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Molecular changes in diabetic foot ulcers

Ching-Jen Wang [a,*], Jih-Yang Ko [a], Yur-Ren Kuo [b], Ya-Ju Yang [a]

[a] The Department of Orthopedic Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan
[b] The Department of Plastic and Reconstructive Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan

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ABSTRACT

Aim: This study investigated the molecular changes of extracorporeal shockwave therapy (ESWT) and hyperbaric oxygen therapy (HBOT) in chronic diabetic foot ulcers.
Methods: A cohort study consisted of 39 patients (44 ulcers) in the ESWT group and 38 patients (40 ulcers) in the HBOT group with similar demographic characteristics. The ESWT group received shockwave therapy twice per week for total six treatments. The HBOT group received hyperbaric oxygen therapy daily for total 20 treatments. Biopsy was performed from the periphery of the ulcer before and after treatment. The specimens were immuno-stained, and the positive immuno-activities of vWF, VEGF, eNOS, PCNA, EGF and TUNEL expressions were examined and quantified microscopically.
Results: Significant increases in vWF, VEGF, eNOS, PCNA and EGF expressions and a decrease in TUNEL expression were noted after ESWT ($P < 0.05$), whereas the changes after HBOT were statistically not significant ($P > 0.05$). The differences of vWF, VEGF, eNOS, PCNA, EGF and TUNEL expressions between the two groups were comparable before treatment ($P > 0.05$), however, the differences became statistically significant after treatment ($P < 0.05$) favoring the ESWT group.
Conclusion: ESWT showed significant increases in angiogenesis and tissue regeneration over HBOT in diabetic foot ulcers.

1. Introduction

The international consensus and practical guidelines on the management of chronic diabetic foot ulcers (DFU) suggest multi-disciplinary approaches including control of diabetes, orthotic shoe wear, off loading device, wound care and surgery in selected cases [1–6]. However, treatment of DFU remains challenging because of unsatisfactory results from surgical and non-surgical treatments [5,7]. Many adjunctive therapies are designed to improve the care of DFU including negative pressure wound therapy, ultrasound, recombinant human platelet-derived growth factor-BB (rPDGF-BB), acellular matrix product [7–12]. Recently, extracorporeal shockwave therapy (ESWT) and hyperbaric oxygen therapy (HBOT) were shown effective in burn, acute and chronic wounds and diabetic ulcers [13–18], and the therapeutic benefits were attributed to the improvements in topical blood perfusion and cell activity [13–15]. Despite good clinical results, the exact working mechanisms of ESWT and HBOT in DFU were not thoroughly understood. We hypothesized that the effects of ESWT and

* *Corresponding author at*: The Department of Orthopedic surgery, Kaohsiung Chang Gung Memorial Hospital, 123 Ta-Pei Road, Niao-Sung Hsiang 833, Kaohsiung, Taiwan. Tel.: +886 7 733 5279; fax: +886 7 733 5515.
E-mail addresses: w281211@adm.cgmh.org.tw (C.-J. Wang), kojy@adm.cgmh.org.tw (J.-Y. Ko), kuoyr@adm.cgmh.org.tw (Y.-R. Kuo), yaju01@adm.cgmh.org.tw (Y.-J. Yang).
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HBOT in DFU may be linked to molecular changes in angiogenesis and tissue regeneration. This study investigated the molecular changes after ESWT and HBOT in chronic diabetic foot ulcers.

2. Patients and methods

The Institutional Review Board approved this study (ClinicalTrial.gov, NCT01219127). The inclusion criteria included patients with chronic non-healing diabetic foot ulcers for more than 3 months duration. Exclusion criteria included patients with cardiac arrhythmia or a pacemaker, pregnancy and patients with malignancy.

