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Resistance of Human Endometrium to Ferroptosis

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ABSTRACT

Although iron is an essential nutrient, ferroptosis (cell death) occurs in response to excess iron. Iron-induced lipid peroxidation damages to cell membranes and leads to cell death. Menstruation, on the other hand, is a periodic expulsion of blood and detached endometrium (menstrual blood). Some of the endometrium remains after menstruation, exposed to high concentrations of blood-derived iron, however re-proliferates and undergoes decidualization, an essential differentiation for embryo implantation. Since endometrial cells are considered to be highly resistant to iron, this study examined the effect of iron exposure on cell viability and decidualization using endometrial stromal cell line KC02-44D. In fetal-derived trophoblast cell line BeWO, iron treatment inhibited cell proliferation and caused cytotoxicity, while in KC02-44D, on the contrary, cell viability was increased and cytotoxicity was decreased. Significant increases in the decidualization markers PRL, IGFBP1, IL15, HAND2, FOXO1, and HOXA10 were observed after decidualization treatment, however no difference was observed after iron treatment before decidualization. On the other hand, PTGS2, a marker of ferroptosis, was downregulated in the iron-treated before decidualization group. Since PTGS2 is an enzyme that converts arachidonic acid contained in cell membranes to prostaglandins, which induces inflammation, iron is involved in normal decidualization by maintaining the robustness of cell membranes and suppressing excessive inflammation that occurs during decidualization.

Keywords: Endometrium; Ferroptosis; Decidualization

Introduction

Iron is an essential nutrient for our physical and mental health; however, its absorption rate is lower than that of other nutrients, and it is difficult to obtain iron through diet alone. The daily intake of iron recommended by the Ministry of Health, Labor and Welfare in Japan is 10.5 to 11.0 mg for menstruating women, +2.5 mg for women in the early stages of pregnancy, and +9.5 mg for women in the middle and late stages of pregnancy in 2020. However, it is thought that even an ideal diet provides only about 10 mg of iron per day. In addition, women have menstruation, so once a month, a large amount of iron is discharged from the body along with blood loss. As a result, they suffer from symptoms such as "tiredness," "heaviness," "irritability," and "severe stiff shoulders". Therefore, supplementation with iron supplements is a common way to cope with these symptoms. Recently, however, it has become widely known that ferroptosis (cell death), occurs as the body's defense mechanism against excess iron [1]. Ferroptosis is a condition in which the cell membrane, a lipid bilayer membrane, is oxidized by reactive oxygen species induced in cells by high concentrations of iron, causing the cell membrane to deteriorate into lipid peroxide, resulting in the cell's own death [1]. The human endometrium is divided into two layers: the functional layer, which accounts for 60-70% of the total endometrium from the superficial layer, and the basal layer, which is the deepest part of the endometrium. The functional layer consists mainly of glandular cells, stromal cells, vascular endothelial cells, and immune cells, and is where the embryo (fertilized egg) is first implanted.

The interaction of these cells with embryo-derived cells results in embryo implantation. Among them, endometrial stromal cells change from a fibroblast-like morphology to an epithelial cell-like morphology under the stimulation of progesterone secreted by the corpus luteum after ovulation. This differentiation is called decidualization [2]. During decidualization, endometrial stromal cells produce and secrete the known decidual markers, prolactin (PRL), Insulin-like

Growth Factor Binding Protein 1 (IGFBP1), cytokines including interleukin-15 (IL15), chemokines, growth factors, and angiogenic factors [3]. PRL and angiogenic factors act on vascular endothelial cells to promote spiral artery development and vascular stabilization, which facilitates embryo implantation and promotes decidualization. IGFBP1 works on embryo-derived extravillous trophoblast (EVT) cells to promote EVT invasion into the decidua and placentation [2]. On the other hand, if embryo implantation does not occur, a sudden drop in progesterone and estrogen production and secretion from the ovarian corpus luteum causes the endometrium to enter the menstrual phase, and the functional layers of the endometrium peel off with the large amount of blood contained therein (this is collectively referred to as menstrual blood) [4]. However, it is now known that some of the decidual endometrial stromal cells in the functional layer remain in the endometrium after menstruation [5].

