α-lipoic acid can greatly alleviate the toxic effect of AGES on SH-SY5Y cells

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Abstract. The aim of the study was to explore the influence of α-lipoic acid (α-LA) on the cytotoxicity of advanced glycation end-products (AGEs) against SH-SY5Y cells. AGE-bovine serum albumin (BSA) was incubated *in vitro* using SH-SY5Y cells as a target model, and the control group was set. Cells were exposed to AGE-BSA, and α-LA was selectively added to the cells. Cell growth and death was determined by the MTT assay, which measures cellular metabolic rate, lactate dehydrogenase (LDH) leakage rate and cellular axonal length. Immunocytochemistry was employed to detect the expression of β-amyloid (Aβ) protein in cells, and mRNA expression of amyloid precursor protein (APP) and the receptor for AGE (RAGE) were assayed by PT-PCR. The metabolism of MTT was clearly increased, the rate of LDH leakage was significantly decreased, and axonal length was significantly increased in cells treated with α-LA (0.1 g/l) as compared to untreated cells. Furthermore, the expression levels of Aβ protein were also decreased. In addition, α-LA (0.1 g/l) markedly inhibited the expression of RAGE mRNA, and did not influence APP mRNA expression as compared the control group. α-LA (0.1 g/l) was effective at dampening the cytotoxicity of AGE-BSA, a preliminary observation that confirms the ability of α-LA to significantly alleviate the cytotoxicity of AGEs against SH-SY5Y cells.

Introduction

It has been reported that diabetes may be an independent risk factor for dementia (1,2). The risks of cognitive impairment and Alzheimer's disease (AD) in type-2 diabetic patients were 1.5- and 1.6-fold of the normal population, respectively (3). Furthermore, the degree of brain atrophy seen in AD patients complicated by diabetes was more severe than that seen in AD patients, indicating that diabetes could accelerate the brain aging process of AD patients (4).

Advanced glycation end-products (AGEs) are formed by the irreversible reaction between reducing sugar (glucose) and protein or lipid free radicals (5). The levels of AGEs in diabetes patients were <5-fold that seen in non-diabetes individuals, which also plays a very important role in the occurrence and development of chronic diabetes-related complications (6). It was reported that AGEs were present in the senile plaque (SP) and neurofibrillary tangles (NFTS) in the brain of AD patients (7). When AGE accumulation increased, it stimulated glial cells to generate inflammatory factors and increased RAGE expression, making the inflammatory reaction even more severe (8). However, AGEs-RAGE could initiate distinct signaling pathways including p21/Ras, MAP kinase and NF-κB in an oxidative stress-dependent manner, and enhance neuronal expression of β-secretase. This process could further stimulate the generation of some cytokines, growth factors and adhesion molecules, and promote cell proliferation and vascular permeability. In addition, it also caused macrophage migration, stimulated endothelin formation, and promoted the synthesis of proteoglycans and fibers, which thus influenced β-amyloid (Aβ) protein secretion (9).

AD was first described by and named after Alzheimer in 1907, which was a primary degenerative disease that occurred in the elderly population. The typical pathologic changes included SP, NFTS, neuronal loss and axonal abnormalities, including astrocyte and microglial cell responses, and vascular amyloid changes. The major component of SP is Aβ, which is derived from the degradation of the amyloid precursor protein (APP) via the Aβ formation pathway. Thus, the influence of APP expression will indirectly regulate formation of Aβ protein.

Aβ is composed of 39-43 amino acids with a β-pleated sheet structure. Under normal physiological conditions, Aβ is soluble, its formation, degradation and elimination are in dynamic balance. In the brain of the AD patient, if such balance is destroyed, Aβ will form insoluble precipitation deposits by aggregation and might further lead to SP, which is the initiating factor for the occurrence of AD. Therefore, inhibition of Aβ formation plays a highly significant role in

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AD treatment. Thus, identifying an inhibitory drug therapy will also be very important in the treatment of AD.

α-lipoic acid (α-LA) is a natural product that was primarily extracted from pork liver by Reed in 1951. It is an indispensable substance in the tricarboxylic acid cycle and serves as a cofactor for pyruvate dehydrogenase. α-LA is metabolized to dihydrolipoic acid *in vivo*, and exists in the forms of LA and dihydrolipoic acid with synergistic effects that form a universal antioxidant. α-LA not only eliminates reactive oxygen and reduce oxidative stress levels, but it also increase GSH concentrations in tissues and prevents sharp decreases in GSH after oxidative damage of tissues (10).

It has been demonstrated that α-LA could improve the learning and memory functions in a rat model (11,12), and increased glucose metabolism and insulin sensitivity. Moreover, it could inhibit the developments of diabetic cardiovascular disease, diabetic cataract, diabetic peripheral nerve and autonomic neuropathy. Hager *et al* (13) attempted to use α-LA in the treatment of AD. In that study, 9 patients were orally administrated LA for 1 year, which was the first attempt in the LA-mediated treatment of AD in the clinic. It was found that the cognitive function of the patients was greatly improved by neuropsychological tests (13). However, the mechanism responsible for the ability of LA to protect neurons remains unclear.

