Co-Transplantation of Endothelial Progenitor Cells and Mesenchymal Stem Cells Promote Neovascularization and Bone Regeneration

Hadar Zigdon-Giladi, DMD, PhD;* Tova Bick, PhD;† Dina Lewinson, PhD;† Eli E. Machtei, DMD‡

ABSTRACT

Background: Bone formation relies on sufficient blood supply and osteoprogenitor cells.

Purpose: The study aims to evaluate the influence of endothelial progenitor cells (EPCs) in combination with mesenchymal stem cells (MSCs) on early vascularization and intramembranous bone regeneration.

Materials and Methods: Vertical bone regeneration was tested in rat calvarium guided bone regeneration model. Gold domes were filled with a mixture of 5×10^5 osteogenic transformed MSC and 5×10^5 EPC (EPC/MSC) that were mixed with β-tricalcium phosphate (βTCP) scaffold. Domes filled with βTCP alone served as control. Rats were sacrificed after 4 or 12 weeks. Histomorphometry was used to determine blood vessel (Bv) density, vertical bone height, and bone area in the regenerated tissue.

Results: At both time points, new augmented hard tissue filled the space under the dome, and Bv density was higher in the EPC/MSC transplanted group vs control. However, bone height and bone area were similar among the groups 4 weeks posttransplantation, but were doubled in the EPC/MSC transplanted group 12 weeks posttransplantation.

Conclusions: EPC/MSC transplantation increases Bv formation in the early stages of healing that precedes enhancement of extracortical bone regeneration in later stages.

KEY WORDS: bone regeneration, bone tissue engineering, cell therapy, endothelial progenitor cells, mesenchymal stem cells, neovascularization

INTRODUCTION

Extracortical bone formation is a challenging task, with a high and increasing clinical demand. Several surgical approaches were offered and tested to enhance vertical bone regeneration. Those methods are technique sensitive, have high complication rates, and their results are inconsistent and unpredictable.[1] Therefore, in areas of severely resorbed alveolar bone, short dental implants are still recommended.[2] During the last decade, a major progress has been made in the field of bone regeneration: cell-based biotechnology studies that combine osteoconductive scaffolds, growth factors, or cells are carried out.[3,4]

One prerequisite for tissue regeneration is a readily available population of proliferating, differentiating, and migrating cells.[5] In adults, these stem or progenitor cells include mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs). As MSCs are able to differentiate into osteoblasts (in vivo and in vitro), they are considered as the principal cells that are responsible for the osteogenesis process.[6] Moreover, the capacity of MSC to secrete growth factors, proteins, and cytokines designate MSCs among the most suitable tools for paracrine contribution for bone regeneration.[7] Several

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DOI 10.1111/cid.12104

preclinical studies showed that MSC transplantation enhanced bone formation.[8,9] Clinical studies also confirmed that MSC transplantation resulted in augmented bone formation in maxillofacial surgery and orthopedic bone augmentation procedures.[10,11]

A major impediment in bone augmentation is inadequate blood supply to the bone graft. The potential of vasculature formation within tissue-engineered grafts depends on various factors: scaffold design, vasculogenic potential of cell types, and mechanostimulation on cells to enhance cytokine expression.[12] It was previously reported that peripheral blood-derived EPCs are able to initiate neovascularization.[13] These cells represent a small population with the capacity to proliferate, migrate, and differentiate into cells that line the lumen of blood vessels (Bv).[14] In our previous experiments and in other preclinical studies, local transplantation of EPC improved healing of segmental bone defect.[15,16] Recently, for the first time, a clinical trial using blood-derived progenitor cells healed a tibial nonunion fracture.[17]

In our previous studies we examined bone formation following single-type cell transplantation: EPC, MSC, and osteogenic-transformed MSC (otMSC). As expected, cell transplantation stimulated bone formation, while best results were obtained by otMSC and EPC. As EPC and MSC promote bone formation in different and complementary pathways, we hypothesize that co-transplantation of these cells will further enhance extracortical vertical bone regeneration. Early vascularization of the scaffold will be provided by EPC while MSC will contribute by differentiating into osteoblasts and recruit host EPC and MSC to the regenerated site. The main goal of this study was to enhance extracortical bone formation by combined transplantation of EPC and otMSC . In addition, we evaluated the in vivo neovascularization at early and late phases of intramembranous bone regeneration.