The remaining endometrial stromal cells are exposed to high concentrations of erythrocyte-dependent iron from the disruption of the endometrium during menstruation, but proliferate again in the next menstrual cycle with subsequent estrogen stimulation from the ovary, and differentiate into decidualized cells by progesterone, thereby creating an environment that allows embryo implantation. These reports suggest that endometrial stromal cells are highly resistant to iron, unlike cells in other organs such as cancer cells [6], cardiomyocytes [7], and pancreatic islet of Langerhans beta cells [8] that are known to undergo ferroptosis. Therefore, by using endometrial stromal cell lines KC02-44D [9], this study examined (I) the viability and proliferative capacity of endometrial stromal cells after iron exposure and (II) the effect on decidualization after iron exposure to prove the resistance of endometrial stromal cells to ferroptosis.

Materials and Methods

Culture of the Human Endometrial Stromal Cell Line KCO2-44D and Human EVT Cell Line BeWO

KC02-44D cells [9] and BeWO cells [10] were obtained from the American Type Culture Collection (Manassas, VA, USA) and the JCRB cell bank (Osaka, Japan), respectively. KC02-44D and BeWO cells were cultured in DMEM containing 1% Antibiotics, 10 mM HEPES, 10% fetal bovine serum (FBS), and phenol red (Life Technologies, NY, USA) and Ham's F12 (Life Technologies, NY, USA) containing 1% antibiotics, 10 mM HEPES, and 15% FBS, respectively, at 37 °C and 5% CO2.

CCK8 Assay and LDH Assay

KC02-44D or BeWO cell lines in the logarithmic growth phase were seeded in each well of a 96-well plate with 5,000 cells/100 µL medium and cultured overnight in a CO_2 incubator. A group of cells was prepared with 0 µM, 1 µM, 10 µM, 100 µM, and 1,000 µM of iron (II) ammonium sulfate and cultured in a CO_2 incubator for 24 h. After 24 h, 20 µL of Lysis Buffer for LDH was added to High CTL and the cells were cultured in a CO_2 incubator for 30 min. Fifty µL of the culture supernatant was transferred to another plate, and 5 µL of CCK-8 (Dojin Chemical Laboratory, Kumamoto, Japan) was added to the remaining cells other than the High CTLs and cultured in a CO_2 incubator for 3 hours. To 50 µL of culture supernatant transferred to another plate, 50 µL of Working sol for LDH was added, and the plate was light-shielded and left at room temperature for 30 min. After 30 min, 25 µL of Stop sol was added to stop the reaction. The absorbance at 490 nm in each well was measured with a 2030 ARVO (Perkin-Elmer Japan, YOKOHAMA, JAPAN), and the amount of LDH (originally present in the cells) released into the medium under each condition was calculated using the value of the 0 µM group as 0% to measure cytotoxicity. On the other hand, the absorbance at 450 nm of each well of the plate to which CCK8 was added was measured with a microplate reader (Perkin-Elmer Japan, YOKOHAMA, JAPAN), and the cell viability was examined with the 0 µM group as 100%.

Iron Treatment and Decidualization in KC02-44D Cells

KC02-44D cells were seeded in 24-well plates (CORNING, USA) until confluence (0.4×10^6 cells per well) and then stimulated. Because Phenol red is an estrogen-like agonist [11-13], phenol red-free DMEM containing 10% charcoal-stripped (CS)-FBS (activated charcoal was used to adsorb and remove other hormones in the serum), 10 mM HEPES (pH 7.4), 1% antibiotics, and 1% GlutaMAX (Life Technologies, NY, USA) was used as the control medium. The control group was cultured in the above medium for 7 days; the iron-treated group was cultured in the above medium with 100 nM iron (II) ammonium sulfate for 1 day and in the above medium for 6 days; the decidualization-treated group was cultured in the above medium for 1 day and the above medium with 10-8M estradiol, 10-6M medroxyprogesterone acetate (MPA), an analog of progesterone, and 0.5 mM 8-Bromo-cAMP for 6 days; and the decidualization + iron treatment group was calculated in the above medium with 100 nM iron (II) ammonium sulfate for 1 day and the above medium with 0.5 mM 8-Bromo-cAMP, 10-8M estradiol, and 10-6M MPA for 6 days.

