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Original Article

# Advanced glycosylated end products restrain the osteogenic differentiation of the periodontal ligament stem cell



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## KEYWORDS

AGEs (advanced glycosylated end products);  
PDLSCs (periodontal ligament stem cells);  
Periodontal disease;  
Diabetes mellitus;  
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**Abstract** *Background/purpose:* Many studies have confirmed that periodontal disease interacts with diabetes. The aim of this study was to examine whether the advanced glycosylated end products (AGEs), which are generated by diabetics, have important effects on the osteogenic differentiation of periodontal ligament stem cells (PDLSCs).

*Materials and methods:* In this study PDLSCs were isolated from the periodontal ligaments of extracted third molar teeth. The subjects were divided into two groups, which included the normal control group (N-PDLSCs) and the AGEs-stimulating group (A-PDLSCs). Changes of receptor of AGEs (RAGE) and cumulative ROS in PDLSCs were monitored by western blot and flow cytometry, respectively.

*Results:* In the study AGEs noticeably inhibited the osteogenic differentiation of PDLSCs, with significant lower calcification nodules detected in A-PDLSCs ( $P < 0.01$ ). RAGE expression level and ROS accumulation in A-PDLSCs were clearly higher than those in N-PDLSCs ( $P < 0.01$ ).

*Conclusion:* Our conclusions were that AGEs may cause the apoptosis of stem cells, which could lead to the disorder of bone differentiation function of PDLSCs.

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## Introduction

Periodontitis is a progressive disease characterized by the loss of periodontal tissues, particularly the alveolar bone. The prevalence of adult periodontal disease in China is as high as 80–90%, and is the main cause of adult tooth loss in this country.<sup>1</sup> Previous studies have shown that stem cells exist in the pulp, exfoliated teeth and normal periodontal tissues of adult teeth, of which periodontal ligament stem cells (PDLSCs) are subtypes that can differentiate into osteoblasts, osteocytes, fibroblasts and so on.<sup>2,3</sup> These then produce dental bone-like structures that move along the connective tissue of the periodontal membrane. They also affect typical periodontal ligament-like tissues to regenerate tissues damaged by periodontitis. Its regeneration potential contributes to its self-renewal and multi-differentiation ability, especially osteogenesis differentiation.<sup>4,5</sup> It is suggested that PDLSCs can provide an important cell source for the treatment of periodontal disease mediated by stem cells.<sup>6</sup>

Studies have revealed that, there is a complex internal relationship between periodontitis and diabetes mellitus.<sup>7–14</sup> The two diseases are risk factors for each other and promote the progress of the diseases in conjunction with each other. People with chronic adult periodontitis and type 2 diabetes mellitus have a strong immunoreactivity induced by lipopolysaccharide, which is related to the severity of the pre-existing periodontal disease, revealing the cause of periodontitis susceptibility in a diabetic population.<sup>15</sup> High glucose levels can promote lipopolysaccharide to induce gingival epithelial cells to secrete inflammatory factors, and also can increase the expression of toll-like receptor 4 (TLR4) in gingival epithelial cells resulting in periodontal tissue loss.<sup>16,17</sup> Detrimental periodontal tissues can cause interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) to be released in large amounts, which in turn leads to insulin resistance.<sup>18</sup> The TNF- $\alpha$  level of periodontitis patients plays an important influence on diabetes mellitus as a disease. Meanwhile, TNF- $\alpha$  can also promote bone absorption and inflammation.<sup>19–21</sup> IL-6 can enhance the expression of vascular endothelial growth factor in periodontal tissues, leading to the increase of inflammatory cells in periodontal tissues, aggravating the inflammatory response and inhibiting the growth of periodontal ligament cells. Periodontal therapy can decrease the inflammation level of the periodontal tissues, and help to reduce the blood glucose level and insulin resistance in patients with type 2 diabetic periodontitis.<sup>22–24</sup> Therefore, the treatment of periodontitis has important significance for diabetes prevention and treatment.