A cohort study consisted of 39 patients with 44 diabetic foot ulcers in the ESWT group and 38 patients with 40 ulcers in the HBOT group. Both groups showed comparable demographic characteristics. The ESWT group received shockwave therapy twice per week for total six treatments. The HBOT group received hyperbaric oxygen therapy daily for total 20 treatments. The technical details of ESWT and HBOT were described in previous study [15]. Biopsy was performed from the periphery of the ulcer before and after treatment. The specimens were subjected to immunohistochemical analysis. To evaluate the effects of neo-angiogenesis and tissue regeneration, immunohistochemical stains were performed with respective reagents for von Willebrand factor (vWF), vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), proliferating cell nuclear antigen (PCNA), epidermal growth factor (EGF) and terminal deoxynucletidyl transferase mediated UTP nick end labeling (TUNEL). The molecular changes of vWF, VEGF, eNOS, PCNA, EGF and TUNEL expressions before and after treatment were examined and quantified microscopically with immunohistochemical analysis.

2.1. Immunohistochemical analysis

Immunohistochemical analyses were performed to evaluate the effects of ESWT and HBOT on the molecular changes in neo-angiogenesis and tissue regeneration. Specimens were further analyzed with immunohistochemical stains for vWF, VEGF, eNOS, PCNA, EGF and TUNEL. The specimens were fixed in 4% PBS-buffered formaldehyde and embedded in paraffin wax. Specimens were then cut longitudinally into 5-μm thick sections and transferred to poly-lysine-coated slides. Sections of the specimens were immunoassayed with specific reagents for vWF, VEGF, eNOS, PCNA, EGF and TUNEL to identify new vessel formation, cell activities including cell apoptosis (Santa Cruz Biotechnology Inc, CA, USA). Immuno-reactivity in the specimens was demonstrated using a horseradish peroxidase (HRP)-3′-, 3′-diaminobenzidine (DAB) cell and tissue staining kit (R&D Systems, Inc. Minneapolis, MN, USA). Immunoactivities were quantified from five images of the same specimen using a Zeiss Axioskop 2 plus microscope (Carl Zeiss, Gottingen, Germany). All images of each specimen were captured using a Cool CCD camera (SNAP-Pro c.f. Digital kit; Media Cybernetics, Sliver Spring, MD, USA). Images were analyzed using an Image-Pro® Plus image-analysis software (Media Cybernetics, Sliver Spring, MD, USA). The percentage of immuno-labeled positive cells over the total cells in each area was counted and the average results were used for analysis.

Table 1 – The values of vWF, VEGF, eNOS, PCNA, EGF and TUNEL expressions before and after treatment.

		Before treatment Mean ± SD (range)	After treatment Mean ± SD (range)	P-Value-1
vWF	ESWT	13.5 ± 9.2 (0–20)	49.7 ± 10.7 (43–62)	0.038
	HBOT	19.4 ± 9.58 (5–28)	21.17 ± 11.7 (5–30)	0.893
	P-Value-2	0.413	0.024	
VEGF	ESWT	39.3 ± 7.68 (26–50)	65.7 ± 13.5 (36–91)	<0.001
	HBOT	44.1 ± 8.4 (33–55)	45.8 ± 4.1 (35–60)	0.090
	P-Value-2	0.155	<0.001	
eNOS	ESWT	22.4 ± 6.53 (13–36)	54.64 ± 16.3 (17–80)	0.001
	HBOT	27.5 ± 7.6 (24–41)	37.1 ± 22.1 (20–87)	0.400
	P-Value-2	0.149	0.009	
PCNA	ESWT	27.8 ± 10 (14–40)	60.5 ± 21.9 (13-98)	0.001
	HBOT	28.9 ± 8.2 (20–45)	36.7 ± 11.9 (23–68)	0.061
	P-Value-2	0.781	0.001	
EGF	ESWT	29.1 ± 10.5 (11–44)	68.9 ± 11.1 (51-88)	0.002
	HBOT	32.5 ± 13.8 (5–53)	40.8 ± 14.8 (25–72)	0.051
	P-Value-2	0.651	<0.001	
TUNEL	ESWT	67.6 ± 9.1 (49–76)	31.9 ± 16.4 (12–68)	0.005
	HBOT	63.0 ± 12.3 (45–81)	57.6 ± 23.7 (24–95)	0.161
	P-Value-2	0.427	0.001	

vWF, von Willebrand factor; VEGF, Vessel endothelial growth factor; eNOS, endothelial nitric oxide synthase; PCNA, proliferation cell nuclear antigen; EGF, epidermal growth factor TUNEL, transference-mediated digoxigenin-deoxy-UTP nick end labeling; P-value-1, comparison of data before and after treatment within the same group. P-value-2, comparison of data between ESWT and HBOT.