Extraction of Total RNA, Reverse-Transcription Reaction, and Quantitative PCR

Total RNA was extracted from KC02-44D cells (0.4×10^6 cells) cultured for 7 days with Sepasol®-RNA I Super G (Nacalai tesque., Osaka, Japan). ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co., Osaka, Japan) was used for reverse transcription of total RNA to cDNA. Quantitative PCR was conducted with cDNA, Thunderbird SYBR Next qPCR mix (Toyobo Co.), and primers using a Light Cycler96 (Roche, Basel, Switzerland). For PCR, after preincubation (95 °C 30 s), 45 cycles of 2-step amplification (95 °C 5 s, 60 °C 30 s) were conducted, followed by a melting reaction to confirm the primer specificity. Table 1 shows the gene names and primer sequences. As a housekeeping gene, we used Hypoxanthine phosphoribosyltransferase 1 (HPRT1) and calculated relative expression levels from the threshold cycle (Ct) values of each gene from each sample using the ΔΔCt method [14].

Table 1: Primer List.

Gene Symbol	Definition Primer	Name	Sequence (5′ to 3′)
HPRT1	Hypoxanthine Phosphoribosyl transferase 1	895F	CTAGTTCTGTGGCCATCTGCTTAG
		1034R	GGGAACTGATAGTCTATAGGCTCATAGTG
PRL	Prolactin	374F	ATTCGATAAACGGTATACCCATGGC
		623R	TTGCTCCTCAATCTCTACAGCTTTG
IGFBP1	Insulin-like Growth Factor Binding Protein 1	636F	CTATGATGGCTCGAAGGCTC
		791R	TTCTTGTTGCAGTTTGGCAG
IL15	Interleulin 15	165F	GTTCACCCCAGTTGCAAAGT
		351R	CCTCCAGTTCCTCACATTC
HAND2	Heart and Neural Crest Derivatives expressed 2	1479F	AGAGGAAGAAGGAGCTGAACGA
		1552R	CGTCCGGCCTTTGGTTTT
FOXO1	Fork head box protein O1	2336F	ATGTGTTGCCCAACCAAAGC
		2475R	TTGGACTGCTTCTCTCAGTTCC
HOXA10	homeobox A10	963F	GATTCCCTGGGCAATTCCAAAG
		1083R	ACAGAAACTCCTTCTCCAGCTC
ESR1	estrogen receptor 1	1514F	TGCTGGCTACATCATCTCGGT
		1665R	GACTCGGTGGATATGGTCCTTC
ESR2	estrogen receptor 2	617F	CTAACTTGGAAGGTGGGCCTG
		767R	AGCGATCTTGCTTCACACCA
PGR-AB	progesterone receptor A/B	2484F	CCTTTGGAAGGGCTACGAAGT
		2593R	GAGCTCGACACAACTCCTTTTTG
PGR-B	progesterone receptor B	28F	CGACCCAGGAGGTGGAGAT
		133R	GAGGGAAAAGGGAAGGAGGAG
B2M	Beta 2 microglobulin	226F	GAAGTTGACTTACTGAAGAATGGAGAGAG
		321R	GTAGTACAAGAGATAGAAAGACCAGTCC
EF1A	Elongation Factor 1 alpha	643F	TCTGGTTGGAATGGTGACAACATGC
		971R	AGAGCTTCACTCAAAGCTTCATGG
CXCL8	C-X-C motif chemokine ligand 8	118F	TTGGCAGCCTTCCTGATTTC
		231R	TTTGGGGTGGAAAGGTTTGG
F2R	coagulation factor II thrombin receptor	626F	TGTGCTGTTTGTGTCTGTGC
		728R	AAATGCTGCAGTGACGAAGC
CCL2	C-C motif chemokine ligand 2	224F	AATCACCAGCAGCAAGTGTC
		357R	TCGGAGTTTGGGTTTGCTTG
PTGS2	prostaglandin-endoperoxide synthase 2	1488F	ACCGCAAACGCTTTATGCTG
		1575R	TAGAGTGCTTCCAACTCTGCAG
BCL2L1	BCL2 Like protein 1	665F	ACGAGTTTGAACTGCGGTAC
		793R	CTGCTGCATTGTTCCCATAGAG

Statistical Analysis

After confirming the normality of each group by performing the Shapiro-Wilk test on the data obtained for each group, a two-tailed Welch's t-test was done to estimate the difference between the means of the two groups. Bonferroni correction was, then, performed to avoid a type 1 error according to multiple testing. The IBM SPSS Statistics software (version 29.0; IBM Corp., Armonk, NY, USA) was used for statistical analyses.

