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Original Article

Advanced glycation endproducts regulate smooth muscle cells calcification in cultured HSMCs

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Abstract: Objective: To investigate the mechanism of Advanced glycation end products (AGEs) promoting the calcification of smooth muscle cells. Methods: The successfully cultured smooth muscle cells were divided into three groups: normal culture group (group A), calcified culture group (group B), calcification + AGEs group (group C); the concentration of intracellular calcium ion was detected in each group; the promotion of AGEs on the calcification of HSMCs was confirmed by VON KOSSA staining; and the expressions of β-catenin, RAGE, β-catenin, OPG and E-cadherin protein were detected by immunofluorescence and western blot. Results: The morphology of the cells in each group showed that the amount of calcified plaques in calcification + AGES group were significantly higher than the calcification group. VON KOSSA staining showed that with increasing concentrations of AGE-BSA, the amount of its calcification gradually increased. Calcium concentration in Calcification + 20 mg/L AGEs group was significantly higher, followed by 40 mg/L AGEs group. The expression of β-catenin increased with the increasing concentrations of AGEs. Conclusion: AGEs can promote the calcification of human femoral artery smooth muscle cells, with a concentration gradient effect. With increasing concentrations of AGEs, the expression of RAGE increased, indicating that AGEs-induced HSMCs proliferation was correlated with RAGE expression.

Keywords: HSMCs, calcification, AGEs, RAGE, β-catenin, OPG

Introduction

Excessive proliferation and migration of smooth muscle cells in the artery are the main reasons for arteriosclerosis obliterans and restenosis after surgical intervention. A growing number of studies have shown that the pathology of cardiovascular disease is based on the migration and proliferation of vascular smooth muscle cells (VSMCs), which is the central link in the evolution and outcome of atherosclerosis [1]. Advanced glycation end products (AGEs) stimulated vascular smooth muscle cells, leading to changes in the phenotypes of smooth muscle cells and endothelial migration; "synthetic" smooth muscle cells secreted VEGF, IGF-1 and other factors, inducing the migration and proliferation of smooth muscle cells. AGEs combined the report of advanced glycosylation end products (RAGE) receptors on the membrane of smooth muscle cells, inducing oxidative stress and promoting the activation of redox-sensitive transcription factors, such as activator protein 1 (AP-1), nuclear factor-κB (NF-κB), IL-6 and monocyte chemotactic protein 1 (MCP-1), thus speeding up the process of atherosclerosis [2]. Previous study reported that AGEs can enhance the migratory activity of VSMCs by combining with the membrane RAGE receptors [3]. AGEs deposited on the macrovascular of diabetic patients or aging bodies, and expressed in inner, middle and outer membranes of the aorta. This deposition may induce intimal thickening, declines in elastic fibers, luminal stenosis and AS formation. Tanikawa et al. [4] found that, AGEs may promote the calcification of vascular smooth muscle cells through mediating RAGE/MAPK pathway.

Meanwhile, recent studies found that Wnt/β-catenin signaling molecules played an important role in embryonic bone growth, bone mass balance after the birth, fracture healing and many other bone remodeling processes by reg-

