Original Article *Iran J Ped Hematol Oncol. 2021, Vol 11, No 3, 172-182*

The Effect of Platelet Lysate on Expansion and Differentiation Megakaryocyte Progenitor Cells from Cord Blood CD34$^+$ enriched Cells

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Received: 14 December 2020 **Accepted:** 09 June 2021

Abstract

Background: Umbilical cord blood hematopoietic stem cells (UCB-HSCs) are an attractive source for transplantation. The generation of megakaryocyte-committed cells could lead to shorten period of thrombocytopenia after HSCs transplantation. Platelet lysate (PL) unlike fetal bovine serum (FBS) can prevent immune problems as well as avert transmission of certain diseases to the recipient. In this study, the authors aimed to assess the effect of PL on UCB CD34$^+$ cells expansion and megakaryocyte differentiation.

Materials and Methods: In this experimental study, PL prepared and the subsequent isolation of UCB CD34$^+$ cells were done by magnetic cell sorting. The isolated cells were cultivated in Iscove's Modified Dulbecco's medium (IMDM) supplemented with PL or FBS. Cell expansion was evaluated using Trypan blue. Furthermore, Flow cytometry using monoclonal antibodies (CD41-FITC and CD42b-PE) and the expression of specific genes including GATA1, GATA2, FLI1, NFE2, and RUNX1 via real-time PCR were performed to evaluate the megakaryocyte differentiation.

Results: The results showed that PL insignificantly enhanced UCB CD34$^+$ cell expansion (32.83± 8.47 fold in FBS and 41.67± 10.31 fold in PL containing media). Besides, flow cytometry results showed that expression of CD41 was increased markedly (37.81± 4.78 fold in FBS and 45.78 ± 7.37 in PL containing media, P-value <0.05) but the elevation of CD42b (10.53 ± 2.13 and 13.20 ± 2.06 in FBS and PL containing media, respectively) was not significant (P-value = 0.051). The results of real-time PCR demonstrated a notable increase in GATA binding protein 1 (1.58, P-value <0.01), GATA binding protein 2 (2.45, P-value <0.001), RUNX family transcription factor 1 (1.60, P-value <0.01), Fli-1 proto-oncogene (1.87, P-value <0.001) in PL supplemented media, however, the increase of Nuclear Factor-Erythroid 2 gene expression was not significant in PL supplemented media (P-value = 0.11).

Conclusion: PL improved UCB CD34$^+$ cells expansion and megakaryocyte differentiation compared to FBS.

Keywords: Cord Blood Stem Cell Transplantation, Hematopoietic Stem Cells, Megakaryocyte Progenitor Cells, Platelet-Derived Growth Factor

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[DOI: 10.18502/ijpho.v11i3.6563]

Introduction

Hematopoietic stem cells (HSCs) are a small population of nucleated cells that have the potential to differentiate into mature cells of all hematopoietic lineages and self-renewal capacity for maintenance of HSC pool in bone marrow (BM) (1, 2).

HSCs can be separated from various sources such as BM or mobilized peripheral blood (MPB) progenitor cells and umbilical cord blood (UCB) for the treatment of hematologic disorders (3, 4). Among different sources of HSCs, UCB is an attractive source for transplantation due to superior proliferative capacity, low risk for

