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Short Peptides Protect Oral Stem Cells from Ageing

Bruna Sinjari[1] · Francesca Diomede[1] · Vladimir Khavinson[2,3,4] · Ekaterina Mironova[2] · Natalia Linkova[2,5] · Svetlana Trofimova[2,5] · Oriana Trubiani[1] · Sergio Caputi[1]

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Abstract

Primary stem cells, after several cell divisions, enter into a senescence state, that is characterized by alterations to spindle-shape typical morphology. This concern is one of the main problems in the use of human mesenchymal stem cells (hMSCs) in clinical applications which demand cells in large numbers. Short peptides had geroprotective properties and stimulated stem cell differentiation. The aim of the study is to demonstrate the role of AEDG and KED peptides in maintaining oral hMSCs morphology and functions over long-term expansion. 2 types of hMSCs were investigated: human periodontal ligament stem cells (hPLSCs) and human gingival mesenchymal stem cells (hGMSCs). Cells at the 25th passage were divided into 3 groups: 1 – control (without adding peptide), 2 – treated with AEDG peptide, 3 – treated with KED peptide. Cell cultures were analyzed by an immunofluorescence method and RT-PCR on the p16 and p21 senescence markers expression. AEDG peptide decreased p16 and p21 mRNA expression by 1.56–2.44 times in comparison with the control group. KED peptide decreased p16 and p21 mRNA expression by 1.82–3.23 times in comparison with the control group. These results were confirmed by immunofluorescent visualization. AEDG and KED peptides could be used as supplementary substances in a culture medium to delay the expression of senescence markers in long term stem cell cultivation in order to promote the large-scale in vitro expansion necessarily required for stem cell therapy clinical application. The data obtained confirm the geroprotective effect of AEDG and KED peptide, which was shown early in animal and cells models.

Keywords AEDG peptide · KED peptide · Stem cells · Cell senescence · p16 · p21 · Geroprotection

Introduction

In vitro cell growth is a mechanism that gradually induces the loss of cellular function. Human mesenchymal stem cells (hMSCs) cultured for long-term passage lose self-renewal capacity, multi-lineage differentiation potential and go into a replicative senescence state. This concern is one of the main problems in the use of hMSCs in clinical applications which demand cells in large numbers. Primary somatic cells, after several cell divisions, enter into a senescence state, that is characterized by alterations to spindle-shape typical morphology [1, 2]. Otherwise, they lose their multilineage differentiation potential and the capacity to migrate, becoming unsuitable for clinical use [3–5]. For stem cell transplantation, low grade of cell senescence plays a key role in the large-scale expansion of hMSCs [6, 7]. hMSCs are widely used in regenerative medicine as well as in age associated diseases [8]. They should have multi-differentiation potential, be easily accessible and be obtainable by minimally-invasive or non-invasive collection procedures. Bone marrow-derived mesenchymal stem cells were the first MSCs obtained in vitro, however they require an invasive technique to obtain [9]. Dental tissue serves as an alternative source for MSCs showing a higher colony forming unit and proliferation rate efficiency, along with a similar gene expression profile for genes related to mineralization and to their neural crest origin [10]. Periodontal ligament,

Bruna Sinjari and Francesca Diomede contributed equally to this work.

✉ Natalia Linkova
miayy@yandex.ru

[1] Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio" Chieti-Pescara, Chieti, Italy

[2] Department of Biogerontology, Saint Petersburg Institute of Bioregulation and Gerontology, St. Petersburg, Russia

[3] Department of Geriatrics, Propaedeutics and Nursing Management, Mechnikov North-Western State Medical University, St. Petersburg, Russia

[4] Pavlov Institute of Physiology, Russian Academy of Sciences, St. Petersburg, Russia

[5] Department of Therapy, Geriatrics, and Anti-Aging Medicine, Academy of Postgraduate Education, Moscow, Russia

soft connective tissue, showed the presence of human periodontal ligament stem cells (hPLSCs) and has an important role in homeostasis maintenance and in wound healing of the periodontal area [11, 12]. Human MSCs isolated and cultured from periodontal ligament showed the same functional and morphological features of bone marrow-derived MSCs [13]. Also human Gingiva-derived MSCs (hGMSCs) are a novel type of pluripotent MSCs described in recent literature [14]. GMSCs exhibited self-renewal, a multipotent differentiation potential and immunomodulatory capacities as hMSCs [15–18]. Gingival tissue, as a novel postnatal stem cells source, has been attracting increased attention due to their easy accessibility [19]. GMSCs are easy to collect and isolate. This type of cell has a high proliferative capacity, homogeneity and stable phenotype [20]. Therefore, hPDLSCs and hGMSCs are considered to be optimal cell resource candidates for cell-based therapies [21, 22].

