Epimedium Aqueous Extract Improves Osteoporosis and Regulates PPARγ, GSK-3β and β-Catenin Expression in Ovariectomized rats

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Abstract:
Context: Epimedium brevicornu Maxim., a Chinese herb, has long been used to treat osteoporosis. Its anti-osteoporosis effect may be related with the Wnt/β-catenin signaling pathway.
Objectives: To investigate the effects of aqueous extract of Epimedium on ovariectomized rats via regulating the Wnt/β-catenin signaling pathway in the treatment of osteoporosis.
Materials and methods: After ovariectomy, three-month-old female Sprague Dawley (SD) rats (n = 84) were randomly divided into six groups: Sham, ovariectomized (OVX), positive group (10 mg/kg/day alendronate sodium tablets), AE-Low, AE-Middle and AE-High group (0.11, 0.33 and 0.99 g/kg/day aqueous Epimedium extract respectively, based on the Epimedium clinical dose and the equivalent dose ratio of human-animal surface area). After 12 weeks treatment, bone mineral density (BMD), biomechanical testing, bone micro-architecture was observed, and serum osteocalcin (OCN), osteoprotegerin (OPG), mRNA and protein expression levels were measured.
Results: In comparison to OVX, treatment with AE had positive effects on BMD, trabecular number (Tb.N), trabecular thickness (Tb.Th) and changes in serum markers of bone turnover as well as improved the maximum load. Moreover, the AE treatment group could significantly reverse the increase of peroxisome proliferators-activated receptor γ (PPARγ) and glycogen synthase kinase 3β (GSK-3β) and the decrease of runt-related transcription factor 2 (Runx2) and β-catenin mRNA expression due to ovariectomy.
Discussion and conclusions: AE was shown to exert anti-osteoporotic effect on ovariectomized rat through regulating the key proteins in the Wnt/β-catenin signaling pathway, which may provide a potential mechanism to study its role in further population research.

Key words Epimedium, Osteoporosis, Ovariectomy, Wnt/β-catenin signaling pathway.

INTRODUCTION
Osteoporosis (OP) has aroused much concern around the world with its increase in the aging population[1]. OP is characterized as a bone metabolic disorder in which bone mass is lost and bone micro-architecture degenerates. The development of OP is due to an imbalance between bone resorption and bone formation[2,3]. OP can cause back pain or fractures of the spine and femoral neck and result in a heavy financial burden to society and the family[4].
It seriously endangers people’s health, leading to fractures which cause an increased risk of morbidity and mortality\(^5\). To date, there are no completely satisfactory drugs to treat or cure this disease. In East and Southeast Asian countries such as China and Korea, herbs are often used to prevent OP and other bone diseases\(^6,7\).

*Epimedium brevicornu* is a perennial herb that can strengthen the bone. Its anti-osteoporotic effect is very prominent\(^8\). In China, *Epimedium* has been used to prevent bone disease for thousands of years. It has been reported that *Epimedium* can be used as an alternative medicine to treat postmenopausal osteoporosis\(^9\). Recent research suggested that icariin from *Epimedium* can increase bone mass and improve bone micro-architecture by promoting osteoblast differentiation, strengthening osteoblast activity and inhibiting adipogenic differentiation in some experimental studies\(^10,11\). Besides, it has been reported that the Wnt/β-catenin signaling pathway plays a critical role in bone development and in the pathogenesis of osteoporosis\(^12\). β-catenin acts as a key mediator in this pathway. When Wnt signalling is activated, β-catenin accumulates in the cytoplasm and is translocated to the nucleus, activating the Wnt downstream target genes and effectors\(^13\). It has not been reported that whether *Epimedium* can act on the Wnt/β-catenin signaling pathway to influence bone metabolism. Here, we hypothesize that *Epimedium’s* anti-osteoporotic effect may be related to the Wnt/β-catenin signalling pathway.