transmission of pathogenic agents, low incidence of graft versus host disease (GVHD), relative availability, and ease of isolation (5). HSCs number in a single cord blood (CB) unit is acceptable for hematopoietic reconstruction in children. However, UCB-HSCs have some disadvantages, for instance, the HSCs number in a single CB unit is insufficient for an adult patient so this can lead to a delayed or failed reconstruction of the hematopoietic system (5, 6). UCB-HSCs are immature, therefore, more cell divisions are needed before differentiation. Following UCB transplantation, it could delay platelet recoveries (6). Allogenic platelet transplantation after HSCs transplantation has some disadvantages including bacteremia, alloimmunization, high-cost, and febrile reactions. Using megakaryocyte progenitor along with the immature hematopoietic stem/progenitor cells (HSPCs) during the first few months after transplantation can support short-term platelet production (7, 8). Cultivation of HSCs and generation of megakaryocyte-committed cells could lead to rapid cell reconstitution and shorten the period of thrombocytopenia after HSCs transplantation (6, 7). Differentiation of cells toward the megakaryocyte-platelet linage requires the expression of specific glycoproteins such as CD41 (glycoprotein IIb), CD42b (glycoprotein Ib), and CD61 (glycoprotein IIIa) (9-12). These glycoproteins are involved in platelet aggregation/function and facilitate the identification and isolation of megakaryocyte cells (13). Cell culture is required for cell proliferation in vitro. Some mediums are serum-free which have some disadvantages including high cost and need a protein/growth factor supplement, and some others, need supplementation with animal or human serum (14, 15). In cell culture, fetal bovine serum (FBS) supplement has been considered as the gold standard media, however, clinical use of FBS has some limitations including transmission possibility of pathogens, zoonotic contamination (especially prions, bovine diarrhea virus, mycoplasma, creutzfeldt Jacob virus, viruses, and parvovirus), high cost, xenogeneic immune reactions, antibodies production against non-native proteins, inflammation, production of non-homologous immune response (which leads to the transplant rejection), batch-to-batch variation, fetus scarification during sample collection, and ethical issues regarding the animal welfare (15-19). Due to the drawbacks of serum-free culture medium as well as animal serum, it is imperative to search for an alternative human resource. These alternatives are autologous and allogeneic serum albumin, human serum, umbilical cord blood serum, plasma derivative material, platelet derivatives, and acellular Wharton's jelly (15, 20). A high concentration of growth factors available in platelet lysate (PL) makes it a proper replacement for animal serum in cell culture (21). PL granules, specially α-granules, release various cytokines and growth factors including chemokine ligand 2(C¬XCL2), CXCL4 (PF4), CXCL10, CXCL12 (SDF-1), CXCL1, CXCL3, CXCL5, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), connective tissue growth factor (CTGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), and basic fibroblast-derived growth factor (b-FGF). Compared to FBS, the promotion of proliferation and differentiation of cells observed in PL may be due to the abundance of growth factors and cytokines with synergic effect (21-24). In the current study, the effect of PL as a substitute for PBS on the HSCs culture and megakaryocyte differentiation has been investigated.

Materials and Methods

PL procurement

In this experimental study, PL preparation was performed via freeze/thaw protocol

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[DOI: 10.18502/ijpho.v11i3.6563]

from platelet bags obtained from the Iranian Blood Transfusion Organization. Briefly, 10 bags of platelet (isolated from peripheral blood) were collected, and PL was prepared by freeze/thaw protocol. The pooled platelet bags were frozen at -80°C/24 hour and subsequently thawed three times at 37 °C to release PDGFs available in platelets. Then, bags were centrifuged at 4000 g/15 min at 4 °C, and to isolate leukocytes along with platelet fragments, the supernatant was filtered by 0.2µm filter. For future use, PL was stored at -80°C (25).

UCB CD34+ cell selection

After receiving informed consent, CB samples were obtained from Al-Zahra hospital (Tabriz, Iran) in a heparinized syringe from full-term deliveries. Research grade Ficoll–Paque (Bahar Afshan, Iran) was used for separating mononucleated cells (MNCs) within 8 hours after delivery. Then, immuno-magnetic microbead and MiniMACS columns (Miltenyi Biotech, USA) were used for the positive selection of $CD34^+$ cells with a purity above 95%. Finally, cells were counted and cell viability was assessed (cells viability>95%) by Trypan Blue 0.4% solution (Sigma, USA).

UCB CD34+ cell culture

To determine the PL effects on megakaryocyte differentiation and expansion of UCB $CD34^+$ cells, UCB $CD34^+$ cells were cultured as described earlier with minor modifications (26). Briefly, purified UCB $CD34^+$ cells cultured in Iscove's Modified Dulbecco's medium (IMDM, Gibco, USA) supplemented with 10% FBS (FBS-IMDM) or PL (PL-IMDM), 20 ng/ml thrombopoietin (TPO), 50 ng/ml stem cell factor (SCF), 20ng/ml Interleukin (IL)-6 and 5ng/ml IL-3 in 6-wells plates. The cells were incubated in 5% CO_2 humidified air at 37 °C for 8 days and the culture medium was exchanged on day 4.