Short peptides are a wide range of molecules that are capable of interacting with DNA to show the behavior of signaling factors [23, 24]. Some short peptides had geroprotective properties and stimulated stem cell differentiation. AEDG peptide (Ala-Glu-Asp-Gly) induced the activity of telomerase and telomere elongation in human fibroblast culture [25, 26], which can be due to reactivation of the telomerase gene in somatic cells and indicates the possibility of prolonging the life span of a cell population. AEDG peptide also prolonged animal life span [27]. KED peptide (Lys-Glu-Asp) had a vasoprotective activity in senescence endothelium cultures. The vasoprotective effect of KED peptide in endothelium cultures has been realized through epigenetic increased regulation of Ki67 and decreased regulation of p53 proteins synthesis [28, 29]. Moreover, KED peptide normalized blood circulation in elderly patients with atherosclerosis [30]. Also shown was that KED peptide and a mixture of KED, AEDG, KE and AED peptides promoted the neuronal differentiation of hPDLSCs. The effect of peptides on neuronal differentiation of hPDLSCs was studied by immunofluorescence and western blot analysis. Growth Associated Protein 43 (GAP43), which implements neurotransmission mechanisms and neuroplasticity, demonstrated an increased expression in hPDLSCs cultured with a mixture of all peptides studied and with KED alone. The peptides mixture and KED increased the expression of Nestin (neurofilament protein) in hPDLSCs cultures; this protein expresses in early neuronal precursors [31]. Thus, short peptides are considered to be bioregulators in the maintenance of structural and functional homeostasis of cell populations. They are necessary for the development of cells, and for the interaction and functioning of cells. The aim of the study is to demonstrate a novel role of AEDG and KED peptides to maintain oral hMSCs morphology and functions over long-term expansion.

Materials and Methods

Cell Cultures

The study was approved by the Ethical Committee of the University "G. d'Annunzio", Chieti and Pescara (PI: Prof. Trubiani Oriana; N266/2014).

Human Periodontal Ligament Stem Cells (hPDLSCs) Culture Establishment

hPDLSCs were collected from periodontal ligament biopsies performed on volunteers exempt from systemic and oral diseases. Tissue fragments were obtained by scraping the roots of non-carious third molar teeth at the horizontal fibers of the periodontal ligament. Biopsies were placed in a Petri dish with Mesenchymal Stem Cell Growth Medium- Chemically Defined (MSCGM-CD, Lonza, Basel Switzerland) and cells spontaneously migrated after 2 weeks [32]. In all experiments 2nd passage cells were plated at 1×10^3 cells/cm^2 density.

Human Gingival Mesenchymal Stem Cells (hGMSCs) Culture Establishment

hGMSCs were collected and isolated from human gingival biopsies during surgical procedure from patients with general good health conditions. Tissue samples were placed in a Petri dish and maintained with MSCGM-CD medium (Lonza) in an incubator at 37 °C and 5% CO_2. The medium was replaced every 2 days. Cells were spontaneously migrated from tissue after 2 weeks of cultivation [33].

Cell Characterization

To evaluate the mesenchymal features of hPDLSCs and hGMSCs, cytofluorimetric detection and mesengenic differentiation was performed. Cytofluorimetric analysis was assayed as previously described [34]. Expression of Oct3/4, Sox-2, SSEA-4, CD14, CD29, CD34, CD44, CD45, CD73, CD90, and CD105 markers were evaluated on hPDLSCs and hGMSCs. The analysis was performed using FACStarPLUS flow cytometry system and FlowJo™ software (TreeStar, Ashland, OR, USA).

