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ORIGINAL ARTICLE

Gene expression in human mesenchymal stem cell aging cultures: modulation by short peptides

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Abstract

Effects of the short peptides Ala-Glu-Asp (AED), Lys-Glu-Asp (KED) and Lys-Glu (KE) on the expression of *IGF1*, *FOXO1*, *TERT*, *TNKS2,* and *NFκB* genes were studied in human embryo bone marrow mesenchymal stem cells (line FetMSCs) variously aged in "passages" or "stationary" cultures. Both cell aging models were similar in gene expression. The main difference was in the *TERT* gene expression level, which showed an eightfold increase at the "stationary" aging. *IGF1* gene expression levels were very similar in both cell culture aging models, being enhanced by 3.5–5.6 fold upon the addition of the peptides. The *FOXO1* gene was expressed twice more actively in the "stationary" than in the "passages" aging model. KED peptide inhibited *FOXO1* gene expression by 1.6–2.3 fold. KE peptide increased *FOXO1* gene expression by about two-fold in the "stationary" aging model but did not affect it in the "passage" aging model. The most striking difference in the peptide effect on cell aging between "passages" and "stationary" aging models was in the KED effects on *TNKS2* gene expression; this expression was inhibited by KED in the "passages" model, while stimulation was observed in the "stationary" model. AED, KED, and KE stimulated expression of the *NFκB* gene in both models. Thus, the peptides studied at nanomolar concentrations modulate the expression of some genes known to be involved in cell aging.

Keywords Short peptides · Genes · Cell aging · Human mesenchymal stem cells

Introduction

It is widely known that tissue regenerative potential in adult humans decreases with age. This decrease can be explained by the weakening of the stem cell proliferative activity. Although regenerative ability is one of the main characteristics of stem cells, their epigenetic aging in vivo occurs parallel to the body aging [1]. Mesenchymal stem cells (MSCs) have large therapeutic potential due to their ability to differentiate into various types of functionally active cells [2]. MSCs content in most tissues is low, so their practical application involves preliminary cell reproduction by cultivating in vitro. However, MSCs proliferation ability in cell cultures is limited. Thus, the mitotic activity of bone marrow MSCs decrease, and their differentiation ability is lost by the 10th passage (which corresponds to about 28 cell population doublings) [3]. After a certain number of divisions, MSCs enter the so-called senescent state characterized by specific morphological modifications and complete loss of the proliferation ability. This phenomenon known as the "Hayflick limit" is usually considered to be an in vitro analog of the natural cell aging in vivo. In mammals, all cells of an organism age at the same rate, irrespective of their proliferative activity [1]. Apparently, in cell cultures, transition into the senescent state reflects some intracellular processes. The decrease in the stem cell proliferative activity at cell aging in vivo can result from similar internal processes or external causes, such as changes in the composition of their niche and systemic milieu.

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At MSCs cultivation, specific changes in gene expression occur starting from the first passages and gradually increasing as the cells approach their senescent state [4]. In general, these changes are similar to differences in gene expression profiles between the MSCs samples obtained from donors of various ages [5]. Therefore, cell aging in "passages" can be considered as a model of their natural aging in vivo. Obviously, aging in the whole body context cannot be reduced to intracellular mechanisms, but also depends on their interaction with the cellular micro-environment. In adult body tissues, the stem cell activity is usually quite low, being regulated in accordance with the current requirements of cell renewal. A special niche surrounding the stem cells keeps most of them in a quiescent state, which ensures slower expenditure of their replication potential.

Cell senescence can be associated with a shortening of telomeres or accumulation of various kinds of chromosomal damage. Apparently, this state should be epigenetically controlled [6]. This view is supported by the existence of cells with unlimited division potential, such as embryo stem cells and induced pluripotent stem cells [7], as well as by the ability of certain pharmacological agents to decelerate replicative aging [8, 9]. Moreover, there are reasons to believe that senescence comprises a series of progressive and phenotypically different states that are caused by different factors and have various molecular mechanisms [10]. In the adult mammal body, many cells are post-mitotic. According to some features (heterochromatinization, inflammatory interleukin synthesis, enhancement of β-galactosidase activity), post-mitotic neurons in different brain regions are similar to senescent cells. Senescent cell state can be regarded to be physiologically irreversible; the only known possibility to reverse this state is through such extraordinary experimental procedures as the reprograming into pluripotent cells. In contrast, the quiescent state can be easily reversed: transition to mitosis is induced by standard physiological stimuli [11]. The most common and long-known causes of cell transition into the quiescent state (phase G) include lack of nutrients and contact inhibition.