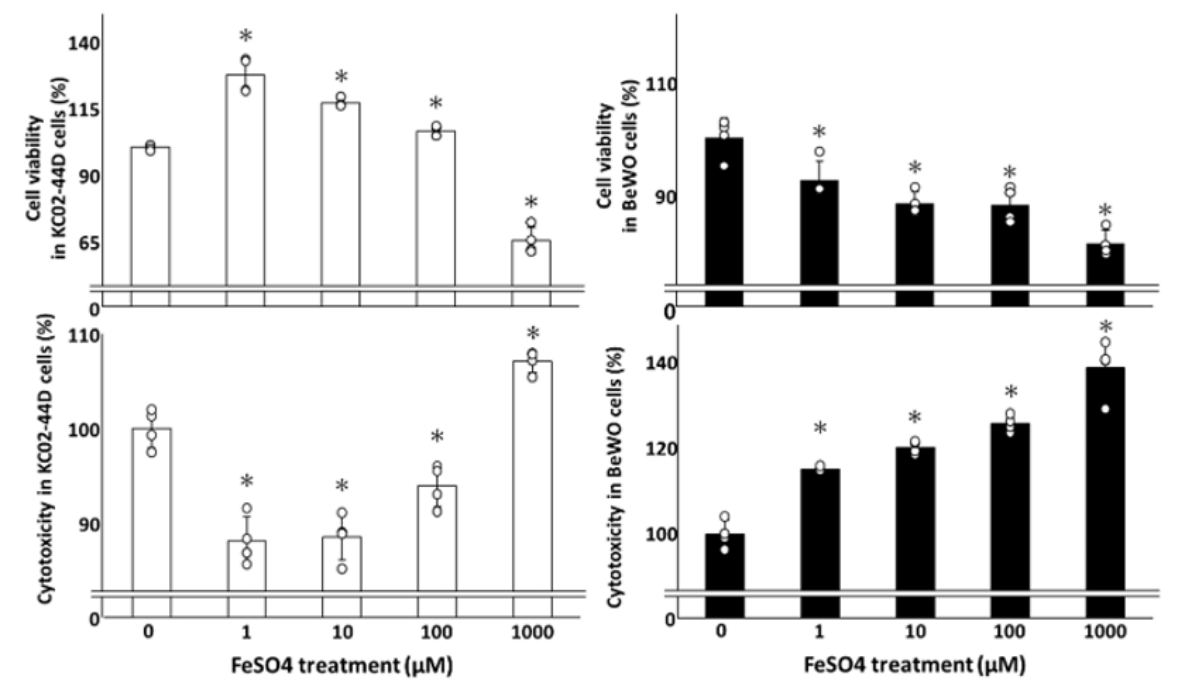
Results

Cell Viabilities

Cell viability in KC02-44D cells was examined at different concentrations of $FeSO_4$ and was significantly increased at 1 μM, 10 μM, and 100 μM compared with 0 μM ($p < 0.05$). In contrast, the cell viability was significantly decreased at 1,000 μM in KC02-44D cells ($p < 0.05$) (Figure 1). Cell viability in response to $FeSO_4$ was subsequently examined using BeWO cells as a control, and found that viability was significantly decreased ($p < 0.05$) at concentrations ranging from 1 to 1,000 μM compared with 0 μM (Figure 1).

Cytotoxicity of $FeSO_4$ Against Each Cell Line

The cytotoxicity of $FeSO_4$ in KC02-44D cells was examined in terms of the amount of extracellular LDH released from the cells, and a significant decrease was observed at 1 μM, 10 μM, and 100 μM and a significant increase at 1,000 μM ($p < 0.05$) (Figure 1). In contrast, there was a significant increase in the amount of free LDH induced by $FeSO_4$ in BeWO cells at all concentrations relative to 0 μM ($p < 0.05$) (Figure 1).



Note: KC02-44D and BeWO cells were treated with $FeSO_4$, and cell viability was determined by Cell Counting Kit-8 assay and cytotoxicity by L-lactate dehydrogenase assay.