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2.2. Statistical analysis

The data before and after treatment within the same group were compared statistically using a paired *t*-test. The differences between the two groups were compared statistically using Mann–Whitney *U*-test. The statistical significance was set at $P < 0.05$.

3. Results

The results of immunohistochemical analysis for vWF, VEGF, eNOS, PCNA, EGF and TUNEL expressions are summarized in Table 1. The microscopic features of immunohistochemical stains are shown in Fig. 1 for vWF and VEGF, Fig. 2 for eNOS and PCNA and Fig. 3 for EGF and TUNEL expression. Significant increases in vWF, VEGF, eNOS, PCNA and EGF, and a decrease in TUNEL expressions were noted after treatment with ESWT ($P < 0.05$), whereas the molecular changes after HBOT were statistically not significant. The differences of vWF, VEGF, eNOS, PCNA, EGF, and TUNEL expressions between the two groups were comparable before treatment ($P > 0.05$), however, the differences became statistically significant ($P < 0.05$) after treatment favoring the ESWT.

4. Discussion

Wound healing stages include inflammation, proliferation, epithelization, and remodeling. Non-healing wounds occur when this process is interrupted or out of sequence, often the case in diabetic ulcers [5]. At a molecular level, failure of wound healing may result either from deficient supply or functional inhibition of growth factors such as those investigated during this study. The current study demonstrated significant increases in vWF, VEGF, eNOS, PCNA and EGF and a decrease in TUNEL expression after treatment with ESWT as compared to HBOT in chronic diabetic ulcers. It appears that ESWT significantly increased angiogenesis (vWF, VEGF and eNOS) and cell proliferation (PCNA and EGF) and decreased cell apoptosis (TUNEL) leading to tissue regeneration and wound repair. These findings are supported by other studies that ESWT enhanced skin flap survival and diabetic wound healing via increasing angiogenesis and topical blood perfusion in animals [14]. The results of the current study suggest that ESWT may have the ability to improve wound healing by increasing angiogenesis and cell activity in the wound environment, normalizing the rate of apoptosis, and making positive changes to growth factor and cytokine levels. This