Cell expansion assay

To evaluate the UCB $CD34^+$ cells expansion, the culture was collected and the cells counted using the Trypan Blue (Gibco, USA) on day 8. To determine expansion fold, counted viable cells on day 8 were divided by the cells seeded on the first day.

2.5 Immunophenotypic marker analysis

Megakaryocyte lineage markers were evaluated by the flow cytometry (FACSCalibur, Becton Dickinson, US) on day 8 in both culture conditions. Cells were incubated with monoclonal antibodies including CD41-FITC, and CD42b-PE (both from Beckman Coulter, France) for 20 minutes at room temperature. BD FACSCalibur flow cytometer (BD Biosciences, USA) was used to conduct immunophenotyping and the subsequent analysis was carried out by FlowJo v10 (Tree Star Inc., USA).

Quantitative RT-PCR (qRT-PCR)

The result of PL on megakaryocyte differentiation was evaluated by assessing the expression of specific genes including GATA1, GATA2, FLI1, NFE2, and RUNX1 by real-time PCR. For this purpose, on day 7, total RNA was isolated by RNA isolation kit (Yekta Tajhiz Azma, Iran), and cDNA synthesis was performed using cDNA Synthesis Kit (Takarabio, japan). For GATA1, GATA2, FLI1, NFE2, and RUNX1 genes, qPCR samples were incubated at 95°C for 10 min (pre-incubation) then exposed to denaturation for 40 cycles at 95°C for 5 sec, annealing for 10 sec at 60°C, and extension for 10 sec at 72°C. The specificity of each PCR product was checked by melting curve analysis. PCR efficiency and exponential amplification were assessed by LightCycler software and determined by respective standard curve slopes. To normalize the data, the expression of GAPDH was assessed. Relative gene expression was determined using the following equation:

Relative gene expression = $2^{-\Delta\Delta ct}$

On day 7, UCB $CD34^+$ cells cultured in IMDM supplemented with 10% FBS were

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[DOI: 10.18502/ijpho.v11i3.6563]

the control group and UCB $CD34^+$ cells cultivated in PL-IMDM were the sample groups. Gene-specific primers (Sinaclon, Iran) are shown in Table I.

Ethical Consideration

Ethics approval was awarded by the Tabriz University of Medical Sciences Ethics Committee (Ethics No. IR.TBZMED.REC.1396.1282).

Statistical analysis

GraphPad Prism v8.0.2 scientific software was used to analyze and process the data. Variables were expressed as mean ± standard deviation, and P-value< 0.05 was considered significant.

Results

PL increased UCB $CD34^+$ cells expansion

To investigate the UCB $CD34^+$ cells expansion, on day 8, cells counted and the expansion fold was plotted (Figure 1). The mean fold increase (FI) of UCB $CD34^+$ cells cultivated in two separate mediums containing PL and FBS was 41.67± 10.31 and 32.83± 8.47 fold, respectively. However, the difference of observed fold increase between the groups was not statistically meaningful.

PL enhanced megakaryocyte differentiation

The megakaryocyte lineage marker was evaluated by flow cytometry on day 8 (Figure 2). Figure 2 shows the immunophenotyping results of cells cultivated in two separate medium containing PL and FBS; Figure 2A demonstrates CD41 and CD42b expression in HSCs at day 0, Figure 2B shows CD41 and CD42b expression in cells cultivated in FBS-IMDM at day 8, and figure 2C illustrates CD41 and CD42b expression in cells cultivated in PL-IMDM at day 8. The immunophenotypic result showed that in comparison with FBS-IMDM, megakaryocyte lineage markers including CD41 and CD42b were elevated in cells cultured in PL-IMDM (Figure 3). The increase of CD41 marker was statistically significant (P-value<0.05), however, the increase of CD42b was not significant among cells cultured in the PL-IMDM. Altogether, CD41 and CD42b expression increased in megakaryocyte maturation. Table II shows the percent of cell surface markers expression in both cultured media.