MATERIALS AND METHODS

The experimental procedures were approved by the committee for the supervision of animal experiments at the Faculty of Medicine, Technion (I.I.T.) no. IL0530412.

Isolation, Expansion, and Characterization of Peripheral Blood EPC

Pooled peripheral blood (20–30 mL) were obtained from the heart of five male Lewis inbred rats. Blood was collected into sterile heparinized tubes and EPCs were isolated and cultured as previously described.[15,18] Tube formation was observed following seeding on Matrigel-coated plates and cells were also characterized by flow cytometry (flow cytometry analysis) analysis.[15,18]

Isolation, Expansion, Characterization, and Osteogenic Differentiation of Rat Bone Marrow MSC (bmMSC)

Bone marrow was flushed out from tibiae that were removed from five Lewis inbred male rats and pooled. Isolation, culture, and characterization were previously described.[8] For osteogenic differentiation, 70% confluent MSCs were grown for 14 days in Dulbecco's modified Eagle's medium osteogenic differentiation media containing dexamethasone 10^{-7} M, ascorbic acid 5×10^{-5} M, and β-glycerophosphate 10^{-2} M (Sigma Chemical Co., St. Louis, MO, USA).

Coating of βTCP with Fibronectin

In accordance with the results obtained in our previous study,[19] β-tricalcium phosphate (βTCP) was used as scaffold for the present study. To enable attachment of cells, on the day of surgery, βTCP granules (Poresorb-TCP®, Lasak Ltd, Prague, Czech Republic) were coated with fibronectin as described by Seebach and colleagues.[16] Briefly, for each rat, 0.2 g βTCP granules was placed as a dense monolayer in each well of a 24-well plate, mixed with 50 μg fibronectin and incubated for 30 minutes at 37°C.

Cell Transplantation

Male Lewis rats (300 g) were anesthetized by intramuscular injection of 100 mg/kg body weight (bw) Ketamin (Ketaset, Fort Dodge, IA, USA) and 5 mg/kg bw Xylasin (Eurovet, Cuijk, Holland). Cephalexin (50 mg/kg) bw (Norbrook Laboratories, Newry, BT35 6QQ, Northern Ireland) and 0.3 mg/kg bw Boprenorphine (Vetamarket-Petach tikva, Israel) were injected s.c. preoperatively and 3 days postoperation. Surgical procedure was performed as previously described.[8,18] Briefly, a U-shaped incision served to raise a full-thickness skin flap and exposure of the parietal bone. Five perforations (1 mm diameter) of the cortical bone were performed to allow passage of blood, cells, and nutrients from the bone marrow into the space under the dome. Just prior to transplantation, 1:1 mix of 5×10^5 EPC and 5×10^5 otMSC suspended in 50 μL

endothelial basal medium (EBM-2) (EPC/MSC, $n = 10$) or 50 μL EBM-2 (control, $n = 10$) were mixed with 0.2 g fibronectin-coated βTCP particles and filled rigid gold domes (7 mm radius, 5 mm height). The domes were secured to the calvarium using fixation screws. The flaps were repositioned and sutured. Each rat was kept in a separate cage and fed rat chow and water ad libitum for 4 weeks ($n = 2$ for each group) or 12 weeks ($n = 8$ for each group). Then rats were sacrificed by CO_2 asphyxiation and the domes were removed. The part of the calvarium surrounding the regenerated area was sawed out and specimens were fixed immediately in 10% neutral-buffered formalin for 2 days and analyzed by histology and histomorphometry.

Histological and Histomorphometric Analysis

Specimens were decalcified in 10% EDTA (Sigma-Aldrich, MS, USA) for 3 weeks, cut in half at the midline, embedded in paraffin, and sectioned (5 μm). For determination of bone morphology, sections were stained with hematoxylin and eosin H&E. Four stained sections (~20 μm apart) from each specimen were captured by a digital camera (Olympus DP70, Olympus, Tokyo, Japan) with a calibration scale and analyzed morphometrically using image *J* software (NIH, Bethesda, MD, USA). Three parameters were measured: (1) vertical bone height: maximal bone height (in mm) measured form the base of the calvarium to the crest of the newly formed bone; (2) bone area: area of newly formed bone in the augmented tissue under the dome (above the original calvaria); (3) Bv density: paraffin-embedded serial sections were stained with H&E. Luminal structures perfused with red blood cells were identified as Bv. Bv density was calculated by dividing the total number of Bv by the area (260×444 μm) of each section (vessels/mm^2). Ten sections were evaluated for each specimen by two blinded examiners.