To assess the ability to differentiate into osteogenic and adipogenic lineage commitment, hPDLSCs and hGMSCs were maintained under osteogenic and adipogenic conditions for 21 and 28 days, respectively, as previously reported [35]. To evaluate the formation of mineralized precipitates and lipid vacuoles, after the differentiation period, alizarin red and adipo oil red staining was performed on undifferentiated and differentiated hPDLSCs and hGMSCs. Inverted light microscopy Leica DMIL (Leica Microsystem, Milan, Italy) was used for sample observation. To validate the ability to differentiate

into osteogenic and adipogenic lineages, the expression of RUNX-2, ALP, FABP4, and PPARγ were evaluated by real time polymerase chain reaction (RT-PCR) as reported by Diomede et al. [36]. Commercially available TaqMan Gene Expression Assays (RUNX-2 Hs00231692_m1; ALP Hs01029144_m1; FABP4 Hs01086177_m1; PPARγ Hs01115513_m1) and the Taq-Man Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were used according to standard protocols. Beta-2 microglobulin (B2M Hs99999907_m1) (Applied Biosystems) was used for template normalization. RT-PCR was performed in three independent experiments, and duplicate determinations were carried out for each sample.

Experimental Design

Experiment cells were cultured until the 25th passage and then they were divided into 6 groups: 1 – control of hPDLSCs (without adding peptide), 2 – hPDLSCs treated with AEDG peptide, 3 – hPDLSCs treated with KED peptide, 4 – control of hGMSCs (without adding peptide), 5 – hGMSCs treated with AEDG peptide, 6 – hGMSCs treated with KED peptide. Peptides were diluted in a sodium phosphate-buffered saline (PBS) buffer in concentration 0.01 μg/mL. Peptides were added to the cell medium and replaced every 3 days [31]. The cells were placed at 37 °C in a humidified 5% CO_2 incubator. On the 7th day of peptide treatment, cell cultures were analyzed by immunofluorescence method and RT-PCR on the p16 and p21 senescence markers expression.

Immunofluorescence Analysis

Cells were fixed for 30 min at room temperature, with 4% of paraformaldehyde in 0.1 M PBS, 40 pH 7.4, and permeabilized with 0.1% of Triton1-X100 for 10 min, followed by blocking with 5% skimmed milk in PBS for 30 min. Samples were incubated with a primary mouse monoclonal antibody, anti-p16 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-p21 (1:200; Abcam, DBA, Milan, Italy) as a primary antibody and an anti-mouse Alexa Fluor 568 probe (Molecular Probes) as a secondary antibody. All samples were incubated with Alexa Fluor 488 phalloidin green fluorescence conjugate (1:200, Life Technologies, Milan, Italy), as a marker of cytoskeleton actin and with TO-PRO to stain the nuclei. Samples were observed using a Zeiss LSM800 META confocal (Zeiss, Jena, Germany) connected to an inverted Zeiss Axiovert 200 microscope equipped with a Plan Neofluar oil-immersion objective (40×/1.3 NA) [37]. Images were collected using an argon laser beam with excitation lines at 488 nm and a helium–neon source at 543 and 633 nm.

Real Time Polymerase Chain Reaction

Neurogenic markers were evaluated by RT-PCR. Total RNA was isolated using the Total RNA Purification Kit (NorgenBiotek Corp., Ontario, CA, USA) according to the manufacturer's instructions. The M-MLV Reverse Transcriptase reagents (Applied Biosystems) were used to generate cDNA. RT-PCR was carried out with the Mastercycler ep realplex real-time PCR system (Eppendorf, Hamburg, Germany). Commercially available TaqMan Gene Expression Assays (p16 Hs00923894_m1; p21 Hs01040810_m1) and the Taq-Man Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were used according to standard protocols. Beta-2 microglobulin (B2M Hs99999907_m1) (Applied Biosystems, Foster City, CA, USA) was used for template normalization [38]. RT-PCR was performed in three independent experiments, and duplicate determinations were carried out for each sample.

Statistical Analysis

Data were analyzed using GraphPad Prism version 6.0 software (GraphPad Software, La Jolla, CA, USA). The factors under investigation were the mRNA expression. Data were expressed as means and standard deviation. Two-way analysis of variance tests was performed. Tukey tests were applied for pairwise comparisons. A value of $p < 0.01$ was considered statistically significant in all tests.