Earlier we have shown that AED, KED, and KE short peptides regulate proliferation, apoptosis, and remodeling of the intercellular matrix in rat skin fibroblast cultures upon their aging in "passages" [12], induce human periodontal attachment [13, 14], and reduce the expression of the replicative aging markers in these cells [15]. KED peptide increased p16 and p21 mRNA expression in human periodontal ligament stem cells and human gingival mesenchymal stem cells in "passages" aging models [15]. KED peptide is a more effective activator of human periodontal ligament stem cells neuronal differentiation in comparison with KE and AED peptides. KED, AED, and KE peptides stimulate mRNA of Nestin and GAP43 expression in human periodontal ligament stem cells [13]. The molecular mechanism of the KE peptide aging was suggested that involved selective binding to the duplex DNA at TCGA sequences [16]. Comparative studies of KED, AED, and KE influences on gene expression in "stationary" and "passages" cell aging models have never been carried out before. In the current work, we have studied the effects of AED, KED, and KE on gene expression in the cultures of human mesenchymal stem cells in "passages" and "stationary" aging models.

Materials and methods

Cell cultures

Human embryo bone marrow mesenchymal stem cells FetMSCs (Institute of Cytology, RAS, St. Petersburg, Russia) were used [17]. The cells were cultivated in a CO_2 incubator at 37 °C in the medium with 87.5% DMEM, 10% ESCs, 1.5% HEPES-buffer, 1% penicillin and streptomycin solution, and α-glutamine. For reseeding (after achieving 80–85% confluence) the cells were detached using 0.25% trypsin and 0.02% versen solution (1:1, Sigma), and diluted threefold ($4–5 \times 10^4$ cells/cm^2 final density).

Experimental design

The cells were grown up to the 14th passage with the solution of one of the studied peptides (AED, KED, KE) added to the growth medium at every reseeding to the 20 ng/ml final concentration. This concentration was shown to be most effective in our preliminary experiments. For control cells, an equivalent amount of saline was added. In the 14th passage, visible aging features were observed, including growth inhibition, increased number of dead cells, and increased beta-galactosidase activity. Consequently, these cultures were considered to be aged (close to exhausting of their proliferative potential). To obtain cell aging in the "stationary" model, at the 14th passage, cells were left to grow without reseeding until a monolayer was formed (over 80% confluence) and for 5 more days. Thus, the geroprotective peptide effects were studied in two aging models—in "passages" (with cells reseeded and approaching the Hayflick limit) and in "stationary" (with contact-inhibited cells left for some time in monolayer culture) [18]. The applicability limits and conditions of these models are widely discussed in the current literature [18, 19]. The generally accepted practice is to select the most appropriate cell cultivation and aging conditions when working with any cell culture. A preliminary study aimed at choosing optimal MSCs aging conditions was performed in the present work.

Real-time polymerase chain reaction

After the cell incubation, the RNA-stabilizing reagent Intact-tRNA ("Evrogen," Moscow) was added to cell monolayers directly in culture dishes. RNA extraction was performed using the RNeasy Mini Kit ("Qiagen," Germany) according to the instruction provided by the manufacturer. The first cDNA strand was synthesized with the RevertAid First Strand cDNA Synthesis kit ("Thermo Fisher Scientific Inc.," USA), using 100 ng of RNA per 20 µl of the reaction mixture. The 1 µl aliquots of the reverse transcription mix were directly used as matrices in 25 µl quantitative PCR reactions, which were carried out on the DT-322 real-time PCR system ("DNK-Technologii," Russia) using qPCRmix-HS SYBR+ROX kit ("Evrogen," Russia). Oligonucleotide primers were constructed using the online-service NCBI Primer-Blast. In the primer pairs used, one corresponding to the segments in two adjacent exons. The *GAPDH* mRNA was used as a reference. Its concentration was taken for 1 in all samples. Three independent cell samples of each group (biological parallels) were used in the experiments. For each sample of cDNA, a minimum of three parallel PCRs was performed in adjacent wells (technical parallels).