The present study was designed to investigate the therapeutic effect of aqueous extracts of *Epimedium* (AE) in ovariectomized rats and to explore whether AE can treat OP via regulation of some proteins of the Wnt/β-catenin signaling pathway through promoting osteogenic differentiation. In this study, we used the model of osteoporosis in ovariectomized rats\(^14\) to observe the effect of AE on key proteins of the Wnt/β-catenin signaling pathway involved in bone metabolism and bone tissue. Ultimately, our goal is to discover possible mechanism of the effect of AE on bone metabolism, thus providing direct experimental evidence in ovariectomized rats of the beneficial effect of AE that can be applied for the prevention and treatment of osteoporosis.

**MATERIAL AND METHODS**

**ANIMALS**

Specific pathogen-free (SPF) Sprague-Dawley (outbred) 3-month-old female rats (250.0 ± 12.0 g) were purchased from Guangdong Medical Laboratory Animal Center. [Approval No. Guangdong SCXK (2013-0002)]. Food and water were available *ad libitum*. The rats were housed separately and allowed to acclimate for 2 weeks in a controlled temperature of approximately 23°C. All standard sterile animal feed was provided by Jinan University Medical Center and in accordance with the Guide for the Care and Use of Laboratory Animals. The present study was approved by the Animal Ethics Committee of Jinan University (Guangzhou, China).

**REAGENTS**

Raw herb of *Epimedium* was bought from Guangzhou Nanbeihang Chinese Herb Slice Co., LTD (Guangzhou, China) and quality control was according to the standard of Pharmacopeia of People’s Republic of China. *Epimedium* was originated from Guizhou province of China and was identified by Menghua Wu (a teacher of Lingnan Research Centre of Traditional Materia Medica, Jinan University) as *Epimedium brevicornu* (Voucher No.20160627). Aqueous extract of *Epimedium* was extracted by Guangzhou Jingxiutang Baiyunshan Co., LTD (Guangzhou, China). The aqueous extract of *Epimedium* (No. 201606) is deposited at the Chinese Medicine Reasearch Laboratory of Pharmacy College, Jinan University, China. Compounds of epimedin A, epimedin B, epimedin C and icariin (purity > 98%) were purchased from Chengdu Pufeide Biotech Co., LTD (Chengdu, China). Alendronate sodium tablets were purchased from MSD (Hangzhou, China). ELISA kits for serum osteocalcin (OCN) and osteoprotegerin (OPG) were purchased from Cloud-Clone (Houston, TX, USA). Antibodies against GAPDH, β-catenin, and HRP were purchased from Cell Signaling Technology (Danvers, MA, USA). Prime-Script™ RT reagent Kit with gDNA Eraser and SYBR® Premix Ex Taq™ were purchased from Takara Bio Inc. (Dalian, China).
SAMPLE PREPARATION

Epimedium was soaked for 1h in distilled water, then exacted in water twice, mixed and condensed to 1 g/mL Epimedium aqueous extract. The extract (1 mL) was dissolved with methanol into 10 mL volumetric flask and filtered through 0.45 μm membrane, used as sample solution. Epimedin A, epimedin B, epimedin C and icariin (1.1, 0.5, 3.1, 1.4 mg respectively) were placed in 1 mL volumetric flask, then add methanol to dissolve the volume to the mark and shake. Mixed the precise amount of the reference solution 0.2 mL and placed into 1 mL volumetric flask, add methanol to the mark and used as mixed standard reference solution.

HPLC ANALYSIS

HPLC analyses were performed on an Agilent Series 1260 HPLC system with a diode array detector and Agilent Chemstation software (Agilent, Palo Alto, CA, USA). All the separations were carried out on a Lichrospher-C18 column (250 mm × 4.6 mm I.D. μm). The mobile phase consisting of 25% acetonitrile (A) and 0.1% acetic acid water (B). The flow rate was 0.6 mL/min and injection volume was 10 μL. The column temperature was 25°C and detected at 270 nm. HPLC peaks were identified by comparison with the retention times and UV spectra of standard compounds.