Results

Cell Characterization

hPDLSCs and hGMSCs showed a positivity for the following surface molecules CD13, CD29, CD44, CD73, CD90 and CD10, while they were negative for CD14, CD34 and CD45 (Fig. 1a). To evaluate the mesenchymal feature cells were induced to adipogenic and osteogenic lineage commitment. To define hPDLSCs and hGMSCs mesenchymal stem cells, the ability to differentiate into ostegenic and adipogenic lineage has been assayed. hPDLSCs and hGMSCs cultured under osteogenic conditions showed a high expression of RUNX2 and ALP when compared to the control cells (Fig. 1b). Alizarin red S staining showed a positive red stain to mark the calcium deposition after 21 days of induction (Fig. 1c1, c2). Cells differentiated to the adipogenic lineage showed an up regulation of FABP4 and PPARγ compared to undifferentiated samples (Fig. 1d). The adipo oil red O staining showed a positive stain to lipid droplets localized at cytoplasmic level (Fig. 1e1, e2).

a

	hPDLSCs		*hGMSCs*	
Antigen	**Phenotype**	**MFI ratio ± SD**	**Phenotype**	**MFI ratio ± SD**
CD13	+++	154.6±28.2	+++	173.4±21.7
CD29	+++	114.4±15.8	+++	195.2±22.3
CD44	+++	164.7±31.4	+++	167.8±27.3
CD73	++	31.8±1.6	++	29.4±2.9
CD90	+++	270.7±31.8	+++	396.4±28.2
CD105	+	9.5±2.7	+	9.1±1.3
CD14	-	ND	-	ND
CD34	-	ND	-	ND
CD45	-	ND	-	ND

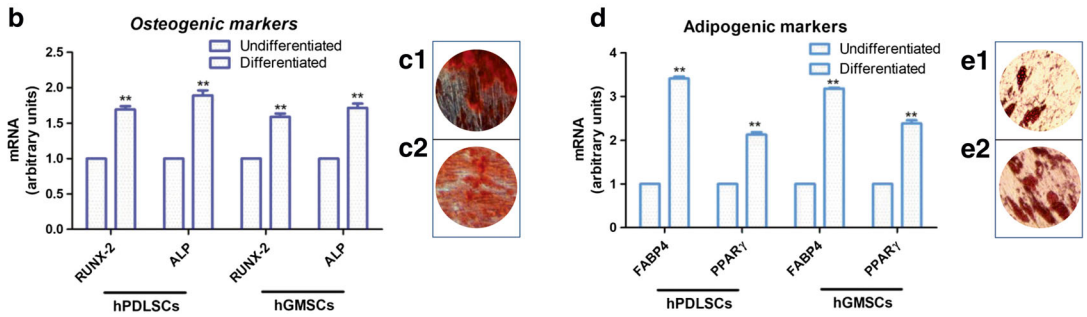

Fig. 1 hPDLSCs and hGMSCs cell population characterization. a Flow cytofluorimetry analysis; − indicates negative expression (0%); + indicates moderate expression; ++ indicates positive; +++ indicates high expression (100%); MFI ratio is the average of three different biological samples ± standard deviation. **b** RT-PCR graph of RUNX2 and ALP. Alizarin red S staining in **c1** hPDLSCs and **c2** hGMSCs culture. **d** RT-PCR graph of FABP4 and PPAR . Oil red O staining in **e1** hPDLSCs and **e2** hGMSCs culture. Scale bar: 10 μm. Mag: 10X. ** - $p < 0.01$ in comparison with the corresponding value in the "undifferentiated" group.

Immunofluorescence Analysis

hPDLSCs and hGMSCs cultured until 25th passage were treated with AEDG and KED peptides for 1 week. After the treatment period, cells were processed to immune-staining and then observed using confocal microscopy to evaluate the modulation in the expression of p16 and p21. Untreated hPDLSCs and hGMSCs showed a positive staining at nuclear level for p16 and p21. At the same time hPDLSCs and hGMSCs treated with AEDG and KED peptides showed a low positivity for the same considered markers (Figs. 2 and 3).

Gene Expression

mRNA levels of p16 and p21 senescence related genes in hPDLSCs and hGMSCs were analyzed by RT-PCR. AEDG peptide decreased p16 and p21 expression in hPDLSCs by 1.56 and by 2.22 times in comparison with the corresponding control. KED peptide decreased p16 and p21 expression in hPDLSCs by 1.95 and by 2.17 times in comparison with the corresponding control. AEDG peptide decreased p16 and p21 expression in hGMSCs by 1.92 and by 2.44 times in comparison with the corresponding control. KED peptide decreased p16 and p21 expression in hGMSCs by 3.23 and by 1.82 times in comparison with the corresponding control (Fig. 4).