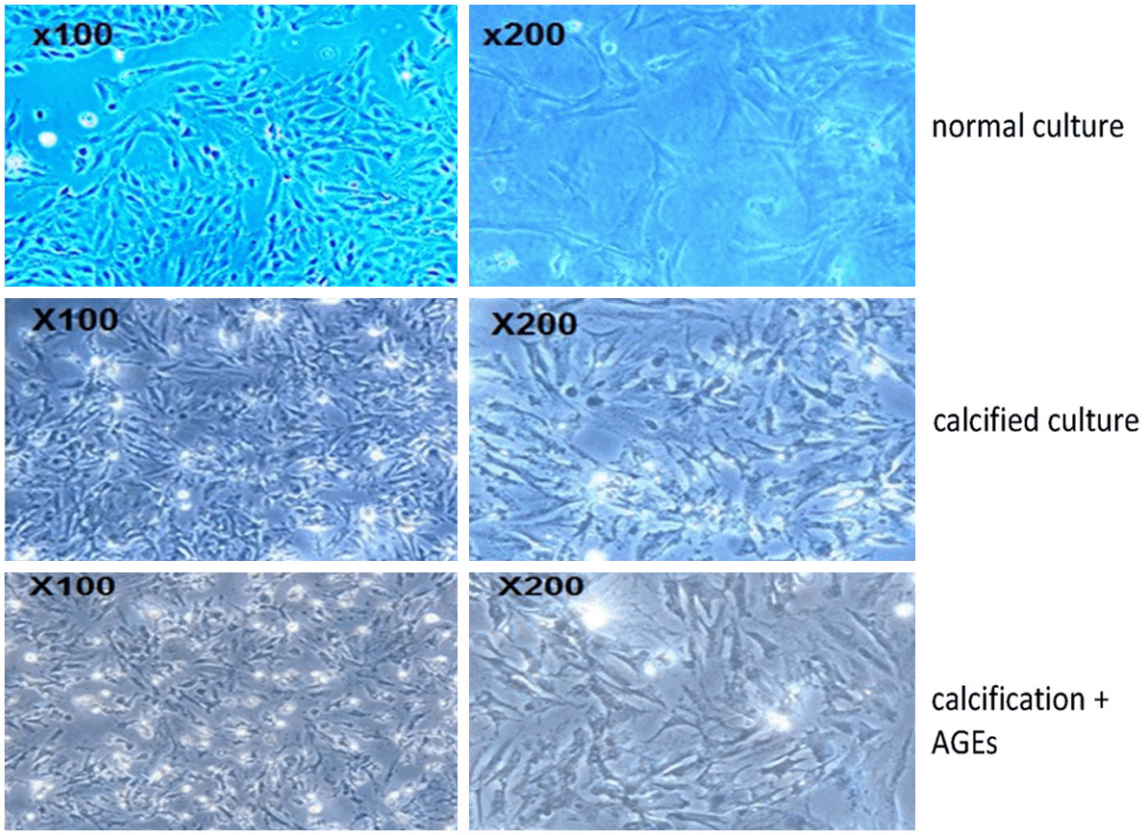

Figure 1. Cell morphology in each experimental group.

ulating the proliferation, differentiation and function of osteoblasts [5]. Activation of Wnt/β-catenin signals promoted the expression of proteins related to bone formation, such as Runx2, OPG, Osx, BMP, cyclinD1 and MMP7. Therefore, Wnt/β-catenin signaling pathway is considered to be the most critical signaling pathway in the process of bone formation [6]. Furthermore, studies found that in tunica media, under the effect of the many factors, smooth muscle cells proliferated, migrated and transformed to osteocytes; the transformed osteoblasts had the characteristics of smooth muscle cells, and the key transcription factor for the differentiation of osteoblast-like cells was also highly expressed in the vascular smooth muscle of cattle. These results strongly suggested that: calcification in the calcification of tunica media in diabetic patients, wnt/β-catenin signaling pathway was the most critical signaling pathway [7].

This study further confirmed the role of β-catenin in the calcification of tunica media mainly through investigating the expression changes of β-catenin and its downstream signaling proteins, such as OPG and E-cadherin, in order to explore the preliminary mechanism of AGEs promoting the calcification of HSMCs in femoral artery.

Materials and methods

Isolation and culture of HSMCs

Normal femoral artery was offered by Dr. Wang Mian, Vascular Surgery Affiliated Hospital of Zhongshan University, which was taken from normal donors. Under a sterile environment, the outer and inner membranes were scraped with scalpel blades; the media layers were cut into 1-2 mm blocks and individually affixed to 25 cm^2 flasks with tweezers; the flasks were slowly flipped, and 10 ml DMEM containing 20% fetal bovine serum and 100 U/L penicillin and streptomycin was added; cells were incubated in an incubator (37°C, 5% CO_2); Two hours later, the flasks were gently flipped to adequately cover tissue blocks. After incubation in the incubator for about two weeks, the growth of smooth muscle cells was observed;