ANIMAL EXPERIMENT

3-month-old female SD rats (n = 84) were randomly divided into ovariectomized-A (OVX-A) group (n = 70) and Sham group (n = 14). The osteoporotic model was established by ovariectomy. After 12 weeks, the successful induction of OP was confirmed by measuring the bone mineral density (BMD). Then, the rats in the OVX-A group (n = 70) were randomly divided into the following groups: OVX (n = 14), AE-Low (AE-L, n = 14), AE-Middle (AE-M, n = 14), AE-High (AE-H, n = 14) and Positive (n = 14). The AE group rats were administered AE by gavage at 0.11, 0.33 or 0.99 g/kg every day (base on the Epimedium clinical dose and the equivalent dose ratio of human-animal surface area). Rats in the Positive group were administered alendronate sodium tablets by gavage at 70 mg/kg every day. All others were given distilled water by gavage at 10 mg/kg/day. After 12 weeks treatment, the rats were anaesthetized by intraperitoneal injection of 2% sodium pentobarbital. The BMD was measured and blood samples were collected and finally all the rats were sacrificed and the 4th and 5th lumbar vertebrae and the bilateral femora and tibiae were harvested.

MEASUREMENT OF BONE MINERAL DENSITY (BMD)

Rats were anaesthetized by the intraperitoneal injection of 2% sodium pentobarbital. Dual-energy X-ray absorptiometry (DEXA; GE Lunar, GE Healthcare, Madison, WI, USA) was used to measure the BMD of the 4th and 5th lumbar vertebrae and the bilateral femur.

MICRO-COMPUTED TOMOGRAPHY (µCT) ANALYSIS

To determine the three-dimensional bone structure of femur, µCT (µ-CT80, Scanco Medical AG, Bassersdorf, Switzerland) was used to scan the different cross-sections. Quantitative analysis was performed using Micro-View software (http://microview.sourceforge.net/web-index.html). The analysis parameters included trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation/spacing (Tb.Sp) and structural model index.

SERUM MAKERS OF BONE TURNOVER

Under anesthesia, blood samples were taken from the abdominal aorta and the serum was collected by centrifugation and preserved at -20°C. The levels of OCN and OPG were measured. OCN was determined using a Rat BGP/OCN ELISA kit and OPG with a Rat OPG ELISA kit. The serum samples were centrifuged at 5,000 g at 4°C for 30 min.
MEASUREMENT OF BIOMECHANICAL PARAMETERS

All the rats were sacrificed and the 4th and 5th lumbar vertebrae and tibiae were harvested. Each rat tibiae was placed on two fulcra of a multifunctional biomechanical analyser and the middle of the backbone was pressurized at the point of compression. The moment that the tibiae ruptured, the computer recorded the load-displacement curve and we read the maximum bending load from the curve. To evaluate the 5th lumbar vertebrae, each one was placed on the multi-functional biomechanical analyser, then compressed to achieve maximum load limit, when the computer recorded the load-displacement curve and we read the maximum compression load from the curve.

REAL-TIME PCR

Each femur was cut into pieces and ground in a mortar under liquid nitrogen. After sufficient milling, the corresponding volumes of TRIzol Reagent were added for RNA extraction. The extracted RNA was quantified using a Biophotometer and its purity was verified by measuring absorbance at wavelengths of 260/280. A PrimeScript™ RT reagent Kit with gDNA Eraser was used to remove genomic DNA. The purified mRNA was then reverse transcribed into cDNA using SYBR Premix Ex TaqII. GAPDH was used as the internal control. Primers are listed in Table 1.

Table 1. Primers sequence.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>β-Catenin forward</td>
<td>5‘-ACTCTAGTGACGCTTCTGGTTCTG-3’</td>
</tr>
<tr>
<td>β-Catenin reverse</td>
<td>5‘-CTCGGTAATGCTCCTCTTCA -3’</td>
</tr>
<tr>
<td>GSK-3β forward</td>
<td>5‘-ACACCTGCCCCCTCTACATTTACC-3’</td>
</tr>
<tr>
<td>GSK-3β reverse</td>
<td>5‘-ATTGGTCTGCCACGGTCTCCA-3’</td>
</tr>
<tr>
<td>PPAR-γ forward</td>
<td>5‘-TGTGGACCTCTCTGTGATGG-3’</td>
</tr>
<tr>
<td>PPAR-γ reverse</td>
<td>5‘-CATTGGTTCAGCTCTTGTGA-3’</td>
</tr>
<tr>
<td>Runx2 forward</td>
<td>5‘-AGCCGAGGCAACAGTTT-3’</td>
</tr>
<tr>
<td>Runx2 reverse</td>
<td>5‘-CCTAAATCTGAGGCGGTCA-3’</td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>5‘-CAACCGGAAAACCATCACA-3’</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>5‘-ACGCCAGTAGAATCCACACGAT-3’</td>
</tr>
</tbody>
</table>

