTACCCCTGGGCTTGGAG-3’</td>
</tr>
<tr>
<td>Skp2</td>
<td>5’-CTCAACTACCTCCAACACCTATC-3’</td>
<td>5’-TCTGGCACAGTAATCTCCAAA-3’</td>
</tr>
<tr>
<td>P27</td>
<td>5’-CGCTTAACCTGAGGACACCAG-3’</td>
<td>5’-GAATCGTCGGTTGCAGGTC-3’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5’-CATCCTGGCTTGGGACCTGG-3’</td>
<td>5’-TAATGTCACGCAGATTCC-3’</td>
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**Cell culture and irradiation treatment in vitro**

Aortic human vascular smooth muscle cells (hVSMCs) were maintained in vitro. hVSMCs were purchased from Institute of Biochemistry and Cell Biology, Central South University Xiangya School of Medicine (Changsha, China), and the cells were cultured in DMEM media supplemented with 20% fetal bovine serum, at 37°C in a humidified incubator with 5% CO₂. The hVSMCs exhibited a typical “hill and valley” growth pattern and the identity was confirmed by morphological examination. Medium was replaced twice a week. The cell became confluent, and subculture using trypsinization was performed. Confluent cell at passage numbers 3-5 was used for the irradiation treatment.

At room temperature, 3-5 generation cultured hVSMCs were irradiated by 6 MV-X-ray linear accelerator with various irradiation doses (0 Gy, 2 Gy, 8 Gy, 16 Gy, 24 Gy and 30 Gy).

**EDU incorporation assay**

The cells were harvested 12 hours, 24 hours and 48 hours after irradiation for light microscopic observation. The incorporation of 5-ethyl-2′-deoxy-uridine (EDU) into actively proliferating hVSMCs at 24 and 48 hours after irradiation was evaluated using a Cell-Light™ EDU Cell Proliferation Detection kit (RiboBio, China), following the manufacturer’s instructions. Cellular immunostaining was observed with an epifluorescence microscope (Motic, Germany). Digital images were acquired and analyzed with Image J software.

**Cell cycle analysis and cell apoptosis assay**

DNA content of hVSMC from all groups, treated with irradiation for 24 h was measured by Flow Cytometry (BD, USA). Propidium iodide (PI) staining was used to determine the cell cycle by flow cytometry. In each group, the cells were harvested by trypsinization followed by centrifugation at 800 rpm for 5 min. Next, cold 75% ethanol was added to cells for resuspension. Finally, 500 µL PI stain solution (250-500 µg/mL RNase A) was added to samples, which were analyzed on a FACScan within 30 min. Data were acquired from 10,000 cells and processed using Lysis II software (Becton-Dickinson).

Cell apoptosis was detected by Annexin V-fluorescein isothiocyanate and PI double-staining, and 1–5 × 10⁵ cells were harvested from each group for apoptosis assay. Following centrifugation at 2000 rpm for 5 min, the pellet was resuspended in 500 µL 1x binding buffer with 5 µL Annexin V and 5 µL PI (final concentration, 10 µg/mL). After incubation for 15 min in the dark, samples were subjected to apoptosis assay by flow cytometry, followed by data analysis using Lysis software. In total, ≥10,000 events were analyzed for each sample.

**Western blot and qRT-PCR analysis in vitro**

Western-blot and qRT-PCR were performed to detect the protein and mRNA expression of PCNA, Skp2 and p27kip1 in various cell groups at 24 and 48 hours after irradiation. They were performed as described previously in the in vivo study.

**Statistical analysis**

Data were analyzed by SPSS19.0 statistical software, and expressed as means±SD. Statistical significance was estimated by the one-way analysis of variance (ANOVA) for the comparison of two or several groups; p<0.05 was considered statistically significant.

**Results**

**Radiation suppresses neointimal hyperplasia in the graft**

The dogs were anesthetized and subjected to prosthetic vascular graft replacement. At 2 weeks after surgery, the anastomotic stomas were treated with four daily fractions of 7 Gy. H&E staining showed that there was a well-defined smooth muscle layer with little or no observed disruption (Fig.1A-D). The graft neointima and anastomotic vessel wall of the radiotherapy group (the proximal intimal: 143.99 ± 19.916 μm; the distal intimal: 170.51 ± 14.860 μm) were significantly thinner than those of the control group (the proximal intimal: 163.41 ± 8.866 μm; the distal intimal: 230.95 ± 12.753 μm) (p<0.01). The proximal intima of the graft was thinner than the distal in both groups (p<0.01; Fig.1E).