PL enhanced megakaryocyte gene expression

The effect of PL on megakaryocyte gene expression including GATA1, GATA2, FLI1, NFE2, and RUNX1 was evaluated (Figure 4). qPCR results revealed that GATA1, GATA2, FLI1, and RUNX1 gene expression of PL-IMDM cultured cells significantly enhanced compared to FBS-IMDM (P<0.05). The elevated expression level of NFE2 was not statistically notable in PL-IMDM cultured cells.

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Table I: The primer sequences used in real-time PCR.

Target gene		Primer Sequence	Product length (bp)
RUNX1	Fo:	CCGGGAGCTTGTCCTTTTCC	174
	Re:	GTGCTGTGTCTTCCTCCTGC	
NFE2	Fo:	GATCCTCGTCCAGCAGTGTC	109
	Re:	TGGCTCTAGAAACCTGTGGTG	
FLI1	Fo:	CAGGAAGTGGAATTGAGGCTCT	153
	Re:	GTGGGAGGGGGTTGATCTTG	
GATA1	Fo:	CACGACACTGTGGCGGAGAAAT	140
	Re:	TTCCAGATGCCTTGCGGTTTCG	
GATA2	Fo:	CTGTTCAGAAGGCCGGGAG	149
	Re:	TTCGCTTGGGCTTGATGAGT	
GAPDH	Fo:	ACCCATCACCATCTTCCAGGAG	159
	Re:	GAAGGGGCGGAGATGATGAC	

Table II: Expression of megakaryocyte surface markers at day 8 in PL-IMDM or FBS-IMDM cultured cells

Marker	FBS	PL
CD41⁺	37.81± 4.78	45.78 ± 7.37
CD42b⁺	10.53 ± 2.13	13.20 ± 2.06
CD41⁺/CD42b⁺	9.54 ± 2.06	12.10 ± 2.04

IMDM: Iscove's modified Dulbecco's medium; FBS: fetal bovine serum; PL: platelet lysate; Data are presented as mean ± standard deviation. Data are obtained from six independent experiments.

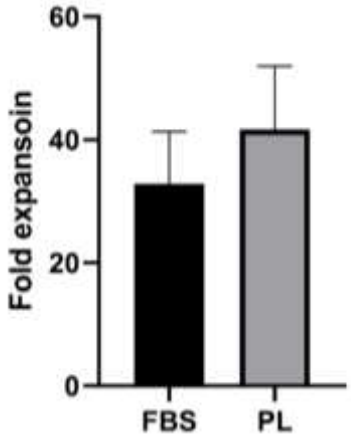


Figure 1. Fold increase in cells cultured in PL-IMDM and FBS-IMDM. HSCs cultivated in PL-IMDM or FBS-IMDM and harvested at day 8 and counted. The study performed in six independent experiments and data are presented as mean ± standard deviation. PL: platelet lysate; FBS: fetal bovine serum; IMDM: Iscove's modified Dulbecco's medium; HSCs: hematopoietic stem cells.

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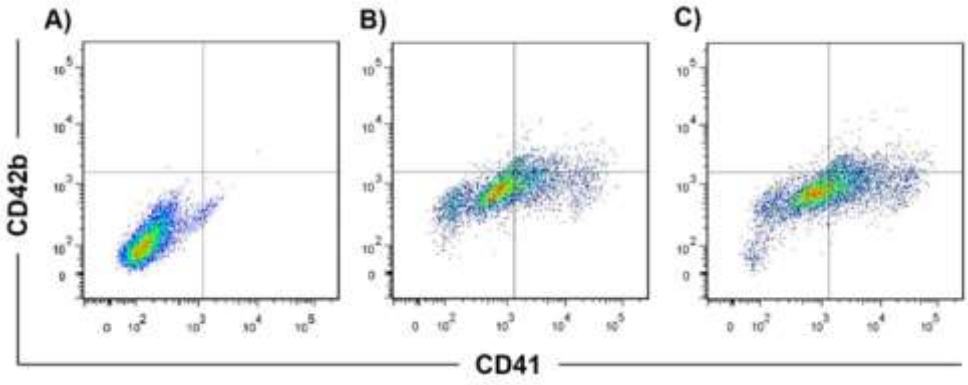


Figure 2. Immunophenotyping results of cells cultured in PL-IMDM or FBS IMDM. A. CD41 and CD42b expressions in HSCs at day 0 B. CD41 and CD42b expressions in cells cultured in FBS-IMDM at day 8. C. CD41 and CD42b expressions in cells cultured in PL-IMDM at day 8. PL: platelet lysate; FBS: fetal bovine serum; IMDM: Iscove's modified Dulbecco's medium; HSCs: hematopoietic stem cells.