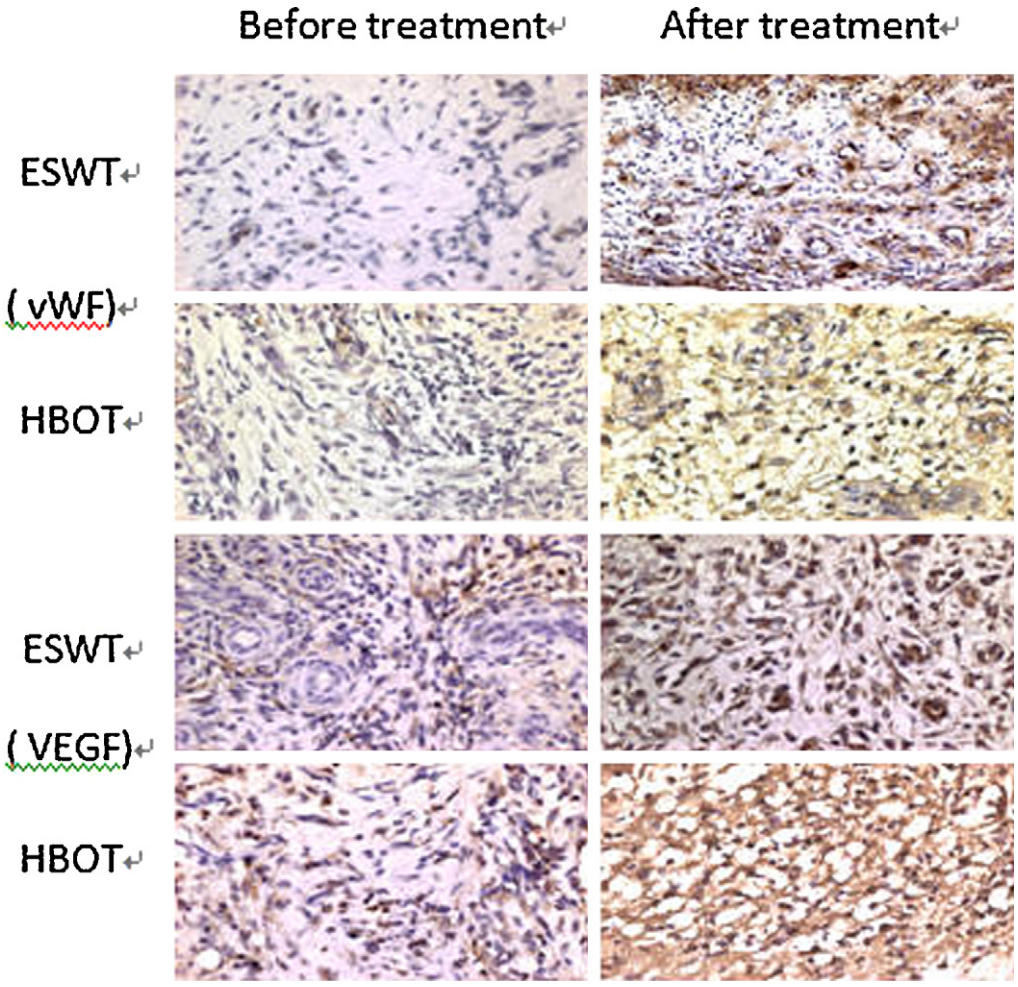

Fig. 1 – Microscopic features of vWF and VEGF expressions showed significant increases after treatment with ESWT (A), whereas the changes after HBOT are not significant (B).

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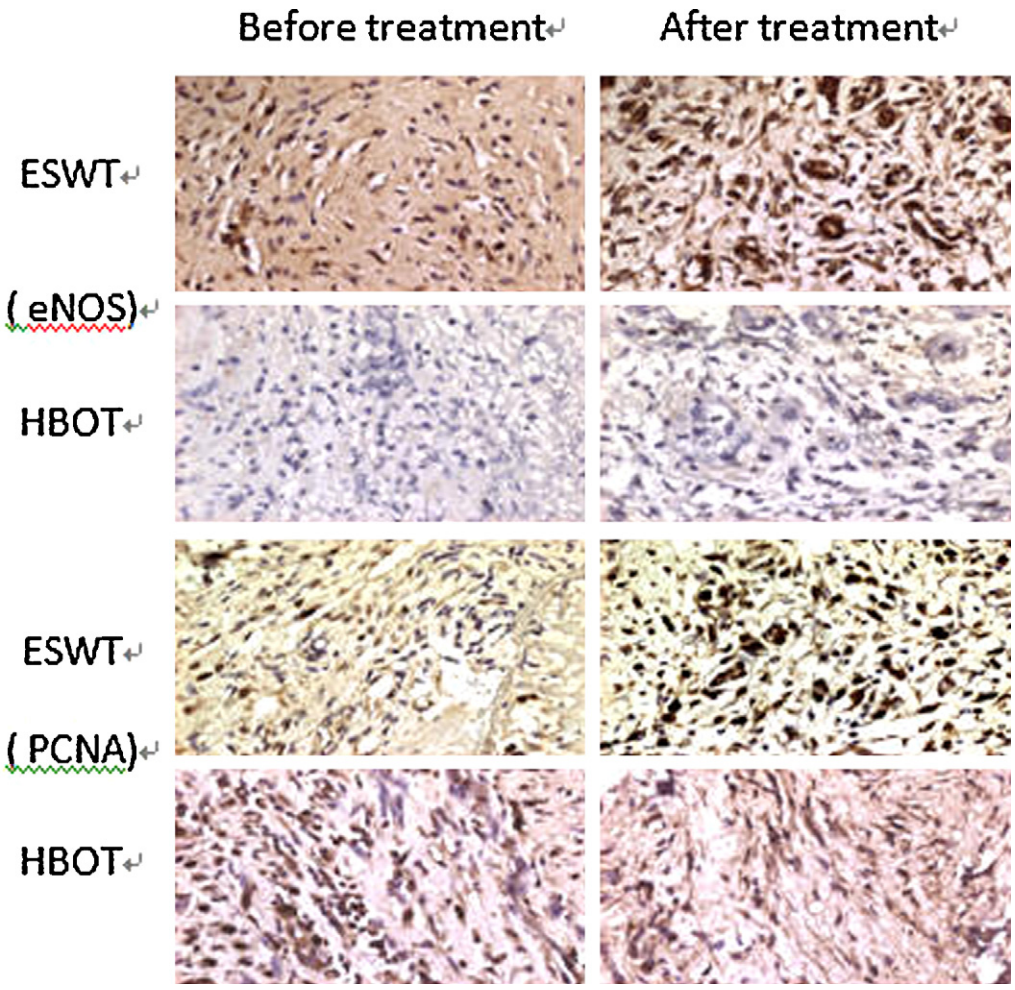