Statistical Analysis

StatPlus® (AnalystSoft, Vancouver, BC, Canada) and JMP 10.0 (SAS Institute, Cary, NC, USA) statistical packages were used. Descriptive statistics which included means and medians, ranges, and standard error were initially tabulated. After testing for normality and equal variance, differences between mix and control groups were analyzed using *t*-tests. A significance level of $p < .05$ was used.

RESULTS

All rats survived the surgeries without serious surgical and postsurgical complications. In all cases, new tissue filled the space under the gold dome.

In Vivo Neovascularization Increase following EPC and MSC Co-Transplantation

The effect of EPC/MSC on neovascularization was examined 4 weeks (early bone formation) and 12 weeks (late bone formation) posttransplantation. Intergroup analysis showed that EPC/MSC transplantation significantly increased neovascularization in both time points. Bv density was elevated by 75% compared with control (EPC/MSC 4.35 ± 0.38 vs control 2.41 ± 0.3 Bv/mm^2, $p \leq .0001$) at the early stage of bone formation; and to a lesser extent, only by 33% at the late stage of bone regeneration (EPC/MSC 3.73 ± 0.21 vs control 2.64 ± 0.37 Bv/mm^2, $p \leq .01$, compared to control) (Figure 1).

Bone Formation Enhancement following EPC and MSC Co-Transplantation

Histological analysis revealed that the newly formed bone originate from the calvaria (Figure 2). Islands of new bone raised in a vertical direction between tricalcium phosphate (TCP) residues up to the top of the dome (Figures 2 and 3). New vessels were observed in close proximity to these islands of new bone as well as in areas remote from the original calvaria. Osteoblasts and osteoclasts were also detected next to new bone and residual scaffold (Figure 3).

Histomorphometric analysis 4 weeks following surgery (at early bone formation) showed similar vertical bone height in EPC/MSC and control groups (range 1.2–1.8 mm). Vertical bone height increased from 4 weeks to 12 weeks in both groups. However, significant differences in bone measurements were observed between the groups at 12 weeks: vertical bone height was higher in the EPC/MSC group (3.75 ± 0.34 mm) compared with control (1.8 ± 0.25 mm) $p \leq .006$. Bone area was also higher in the EPC/MSC group (11.33 ± 1.33 mm^2) compared with control (6.11 ± 1.56 mm^2) $p \leq .02$ (Figure 4).

DISCUSSION

In this study we enhanced extracortical bone regeneration by co-transplantation of otMSC and EPC mixed with TCP scaffold, in a rat calvaria GBR model. We also