Discussion

Tissue engineering and regenerative medicine require the use of hMSC. Self-renewal, multipotency, large-scale in vitro expansion with a low grade of senescence are considered crucial factors for clinical applications. hMSCs from oral tissues are widely used not only in maxillofacial regenerative applications but also for other purposes [8]. For stem cell transplantation the main concern is in vitro long-time cultivation to obtain a large quantity of stem cells. The long time cultivation in vitro of hMSCs may cause an alteration in the expression of embryonic stemness markers and in the capacity of proliferation and differentiation [6, 7].

Short peptides play an important role in the transmission of biological information, as autocrine hormones and neuropeptides. They guarantee the maintenance of the cellular homeostasis [39, 40]. Peptides were considered as bioregulators that maintain the major physiological functions providing an important place in the complex chain of homeostasis processes leading to the ageing of cells, tissues and organs [40]. In the

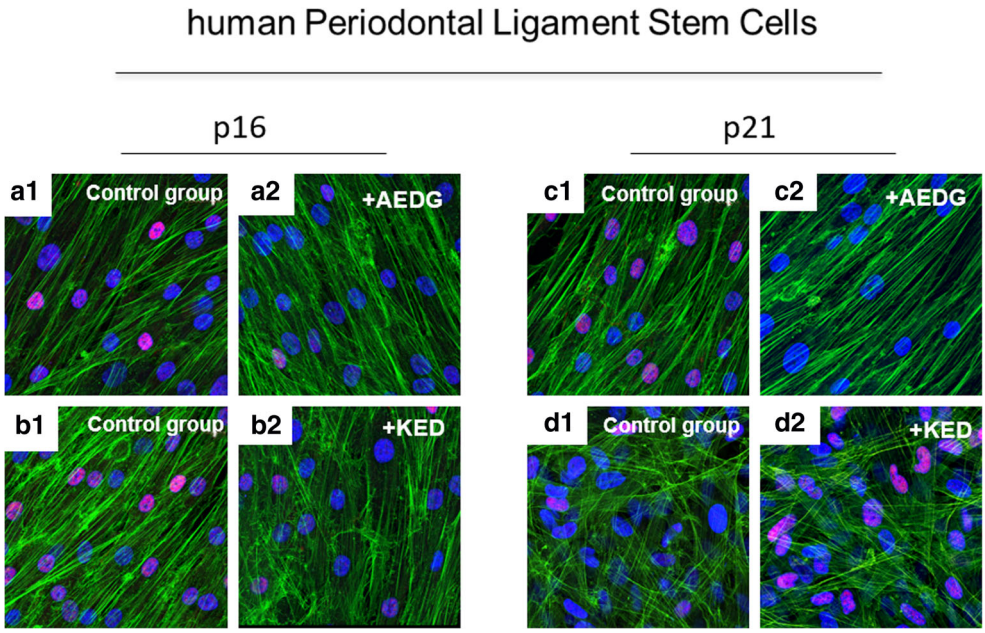

Fig. 2 The influence of AEDG and KED peptides on p16 and p21 protein syntesis in hPDLSCs. P16 expression in **a1** untreated hPDLSCs and **a2** AEDG peptide treated hPDLSCs; **b1** untreated hPDLSCs and **b2** KED peptide treated hPDLSCs. P21 expression in **c1** untreated hPDLSCs and **c2** AEDG peptide treated hPDLSCs; **d1** untreated hPDLSCs and **d2** KED peptide treated hPDLSCs. Scale bar: 10 μm. Mag: 20X

present research study, oral stem cells, hPDLSCs and hGMSCs cultures have been used to evaluate the in vitro protective effects of AEDG and KED peptides on long term passage cell culture.

Oral derived MSCs are easy to isolate and manipulate and they also show high immunomodulatory properties due to their paracrine effects. Paracrine effects are able to promote tissue regeneration, and inhibit fibrosis and apoptosis. They also modulate the immune system and the inflammatory process; properties that could enhance their clinical utility [41]. The PDLSCs and hGMSCs were defined mesenchymal stem cells following Dominici's criteria, in fact they are able to differentiate and adhere to a plastic substrate, to differentiate into adipogenic and osteogenic commitment when induced and they also express surface stemness markers, while showing a negative expression for hematopoietic specific surface molecules [42–44]. Long time cultivation of hPDLSCs and hGMSCs can induce cellular senescence. The use of long term passage cells seems to be unsafe for cell transplantation [45, 46].