Briefly, the reaction was carried out in a total volume of 20 μL comprising 10 μL SYBR Premix Ex Taq II, 6 μL of RNuclease-free water, 0.8 μL each of forward and reverse primers (0.4 μM) and 2 μL of cDNA. PCR was carried out during the exponential phase with an initial denaturation of 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 34 s. RT-PCR values were calculated by the △△CT method and normalized to GAPDH mRNA levels. ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used to analyse the results.

WESTERN BLOT

Proteins were extracted from the femur of the Sham group, OVX group and AE-H group. The concentration of total protein was measured using a BCA protein assay kit. An equal amount of protein was loaded onto each lane and electrophoresed on 10% SDS-PAGE gels and then transferred to PVDF membranes. The membranes were cut into strips according to the corresponding protein weight. After blocking in 5% fat-free milk for 1 h, the strips were incubated with primary antibodies overnight at 4°C, washed with TBST and then incubated with the appropriate secondary antibody at room temperature for 1 h. The membrane strips were then washed three times with TBST and visualized using ECL luminous liquid and detected with a western blotting system. Image Lab software (Bio-Rad, Hercules, CA, USA) was used for grayscale analysis.
**STATISTICAL ANALYSIS**

Data were analysed using SPSS 20 (IBM Corp., Armonk, NY, USA). Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test. A value of $P < 0.05$ was considered to be significant and of $P < 0.01$ was considered very significant. All values are expressed as mean ± SD.

**RESULTS**

**CHEMICAL COMPOUNDS IN **EPIMEDIUM** AQUEOUS EXTRACT**

HPLC profile from Figure 1A illustrated that AE shows major single and sharp peaks at 17.049, 19.320, 21.959, 23.749, 26.035, 42.961, 51.074 min. From Figure 1B, mixture of marker purified standard compounds has single and sharp peaks at 17.639 min, 19.911, 22.679 and 26.771 min. According to the retention time of reference solutions, it turned out that compounds in AE include epimedin A, epimedin B, epimedin C and icariin.

**AE can improve BMD in ovariectomized rats**

At 12 weeks after ovariectomy, the BMD of the 4th and 5th lumbar vertebrae and femur was measured by DEXA (Figure 2). The BMD of different bones in the OVX group was significantly lower than that of the matched bones in the Sham group ($P < 0.05$). After 12th weeks of treatment, the BMD of the 4th and 5th lumbar vertebrae and the femur was measured. The BMD of different bones in the AE and the Positive groups were significantly higher than that of those in the OVX group ($P < 0.05$).

**AE can improve bone microstructure in ovariectomized rats**
Measurement of BMD at 12 weeks after ovariectomy and after 12 weeks treatment. Results are represented as mean ± SD (n = 6, 14; Sham, OVX group is 6, treatment group is 14). *P < 0.05 vs. Sham group; ▲P < 0.05 vs. OVX group.

In µCT analysis, the images clearly showed three-dimensional bone structure of femur (Figure 3). Compared to the Sham group, trabecular number (Tb.N) and trabecular thickness (Tb.Th) in the OVX group significantly decreased (P < 0.01), and trabecular separation (Tb.Sp) in the OVX group significantly increased (P < 0.01). Compared to the OVX group, Tb.N in the AE and Positive groups significantly increased (P < 0.01). Tb.Th in the AE-H and Positive groups increased (P < 0.05, P < 0.01), but there were no significant differences in the AE-L or the AE-M group. Tb.Sp in the AE-L, AE-H and Positive groups decreased (P < 0.05, P < 0.01, P < 0.01, respectively), but there were no significant differences in the AE-M group.