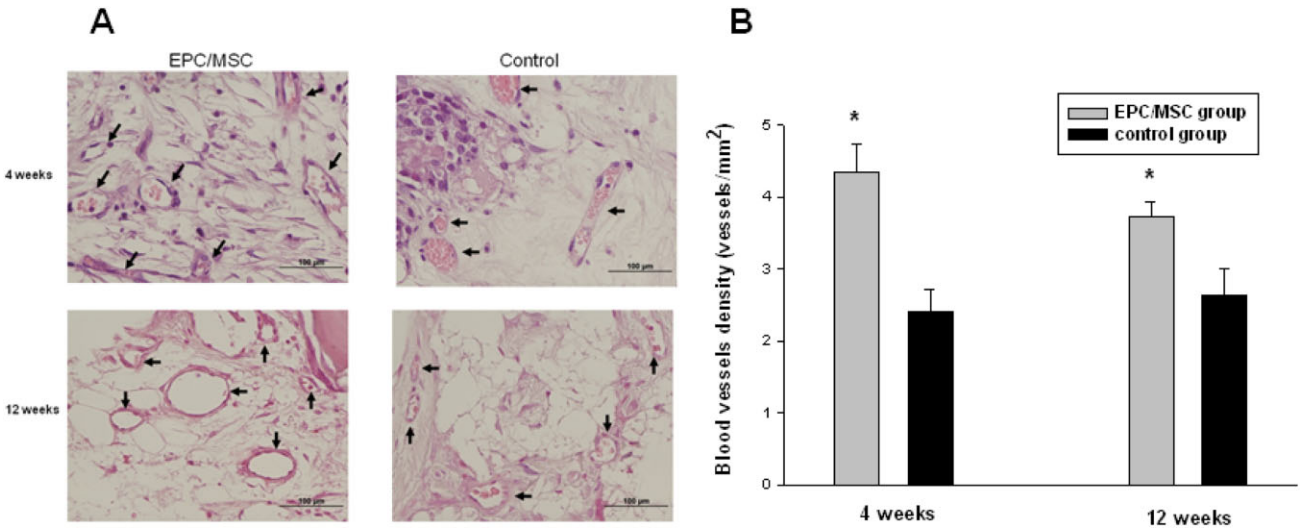

Figure 1 *A*, Representative (H&E) blood vessel density of EPC/MSC (left compared to control (right) groups, at 4 weeks (upper panels) compared to 12 weeks (lower panels) posttransplantation. Black arrows point at blood vessels. *B*, Blood vessels density, (*$p \leq .05$ control vs EPC/MSC group). EPC, endothelial progenitor cell; H&E, hematoxylin and eosin; MSC, mesenchymal stem cell.

showed that transplantation of EPC and MSC significantly increased neovascularization at an early phase of intramembranous bone regeneration.

In a previous study, we have established a GBR model in rat calvaria and compared extracortical bone formation following transplantation of four commercially used scaffolds under a rigid dome. As TCP showed the best results (low inflammation and the highest bone regeneration), it was used in our following studies.[8,18] As TCP allowed only minimal bone regeneration, we added bmMSC, otMSC, or EPC to the TCP in order to further improve bone regeneration. As we expected, following cell transplantation, extracortical bone formation was significantly increased compared with TCP alone. The best results were obtained following transplantation of EPC and otMSC.[8,18]

MSC and EPC contribute to bone regeneration by different mechanisms. Previous studies demonstrated that MSCs participate in bone regeneration directly by differentiating into osteoblasts[20] and indirectly by a paracrine pathway, that is, secretion of growth factors and cytokines.[21]

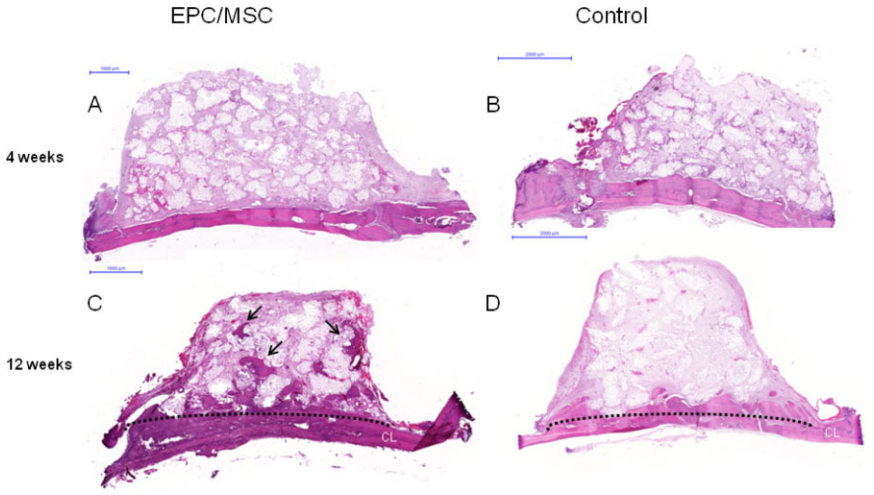

Figure 2 Representative (H&E) histological analysis of EPC/MSC (A, C) and control (B, D) transplanted groups, 4 (A, B) and 12 (C, D) weeks posttransplantation. Newly formed tissue that filled the space under the gold dome is continuous with the original calvaria (CL). The dotted line demonstrates the upper border of the original calvaria. Black arrows pointed at new bone islands. EPC, endothelial progenitor cell; H&E, hematoxylin and eosin; MSC, mesenchymal stem cell.