Statistical analysis

For statistical processing, Microsoft Office Exel 2010 program was used. Experimental values were compared pairwise with the controls according to the paired Student's *t* test.

Results

The insulin-like growth factor 1 (IGF1) gene is expressed at moderate and similar levels in the "passages" and "stationary" aging model MSCs cultures (Fig. 1). Each of the three peptides that we used stimulates gene expression and does it virtually equally in "passages" and "stationary" cultures. At the "passage" aging, AED, KED, and KE peptides increase the *IGF1* gene expression by 4.6, 3, and 3.5 fold, respectively, compared with the control (Fig. 1). At "stationary" aging, AED, KED, and KE peptides increase the *IGF1* gene expression by 5.6, 2, and 5.2 fold compared with the control (Fig. 1).

In MSCs "passages" aging culture, the *FOXO1* gene is expressed more actively than the *IGF1* gene (Fig. 2). AED and KE peptides do not affect its expression level, while KED reduces it by 1.6 fold. In MSCs "stationary" aging culture, *FOXO1* is expressed 1.5 times more actively than in "passages" aging culture (Fig. 2). KED peptide decreases *FOXO1* expression by 2.3 fold (Fig. 2), while KE peptide increases it by 2.3 fold (Fig. 2).

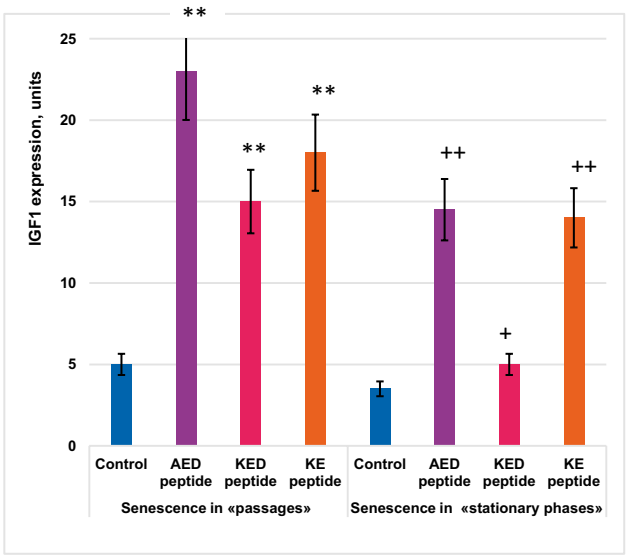

Fig. 1 Short peptide effects on *IGF1* gene expression in MSCs in "passages" and "stationary" aging models. Statistically significant differences between control and peptide-treated cells in passage cultures are shown by **—$p < 0.01$; those between control and peptide-treated cells in stationary cultures—by: +—$p < 0.05$; ++—$p < 0.01$. The vertical axis—*IGF1* gene expression normalized by GAPDH, $\times 10^6$, m ± 2SD

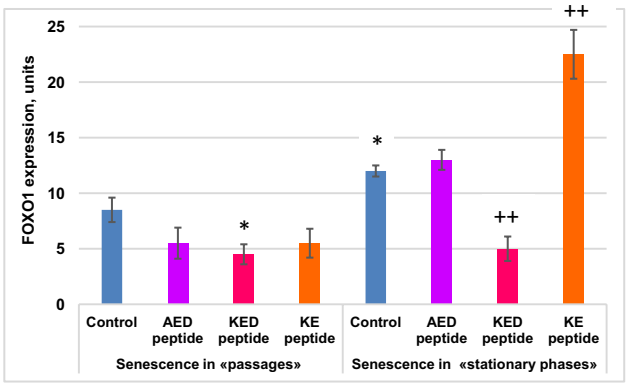

Fig. 2 Short peptide effects on *FOXO1* gene expression in MSCs in "passages" and "stationary" aging models. Statistically significant differences between control and peptide-treated cells in passage cultures are shown by *—$p < 0.05$; those beteen control and peptide-treated stationary cultures—by ++—$p < 0.01$. The vertical axis—*FOXO1* gene expression normalized by GAPDH, $\times 10^6$, m ± 2SD

