The Expression Level of TGF-β1, TGF-β3 and VEGF in Transplanted Oral Mucosal and Cutaneous Wounds

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Keywords: TGF-β1, TGF-β3, VEGF, Wound healing, Oral mucosa, Cutaneous wounds.

Abstract

Oral mucosal wounds heal faster with minimal scar compared with cutaneous wounds. In order to reduce the effect of environmental factors and find the expression difference of growth factors in oral and dermal wound, this study created the following rat wound model. The oral mucosa was firstly transplanted to left abdominal skin and after the wounds healed, a line-like full-thickness excisional wound was created on the mucosal tissue site and the right abdominal skin. Full-thickness tissue biopsies were collected from the wounds including 5 mm of the surrounding tissue at 12 hrs, 1 d, 3 ds, 5 ds and 7 ds after wounding. Quantitative real time PCR and immunohistochemistry were used to analyze the expression level of TGF-β1, TGF-β3 and VEGF. In order to find the reaction of oral and dermal fibroblasts to different growth factors, the effects of TGF-β1, TGF-β3 and VEGF on migration and proliferation of both cells were also evaluated in vitro. Results showed that reduced TGF-β1 expressions were found in transplanted mucosal wounds at most time points post injury compared with cutaneous wounds. The TGF-β3 expressions were higher at 3 d while lower at 7 d post injury in transplanted mucosal wounds. VEGF levels generated in transplanted mucosal wounds remained at low levels at 5 d and 7 d post injury. In vitro study found that both oral and dermal fibroblasts migration could be promoted by TGF-β1 and TGF-β3, while the migration of oral fibroblasts could not promoted by VEGF suggesting that oral fibroblasts reacted differently to growth factors. The in vitro proliferation experiment showed that both cells could be promoted by the three growth factors at different concentrations. Our results suggested that the rapid healing and the absence of scars in oral mucosa are directly related to its intrinsic characteristics and not to environmental factors.

Key Words: Inflammation; Healing; Wounds; Remodelling

Introduction

Wound healing in adult skin is characterized by extensive granulation tissue formation followed by wound contraction and scar formation [1]. In exposed areas, the body scar tissue causes aesthetic problems, and around the joints it may impair function [2]. The mechanical effects of scar tissue in children can even restrict growth [3]. The clinicians have a common impression that human oral mucosal wounds heal faster with minimal scar formation compared with skin wounds. The animal model studies have indicated that wounds in the tongue heal quickly with little inflammation and different cytokine expression compared with skin [4-6]. Various reasons that contribute to minimal scarring in the oral cavity have been suggested, which include the distinct fibroblast phenotype, the presence of bacteria stimulating wound healing, the moist environment and growth factors present in saliva. Animal studies have shown that saliva application enhances skin wound healing and reduce inflammation [7]. This effect of saliva is attributed mainly to its relatively high concentration of growth factors and topical use of artificial saliva has been suggested as a treatment for skin burn wounds [8].

Wound healing is a dynamic process of the interaction between growth factors and the cells in the wound beds. Transforming growth factor beta (TGF-β) is a family of growth factors with multifunction during wound healing. There are three isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) that are involved in wound healing and the functions appear to be overlapping [9]. A number of processes in wound healing including inflammation, stimulating angiogenesis, fibroblast proliferation, collagen synthesis, the deposition and remodeling of new extracellular matrix are thought to be related to the function of TGF-β family [5]. TGF-β also regulates wound re-epithelialization and connective tissue regeneration [10,11]. So, TGF-β family plays a key role in the process of wound healing, including scar formation. According to former studies, the expression of TGF-β1 was significantly lower in oral wounds compared with cutaneous wounds at both 24 hours and 48 hours after wounding. These data suggested the potential role of decreased TGF-β1 levels in the reduction of scar formation in oral mucosa. However, there was a significant increase of TGF-β3 in oral mucosal wound suggesting that an increase in TGF-β3 compared with TGF-β1 levels may aid in scar prevention.