The prevalence of periodontitis in patients with type 2 diabetes mellitus for more than 5 years exceeds 85%.<sup>7</sup> Studies have shown that advanced glycosylated end products (AGEs) deposit locally in gingival tissues in diabetic patients with long-term hyperglycemia.<sup>25</sup> AGEs are the end-stage products of non-enzymatic saccharification, which play an important role in the pathogenesis of diabetes mellitus.<sup>6</sup> The receptor of AGEs (RAGE) take essential part in accelerating the process of diabetic periodontitis and alveolar bone loss.<sup>1–3</sup> According to research on the AGEs-RAGE system may be a dominant pathway leading to the

vicious cycle of tissue damage and repair in diabetic periodontitis.<sup>25</sup> Periodontitis can decrease the expression of KAT2A gene in PDLSCs, activating the classical Wnt pathway.<sup>26</sup> And the microenvironment of chronic periodontal inflammation can reduce the differentiation potential of PDLSCs. Both can inhibit the osteogenic differentiation of PDLSCs and promote the processes of periodontitis.<sup>27,28</sup> Diabetes mellitus and chronic adult periodontitis are already recognized as being closely related. However, the interaction between AGEs and PDLSCs has not been clarified, so this study will explore this issue.

## Materials and methods

### Materials

Periodontal tissues were obtained from the extracted third molar teeth at the department of Oral and Maxillofacial surgery in the First Affiliated Hospital of Hainan Medical University. Totally 24 teeth used in the experiment were extracted from young men between the ages of 20 and 30. The teeth were free from infection, and patients were free from systemic diseases. The study was carried out under ethical guidelines and with the consent of each patient involved in the study. The tissue cleaner was PBS containing 10% FBS. The primary cell culture medium was DMEM containing 10% FBS. Basal medium of STEMPRO osteocyte contained 10% osteogenesis supplement. The cell digestion solution was 0.25% EDTA trypsin. All of the above cell culture reagents were purchased from GIBCO. Fluorescence direct labeling antibodies including STRO-1, CD146, CD34 and CD45 were purchased from the Abcam company. Primary antibody of RAGE and HRP labeled second antibody were purchased from SANTA CRUZ and CST, respectively. AGE-BSA was purchased from CALBIOCAM, and carboxy-H2DCFDA ROS Kit was purchased from Invitrogen.

### The harvesting of PDLSCs

Soon after extracting the third molar teeth, the epithelial tissues and other connective tissues were surgically removed. The periodontal tissues from the middle one third part of the tooth root were harvested and prepared into around 1 mm<sup>2</sup> tissue blocks. Then the tissue blocks were placed in a culture dish with 10% FBS and incubated at 37 °C with 5% CO<sub>2</sub> in an incubator for 2 h. Once the tissue blocks attached to the bottom of the culture plate, the primary cell culture medium were added. We changed the medium every two days and cultured the cells for 1–2 weeks. After cell migration from tissue blocks, single cell cloning was labeled and cultured for 1 week, the cells were kept for routine subculture.

### The identification of PDLSCs

PDLSCs were cultured to the 2nd to 3rd generation. Then we digested the cells, resuspended them with PBS containing 5% FBS and adjusted the density to 1 × 10<sup>6</sup>/mL. Fluorescence directly labeled stem cell surface marker antibodies were used to confirm the PDLSCs, of which

STRO-1 and CD146 were positive and CD34 and CD45 were negative by flow cytometry.

### The detection of osteogenic differentiation

PDLSCs were cultured to the 3rd to 5th generation and divided into 4 groups, STEMPRO osteocyte basal medium contained 10% osteogenesis supplement was used. In the process of cell differentiation, AGE-BSA (0, 50, 100 and 200ug/mL) was added to stimulate the PDLSCs for 2 weeks. Then 2% alizarin red reagent was applied for staining to detect the osteogenic differentiation of PDLSCs.

### Western blot examination

AGE-BSA (0, 50, 100 and 200ug/mL) was used to stimulate the PDLSCs for 2 weeks. Cell Proteins were collected using cell protein lysate. To detect the RAGE expression level, the proteins were separated via SDS-PAGE and transferred to PVDF membrane. The dilution ratio for primary antibody of RAGE and HRP labeled second antibody was 1:500 and 1:10000, respectively. The density of the protein bands was calculated by Quantity-one software.

### The detection of ROS generation

AGE-BSA (200ug/mL) was added to stimulate PDLSCs for 24 h. Cells were flushed with warm HBSS twice. H2DCFDA probes were non-fluorescent in their initial form and the conversion of H2DCFDA involved oxidation. After the H2DCFDA (25uM) was added, PDLSCs were incubated for 60 min at 37 °C, avoiding light. Then Hoechst33342 (1.0 uM) was added for further flow cytometry test. Fluorescence of the H2DCFDA-treated PDLSCs could depend and reflect on the intracellular ROS level.