**Radiation inhibits proliferation of VSMC**

Neointimal hyperplasia involves VSMC activation, proliferation, and migration. The above results have shown that radiation leads to decreased neointima thickness. Therefore, we investigated the effects of radiation on the proliferation and apoptosis of hVSMCs.

We treated VSMCs with various doses of irradiation and subsequently examined the cells under a light microscope. In non-irradiated groups, VSMCs had a good growing state and were woven into mesh-like cells. However, the number of normal cells significantly reduced at 48 hours after irradiation (Fig. 2).

The results of EDU showed that the rate of hVSMCs proliferation is higher in the 2 Gy irradiation group than in the control group. The 8 Gy irradiation group’s proliferation rate was significantly higher after 48 hours, but the other radiation groups’ proliferation rates were obviously lower than those in the control group (p<0.01) (Fig. 3).

**Radiation promotes apoptosis of VSMC**

The PI detected using the ratio of VSMC G1 cell cycle and S cell cycle in the irradiation group and the control group was obviously different (p<0.01). Compared with the control group, the percentage of apoptosis in the irradiation group increased with dose increment (Fig. 4).

Annexin V-FITC showed that the early and late apoptotic rates in each irradiation group increased significantly more than those in the control group in a dose-dependent manner (p<0.01). The proportion of survived cells in each irradiation group was significantly decreased compared with the control group, in a dose-dependent manner (p<0.01) (Fig. 5).
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Fig. 1 - Histological sections of the ePTFE graft (hematoxylin and eosin). (A) Neointima of the proximal anastomosis for the graft in the control (0 Gy) group. (B) The proximal anastomosis with thinner neointima for the graft in the (28 Gy) radiotherapy group. (C) Neointima of the distal anastomosis for the graft in the control group. (D) The distal anastomosis with thinner neointima for the graft in the radiotherapy group. (E) Five weeks after surgery, neointima thickness of graft for 28Gy irradiated-treated group was significantly thinner than that of the control group. Neointima thickness in the proximal anastomosis of the graft was thinner than distal anastomosis in both groups. ** p<0.01. N = neointima; L = vessel luminal; ePTFE = expanded polytetrafluoroethylene; pro = proximal anastomosis of the graft; dis = distal anastomosis of the graft. Scale bar, 100 µm.

Radiation up-regulates expression of p27kip1, but down-regulates expression of PCNA and Skp2 in the graft and VSMC

To understand the mechanism for radiation affecting proliferation and apoptosis of VSMCs, we examined the expression of PCNA, Skp2 and p27kip1, which are involved in the proliferation and apoptosis process.

Western-blot analysis of the in vivo study clearly showed that the expression of p27kip1 was elevated, but expression of PCNA and Skp2 were significantly down-regulated in the neointimal area after irradiation (Fig. 6A, B, C).

Compared with the control group in vitro, the cellular protein levels of Skp2 and PCNA in the 2 Gy group and 8 Gy group were followed by an ascending order. In the 16 Gy group, 24 Gy group, 30 Gy group, the protein levels of Skp2 and PCNA were followed by a decreasing trend. And the significant time-dependent effect was observed in irradiated groups.
Fig. 2 - Morphological changes in each group cell under a light microscope. Representative examples of morphological changes induced by irradiation (0 Gy and 30 Gy) treatment for 12 h and 48 h. The number of normal cells significantly reduced for cell swelling, volume increased, form irregular, even cell membrane ruptured at 48 hours after irradiation. Scale bar: 200 µm.

The cellular protein level of p27k\(^{\text{kip1}}\) in each irradiated group was markedly increased in a time- and dose-dependent manner (Fig. 6 D, E, F).