In an *in vitro* study, Zhang *et al* found that LA could combat the damage that was mediated by the Aβ peptide and H_2O_2 on mouse neurons by activating the PKB/Akt signaling pathway (14). In the latest study, it was also found that α-LA inhibited Aβ protein aggregation, reduced Aβ fiber formation and depolymerized the aggregated Aβ (15). Nevertheless, studies of inhibiting Aβ formation remain very limited.

In the current study, we employed α-LA to treat cells, following which, we observed the protective effect on neurons and explored the possible mechanisms involved in an attempt to provide a theoretical basis for its application in the near future.

Materials and methods

The source of the cell-line and materials. SH-SY5Y wells were provided by The Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). D-glucose 1.1.2 (Amresco, LLC., Solon, OH, USA); MEM culture medium (HyClone, Logan, UT, USA); fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd., Hangzhou, China); 0.1% pancreatic enzymes, 0.02% EDTA, BSA and MTT (Beifangtongzheng); lactate dehydrogenase (LDH) test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); RT-PCR reagents (Sangon Biotech Co., Ltd., Shanghai, China); rabbit anti-human Aβ (Wuhan Boster Biological Technology, Ltd., Wuhan, China); SP immumohistochemical staining kit and DAB colour developing reagent kit (ZSGB-BIO, Beijing, China); and α-LA (Yabao Pharmaceutical Group Co., Ltd., Beijing, China).

Incubation of SH-SY5Y cells. SH-SY5Y cells were cultured in MEM medium that was supplemented with 10% FBS, 1% double-antibody (penicillin and streptomycin) and incubated at 37˚C and 5% CO_2 in air in a culture flask. The medium was replaced every two days. The cells were digested with 0.1% pancreatic enzymes (containing 0.02% EDTA) and seeded at a homogeneous density in a new flask.

Cell counting. The cells were digested as described above, collected and centrifuged at 700 rpm for 5 min. The supernatant was removed, and medium was added. Next, cells were pipetted up and down into a cell suspension. An aliquot of the suspension was taken and mixed with an equal volume of trypan blue. Trace volume of the suspension was dropped on one side of the coverslip in a counting plate, and the suspension did not overflow out of the coverslip. An objective lens x10 magnification was used to count the cells in the four squares of the counting chamber. If cells were found on the center line, the cell found on the left and top left was counted. The cell density was calculated as follows: Cell number/ml original medium = (sum of cell number in 4 squares/4) $x10^4$ x2.

Preparation for AGE-BSA. FBS 5 g/l, D-glucose 0.5 M, penicillin 100 μ/l, streptomycin 100 μ/l and EDTA 1 mmol/l were dissolved in PBS (pH 7.4), then filtered by 0.22 μm microfiltration membrane and incubated at 37˚C avoiding light for three months. The systems with the same FBS but under conditions of different glucose concentrations were used as control. PBS (pH 7.4) was used to dialyze the unbound glucose before use. The solution was stored at -20˚C. AGEs was detected by a fluorospectrophotometer (4).

Grouping. First, the cells were divided into three groups randomly. Group 1 was the control group without any processing; group 2 was treated by different BSA concentrations (terminal concentrations of 0, 20, 40, 80, 160 and 320 μg/ml) for 48 h; and group 3 was treated by different AGE-BSA concentrations (terminal concentrations of 0, 20, 40, 80, 160 and 320 μg/ml) for 48 h. According to the results of the above experiment, group 4 was added, which represented cells treated by different α-LA concentrations (terminal concentrations of 0.01, 0.1 and 1 g/l) to intervene in cells 30 min prior to AGE-BSA (160 μg/ml) treatment, which was then added to treat the cells for 48 h. According to the result of group 4, group 5 was added, which included treatment by α-LA (0.1 g/l) to intervene cells 30 min prior to AGE-BSA (80 μg/ml) treatment for 48 h. The cell growth state were assayed by MTT metabolic rate, LDH leakage rate and cell axonal length. Immunocytochemistry was used to detect Aβ protein expression. Further, mRNA expression of APP and RAGE were tested by RT-PCR wherein the AGE-BSA concentration was chosen that slightly influenced cell growth. The concentration in each control group was found to be consistent with group 3.

MTT assay. SH-SY5Y cells were seeded at a density of $5x10^4$/ml in 96 well-plates and incubated in 200 μl medium. Serum-free medium was added to replace the original medium after 24 h of culture. The cells were randomly divided into five groups. Group 1 was added with different BSA concentrations (0, 20, 40, 80, 160 and 320 μg/ml) for 48 h; group 2 was treated by different AGE-BSA concentrations (0, 20, 40, 80, 160 and 320 μg/ml) for 48 h. Group 3 was treated by different α-LA concentrations (0.01, 0.1 and 1 g/l) to intervene in cells 30 min earlier and then AGE-BSA