**The effect of AE on the serum levels of osteocalcin and osteoprotegerin**

![Fig. 2 Measurement of Bone mineral density (BMD).](image)

![Fig. 3 Micro-computed tomography (µCT) analysis.](image)
(A) Representative images of three-dimensional bone structure of femur were derived from µCT in the Sham group (a), OVX group (b), Positive group (c), AE-L group (d), AE-M group (e) and AE-H group (f). (B) Histological analysis of femur. Data are represented as mean ± SD (n = 10). *P < 0.01 vs. Sham group; ▲ P < 0.05 vs. OVX group, ▲▲ P < 0.01 vs. OVX group.

As shown in Figure 4, the serum level of OCN and OPG in the OVX group was lower than in the Sham group (P < 0.01). Compared to the OVX group, the levels of OCN and OPG in the Positive and AE groups was significantly increased (P < 0.01).

**AE can enhance bone strength in ovariectomized rats.**

![Fig. 4 The level of serum osteocalcin (OCN) and osteoprotegerin (OPG).](image)

Data are represented as mean ± SD (n=10); *P < 0.01 vs. Sham group; ▲▲ P < 0.01 vs. OVX group.

As shown in Figure 5, biomechanical testing showed that the maximum load of the 5th lumbar vertebra in the OVX group was significantly lower than that in the Sham group (P < 0.01). Compared to the OVX group, the maximum load of the 5th lumbar vertebra in the Positive and AE groups significantly increased (P < 0.01). The results from the three-point bending test indicated that the maximum load of the tibia in the Sham group was higher than in the OVX group. Compared to the OVX group, the maximum load of the tibia increased in both the Positive and AE groups (P < 0.01).

**The effect of AE on the mRNA expression of Runx2, β-catenin, PPARγ and GSK-3β.**
The effect of AE on the protein expression level of β-catenin.

Western blot analysis showed that the protein expression level of β-catenin in the OVX group was significantly lower than in the Sham group (P < 0.05). Compared to the OVX group, the protein expression level of β-catenin in the AE-H group was significantly increased (P < 0.05) (Figure 7).
DISCUSSION
Recent studies showed that Epimedium has aphrodisiac properties and can be used as a tonic, which plays an important role in enhancing reproductive function and has osteoprotective, neuroprotective, cardioprotective, anti-inflammatory and immuno-protective effects[15]. According to the practice of traditional Chinese medicine, we used the aqueous extraction method to prepare Epimedium extract for research. Studies have indicated that the active components of Epimedium consist of 23 compounds [16]. From our results, AE has more than 4 compounds including epimedin A, epimedin B, epimedin C and icariin. In this study, Alendronate was selected as a positive control. Alendronate sodium tablets are commonly used in the treatment of osteoporosis in clinical, which is characterized by strong inhibition of bone resorption activity without inhibition of bone mineralization[17]. It exerts action through inhibiting osteoclast activity and indirectly inhibiting bone resorption [18]. Although alendronate sodium is an active molecule indicated as a first-line regimen for osteoporosis treatment, the usage of this drug still be limited due to some shortcomings such as low oral bioavailability and gastrointestinal mucosa irritation [19].

It was reported that Epimedium can directly promote osteoblast proliferation[20] as well as inhibiting osteoclast activity, thereby increasing bone mass and improving bone microstructure. We also found that AE could increase BMD value, enhance bone strength and improve bone microstructure. Our results suggested that the rats given alendronate sodium or AE had increased BMD value after 12 treatments. Bone strength is another important index in the evaluation of bone fracture. Bone biomechanical tolerance of the OP rats decreased significantly after ovariectomy, but improved after AE treatment similar to positive control. Bone histomorphology allows effective observation of changes in bone microstructure, which is an important factor affecting bone mass, which in turn also reflects bone strength[21]. When osteoporosis occurs, the bone trabeculae become smaller and thinner and the trabecular spacing increases, causing the bone marrow cavity to become disorganized.
and bone mesh fibers to be irregularly arranged. In this study, AE was shown to able to effectively increase the BMD, the number of bone trabeculae and trabecular thickness as well as reduce trabecular separation and enhance bone strength in OVX rats, thereby reducing the loss of bone mass. Moreover, OPG and OCN are widely accepted as serum markers of bone turnover[22]. Changes in serum biochemical parameters reflect changes in bone metabolism in the body, which can be used to evaluate bone turnover and reflect bone resorption and bone formation[23]. Our results showed that OCN and OPG were both significantly increased in the AE group compared with the OVX group, which indicated that AE had the effect of promoting bone formation. Thus, AE has a very good therapeutic effect in protecting the postmenopausal bone from postmenopausal osteoporosis, with the AE-H group showing the best response in preventing OP.