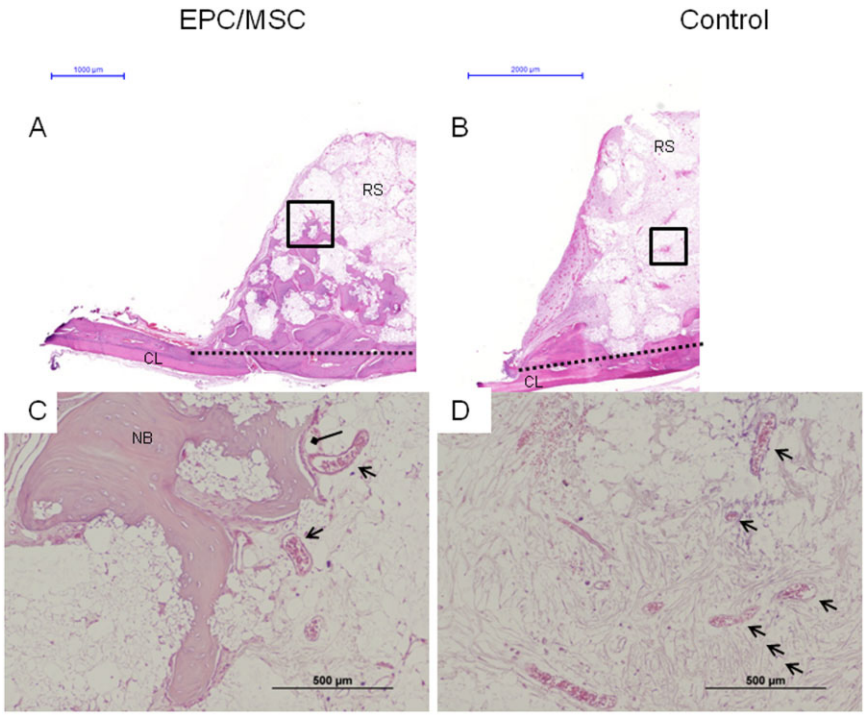

Figure 3 Representative (H&E) histological analysis of EPC/MSC (A) and control (B) transplanted groups 12 weeks posttransplantation. Newly formed tissue that filled the space under the gold dome is continuous with the original calvaria (CL). The dotted line demonstrates the upper border of the original calvaria. (C) and (D) present a higher magnification of the square region in (A) and (B), showing a highly vascularized connective tissue. Flat black arrows point at blood vessels, square black arrow points at palisading osteoblasts. EPC, endothelial progenitor cell; H&E, hematoxylin and eosin; MSC, mesenchymal stem cell; NB, new bone; RS, residual scaffold.

It is well documented that angiogenesis is a prerequisite for bone regeneration. EPCs contribute to bone regeneration by vasculogenesis and angiogenesis as well as by a paracrine effect: EPCs secrete cytokines such as vascular endothelial growth factor that act as prototypical angiogenic growth factors that mobilize bone marrow-derived EPC of the host,[22–24] which in turn contribute to postnatal neovascularization via vasculogenesis.[25] EPC may additionally contribute to bone formation by differentiating into osteogenic cells.[5]