$FeSO_4$: Iron (II) ammonium sulfate.

*: $p < 0.05$ by using Welch's T-test with Bonferroni correction against control.

Figure 1: Cell Viability and Cytotoxicity by $FeSO_4$ Treatment.

Effects of $FeSO_4$ in Decidualization

The effect of pre FeSO4 exposure on decidualization of KC02-44D cells was examined by measuring decidual markers by qPCR. The expression of PRL, IGFBP1, IL15, HAND2, FOXO1, and HOXA10 was significantly elevated in the decidualization group and decidualization plus pre 100 μM FeSO4 treatment group ($p < 0.05$, Table 2), however there were no differences between decidualization groups, nor were there significant changes in the control group when 100 μM FeSO4 was added (Table 2). The expression of estrogen receptor (ESR) 1, ESR2, progesterone receptor (PGR)-AB, and PGR-B in KC02-44D cells did not differ significantly among the four groups (Table 2). There were also no significant differences in the expression of the housekeeping genes, elongation factor 1 alpha (EF1A) and beta-2-microglobulin (B2M) among the four groups (Table 2). Next, the expression of ferroptosis markers was examined by qPCR. CXCL8 expression was significantly upregulated in two decidualization groups compared to the control group ($p<0.05$), however was not different from the control group plus 100 μM FeSO4 (Table 2). Coagulation factor II thrombin receptor (F2R) expression was significantly elevated ($p<0.05$) in the decidualization treatment group versus the control group. On the other hand, iron treatment followed by decidualization did not significantly increase F2R expression relative to the control group. CCL2 expression was not significantly different among the four groups (Table 2). Prostaglandin-endoperoxide synthase 2 (PTGS2) expression was significantly elevated ($p<0.05$) in the decidualization-treated group versus the control group ($p < 0.05$). The addition of iron before decidualization significantly suppressed PTGS2 expression compared with the control group ($p<0.05$). The addition of 100 μM FeSO4 to the control group did not significantly change PTGS2 expression (Table 2). BCL2L1 expression did not differ significantly among the four groups (Table 2).

Table 2: Gene Expression by Iron Treatment in KC02-44D Cells.

Definition	Gene Symbol	Control mean ± SD	Iron Treatment mean ± SD	Decidualization mean ± SD	Decidualization + Iron mean ± SD
Prolactin	PRL	2.6 ± 2.4 †	1.6 ± 0.5†	1647.5 ± 1027.9 *	1746.8 ± 1060.3*
Insulin-like Growth Factor Binding Protein 1	IGFBP1	5.6 ± 3.0 †	4.0 ± 3.0 †	42000.9 ± 26808.2*	55553.9 ± 38643.2*
Interleukin 15	IL15	2.4 ± 1.1 †	3.6 ± 1.5 †	12.4 ± 3.5*	12.9 ± 4.6*
Heart and neural crest derivatives expressed 2	HAND2	4.4 ± 2.4 †	7.3 ± 2.3 †	46.2 ± 16.6*	38.7 ± 19.2*
forkhead box protein O1	FOXO1	2.6 ± 1.3 †	2.6 ± 1.2 †	181.6 ± 43.2*	176.1 ± 58.7*
homeobox A10	HOXA10	1.3 ± 0.4 †	1.8 ± 0.5	2.1 ± 0.4*	1.9 ± 0.6 *
estrogen receptor 1	ESR1	4.4 ± 2.4	5.4 ± 3.8	7.5 ± 3.7	5.4 ± 3.5
estrogen receptor 2	ESR2	3.9 ± 1.9	2.9 ± 1.1	4.1 ± 2.9	3.3 ± 1.2
progesterone receptor A/B	PGR-AB	1.9 ± 0.6	2.4 ± 0.9	2.4 ± 0.4	1.9 ± 0.7
progesterone receptor B	PGR-B	6.0 ± 3.9	3.2 ± 1.8	4.7 ± 2.0	3.9 ± 1.0
eukaryotic translation elongation factor 1 alpha 1	EF1A	1.9 ± 0.7	2.8 ± 1.5	2.7 ± 1.3	2.0 ± 1.1
beta-2-microglobulin	B2M	2.2 ± 0.6	2.4 ± 0.8	1.8 ± 0.8	1.7 ± 0.8
C-X-C motif chemokine ligand 8	CXCL8	2.9 ± 2.6	3.6 ± 4.1	45.2 ± 22.5*	39.5 ± 8.4*
coagulation factor II thrombin receptor	F2R	1.3 ± 0.6 †	1.6 ± 0.5 †	2.5 ± 1.0*	2.1 ± 1.0
C-C motif chemokine ligand 2	CCL2	5.6 ± 2.4	4.3 ± 2.8	5.7 ± 1.4	4.7 ± 1.1
prostaglandin- endoperoxide synthase 2	PTGS2	8.2 ± 8.9 †	7.7 ± 5.1 †	46.2 ± 34.6*	13.1 ± 7.6 †
BCL2 like protein 1	BCL2L1	32.3 ± 23.9	28.5 ± 44.4	27.2 ± 29.7	32.5 ± 22.4