Fig. 2 – Microscopic features of eNOS and PCNA expression showed significant increases after treatment with ESWT (A), whereas the changes after HBOT are not significant (B).

may explain at least in part that ESWT is more effective than HBOT in chronic diabetic foot ulcers [13,15].

The growth factors and cytokines have essential roles in a wound-healing environment [5,19]. An increase in vWF is indicative of new vessel formation. An increase in VEGF is an indication of increased vascular permeability and micro-vascular activity including angiogenic growth of new blood vessels. eNOS is an immune system transmitter and vasodilator associated with wound closure. An increase in PCNA, a precursor to DNA synthesis and tissue repair, indicates an elevation in cellular proliferation assisting wound repair. An increase in EGF is indicative of epithelial cell growth and wound healing. The TUNEL technique detects the rate of cell apoptosis, or cell death. Apoptosis rates are increased in chronic wound beds and necrotic tissues. The TUNEL technique showed a decrease in cell apoptosis following ESWT treatment indicating an increase in cellular viability and a decrease in the rate of cell death.

The mechanism of ESWT in diabetic foot ulcers remains unknown. ESWT is considered to be largely vascular in nature [20–22]. Growth factor deficiency may result from increased protease levels causing degradation of growth factors and extracellular matrix (ECM) components at the wound site [19]. Increasing growth factor level or inhibiting protease activity may be necessary to activate the healing in a persistent chronic non-healing wound [4,5]. The high-energy acoustic waves associated with ESWT causes significant mechanical stresses at a molecular level inducing an inflammatory response that promotes angiogenesis and blood flow into the tissues. Increased angiogenesis and improved blood flow perfusion have a direct effect on ischemic wound that are causative or a contributing co-morbidity in chronic wounds [5,19] Furthermore, an increase in cell activity and a decrease in cell apoptosis are in concert with the wound healing following treatment with ESWT. This immediate reaction is followed by a longer-term healing response that can take weeks or months to achieve full effect. Therefore, the coordination of multiple cellular and molecular events must occur to initiate the growth factor-mediated extracellular matrix (ECM) homeostasis of wound healing.

The exact mechanism of HBOT remains poorly understood. Some studies reported that HBOT has important effects on the