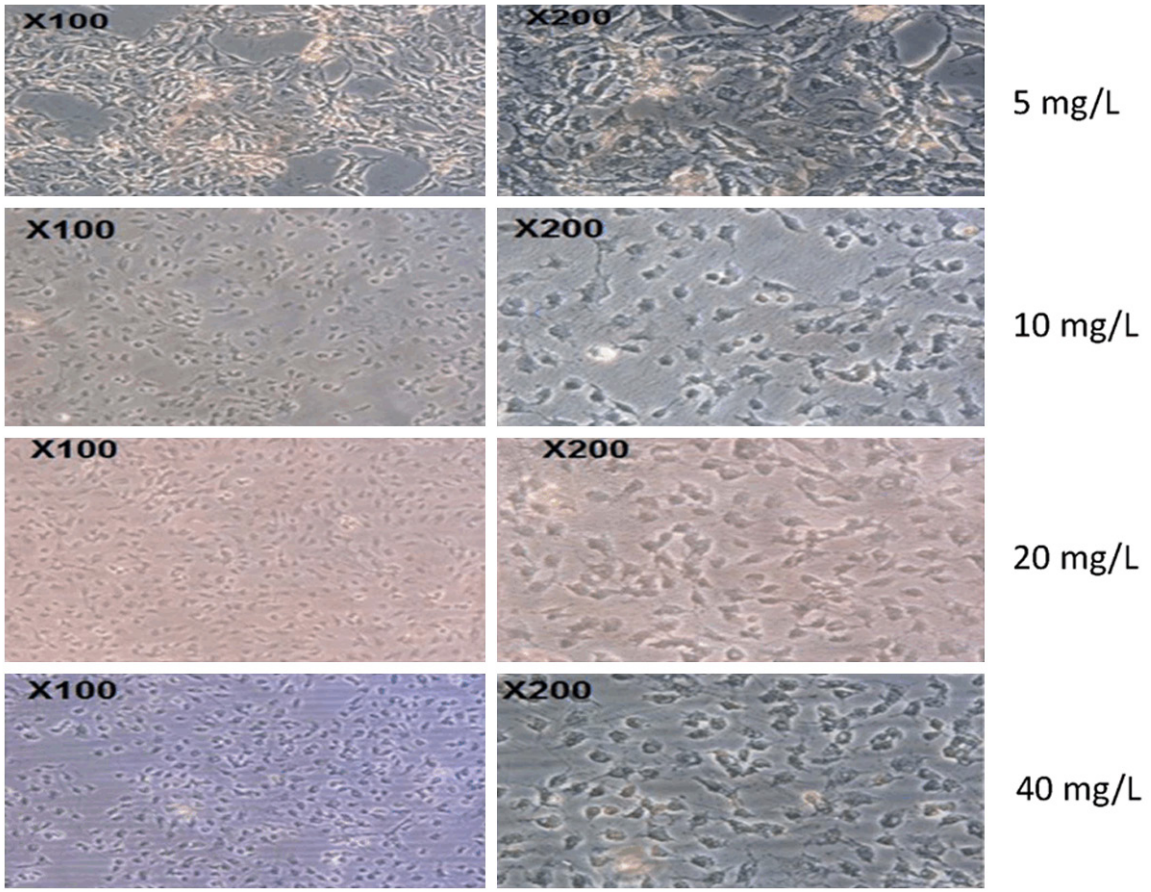

Figure 2. Cell morphology in different concentration of AGEs.

Table 1. Comparisons of intracellular calcium among different groups

Group	Control	Ca^{2+}	Ca^{2+} + AGES (20 mg/L)	Ca^{2+} + AGES (40 mg/L)	AGES (40 mg/L)	AGES (20 mg/L)	AGES (10 mg/L)
Concentration of Ca^{2+}	1.21067± 0.162069	2.81267± 0.731392	3.10600± 0.261019*	3.40667± 0.668398*	2.525± 0.377762*	1.75233± 0.255819	1.28633± 0.055983

Compared to control group, *P < 0.05.

after the flasks were covered, trypsin digestion and passage were performed; cells of 3-8 generations were used in the experiment.

Experimental groups

The cultured smooth muscle cells were randomly divided into three groups: normal culture group (group A), calcified culture group (group B), incubated with calcification medium (according to the literature method, 10 mmol/L β glycerol phosphate and 10 mmol/L pyruvate were added into the normal DMEM medium); calcification + AGEs group (group C): based on the amount of AGEs, it was divided into four subgroups: C1 group: AGEs 40 mg/L, C2 group: AGEs 20 mg/L, C3 group: AGEs 10 mg/L, C4 group: AGEs 5 mg/L.