The process of new blood vessel growth is also a key element of wound healing which is called angiogenesis. The neovascularization in healing is important which facilitates the growth of the cells during wound repair by supplying oxygen and nutrients. Wound angiogenesis is reflected by levels of the pro-angiogenic factors like VEGF (vascular endothelial growth factor) [12,13] VEGF is a 40 kDa-45 kDa
homodimeric glycoprotein that was initially found to regulate the permeability of blood vessels [14]. Studies have found that VEGF could induce angiogenesis in vivo as a potent mitogen for vascular endothelial cells [15]. The role of VEGF in scarless wound healing is not entirely clear. Some fetal wounds studies have shown that VEGF expression is reduced in scarless wounds compared with fibrotic wounds [12]. However, Colwell et al. [16] found that VEGF mRNA levels were higher in E16 scarless excisional rat wounds compared to fibrotic wounds at E18.

Wound healing models include animal experiment and in vitro experimental models. The latter including the scratch assay and transwell test, have been shown to provide the insights into the process of cell migration, and CCK-8 to test the cell proliferation [15,16]. The concentrations of growth factors can be easily altered in vitro and this is the great advantage of these models. In order to gain a better understanding of the molecular and cellular processes involved in wound healing in oral mucosa and skin, both cell culture and animal wound healing models are included in this study. Firstly, the effect of TGF-β1, TGF-β3 and VEGF on the proliferation and migration of isolated fibroblasts obtained from the oral cavity and skin was determined. The hypothesis was that the cell responses of mucosal fibroblasts to growth factors were different from those of dermal fibroblasts. To confirm that the wound healing process in oral mucosa was faster than that in skin, we produced a special animal wound model.

In scarless wound healing model, fibroblasts were seeded into 12-well plates with culture medium until they reached 80% confluence. The monolayers were then scored with a sterile pipette tip to leave a plus shape scratch of approximately 0.4 mm-0.5 mm in width. Culture medium was then immediately removed and changed with optimal concentration of TGF-β1, TGF-β3 and VEGF. The same volume of PBS was added as the control group.

In proliferation assays, the effects of the TGF-β1, TGF-β3 and VEGF on fibroblasts proliferation were determined utilizing CCK-8 assay. Briefly, 2 × 10^5 fibroblasts were seeded into the 96-well plates for 24 h to adhere. Then the medium was changed with different concentrations of TGF-β1, TGF-β3 and VEGF at 0 ng/ml, 1 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, and 100 ng/ml respectively. The same volume of PBS was added as the control group. We chose to evaluate the cell proliferation in its logarithmic growth phase (day 3). All experiments were performed five times.

Animal experimentation

25 male SD rats, weighting approximately 180 g, were obtained from Animal Experimental Center and Animal Biosafety Level 3 Laboratory of Wuhan University (Wuhan, Hubei, China). The rats were housed at Animal Experimental Center of Union Hospital, Tongji Medical College of Huazhong University of Science and Technology (Wuhan, Hubei, China) with 12 h light/dark cycles and fed antibiotic-free food and water ad libitum. Firstly, oral mucosa grafting surgery was done to the animals. Briefly, the animals were generally anesthetized with 10% chloral hydras and 0.9% normal saline (1:2, vol/vol, 0.9 ml/100 g). In each rat, a full-thickness excisional wound (d=1 cm) was created on the left abdominal skin, and an identical wound was created on the oral mucosa of the tongue. The full-thickness mucosal tissue was sutured on the wound of the skin. After about 21 days, when the wounds were healed, a new, line-like full-thickness excisional wound (1.5 cm in length) was created on the mucosal tissue site. As a control, an identical wound was created on the right abdominal skin in the same rat. 25 rats were randomized for 5 time points (12 h, 1 d, 3 d, 5 d and 7 d; n=5) after the wounding. Full-thickness tissue biopsies were collected from the wounds including 5 mm of the surrounding tissue sue at 12 hrs, 1 d, 3 ds, 5 ds and 7 ds after wounding. The half of tissue samples were embedded in 4% paraformaldehyde for histopathology study and the other half in RNA later RNA Stabilization Reagent (QIAGEN; Valencia, CA) for quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis and immunohistochemical analysis