To evaluate the AEDG and KED peptides effect on oral stem cells immunofluorescence analyses were performed to study the expression of p16 and p21 on cells cultured at the 25th passage. AEDG peptide showed the best effect on hPDLSCs and hGMSCs. Cells treated with AEDG peptide showed a low p16 and p21 gene expression and protein

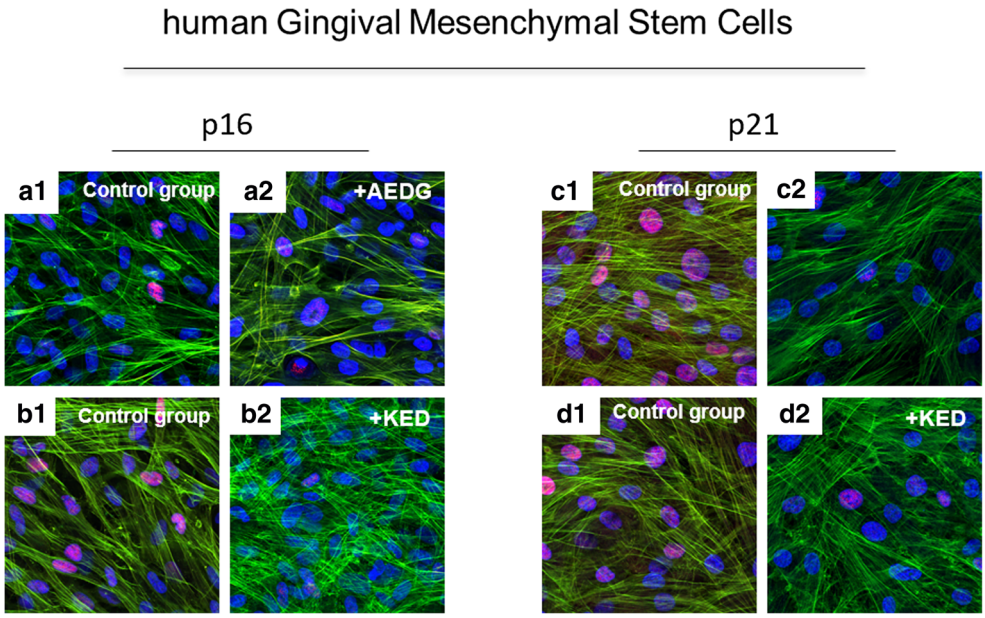

Fig. 3 The influence of AEDG and KED peptides on p16 and p21 protein syntesis in hGMSCs. P16 expression in **a1** untreated hGMSCs and **a2** AEDG peptide treated hGMSCs; **b1** untreated hGMSCs and **b2** KED peptide treated hGMSCs. P21 expression in **c1** untreated hGMSCs and **c2** AEDG peptide treated hGMSCs; **d1** untreated hGMSCs and **d2** KED peptide treated hGMSCs. Scale bar: 10 μm. Mag: 20X