Discussion

VSMC proliferation plays a major role in the development of neointimal hyperplasia. The current preventive strategies for targeting the neointimal formation include pharmacologic agents and brachytherapy (6-8). Previous studies have extensively explored the effectiveness of radiation therapy in reducing neointimal hyperplasia with several animal models, including coronary angioplasty model (9, 10), dialysis access grafts or fistulae model (11, 12), and aortic interposition model (2). Several clinical studies have demonstrated a significant decrease in vascular restenosis following brachytherapy (13, 14), including for instance the BRAVO trial (15). There are two strategies of brachytherapy, namely endovascular radiation and external-beam radiation (16). Endovascular radiation seems to be more selective and accurate; however, external beam radiation is more convenient and less invasive. The feasibility and safety were both addressed in previous studies. Since vascular stenosis remains a critical problem in clinical practice and has huge unmet needs, radiation therapy could play an important role and is worthy of further investigation.

Although these therapies have greatly reduced restenosis, the cellular and molecular mechanisms of neointimal hyperplasia and vascular remodeling remain unknown. Multiple factors are believed to contribute to neointimal hyperplasia in the ePTFE graft, including surgical trauma to the vessel during graft placement, bio-incompatibility of the ePTFE graft material, and mechanical factors at the anastomoses. We observed that the patency and healing of the ePTFE grafts were not affected by a total dose (28 Gy) of postoperative fractionated radiotherapy in canine models. In order to avoid the adverse effects of radiation on the skin incision and the acute inflammation phase after surgery, we chose two weeks after surgery to perform the radiotherapy when VSMC proliferation
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Anastomotic end of the graft has formed a complete lining by H&E staining. Although the source of the neointima was the same in the two anastomoses, the proximal anastomose region was thinner in the grafts, which favors the hypothesis that low wall shear stress leads to more intimal hyperplasia (17, 18).

In this study, the time and dose of radiation selection for dogs were chosen according to actual clinical conditions. The dosage range of external beam radiation generally recommended is between 15 Gy to 30 Gy in animal experiments and clinical applications. The irradiation within this dosage range can inhibit intimal hyperplasia of the anastomotic site, with most of the reports concentrated on the effect of irradiation after angioplasty and stent implantation (19, 20).

The studies have shown that the mechanism of neointimal hyperplasia is similar to wound healing (21). VSMC proliferation and migration are critical to the intimal proliferation and lead to gradual narrowing of the vessel lumen (22). It has been reported that VSMCs represent more than 90% of neointima cells (23). Alpha-smooth muscle actin (alpha-SMA) is located primarily in the microfilament bundles of VSMCs and exerts contractile functions (24). We also found that numerous cells expressing alpha-SMA were detected in the neointima in vascular artificial graft by immunohistochemistry. It is well established that accelerated proliferation of VSMCs are fundamental events in the development of intimal hyperplasia (25-27). Therefore, the treatment of neointimal hyperplasia with vascular radiation appears to work through inhibition of VSMC proliferation. It can be proposed that radiotherapy inhibits neointimal hyperplasia of prosthetic grafts through three pathways: (i) reduces cell migration from the two anastomoses; (ii) prevents cell recruitment, seeding, differentiation of circulating primary cells; (iii) attenuates the cell infiltration and migration from perivascular tissue and neo-adventitia via micropores of prosthetic grafts (28).

PCNA, or cyclin, is a non-histone acidic nuclear protein that plays a key role in the control of eukaryotic DNA replication. This acidic nuclear protein is synthesized in the early G1 and S phases of the cell cycle and significantly increased is known to be at its peak (1).
when they enter the cell cycle. Therefore, the levels of the PCNA behave as a marker for proliferating cells (29). Skp2 is an F-box protein component of the SCFSkp2 ubiquitin-ligase that controls cellular proliferation by regulating the ubiquitination and degradation of several cell-cycle regulatory proteins, including the cyclin-dependent kinase inhibitor (CDKI), p27kip1 (30). P27kip1, which binds to and inhibits phase G1 cyclin/cyclin-dependent kinase (CDK) complexes, is maintained at high levels in quiescent cells and declines to permit cell cycle progression (4). Over-expression of the inhibitory molecules p27kip1 has resulted in decreased neointimal formation in animal arterial injury models (4, 31).