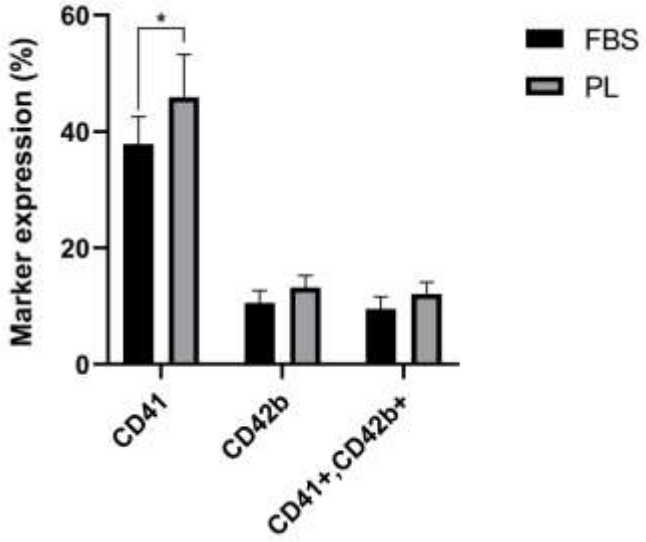


Figure 3. The analysis of flow cytometry on megakaryocyte cluster of differentiation (CD) receptors percentage in PL-IMDM or FBS-IMDM cultured cells. Data are presented as mean ± standard deviation (*P-value<0.05). PL: platelet lysate; FBS: fetal bovine serum; IMDM: Iscove's modified Dulbecco's medium.

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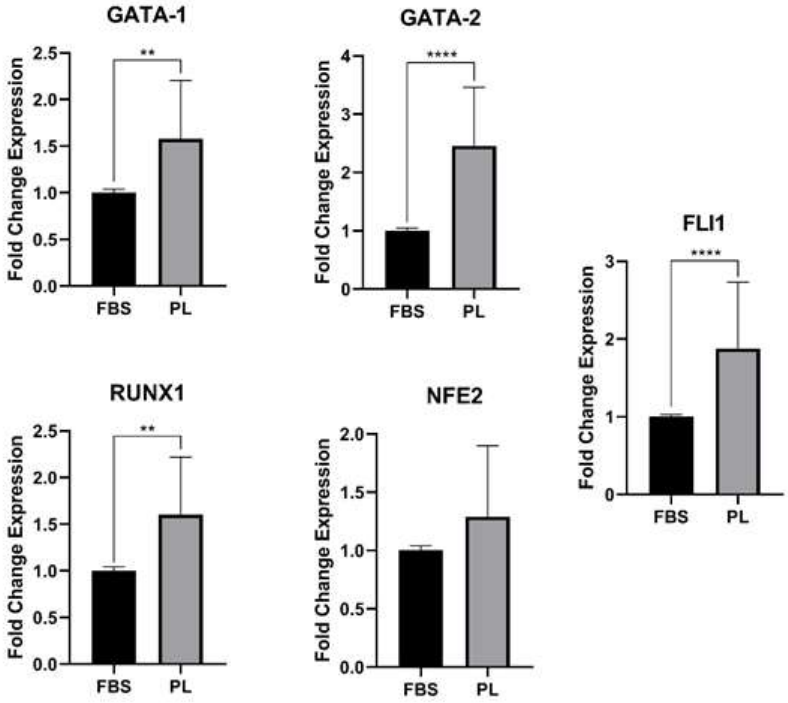


Figure 4. Levels of gene expression on megakaryocytes. Cells were collected after 8 days from PL- or FBS-IMDM. The study consisted of six independent experiments and data are presented as mean ± standard deviation (**P<0.01, ****P<0.0001).