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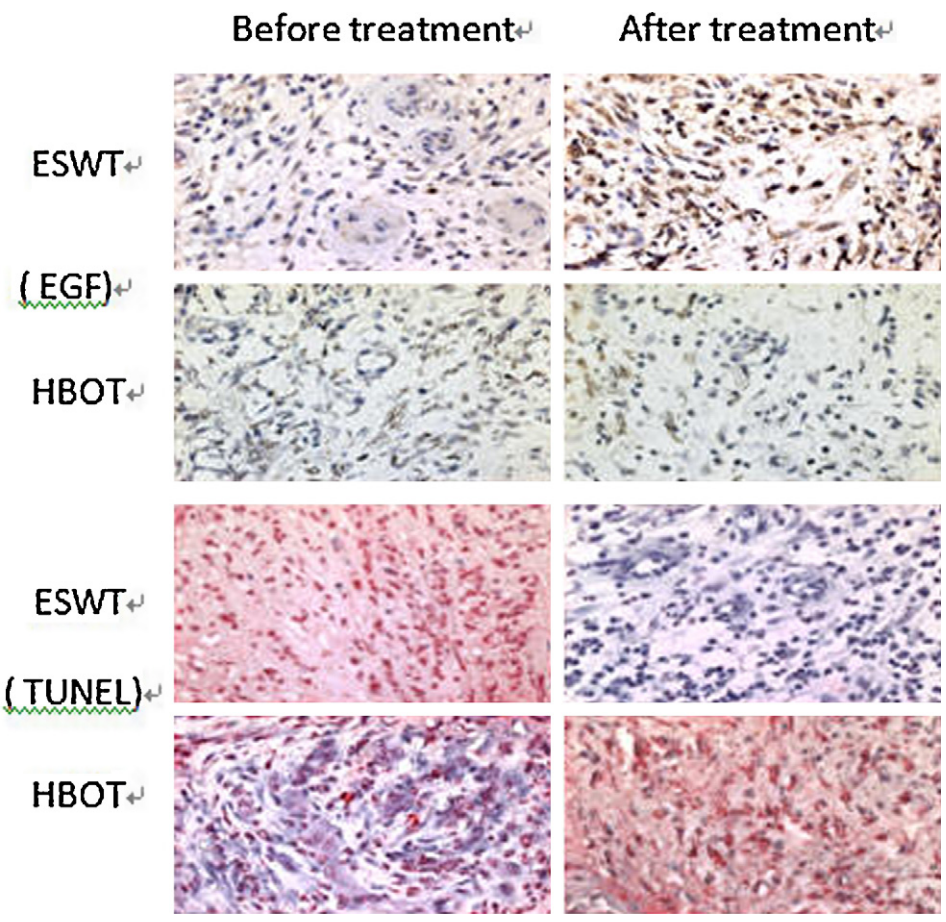

Fig. 3 – Microscopic features of EGF expression showed significant increases after treatment with ESWT (A), whereas the changes after HBOT are not significant (B). The TUNEL expression showed significant decrease after treatment with ESWT (A), whereas the changes after HBOT are not significant (B).

biology of cytokines and other mediators of inflammation [23]. HBOT causes cytokine down-regulation, growth factor up-regulation, and suppresses stimulus-induced pro-inflammatory cytokine production and affects the liberation of TNF-α (tumor necrosis factor alpha) and endothelins [24–29]. VEGF levels are significantly increased with HBOT, whereas the values of PGE2 (prostaglandin E2), COX-2 (cyclo-oxygenase 2) and mRNA expressions are markedly reduced. Therefore, cytokines, prostaglandins (PGs), and nitric oxide (NO) may play a major role in the mechanism of action of HBOT [28–30].

Several limitations to this study should be noted. This study is limited by virtue of a small number of patient population that may create a low power of statistical analysis. Many factors and adjunctive therapies have been advocated to improve the healing of chronic diabetic foot ulcers. This study only investigated the molecular changes after ESWT and HBOT. Furthermore, there is no control group in this study and the natural course of diabetic ulcers is unknown. Additional studies are needed to verify the molecular changes of the control group and after other modalities of treatment.

In conclusion, ESWT demonstrated molecular changes with significant increases in angiogenesis and tissue regeneration over HBOT in chronic diabetic foot ulcers.

Contributions

Ching-Jen Wang, M.D. participated in the study with primary responsibility in conception and design drafting, overview the entire study, and data collection and analysis, literature review, reference search, draft writing and critically revised the manuscript and read proof of the final manuscript.

Jih-Yang Ko, M.D. participated in the study with primary responsibility in patient recruitment, reference search and read proof the final manuscript.

Yur-Ren Kuo, M.D., PhD. participated in the study with primary responsibility in patient recruitment, data collection, reference search, literature review and read proof the final manuscript.

Ya-Ju Yang participated in the study with primary responsibility in assessment of the ulcers, shockwave application, and immunohistochemical stains and. read proof the final manuscript.

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Conflict of interest

The authors declared that they have no conflict of interest. One author (CJW) has served as a member of the scientific advisory committee of Sanuwave until Nov. 2010. The remaining authors declared no conflict of interest.

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