In the present study, we further confirmed an observation in vivo that the expression of PCNA and Skp2 reduced significantly in the radiotherapy group, while the relative expression of p27kip1 markedly increased in the radiotherapy group. These results were consistent with the decrease in vessel wall thickness as well as the neointima of the graft. However, we did not observe the long-term effect. Whether this inhibition is permanent or merely a delay in the hyperplasia process requires further study to longer time points.

These findings in vivo, suggest that suppression of the neointima appears to be dependent on inhibition of the proliferation of VSMCs. But the mechanisms of regulating the

Fig. 6 - Expression of proliferating cell nuclear antigen (PCNA), Skp2 and p27kip1. Skp2 protein level (A), p27kip1 protein level (B) and PCNA protein level (C) of neointimal area in the 28 Gy irradiated-treated and the control group (0 Gy irradiated-treated group) was analyzed by Western blotting in vivo study. The level of p27kip1 protein in the irradiation treatment group was higher than that in the control group, while protein expression of PCNA and Skp2 were significantly down-regulated after irradiation treatment. The histogram shows the mean ± SEM; n = 6/group; **p<0.01 vs. control. Skp2 protein level (D), p27kip1 protein level (E) and PCNA protein level (F) of neointimal area in the radiotherapy groups (2 Gy, 8 Gy, 16 Gy, 24 Gy, 30 Gy irradiated-treated group) and the control group (0 Gy irradiated-treated group) was analyzed by Western blotting in vitro study. The protein levels of Skp2 and PCNA in 2 Gy group and 8 Gy group were followed by an ascending order. In the 16 Gy group, 24 Gy group, 30 Gy group, the protein levels of Skp2 and PCNA were followed by a decreasing trend. The protein expression of p27kip1 in each irradiated group was significantly increased in a time-dependent manner and dose-dependent manner. The figures are representative of three experiments. The histogram shows the mean ± SE of three experiments. **p<0.01 vs. control at 24-hours after irradiation. ##p<0.01 vs. control at 48-hours after irradiation.
cell cycle in VSMCs remain largely unknown and the effects of radiation on specific cell cycle inhibitors are still unclear. In order to identify potential targets, it is important to understand the cellular pathways involved in x-ray radiation response. The machinery regulating cell cycle represents the "final common pathway" of these signaling cascades. The eukaryotic cell cycle consists of four phases: G1, S, G2, and mitosis. The two most dramatic phases are the DNA synthesis (S) phase and mitosis. Entry into the S phase is controlled by the expression and activities of regulatory proteins. Moreover, corresponding CDKIs can negatively regulate cell-cycle progression. EDU can accurately and quickly detect the percentage of cells in S phase with no DNA denaturation. In our aortic interposition model, which is an arterial to arterial configuration model, we focus on general clinical dissemination and the underlying mechanisms may be interchangeable in above two settings. Proved by preclinical and clinical trials, radiation is both effective in inhibiting arterial graft and venous neointimal formation and VSMCs are always the key target cells. Besides, venous neointimal hyperplasia mainly occurs a few centimeters downstream from the anastomosis, which indicates hemodynamic factors are involved in the pathological process. In our study, we also found that the proximal anastomose region was thinner in the grafts, which favors the hypothesis that low wall shear rates lead to more intimal hyperplasia. Corporations between arterial interposition experiments and arteriovenous access models should be able to promote studies of applying radiation therapy in inhibiting neointimal hyperplasia.

Conclusion

In summary, we propose that the dosage and timing of radiation may influence the occurrence and development of neointimal hyperplasia in artificial blood vessels. Therefore, inhibition of VSMC cycle progression by irradiation represents a potentially promising therapeutic strategy for the treatment of neointimal hyperplasia in prosthetic vascular grafts. It has also been suggested that Skp2/p27kip1 could serve as a potential therapeutic target to suppress neointimal formation in prosthetic vascular grafts.

Abbreviations

VSMCs  Vascular smooth muscle cells
hVSMCs  Human VSMCs
PCNA  Proliferating cell nuclear antigen
Skp2  S phase kinase associated protein 2
ePTFE  Expanded polytetrafluoroethylene
PI  Propidium iodide
ANOVA  One-way analysis of variance
alpha-SMA  Alpha-smooth muscle actin
CDKI  Cyclin-dependent kinase inhibitor

Disclosures

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Conflict of interest: None of the authors has financial interest related to this study to disclose.

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