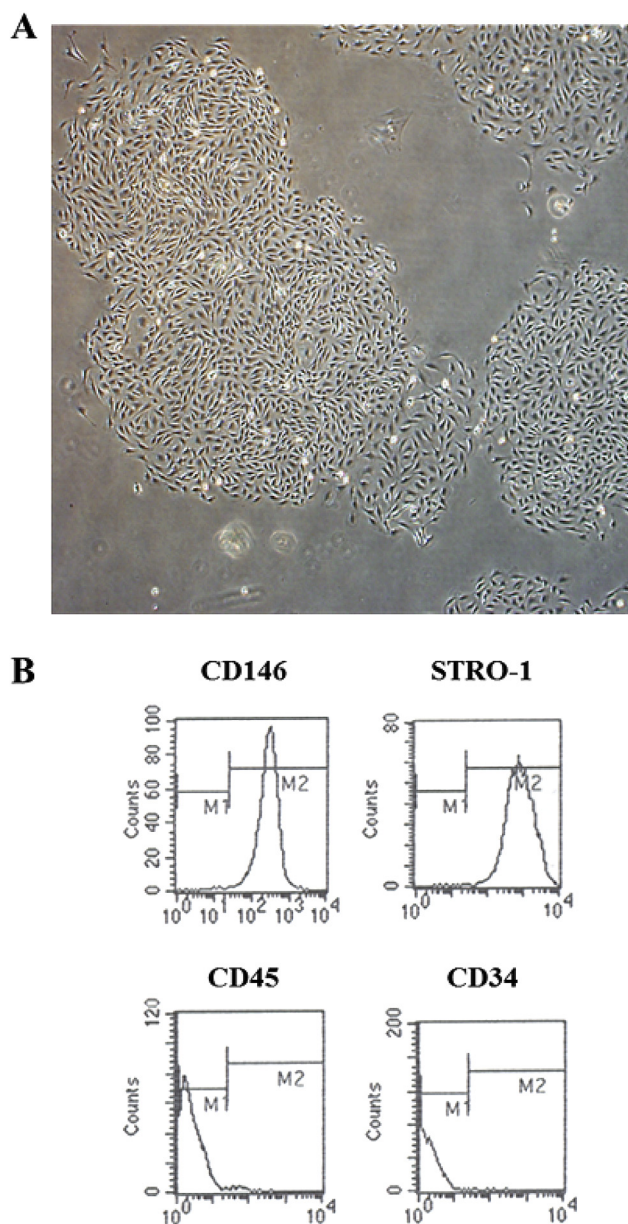
### Data analysis

All the above examinations were repeated three times. SPSS 23.0 statistical software was applied to analyze the results. All data was expressed by means  $\pm$  SEM, and the unpaired, two-tailed Student *t* test was used for comparison between groups.  $P < 0.05$  was considered as statistically significant.

## Results

### Culture and identification of PDLSCs

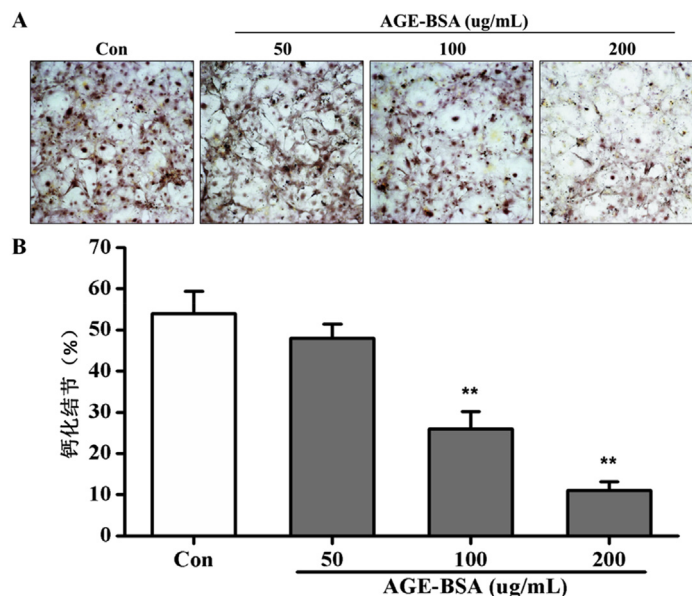
PDLSCs were cloned after culturing for 7 days, and grew like fibrous cells (Fig. 1A). PDLSCs were positive for STRO-1 and CD146, while negative for CD34 and CD45 (Fig. 1B). The identification of primary PDLSCs was confirmed by flow cytometry three times. The average percentage of positive marker cells of CD 146, STRO-1, CD45 and CD34 was 91%, 84%, 13% and 6%, respectively. Different passage of PDLSCs developed for further experiments should be re-identified before use.