Determination of intracellular Ca^{2+} concentrations

According to the method described in the literature, samples were prepared and measured using a spectrophotometer at a wavelength of 570 nm. 800 μl Genmad buffer, 100 μl Genmad reaction solution and 100 μl sample were mixed in a new 1 ml cuvette and incubated for 5 minutes; then the cuvette was placed into the spectrophotometer to obtain absorbance readings. Standard curve was constructed: the ordinate (Y-axis) was the absorbance (OD); the

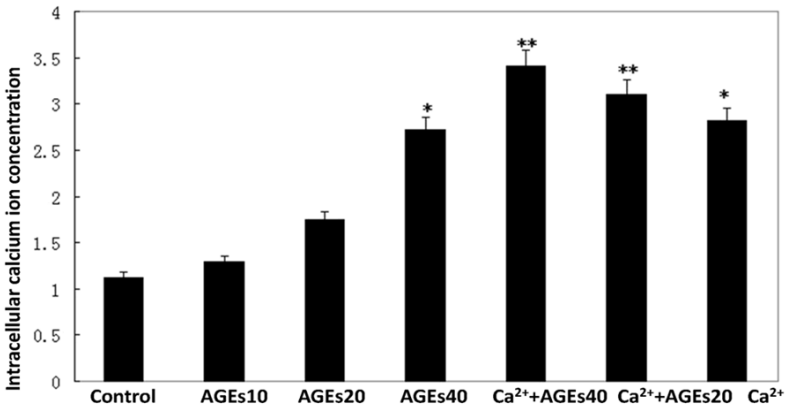

Figure 3. Bar graphs of intracellular calcium concentrations in each group: *$P < 0.05$ compared with control group.

abscissa (X-axis) was the standard calcium concentration (mmol/L); the corresponding calcium concentration was obtained from the standard curve.

Immunofluorescence

(1) Cell morphology observation: smooth muscle cells in each group were placed under an inverted microscope to observe and photograph; (2) The smooth muscle cells of each group were seeded on circular glass slides with a diameter of 13 mm, adherent growing in an incubator (37°C, 5% CO_2); cell growth was observed under a microscope; when the cells covered about 40-50% of the glass slide, the medium was aspirated and PBS washing was performed (3 × 5 minutes); (3) Cells were fixed in 4% paraformaldehyde at 4°C for 15 min, and then rinsed by PBS (5 min × 3) and pre-cooled CH_3OH for three times; (4) Smooth muscle cells were transparent by pre-cooled CH_3OH at -20°C for 5 min, and rinsed by PBS again (5 min × 3); (5) Cells were sealed with 10% goat serum and 5% BSA blocking solution at room temperature for 20 min, and then they were incubated with the primary antibody (1:150 mouse anti-HSM α-actin monoclonal antibody) at 4°C overnight; on the next day, rewarming at room temperature was performed for one hour; (6) was added after PBS washing (5 min × 3), cells were incubated with green fluorescence-labeled goat anti-mouse secondary antibody for 1 hour at room temperature in the dark; PBS washing (5 min × 3) was performed again; (7) DAPI dye stained nuclei for 15 min before ddH_2O cleaning; (8) Mounting Medium was used for sheet-sealing before photographing by fluorescence microscope; samples were stored at 4°C.

Western blotting

The total protein was separated using SDS-PAGE and electrotransferred to a PVDF membrane (Millipore Corporation, Bedford, MA, USA) at 100 V for 60 min. The membrane was immersed in 5% non-fat milk in a TTBS solution [0.2 M TRIS-HCl (pH 7.6), 1.37 M NaCl, 0.1% Tween-20] for 1 h at room temperature. The proteins were incubated with the polyclonal antibodies in 5% non-fat milk in a TTBS solution for 3 h at room temperature and subjected to three 5 min rinses in a TTBS solution. The membrane was then incubated with a horseradish peroxidase-conjugated goat antirabbit antibody (Zhongshan Goldenbridge Biotechnology Co., Ltd, Beijing, China) for 1 h at room temperature, and subjected to three 5 min rinses in a TTBS solution. The blot was developed with a Super ECL Plus kit (Applygen, Beijing, China), and the signal was exposed with X-ray film.