Wnt/β-catenin is the main pathway involved in osteoporosis. When the Wnt signaling is activated, the Wnt protein binds to the transmembrane receptor Frizzled on the cell membrane and the synergistic receptor lipoprotein receptor-related protein 5/6 (LRP5/6), resulting in inactivation of GSK-3β phosphorylation and accumulation of β-catenin. When β-catenin enters the nucleus, it activates the Wnt downstream target genes and up-regulates the expression of Runx2 to promote cell proliferation and activation after interaction with transcription factors in the nucleus. Bone formation activity is enhanced and in turn β-catenin accumulation is reduced. Therefore, β-catenin is the most important influential molecule in the whole Wnt signaling pathway. The fixed quantity and accumulation of β-catenin in the cytoplasm is the key to activation of the Wnt signaling pathway[24, 25]. Its phosphorylation and degradation in the cytoplasm are affected by the key enzyme GSK-3β. Some experiments have shown that GSK-3β, a small molecule inhibitor, can increase bone mass, improve bone mechanical properties and reduce the incidence of fractures[26]. The inhibition effect of GSK-3β in vivo can enhance Wnt signaling, promote osteoblast differentiation and enhance bone metabolism[27].

Runx2 is a specific transcription factor in osteoblasts that controls the expression of a series of osteogenesis-related genes and is a key regulator in the processes of osteoblast differentiation and maturation and osteoclast formation[28]. Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily and play a vital role in lipid and glucose metabolism[29]. PPARγ belongs to the family of PPARs and acts to regulate differentiation of adipocytes and control storage of body fat[30, 31]. In the present study, we found that the expression of β-catenin and Runx2 mRNA decreased significantly after bilateral ovariectomy in rats and that the expression of GSK-3β and PPARγ mRNA increased significantly. After treatment with aqueous extract of *Epimedium*, compared to the OVX group, the mRNA expression of PPARγ and GSK-3β was reduced, while the mRNA expression of Runx2 and β-catenin increased but remained lower than in the Sham group. We therefore speculated that AE could down-regulate the expression of PPARγ mRNA and the phosphorylation of GSK-3β but up-regulate Runx2 mRNA expression, thus boosting β-catenin accumulation and causing its translocation into the nucleus and subsequent activation of the Wnt/β-catenin signaling pathway. The results of western blot suggested that β-catenin protein expression was significantly decreased in OP rats compared with the Sham group, and the expression of β-catenin protein in AE rats was significantly increased after treatment.

According to our experimental results, it can be speculated that AE may act through promoting the phosphorylation of GSK-3β, inhibiting β-catenin degradation and causing accumulation of β-catenin in the nucleus, consequently activating expression of the target gene Runx2 downstream of the Wnt/β-catenin signaling pathway, while inhibiting PPARγ gene expression, thus promoting osteogenic differentiation and inhibiting adipogenic differentiation.

**CONCLUSIONS**

In short, AE can not only improve BMD and bone microstructure in OVX rats, but it also enhances bone strength. It acts to decrease the mRNA expression of PPARγ and GSK-3β and increase the mRNA expression of Runx2 and β-catenin. Meanwhile, AE also increases the protein expression level of β-catenin. Thus, it can be speculated that AE may play a role in the
Wnt/β-catenin signaling pathway, but the specific mechanism needs to be confirmed by further in vitro experiments or transgenic animal experiments. Epimedium could be used as an alternative drug for postmenopausal osteoporosis prevention and treatment after further research.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
All procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Ethics Committee of Jinan University (Guangzhou, China).

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Conflicts of Interest: No conflict of interest.

Statement of originality of work: The manuscript has been read and approved by all the authors, the requirements for authorship have been met, and that each author believes that the manuscript represents honest and original work.

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