In MSCs "passages" aging cultures, *TERT* has a negligible expression activity (Fig. 3). AED and KE peptides do not affect *TERT* expression (Fig. 3), while KED peptide reduces it by about sixfold (Fig. 3). In MSCs "stationary" aging cultures, *TERT* gene expression turned out to be expressed at about eightfold higher level compared with "passages" aging cultures (Fig. 3). AED, KED, and KE peptides reduce *TERT*

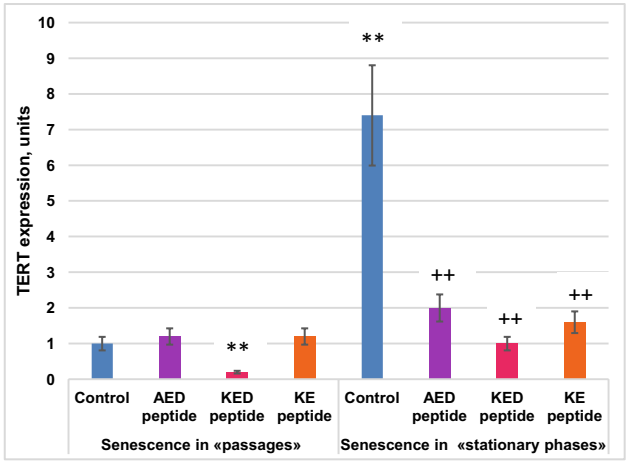

Fig. 3 Short peptide effects on *TERT* gene expression in MSCs in "passages" and "stationary" aging models. Statistically significant differences between control and peptide-treated cells in passage cultures are shown by **—$p < 0.01$; those beteen control and peptide-treated stationary cultures—by ++—$p < 0.01$. The vertical axis—*TERT* gene expression normalized by GAPDH, $\times 10^6$, m ± 2SD

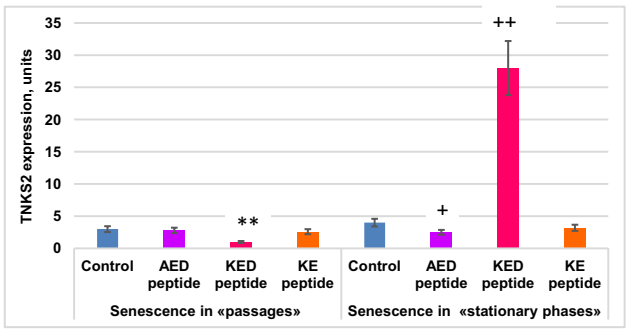

Fig. 4 Short peptide effect on *TNKS2* gene expression in MSCs in "passages" and "stationary" aging models. Statistically significant differences between control and peptide-treated cells in passage cultures are shown by **—$p < 0.01$; those beteen control and peptide-treated stationary cultures—by: +—$p < 0.05$; ++ —$p < 0.01$. The vertical axis—*TNKS2* gene expression normalized by GAPDH, $\times 10^6$, m ± 2SD

expression in "stationary" aging cultures by 4, 8, and 5.3 fold, respectively—virtually down to the level of its expression in "passages" aging cultures.

In MSCs "passages" aging cultures, the *TNKS2* gene is expressed by three-fold more actively compared with the *TERT* gene (Fig. 4). This higher expression can reflect the multifunctional nature of tankyrase 2, which is involved in supporting the telomere structure and other cellular processes. At MSCs "passages" aging, KED peptide reduces *TNKS2* expression by four-fold, while AED and KE peptides do not affect it (Fig. 4). A slightly different picture is observed in MSCs "stationary" aging cultures; the addition of AED peptide reduces *TNKS2* gene expression by approximately twofold (Fig. 4), while KED peptide increases it by sixfold (Fig. 4).

Both in "passages" and "stationary" aging cultures, *NFκB* is expressed at rather low levels (Fig. 5). Its expression is increased in both cultures by AED (Fig. 5), KED (Fig. 5), and KE peptides (Fig. 5).