Statistical methods

All the data were analyzed using SPSS 16.0 software. The continue variables were present as Mean ± SD and tested using one-way ANOVA. $P < 0.05$ was considered significant.

Results

Cell morphology in each experimental group

As shown in **Figure 1**, calcified plaques in calcification + AGEs 20 mg/L group were significantly more than those of the calcified group, and those of the two groups were more than those of the normal group. In the normal culture group, calcified plaques were almost invisible (white spots were the calcified plaques); in calcium culture group, significant calcified plaques can be observed in smooth muscle cells; after adding AGEs, calcified plaques were increased significantly in smooth muscle cells; it can be seen from **Figure 2** that with increasing concentrations of AGEs, intracellular deposition of

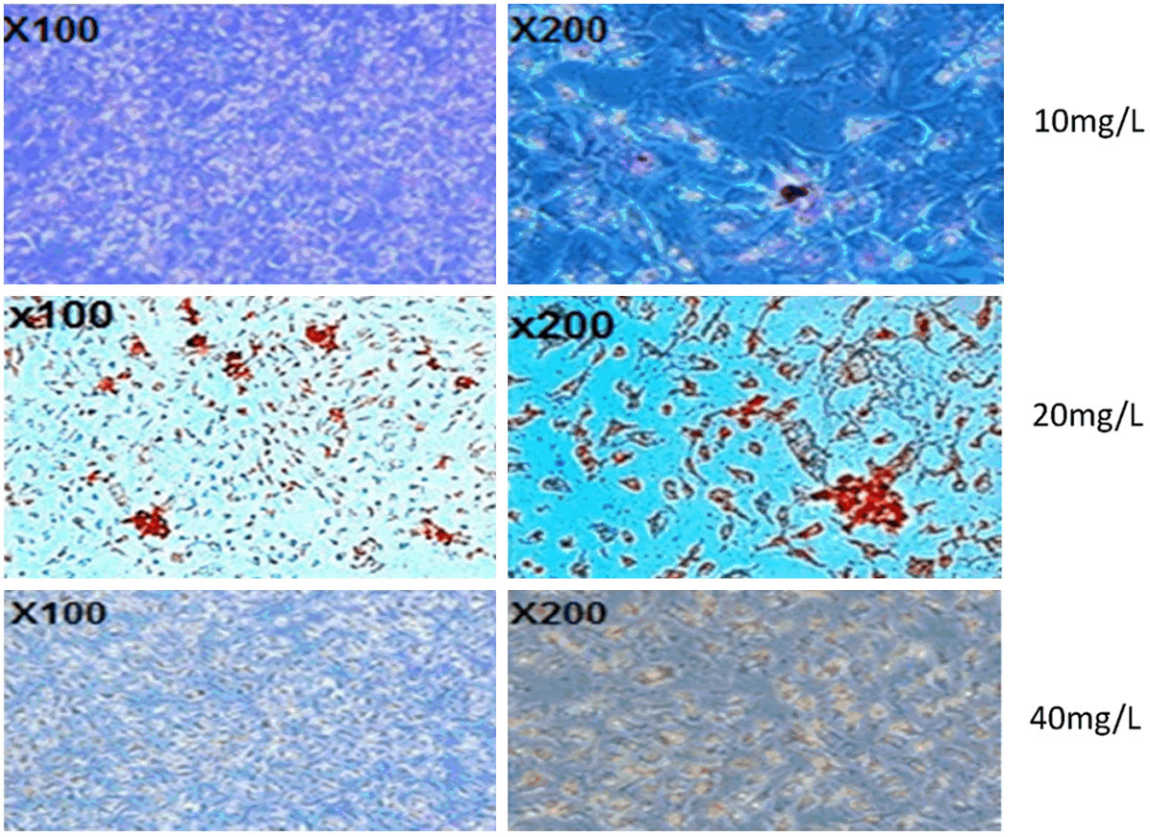

Figure 4. Images of VON KOSSA cells staining: the former was 100 times and the latter was 200 times. Under simple AGEs cultivation, with higher concentrations of AGEs, the number of calcified plaque was increased, respectively.

calcified plaque formed in subgroups of 40 and 20 mg/L AGEs.