For qRT-PCR analysis, half of tissue samples harvested from 5 rats/group were sacrificed at 12 hours and 1 day to 7 days post wounding. Following sacrifice, the tissue (about 100 mg) were obtained and immediately stained in the RNA later RNA Stabilization reagent (QIAGEN; Valencia, CA) at 4° overnight and frozen at 20. Total RNA was extracted from the samples using TRIZOL reagent (Invitrogen Life Technologies; Carlsbad, CA) followed by a clean process according to the manufacturer’s protocols [3]. The value of OD

Materials and Methods

Fibroblast culture, cell migration and proliferation

Human dermal fibroblasts were obtained from the dermis of juvenile human foreskin during circumcision, with consent from the patients, as previously described [17]. Oral fibroblasts were isolated and cultured from gingival biopsies of patients undergoing third-molar extractions [18,19]. All tissue samples were obtained from healthy individuals in compliance with a protocol approved by Medical Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, Hubei, China)  with 300 µl of culture medium in upper chamber and 600 µl medium in lower chamber. After the cells adhered, the medium in the upper chamber was changed with serum-free DMEM/F-12. To determine the respective effects of TGF-β1, TGF-β3 and VEGF, the culture medium in the lower chamber was changed with 10 ng/ml TGF-β1, TGF-β3 or 20 ng/ml VEGF which was indicated in our former experiment [20]. The same volume of PBS was added as the control group. According to our preliminary studies results [17,20], we chose to evaluate fibroblasts migration at 24 h (the migration of fibroblasts was most active at this time point). The migrated fibroblasts were stained with crystal violet digested or digested by trypsin-EDTA and counted under microscopy. All experiments were done five times.

260/OD 280 of RNA samples were all greater than 1.8. The synthesis of cDNA was performed by reverse transcription of RNA (1 µg) using RevertAid First Strand cDNA Synthesis Kit (Fermentas; US) according to the manufacturer’s standard protocol. Real-time PCR analyses were performed in fluorescence thermocycler (Applied Biosystems 7500 Real-time PCR, USA) using specific primers as shown in Table 1. Reactions were performed in triplicate under the following conditions: an initial denaturation at 95°C for 15 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and elongation at 72°C for 33 s.

The cDNA amplification was in a 10 µl reaction mixture containing 50 ng cDNA solution, 5 µl of SYBR premix EX Taq (TAKARA, Japan), 4 pmol each of the forward and reverse primers, and 2 pmol of Rox Reference Dye II (TAKARA, Japan). The primers sequences of TGF-β1, TGF-β3 and VEGF were showed in Table 1.

<table>
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<tr>
<th>Gene</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
<th>Expected size</th>
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<tr>
<td>TGF-β1</td>
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<td>177 bp</td>
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<tr>
<td>TGF-β3</td>
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<td>CACACAGCAGTTCTCCCTCA</td>
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<td>VEGF</td>
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<td>CCATGAGTTCCATGTCGAGA</td>
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<td>GAPDH</td>
<td>GCCACAGTCAGGGCTGAGAA</td>
<td>ATGGTGGTGAAGACGCAGTA</td>
<td>143 bp</td>
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Table 1: Primer sequences for real-time PCR.

Goat anti-EGF (SC-1343) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-TGF beta 1 (ab64715) and anti-TGF beta 3 (ab58586), rabbit anti-FGF basic (ab16828), and anti-VEGF (AB46154) antibodies were purchased from Abcam (Cambridge, MA, USA). Following sacrifice, half of tissue samples harvested from 5 rats/group were fixed in 4% paraformaldehyde for 24 h, routinely processed, and embedded in paraffin by the Department of Pathology of Union Hospital. For immunostaining of extracellular matrix (ECM), TGF-β1 (1:50 dilution), TGF-β3 (1:20 dilution), and VEGF (1:100 dilution) were performed using the Vectastain ABC Elite Kit and Vector DAB Substrate Kit (Vector Laboratories). To this end, samples were fixed, whose nonspecific binding sites were blocked, incubated with the primary antibody as above and with the appropriate biotinylated secondary antibody at room temperature for 1 h. The sections were then incubated with freshly prepared Vectastain ABC reagent at room temperature for 30 min. DAB substrate was prepared according to manufacturer’s instructions and allowed to react with the samples until suitable color development was noted. Color development was terminated by a wash with distilled water. Control staining was performed by replacing the primary antibody with the phosphate buffer saline (PBS) and gave only a very weak nonspecific background staining.