Discussion

IGF1 is one of the most important intermediates in realizing the growth factor effect on many physiological processes in the body of humans and other mammals [20]. In many animals, the reduced insulin/IGF1 signal pathway correlates with an increased lifespan [21]. However, the role of this system in the regulation of aging processes in the human body is still unstudied. It is known that in the centenarian population, the frequency of heterozygous mutations in the insulin-like growth factor receptor gene (*IGF1R*) is substantially increased, which correlates with increased IGF1 level in blood plasma and low activity of its receptor [22]. It should also be noted that the effect of local IGF1 produced in peripheral tissue cells, and the systemic, circulating IGF1 can be different. AED, KED, and KE peptides stimulate *IFG1* gene expression in both aging models. Given the role of IGF1 as the main growth hormone intermediator, it can be suggested that AED, KED, and KE peptides stimulate anabolic (synthetic) processes in aged MSCs.

One more gene that plays an essential role in aging in various animals, from nematodes to mammals, is *FOXO1* [23]. Being the main target of insulin signaling, the FOXO1 transcription factor regulates cell metabolism, and organismal survival [20]. It is actively expressed in insulin-sensitive organs, such as the pancreas, liver, skeletal muscles, and fat tissue, where energy metabolism is regulated [24, 25]. In the pancreas, FOXO1 inhibits the proliferation of β-cell but facilitates their survival. In many experimental studies, the mutual interaction of the redox regulation system and FOXO1 was established, which probably underlies the effect on the terminal differentiation, proliferation, and survival of the target cells [26–29]. Being modified by many types of cell stimulation, FOXO1 expression supports tissue homeostasis through regulating cell proliferation, apoptosis, autophagy, and responses on the oxidative stress [30]. The functional significance of the peptide effects on *FOXO1* gene expression, as well as a more active expression of this gene in "stationary" aging culture, require further studies.

Telomerase activity was shown to prevent the senescent state in cell cultures [31]. Nevertheless, superexpression of the telomerase gene *TERT* does not increase longevity in mice [32]. In most of the normal human somatic tissue cells, this gene is virtually not expressed, whereas its

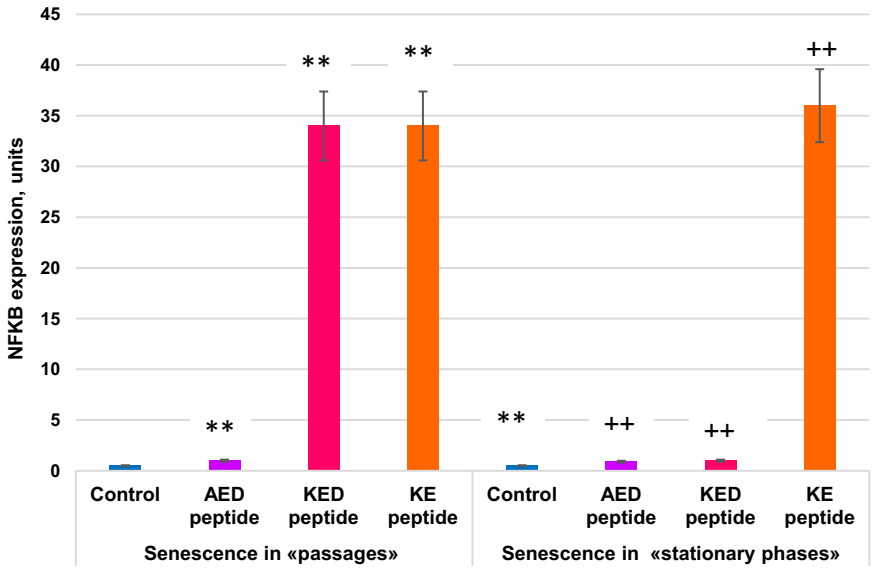

Fig. 5 Short peptide effect on *NFkB* gene expression in MSCs in "passages" and "stationary" aging models. Statistically significant differences between control and peptide-treated cells in passage cultures are shown by **—$p < 0.01$; those beteen control and peptide-treated stationary cultures—by ++—$p < 0.01$. The vertical axis—*NFkB* gene expression normalized by GAPDH, $\times 10^6$, $m \pm 2SD$

expression level in cancer cells is quite high [33]. It can be suggested that the telomeres in somatic tissue cells are initially long enough to provide for the necessary range of their proliferative potential, while weak telomerase expression or its absence altogether serves as a preventive mechanism for neoplastic transformation. The higher expression level of the *TERT* gene in "stationary" aging cultures is somewhat unexpected. The MSCs cultures studied, like those of most human somatic cells, practically do not express telomerase. The gene of tankyrase 2 is expressed much more actively than the *TERT* gene in both MSCs cultures.