**Figure 1** Culture and identification of PDLSCs. (A) Primary culture of PDLSCs was evaluated by light microscope under a 100X magnification. (B) PDLSCs were identified by Flow cytometry, with STRO-1 and CD146 as positive markers and CD34 and CD45 as negative markers.

### AGE-BSA could inhibit the osteogenic differentiation of PDLSCs

We used different doses of AGE-BSA (50, 100 and 200ug/mL) to stimulate PDLSCs and made a test of osteogenic differentiation. AGE-BSA noticeably inhibited osteogenic differentiation of PDLSCs (Fig. 2A). The percentage of osteogenic calcification nodules of PDLSCs in the experimental group was obviously lower than that in the control group (Fig. 2B). AGE-BSA showed dose-dependent inhibition to the osteogenic differentiation of PDLSCs. 100 and 200ug/mL AGE-BSA



**Figure 2** The inhibitory effect of AGE-BSA on the osteogenic differentiation of PDLSCs. (A) With AGE-BSA (50, 100 and 200µg/mL) stimulation for two weeks, the osteogenic differentiation and calcification nodules of PDLSCs was calculated by alizarin red staining. Pictures were taken under a 100X light microscope. (B) Compared with the normal control group (N-PDLSCs), the osteogenic calcification nodules in AGEs-stimulating group (A-PDLSCs) was obviously reduced. The error bars showed means  $\pm$  SEM in triplicate experiments. \*\* $P < 0.01$  by an unpaired two-tailed  $t$  test.

had the most significant effects on inhibiting the osteogenic differentiation of PDLSCs ( $P < 0.01$ ).

### AGE-BSA could increase the expression of RAGE in PDLSCs

With AGE-BSA (50, 100 and 200µg/mL) stimulation, the RAGE expression level of PDLSCs was detected by western blot (Fig. 3A). Compared with the normal control group (N-PDLSCs), the expression of RAGE in AGEs-stimulating group (A-PDLSCs) was obviously enhanced (Fig. 3B). 100 and 200µg/mL AGE-BSA had the most significant effects on promoting the expression of RAGE in PDLSCs ( $P < 0.01$ ).

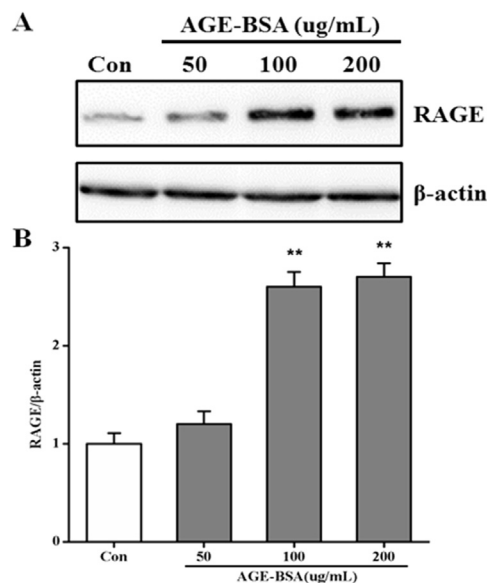
### AGE-BSA could induce ROS accumulation in PDLSCs

Intracellular ROS accumulation in A-PDLSCs was clearly enhanced than in N-PDLSCs (Fig. 4A). With AGE-BSA (200µg/mL) stimulating for 24 h, the fluorescence intensity of ROS in PDLSCs significantly increased ( $P < 0.01$ ) (Fig. 4B).

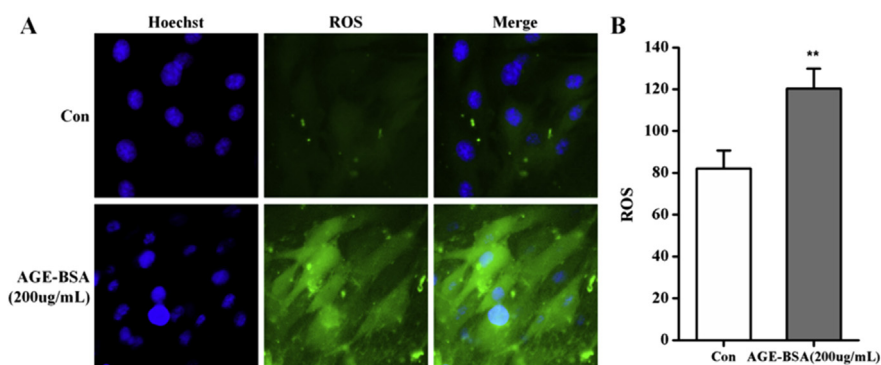
## Discussion

Previous studies on the relationship between chronic adult periodontitis and diabetes mellitus have found that locally deposited AGEs can reduce the production of the extracellular matrix, decrease the expression of collagen, hinder the differentiation and maturation of osteoblasts, mediate the apoptosis and delay the repair of the periodontal tissues.<sup>29–31</sup> Our investigation found that AGEs significantly inhibit bone differentiation of the PDLSCs in a dose-dependant manner. The results supported that compared with non-diabetic patients, periodontal tissue