Statistical analysis

SPSS 16.0 analysis software is used for data analysis. Numeric data are presented as means ± SE (standard error). Two-tailed Student’s t tests were used for statistical analyses. Statistically significant difference was set at p<0.05.

Results

Migration assay of fibroblasts by the stimulation of TGF-β1, TGF-β3 and VEGF

To characterize the effects of TGF-β1, TGF-β3 and VEGF on fibroblasts migration, the responses of fibroblasts to 10 ng/mL TGF-β1, TGF-β3 and 20 ng/mL VEGF were examined by transwell assay. The results shown in Figure 1A indicated that TGF-β1, TGF-β3 and VEGF could promote the migration of dermal fibroblasts. The migration of oral fibroblasts could be promoted by TGF-β1 and TGF-β3, while not promoted by VEGF (Figure 1B).

Simultaneously, the effects of the three growth factors on fibroblasts migration were also further confirmed by scrape wound healing assay (Figure 2). The results showed that TGF-β1, TGF-β3 and VEGF could promote the cell migration of dermal fibroblasts (A). The migration of oral fibroblasts (B) was promoted by TGF-β1 and TGF-β3, but not by VEGF. The result was in agreement with that of the transwell assay.
Figure 2: The scrape wound healing assay of the TGF-β1, TGF-β3 and VEGF on fibroblasts migration. The two sides of the scrape between the fibroblasts were showed with yellow lines. The results showed that the three grow factors compared with PBS (control group) could promote the cell migration of dermal fibroblasts (A). The migration of oral fibroblasts (B), compared with PBS group, was promoted by TGF-β1 and TGF-β3, but not by VEGF.

Proliferation assay of fibroblasts by the stimulation of different concentrations of TGF-β1, β3 and VEGF

To further characterize the effects of TGF-β1, TGF-β3 and VEGF on fibroblasts proliferation and to determine the optimal cytokine concentration, we examined the responses of fibroblasts to different concentrations of TGF-β1, TGF-β3 and VEGF (at 0 ng/ml, 1 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, and 100 ng/ml, respectively). The results at day 3 were chosen to compare the effects of the cytokines at the respective concentrations, as day 3 was a time point of the logarithmic growth phase during which the cells proliferated significantly.

The results shown in Figure 3 indicated that both cells showed a mitogenic response to these three growth factors. Dermal fibroblasts (Figure 3A) proliferation was promoted by the three growth factors at different concentrations. In oral fibroblasts (Figure 3B), the three growth factors promoted the cell proliferation at all concentrations.

TGF-β1 expression in transplanted mucosal and cutaneous wounds

Quantitative real-time analysis showed that significantly reduced TGF-β1 mRNA expression levels were found in transplanted mucosal wounds at 12 h and 7 d post injury when compared with cutaneous wounds. The TGF-β1 expression level was also lower at 3 d post injury, but the difference was not significant (Figure 4).

Immunohistochemistry was used to compare the levels of TGF-β1 protein in transplanted mucosal and cutaneous wound tissues. As shown in Figure 5, TGF-β1 staining was much stronger in cutaneous wound (Figure 5A) than transplanted mucosal wounds (Figure 5B) at 12 h, 1 d, 3 d and 7 d after wounding occurred.