Determination of intracellular calcium concentrations

The Determination of intracellular calcium concentrations showed that the calcium concentrations in calcification + AGEs 20 mg/L group and calcification + AGEs 40 mg/L group were significantly increased; compared to the control group, the intracellular calcium concentrations in calcification group and calcification + AGEs40 mg/L group were also significantly increased ($P < 0.05$), with statistically significant differences (**Table 1**; **Figure 3**). It indicated that AGEs could accelerate intracellular deposition of calcium ions when promoting cell proliferation.

VON KOSSA cell staining

VON KOSSA cell staining result showed that with increasing concentrations of AGE-BSA, the amount of calcified expression increased gradually. At the same time the amount of calcified AGEs (20 mmol/L) cells increased more significantly than that in other calcified group. The number of cytoplasm and nucleus which exhibited pink was also increased. We also found that the number of simple calcified cells decreased significantly, indicating that calcified culture can induce apoptosis (**Figures 4** and **5**).

Western-blot

As shown in **Figure 6**, under calcified culture conditions, the expression of OPG and aggregation of β-catenin in the nucleus were reduced; but after AGEs intervention, RAGE expression was significantly increased dependent on the concentration gradient, and OPG expression and β-catenin accumulation in the nucleus were also enhanced.

Discussion

Diabetes is one of the important risk factors that lead to atherosclerosis. In diabetes, elevated blood glucose, together with plasma pro-