The *TNKS2* gene product, tankyrase 2, is a particular member of poly(ADP-ribose) polymerase family (PARPs), involved in supporting the telomere structure and regulating the Wnt signal pathway activity, as well as glucose metabolism, mitotic cycles and many other processes [34, 35]. Tankyrase 2 is closely homologous to tankyrase 1 (82% identity at the amino acid sequence). The functions of these proteins are mainly, but not entirely, duplicated. This view follows from the fact that double zero-mutants of *TNKS1* and *TNKS2* genes die by the 10th day of embryo development, while singular zero-mutants of any of these genes manifest only insignificant phenotype modifications [36]. This higher expression may be due to the multifunctional nature of tankyrases, which not only support the structure and integrity of telomeres but are also involved in regulating cell metabolism and growth. Given equal expression level of *TNKS2* gene in "passages" and "stationary" aging cultures, these functions appear mostly not associated with cell proliferation. Nevertheless, some peptide effects on the two types of MSCs culture are different. Thus, AED peptide does not affect *TNKS2* in "passages," but inhibits it in "stationary" aging culture. A more drastic difference was observed in KED peptide effects on the two kinds of aging cultures—an inhibition in "passage" and stimulation in "stationary" aging culture. Cell passaging eventually leads to a senescent state in multiple aspects related to the natural physiological mechanisms of cell aging that involve a decrease and dysfunction of metabolic systems [37]. The KED peptide could be suggested to diminish metabolic processes and replicative potential of passaging cells by decreasing *TNKS2* expression, thus increasing their replicative lifespan. On the other hand, the KED-induced increase in expression of the *TNKS2* gene in stationary cells could represent an adaptive mechanism to compensate for the nutrient deficit stress characteristic of stationary cultures. We have shown previously that short peptides are capable of modulating methylation of genes in human cells upon their passage aging [38]. It may well be that KED affects methylation of *TNKS2* upon prolonged passage aging but is without effect upon stationary aging.

NFκB gene encodes the transcription factor known as an inflammatory response regulator [39, 40]. The expression of many genes controlled by this regulator increases with natural aging in mammal tissues. In mouse skin, expression of about 400 genes changes (mostly increases) with aging, of which about a half are direct targets of NFκB. Inactivation of the *NFκB* gene in certain skin areas of old mice with inducible transgenic constructs for 2 weeks resulted in the return of expression levels of 225 (54%) aging-associated genes to the level characteristic of young mice. In terms of gene expression, these skin areas became more similar to the skin of young mice than to the adjacent control skin areas. Expression of molecular cell senescence markers SA-β-gal and $p16^{INK4A}$ was reduced, cell proliferation in the

basal epidermis layer was increased, while the structure and general skin state improved. Thus, *NFκB* gene expression appears to be a prerequisite for the realization of the skin aging program. AED and KE peptides stimulate the expression *NFκB* gene in both models of cell aging. Since NFκB is a transcription factor that regulates inflammation, it can be suggested that the effects of peptides on the *NFκB* gene expression accelerate MSCs aging in both models.

The gene expression patterns studied in the present work appeared to be only slightly different between the two kinds of cellular aging models. A large difference between "passages" and "stationary" models was found only for the *TERT* gene, whose expression turned out to be about eightfold higher in "stationary" than in "passages" culture. Differences between the cell aging models in *NFκB* and *FOXO1* gene expression levels were much smaller but also statistically valid.

Collectively, the data presented show that short peptides could modulate gene expression in both models of MSCs aging. Of course, this conclusion would look more convincing if the quantitative PCR data is supported by Western blot data. Therefore, the results described here could be regarded as preliminary. However, for most genes, mRNA levels are in good accord with protein levels. The findings described here testify to the high biological activity of short peptides. In general, patterns of "passages" and "stationary" aging appear quite similar. In a few instances, the short peptides have different effects on gene expression between cells of "passages" and "stationary" aging models. This variability could reflect differences in reactivity towards the peptides. Anyhow, both kinds of cultures can be used as adequate cell aging models for testing the biological activity of potential geroprotector substances.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Research involving human and/or animal participants Research has not involved human participants or animals.

Informed consent The authors give informed consent for article publication.

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