TGF-β3 expression in transplanted mucosal and cutaneous wounds

The TGF-β3 mRNA expression was higher at 3 d and lower at 7 d post injury in transplanted mucosal wounds compared with cutaneous wounds. The mRNA expression of TGF-β3 at 1 d and 5 d post injury was similar in two groups.
The immunohistochemistry expression of TGF-β3 was shown in Figure 5. The TGF-β3 protein expression was much higher in transplanted mucosal wounds (Figure 5D) at 1 d and 3 ds post injury and lower at 7 d post injury compared with cutaneous wounds (Figure 5C).

**Figure 4:** The design of mouse wound model and Real-time PCR analysis of three grow factors. (A). Healthy oral mucosa (Tongue tissue). (B). The oral mucosa was obtained. (C). The oral mucosa was transplanted to left abdominal skin. (D). 3 weeks later, the wounds were healed. (E). A line-like full-thickness excisional wound was created on the mucosal tissue site and the right abdominal skin. Real-time PCR analysis of the expression of mRNA for TGF-β1 (F), TGF-β3 (G), and VEGF (H) at different stages of wound healing relative to unwounded tissue in SD rats. **p ≤ 0.01.

**VEGF expression in transplanted mucosal and cutaneous wounds**

VEGF mRNA levels generated in transplanted mucosal wounds remained at lower levels at 5 ds and 7 ds post injury than cutaneous wounds. The VEGF expression level was also lower at 12 h and 3 d post injury, but the difference was not significant (Figure 4).

The VEGF protein expression level by Immunohistochemistry also demonstrated similar results. The levels of VEGF in transplanted mucosal wounds (Figure 5F) were less than those of the cutaneous wounds (Figure 5E) at 12 h, 3 ds, 5 ds and 7 ds post injury.

**Discussion**

Wound healing in oral mucosa is clinically distinguished from dermal healing in terms of both its rapidity and lack of scar formation [6,17,18]. Oral mucosa provides an ideal adult study subject for scarless wound healing and therefore quantities of studies have paid attention to this scarless wound healing process. Studies have suggested that a number of cytokines, growth factors, and protease inhibitors contained in saliva are the primary factor that accounts for rapid oral wound healing [18-21]. Bussi and co-workers reported that skin transposed into the oral cavity maintained its morphologic characteristics, such as keratinization, hair follicles and showed an intense inflammatory reaction on dermis [22]. This suggested that the rapid healing and the absence of scars in oral mucosa are directly related to intrinsic characteristics of the tissue and not to the environmental factors such as temperature, salivary flow, the absence of a hemostatic plug, or micro-flora. In order to reduce the influence of the saliva-containing growth factors and wet environment, we created the present animal wound healing model.

**Figure 5:** Immunohistochemical staining of TGF-β1 (B), TGF-β3 (D) and VEGF (F) in transplanted oral mucosal wounds compared with cutaneous wounds. A: TGF-β1 in cutaneous wounds; B: TGF-β1 in transplanted oral mucosal wounds; C: TGF-β3 in cutaneous wounds; D: TGF-β3 in transplanted oral mucosal wounds; C: VEGF in cutaneous wounds; D: VEGF in transplanted oral mucosal wounds.

The TGF-β cytokine family plays a central role in wound healing and scar formation [23]. In this study we demonstrate a strong up-regulation of TGF-β1 and TGF-β3 expression after injury. Firstly, our results showed that TGF-β1 expression levels were much lower in transplanted mucosal wounds in most time-points post injury. A few studies have compared the expression of TGF-β isoforms in oral mucosal vs. dermal wound healing using mouse models [5,6]. It was found that the steady-state expression of TGF-β1 was lower in early stages of gingival wounds compared with the cutaneous wounds. Studies on fetal scarless wound healing showed that levels of TGF-β1 are low in fetal wounds compared with adult skin wounds. TGF-β1 expression was found higher in the deeper dermal wounds than superficial wounds, which may suggest higher TGF-β1 expression was related to hypertrophic scarring [24]. Our data about TGF-β1 expression in transplanted mucosal wounds are consistent with the previous observations.