Note: *P<0.05 vs control. †P<0.05 vs decidualization. SD: standard deviation.

Discussion

In this study, by using endometrial stromal cell lines, we examined the resistance of endometrial stromal cells against ferroptosis after iron exposure. The results showed that even at concentrations at which ferroptosis occurs in other cells [4,6-8] and even at concentrations at which BeWO cells used in this study showed a significant decrease in cell viability and an increase in cytotoxicity, the endometrial stromal cell line KC02-44D showed an increase in cell viability and a decrease in cytotoxicity, respectively. These results suggest that high concentrations of iron are required for postmenstrual endometrial regeneration, especially the proliferation of the stromal cells. Furthermore, the results of expression analysis of decidualization markers suggest that iron exposure has no effect on decidualization, suggesting that iron supplementation may be an active treatment for anemia due to menstruation. On the other hand, exposure to iron prior to decidualization treatment suppressed the increase in F2R expression due to decidualization. Regulation of F2R levels has been shown to ameliorate UVB-induced cell damage [15], suggesting that iron exposure may regulate some cellular damage that occurs during decidualization by regulating F2R.

F2R is a G-protein-coupled receptor and one of four protease-activated receptors involved in the regulation of the thrombotic response. F2R is a G-protein-coupled receptor and one of four protease-activated receptors involved in the regulation of the thrombotic response [16]. It is highly expressed on platelets and vascular endothelial cells and plays an important role in mediating the interaction between coagulation and inflammation, which is critical in the pathogenesis of inflammatory and fibrotic lung diseases [17]. The function of F2R in endometrial stromal cells is unknown. Since high vascular permeability is required for adequate nutrition to the embryo in early pregnancy, it is likely that endometrial stromal cells, along with platelets and vascular endothelial cells F2R in the endometrium, are activated by thrombin and involved in blood coagulation [18], and the suppressed state of F2R seen with iron exposure is considered a better condition for early pregnancy. Estrogen secreted by the ovaries during the follicular phase promotes proliferation of endometrial stromal cells, which also induces expression of PTGS2, which produces Prostaglandin E2 (PGE2) from a known major precursor arachidonic acid [19].

IL11, which is produced by IL1 inhibiting PGE2, is known to be involved in endometrial cell fate determination, and the elevation in PGE2 by PTGS2 suppresses decidualization, which is essential for embryo implantation [20]. Considering these findings and our result that PTGS2 expression is suppressed by exposure to iron before decidualization treatment, it is conceivable that PTGS2 expression is specified by exposure to iron during menstruation and that decidualization during secretory phase is not inhibited by its effect. Indeed, it is known that PTGS2 expression increases during the secretory phase compared to the proliferative phase, but remains constant expression [21]. BCL2L1 is a Bcl2 family molecule and is involved in the mitochondria-mediated apoptosis induction mechanism (Intrinsic pathway) [22]. However, no change in expression was observed in the present study, suggesting that ferroptosis is still inhibited by iron treatment in endometrial stromal cells. In the future, this knowledge will lead to the discovery of novel pathways not found in other cells.

Conclusion

This study demonstrates the resistance of endometrial stromal cells to ferroptosis and shows that there is no risk of aggressive iron supplementation for anemia that occurs during menstruation.

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Competing Interests

The authors declare no conflict of interest. The funders had no role in the study design; collection, analyses, or interpretation of data; writing of the manuscript; or decision to publish the results.

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