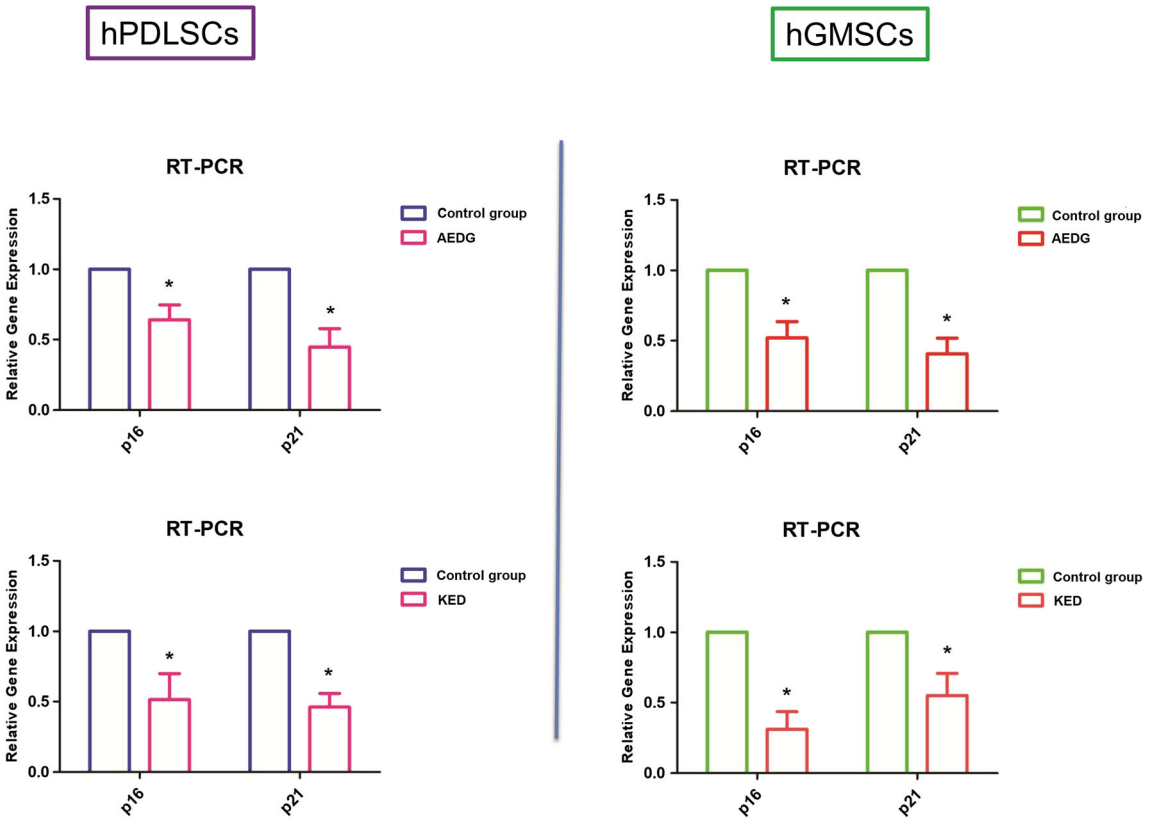

Fig. 4 The influence of AEDG and KED peptides on p16 and p21 gene expression in hPDLSCs and hGMSCs. * - $p < 0.01$ in comparison with the corresponding value in the control group

synthesis when compared to untreated cells cultured at the 25th passage.

AEDG peptide was extracted from the polypeptide pineal gland complex [47] with a strong anti-aging action. AEDG peptide induced telomere elongation, increased telomerase activity [25, 26], inhibited the synthesis of Caspase-3 (apoptosis protein), MMP9, CD98hc (cell aging and dysfunction proteins), increased Ki67 (proliferative protein) in fibroblast cultures during aging [48]. AEDG peptide also induced the decondensation of heterochromatin near the centromeres in cultured lymphocytes derived from elderly and old people [49]. In vivo AEDG peptide reduced the chromosomal aberrations and increased the telomere length, extended mice lifespan and promoted enzyme antioxidant activities [50, 51].

In our experiment KED peptide showed the same results as AEDG peptide, but with a less effect on p21 and p16 gene expression and protein synthesis in hPDLSCs and hGMSCs cultured during the 25th passage. KED is the vasoprotective tripeptide, which stimulated Ki67 proliferative protein and decreased p53 apoptotic protein synthesis in fibroblasts and endothelium cultures during aging [28, 48].

We can suppose that AEDG and KED peptides realize a protective effect on the senescence stem cell by reducing p16 and p21 genes expression and protein synthesis. To validate the RT-PCR quantitative data has been performed. P16 is a protein which is involved in the tumor suppression and plays a critical role in the regulation of cell cycle in G1 phase [52]. On the other hand, the activation of p16 stimulated by oxidative stress or DNA damage is related to cell aging [53]. In several tissues p16 is implicated in the regulation of the self-renewal processes of stem cells and its deregulation may result in aging or tumor development [54]. p21 regulated the cell cycle as an inhibitor. It has been reported that p21 protects adult stem cells from genotoxic stress by preventing inappropriate cycling of damaged stem cells [55]. P21 is considered an essential mediator of p53-dependent cell cycle arrest and moreover p21 is a proliferation inhibitor suggesting an important role in the induction of senescence [56]. In young humans fibroblasts (the 21st passage) can lead to senescence-like growth arrest; they also showed p21 over expression after DNA damage [57]. It was shown that cells lacking the gene encoding for p21 bypass the senescence checkpoint and cells with a p21 deficiency showed an elongated life span without an increase of chromosomal instability [58].

AEDG and KED peptides prevent p16 and p21 gene expression and protein synthesis. They can prolong stem cell division in vitro and prevent its senescence and apoptosis. In conclusion, AEDG and KED peptides could be used as supplementary substances in culture media to delay the expression of senescence markers in long term stem cell culture in

order to promote the large-scale in vitro expansion necessarily required for the clinical application of stem cell therapy.

Compliance with Ethical Standards

Conflict of interests The authors declare no conflict of interests.

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