Our results showed that the TGF-β3 expression level was higher at 3 d and lower at 7 d post injury in transplanted mucosal wounds compared with cutaneous wounds. The mRNA expression levels of TGF-β3 at 1 d and 5 ds post injury was similar in two groups. In former studies, the level of TGF-β3, however, in the gingival wounds
was three times higher than cutaneous wounds at 24 h post wounding and resulted in a higher TGF-β3 to TGF-β1 ratio in earlier stages of gingival wounds. Increased abundance of TGF-β3 relative to TGF-β1 was suggested to protect the healing wounds from scar formation [5,24,25]. Our result about TGF-β3 expression level was different from former studies. The reason maybe because the transplanted mucosal wounds different from normal oral mucosa because of different environment.

Many short-term studies in wound healing models (mostly rodents) have shown a peak in TGF-β1 expression level in the first 24 hr and, in some instances, a second peak on day 7 post wounding in skin wounds [26]. A recent study showed peak TGF-β1 mRNA expression at 7 days after wounding in the pig skin wounds, whereas expression of TGF-β3 increased at later time points [27]. Our study found a peak of TGF-β1 level in at 12 h and a second peak on day 7 post wounding in skin wounds while in transplanted oral mucosa wounds the TGF-β1 level reached its peak on day 5 post wounding. Former study showed that peak mRNA expression of TGF-β3 was found to be around day 14. Our study showed considerable up-regulation in TGF-β3 mRNA levels when we did not identify the peak expression level of TGF-β3 because of the limited observation time points.

Angiogenesis is another major component of wound repair, and it is thought to facilitate the delivery of oxygen and nutrients to the wound site for use by rapidly proliferating reparative cells. VEGF is the single most significant mediator of wound angiogenesis, and its production stimulates capillary growth to provide adequate nutrients, oxygen, and inflammatory cells. Many studies have found angiogenesis to be beneficial for wound repair. The stimulation of angiogenesis can enhance healing rates [28], while a reduction in angiogenesis can impair healing rates [29]. In contrast, some studies have reported enhanced healing with reduced angiogenesis [12]. Although there are many studies examining the angiogenesis process in wound repair, the degree to which angiogenesis actually facilitates wound healing is still not known. Our findings suggested that VEGF levels generated in transplanted mucosal wounds remained at low levels at most time-points post injury than cutaneous wounds. Our results were consistent with those studies about fetal wounds, which suggested that there are reduced levels of angiogenesis in scarless fetal wounds. Scarless fetal wounds had lower levels of VEGF and were less vascular than fibrotic fetal wounds, and the scarless phenotype could be converted to a scar-forming phenotype by adding exogenous VEGF. Injections with anti-VEGF antibodies resulted in nearly a 75% reduction in scar width.

High levels of VEGF have also been described in keloids in the skin [30,31]. The mechanism by which angiogenesis is regulated in oral tissues in our study remains to be elucidated.

Our in vitro study about cell migration showed that TGF-β1, TGF-β3 and VEGF could promote the migration of dermal fibroblasts. The migration of oral fibroblasts could be promoted by TGF-β1 and TGF-β3, while not promoted by VEGF. Our former study also found that VEGF could promote the migration of dermal fibroblasts [32], while the reports about the effect of VEGF on oral fibroblasts migration were very rare. Our results suggested that oral fibroblasts reacted differently to some growth factors in wound healing process. The in vitro proliferation experiment indicated that both dermal and oral fibroblasts could be promoted by the three growth factors at different concentrations. This was consistent with former studies [33,34].

Conclusion

In conclusion, the in vitro study suggested oral fibroblasts reacted differently to growth factors suggesting their different intrinsic characteristics in wound healing process. Our in vivo research result suggested that adult oral mucosa transplanted onto skin heals differently to natural skin wounds. In order to minimize the transplantation on the incisor wound, the incisor wound was made 3 weeks after transplantation. Our finding suggested that the rapid healing and the absence of scars in oral mucosa are directly related to its intrinsic characteristics and not to environmental factors such as temperature, salivary flow, the absence of a hemostatic plug, or microflora.

Conflicts of Interest

The authors do not have any possible conflicts of interest.

Acknowledgments

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