Discussion

The current study investigated the effect of PL on the CB CD34⁺ cells expansion and megakaryocyte progenitor's differentiation. Several studies substituted PL for FBS in cell culture (4, 27-30). Cells cultured in PL have been used for cell therapy (31-33), and their results showed that PL can be an appropriate alternative for FBS in cell culture and cell therapy. Although, in some studies, PL reduced the immunomodulatory effect of MSCs (34), which can be related to different concentrations of chemokines and growth factors in PL that may change cell conditions. PL contains a different concentration of chemokines and growth factors, such as GM-CSF, CXCL12, TGF-β, PDGF, IGF, HGF, FGF, EGF, and VEGF in which HSCs have receptors for these growth factors and may be a viable substitution for FBS. These growth factors, along with other cytokines such as TPO, SCF, FL, and EPO, can increase HSCs proliferation and differentiation (35, 36). Cytokine cocktail can affect HSCs differentiation, for instance, TPO and FL cytokines can induce multi-lineage differentiation of HSCs. Besides, they are suitable for HSCs self-renewal and survival and reduce erythroid differentiation (37).

Several researches have also investigated the HSCs differentiation into megakaryocyte cells *in vitro* (38-42) and *in vivo* (26, 43). *In vivo* results were in contrast with *in vitro* results and showed that the platelet recovery was not sufficient *in vivo*. This can be related to the hematopoietic microenvironment in the mouse or human body.

CD41 and CD42b are considered as megakaryocyte differentiation markers. CD41 is one of the megakaryocyte markers which express in the early stage of megakaryocyte differentiation and increase in maturation. On the other hand, CD42b is

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more expressed in mature megakaryocytes (7, 11, 13).

Megakaryocyte differentiation is largely coordinated by the temporal expression of various transcription factors. *GATA1* promotes erythropoiesis, whereas *GATA2* promotes megakaryopoiesis. Although, *ATA1* is essential for megakaryopoiesis and regulates the expression of CD41. *RUNX1*, *NFE2*, and *FLI1* transcription factors are also essential for megakaryocyte differentiation (4, 7, 44).

In the current study, higher expression levels of CD41 and CD42b were observed in PL-IMDM cultured cells compared to FBS-IMDM. Besides, *GATA1*, *GATA2*, *FLI1*, *NFE2*, and *RUNX1* gene expressions were enhanced in PL-IMDM cultured cells than in FBS-IMDM. These results convinced us to take for granted that PL can enhance megakaryocyte differentiation. *RUNX1* represses the *KLF1* (erythroid-specific transcription factor), which causes a shift in the increased ratio of *FLI1* (megakaryocyte-specific) to *KLF1*. Also, *FLI1* regulates the expression of CD42b and *NFE2* required during the terminal stage of megakaryocyte maturation (44), thus more expression of these genes can be related to more maturation of megakaryocyte. Likewise, we investigated the expansion of HSCs in PL-IMDM and FBS-IMDM. Our results indicated that PL can enhance HSCs expansion, however, this elevation was not statically significant. Noroozi-Aghideh et.al (6) demonstrated that platelet growth factors can induce megakaryopoiesis, however, PL suppressed the expansion of HSCs. Our expansion assay results were not in accordance with the described study. This discrepancy is maybe due to the different method for PL preparation and platelet source, therefore different growth factors and chemokines content which change the HSCs environment. There were some limitations in this study, such as not measuring the other megakaryocyte lineage markers and the ploidy of megakaryocyte cells, lack of performing long-term culture, and evaluation of cultured cell function *in vivo*.

Conclusion

In this study, the effect of PL on UCB $CD34^+$ cells expansion and differentiation into megakaryocyte lineage was investigated. The findings demonstrated that PL can improve UCB $CD34^+$ cells expansion and differentiation into megakaryocytes compared to FBS without worrying about the transfection of xenovirus and any ethical issues. However, the use of PL in $CD34^+$ cells culture still needs further investigation.

Funding

Conflict of intrests

The authors declare no conflict of interest.

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