loss and necrosis is more severe in patients with diabetes mellitus. This is because of a direct correlation between the effects of AGEs and the detrimental effects on PDLSCs.



**Figure 3** The effect of AGE-BSA on RAGE expression in PDLSCs. (A) Evaluation on RAGE expression level with two-week AGE-BSA stimulation via western blot. As an appropriate protein-loading control,  $\beta$ -actin was used for both N-PDLSCs and A-PDLSCs. (B) AGE-BSA (100 and 200µg/mL) could significantly enhance the expression of RAGE in PDLSCs ( $P < 0.01$ ). The error bars showed means  $\pm$  SEM in triplicate experiments. \*\* $P < 0.01$  by an unpaired two-tailed  $t$  test.



**Figure 4** Evaluation on the accumulation of ROS in A-PDLSCs. (A) AGE-BSA (200ug/mL) stimulated PDLSCs for 24 h, the intracellular ROS accumulation was recorded by a 400X fluorescence microscope. (B) The fluorescence intensity of intracellular ROS in A-PDLSCs (200ug/mL, 24 h) was significantly increased than in N-PDLSCs. The error bars showed means  $\pm$  SEM in triplicate experiments. \*\* $P < 0.01$  by an unpaired two-tailed  $t$  test.

In this study, it was also found that AGEs could induce a dose-dependent increase in RAGE expression and an abnormal ROS accumulation level in PDLSCs. RAGE are expressed on a variety of cell surfaces, and previous studies have shown that canonical intracellular signals, including MAPK and WNT signaling pathways were cascaded with the combination of AGEs and RAGE.<sup>17,22,23</sup> It has been found that the MAPK signaling pathway is the key for bone development and homeostasis, and it also plays an important role in bone differentiation of PDLSC.<sup>32,33</sup> The activation of the MAPK signaling pathway is directly related to the secretion of proinflammatory cytokines.<sup>19,21,23,24</sup> Many studies suggest that the high expression of  $\beta$ -catenin in PDLSCs reduces osteogenesis and cellular differentiation via WNT signaling pathway.<sup>34–37</sup> With  $\beta$ -catenin accumulation in the cytoplasm, the stable  $\beta$ -catenin is transferred to the nucleus and mediates the transcription of downstream genes. Furthermore, the accumulation of intracellular ROS is a critical pathological mechanism of diabetes mellitus.<sup>20,22–24</sup> The findings of our study warrant further investigation of this important part of the relationship between diabetes mellitus as a systemic disease and its effect on chronic adult periodontitis and vice versa.

Periodontitis and several systemic diseases are already recognized as being closely related.<sup>38,39</sup> Researchs on the related mechanism have focused on the periodontal tissues response to specific periodontal pathogens, high inflammatory phenotype of monocytes, impaired adhesion, chemotaxis and phagocytosis of neutrophils. Studies similar to ours are uncommon and seldom focused on the osteoblast differentiation of PDLSCs by AGEs. As an autologous source of adult stem cells, PDLSCs have potential therapeutic application value to patients with diabetes mellitus.<sup>2–6</sup> Studies similar to ours are uncommon and relate to other factors. The findings of this study provided some preliminary experimental evidence for the application of PDLSCs in clinical treatment, which also play an important role in the prevention and treatment of periodontal disease in diabetic patients. Moreover, active controlling the incidence of periodontal disease and widely encouraging people to establish the preventive awareness should be promoted to the general population with or without systemic diseases.

Our current results proved that AGEs promote the accumulation of intracellular ROS by increasing the expression of RAGE, which ultimately leads to the increased inflammatory response and disorder in the osteogenic differentiation of PDLSCs. In conclusion, diabetes mellitus and chronic adult periodontitis are being closely related. AGEs may cause the apoptosis of stem cells, which could noticeably inhibit the osteogenic differentiation of PDLSCs.

## Conflicts of interest

The authors declare no conflict of interest.

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