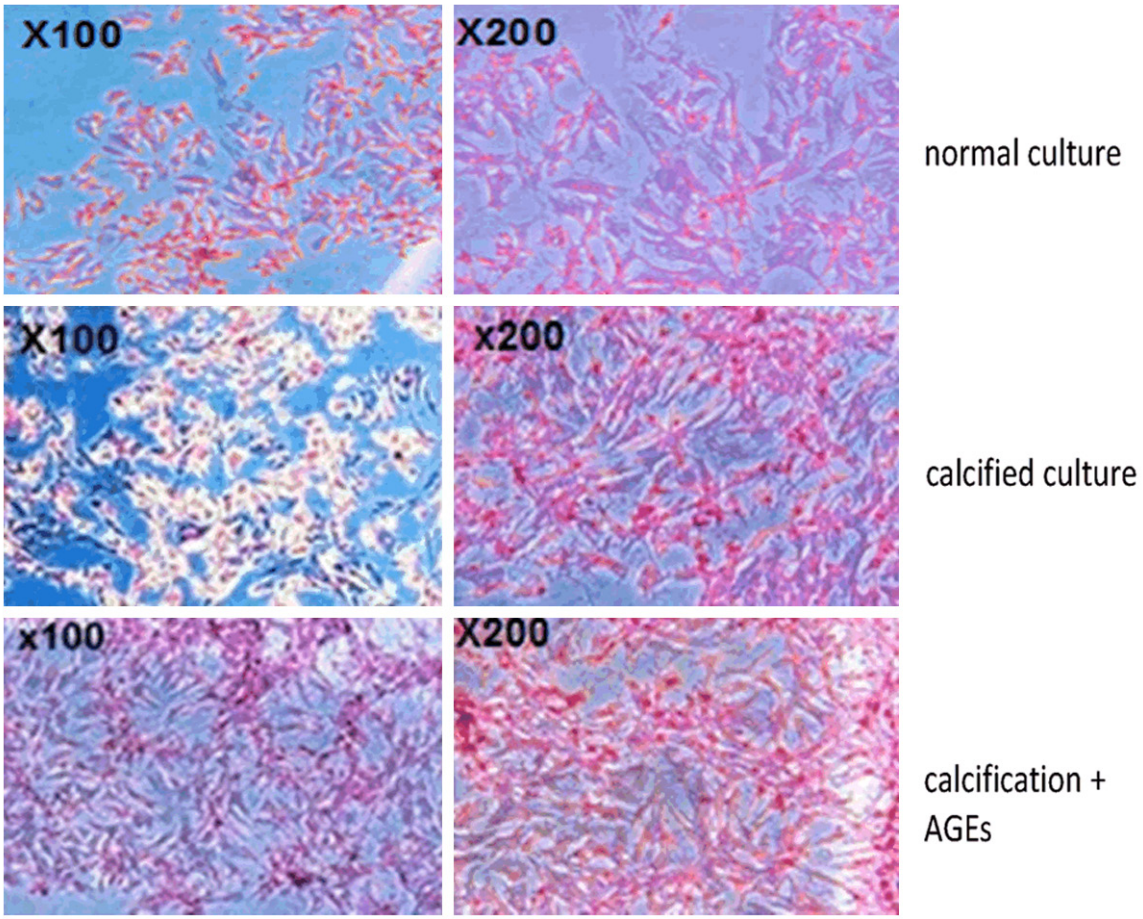

Figure 5. Under normal medium, the formation of calcified plaque barely can be seen. (After VON KOSSA staining and nuclear fast red re-staining, if there was calcium deposition, the cytoplasm and nucleus were stained red or pink). In calcified culture conditions, calcified plaques can be seen significantly in smooth muscle cells; in calcification with AGEs (20 mg/L) cultured conditions, calcium deposition was significantly more than that in other groups. The number of cytoplasm and nucleus which exhibited pink was also increased.

teins, amino acids, free amino terminus of the peptide, through non-enzymatic glycosylation, eventually formed AGEs. AGEs combined with RAGE receptor and promoted inflammatory cell infiltration. It stimulated the proliferation of VSMCs and promoted fibrous tissues proliferation, which also played a key role in the occurrence of AS and development process in Diabetes Mellitus [8]. AGEs promoted HSMCs proliferation with dose and time dependent. With increasing concentrations of AGE-BSA, the study found that AGEs acted on arteries through the expression of its receptor RAGE [9]. AGEs combined with RAGE receptors, and activated multiple signal transduction proteins and transcription factors in downstream, including factors which were related to osteoblasts such as Src, NAD (P) H oxidase, p38MAPK, AP1, NF-κB, Ras/ERK1/2, PI3K/PDK1/Akt [10-12].

Vascular smooth muscle cells (VSMC) proliferation play a key role in diabetic ascular complications [13]. AGEs can stimulate vascular smooth muscle cell proliferation and accelerate the formation of atherosclerosis and coronary restenosis [14]. The risk factor was 3 to 4 times higher in diabetic patients with atherosclerosis than the normal. Under the long-term of hyperglycemia, AGEs may be involved in the occurrence of atherosclerosis [15]. Long-term high blood sugar led to the accumulation of AGEs, thus affecting the bone protein and differentiation of bone cells, which made bone metabolic imbalance and caused osteoporosis. Studies from Yamamoto et al. [16] showed that in diabetic population with osteoporosis, AGEs acted with cell surface receptors and led to the decrease of bone formation and the increase of bone resorption. We found that calcified plaque

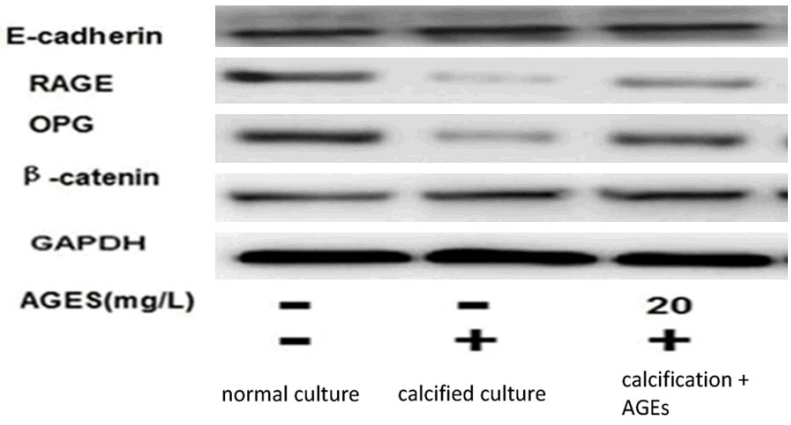

Figure 6. RAGE and OPG expression by Western blotting detection.