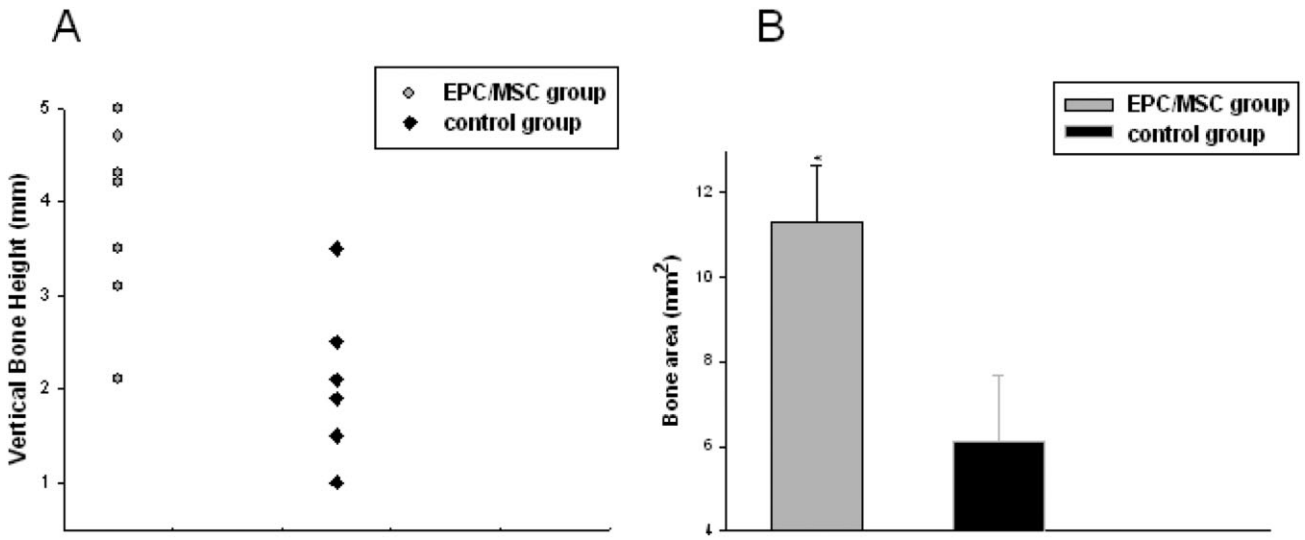

Figure 4 Comparison of vertical bone height (*$p \leq .006$ control group vs EPC/MSC group) (A) and bone area (*$p \leq .02$ control vs EPC/MSC group) (B) 12 weeks posttransplantation. EPC, endothelial progenitor cell; MSC, mesenchymal stem cell.

In this study we found that 1 month postcell transplantation, early vascularization of the newly formed tissue was significantly increased. Three months postcell transplantation, at the late phase of bone regeneration Bv counts were still higher compared with nontransplanted controls, but the differences were less than in the early phase of bone formation. These results are in accordance with previous studies that showed that both EPC and MSC augment Bv formation and reperfusion of ischemic sites.[5,26,27] Additionally, it highlights the role of EPC/MSC in vascularization in the early stages of bone formation.

In order to evaluate the effect of EPC/MSC transplantation on bone regeneration, bone area fraction and bone height were measured. One month posttransplantation, both parameters were not different in EPC/MSC transplanted and nontransplanted controls. However, EPC/MSC doubled bone height and bone area fraction (compared with TCP) at 3 months posttransplantation, similar to the results obtained in our previous studies following transplantation of EPC, otMSC, or bmMSC. We assume that because MSC and EPC contribute to bone formation in different pathways, their co-transplantation will be adjuvant. Unexpectedly, bone height and bone area fraction measurements at 3 months were almost the same in the co-transplanted group compared with single-type cells transplantation.[8,18]

We suggest several explanations to these results: The endogenous neovascularization might be sufficient to sustain osteogenesis. Similar results were obtained by Koob and colleagues[28] in a rat calvaria model, which found increased neovessels formation without significant increase in bone regeneration following co-transplantated human umbilical vein endothelial cells (HUVECs) and bmMSC compared with transplantation of bmMSC. Conversely, Seebach and colleagues[16] and Pang and colleagues[29] demonstrated synergistic effect on bone regeneration when EPCs/MSCs were co-transplanted in a rat long bone critical-sized model. These differences might be attributed to several factors: different bone ossification models (membranous ossification of the calvaria vs endochondral ossification of long bone) and different sub-populations of EPC that were transplanted (early vs late vs mature endothelial cells). It was also suggested that culture conditions influence the osteogenic and vasculogenic potential following cell transplantation. In an recent in vitro novel study, a dynamic culture system of EPC/MSC has been explored for optimization of mineralization and vascularization of a xenograft scaffold.[30] The results showed that dynamically cultured EPC/MSC constructs generated greater mineralization and calcium deposition compared with static conditions.

CONCLUSIONS

The results of this study demonstrate that co-transplantation of EPC/MSC strongly stimulates neovascularization especially at early phase of bone formation, 1 month following transplantation. This increase in neovascularization was followed by significant enhancement of bone formation 3 months post-EPC/MSC transplantation.

ACKNOWLEDGMENTS

We would like to thank Dr. Margarita Filatov and Shami Ivanka for their assistance with cell characterization.

FUNDING SOURCES

This study was supported by Kamin grant 46293, Ministry of Industry Trade and Labor, Israel, and Ofakim grant, Rambam Health Care Campus, Haifa, Israel.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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