can be seen clearly from smooth muscle cells which were cultured in AGEs-BSA with calcification medium, under the ordinary microscope. VON KOSSA cell staining showed that cytoplasm and nucleus were not red in the control group. The number of cells was significantly decreased in calcified group than that in other groups decreased. Cytoplasm and nucleus exhibited pink. The number of cells in calcification plus AGEs (40, 20 mg/L) group was significantly larger than that in the calcified group. The number of cytoplasm and nucleus which exhibited pink was also increased. Through pairwise comparison we found that with increasing concentrations of AGEs, the concentration of intracellular calcium was increased significantly with concentration dependent. There was significant difference between AGES 40 mg/L and AGES 10 mg/L groups with $P < 0.05$. Intracellular calcium ion concentration in the two calcification group adding AGEs 20 mg/L and AGEs 40 mg/L were significantly higher than that in other groups with statistical difference. Meanwhile Von kossa staining result showed that compared with other groups, the number of cytoplasm and nucleus which exhibited pink was increased, which further confirmed that AGEs can promote smooth muscle cell calcified.

These indicated that AGES affected bone protein and bone cells differentiation, which made the bone metabolic imbalance, promoting smooth muscle cell calcified. And then inferred whether the link between AGEs and calcification-related protein existed, or AGEs activated the Wnt/β-catenin signaling pathway and promoted the formation of bone-related proteins, such as: expression of Runx2, OPG, Osx, BMP, cyclinD1, and MMP7.

Our study showed that by staining of smooth muscle cells VON KOSSA we found that cytoplasm and nucleus were not red in the control group. While the number of cells in calcified group decrease significantly than that in other groups, with cytoplasm and nucleus exhibiting pink. The number of plaque deposition was larger in two groups of calcification with AGEs (40, 20 mg/L) than that in other groups. The number of cytoplasm and nucleus which exhibited pink was also increased. With increasing concentrations of AGEs, the number of AGEs (40 mg/L) cells was larger than that in other groups and the number of cytoplasm and nucleus which exhibited pink, compared with other groups, was also increased, indicating that AGEs promoted muscle calcified with concentration-dependent. Our study also found that with increasing concentrations of AGEs and enhanced calcified expression of smooth muscle cell, AGEs promoted vascular smooth muscle cells calcified with concentration dependent. Thus, we concluded that AGEs promoted the deposition of calcium ions in the smooth muscle cells. With interventional concentrations of AGEs increasing, their calcium ion concentration increased and the degree of calcification increased.

β-catenin is associated protein E-cadherin, which is in direct contact with E-cadherin and actin cytoskeleton. It is a mediated protein and is necessary for cell adhesion and movement. It is widely presented on cell membrane, cytoplasm and nucleus. β-catenin and E-cadherin constituted the junctional complex in cell membrane. It mediates same type cell adhered with each other, thereby preventing the activation of downstream β-catenin protein and inhibiting the activation of β-catenin-mediated transcription. Thus they participate in β-catenin signaling pathway associated cell proliferation and differentiation. Previous studies have shown that in the same kinds of cells, when the expression of β-catenin increased and the expression of E-cadherin reduced, the two proteins

achieved a balance. This study found that the β-catenin expression were increased with increasing concentrations of AGEs while the expression of E-cadherin was just opposite β-catenin, which further validated that AGEs/RAGE activated β-catenin signaling pathway, promoting the expression of OPG proteins in the downstream.

Western blot analysis revealed that the expression of β-catenin protein and its downstream OPG were significantly increased, suggesting that wnt/β-catenin signaling pathway played an important role in AGEs promoting smooth muscle cell calcified, which preliminary indicating that it may be mediated effects of RAGE receptor.

In summary, we believe that AGEs can promote femoral artery smooth muscle cells calcified with dose-dependent; with interventional concentrations of AGEs increasing, the expression of RAGE were enhanced, indicating that AGEs induced calcification of HSMCs correlated with the expression of RAGE. AGEs combined with RAGE receptors and they activated wnt/β-catenin signaling pathway, promoting the downstream β-catenin as well as OPG expression increased and smooth muscle cell calcified.

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Disclosure of conflict of interest

None.

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