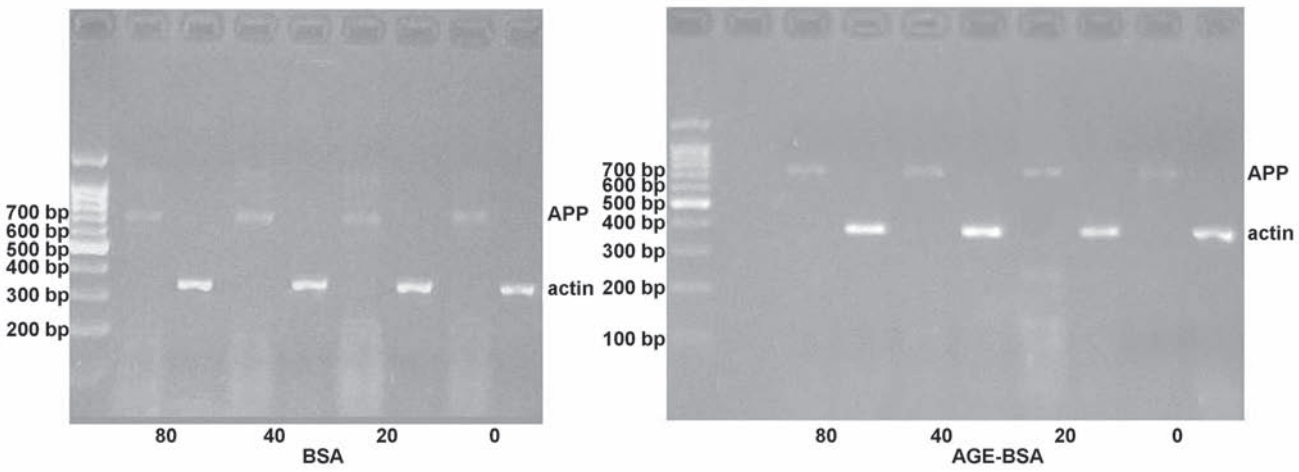

Figure 1. Expression of APP mRNA in SH-SY5Y cells dose-dependently treated by BSA and AGE-BSA. BSA, bovine serum albumin; AGE, advanced glycation end-product; APP, amyloid precursor protein.

(160 μg/ml) was added to treat cells for 48 h. Group 4 was added to introduce treatment of cells with α-LA (0.1 g/l) for 30 mins followed by treatment by AGE-BSA (80 μg/ml) for 48 h. Group 5 was a control treatment in the absence of any treatment. After 48 h of treatment, 5 mg/ml of MTT reagent (20 μl) was added to each well. The cells were further incubated for 4 h at 37˚C, which were then collected, centrifuged and washed once in culture medium without serum. DMSO (150 μl) was added to each well and shaken for 10 min to completely dissolve the formazan crystals. The optical density (OD) at a test wavelength of 570 nm was read by an automatic microplate reader. The cell viability in the control group was set as 100%, and the MTT metabolic rate was calculated by the ratio of OD to the control.

LDH release assay. SH-SY5Y cells were seeded at $5x10^4$/ml in 24 well-plates and incubated in 1 ml medium. Medium without serum was added to replace the original after 24 h. The groups were the same as described for the MTT assay. Culture medium in each group was taken after 48 h, and OD values of the medium were measured by ultraviolet spectrophotometry at 440 nm according to the manufacturer's instruction. Furthermore, the best sample volume was obtained from the standard curve.

Detection for cell axon length. SH-SY5Y cells were seeded at a density of $5x10^4$/ml in 35 mm culture flasks. Serum-free medium was added to replace the original after 24 h. The groups were the same as that described for the MTT assay. Photographic images of cells (random sampling of 30 cells) were taken 48 h after treatment by inverted phase contrast microscopy and the cell axon length was analyzed by pathologic image analysis.

Detection of APP mRNA and AGER mRNA by RT-PCR. The β-actin amplified fragment was 318 bp. Forward primer: 5'-ATCATGTTTGAGACCTTCAACA-3'. Reverse primer: 5'-CATCTCTTGATCGAAGTCCA-3'. The APP amplified fragment was 704 bp. Forward primer: 5'-CACATCCCTGGG CTTACGGT-3'. Reverse primer: 5'-TCAAGTTGGAGGCGG AGTTGC-3'. The RAGE: amplified fragment was 376 bp. Forward primer: 5'-GGCTGGTGTTCCCAATAAGG-3'. Reverse primer: 5'-ACAGGTCAGGGTTACGGTTC-3'. Detection of SH-SY5Y intracellular Aβ levels by immunocytochemistry. Wells with SH-SY5Y cells were seeded at a density of $5x10^4$/ml in 6-well plates. A small slide was placed in the plate before seeding. The assigned groups were as described for the MTT assay.

Statistical analysis. All data were analyzed by SPSS v.13.0 (SPSS, Inc. Chicago, IL, USA), and Microsoft Excel. The data are represented as mean ± SD. The comparisons of measured data among multiple groups were analyzed by one-way analysis of variance to determine the homogeneity test of variance. An α value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Fluorescence identification for AGEs-BSA. After 90 days of incubation of cells with BSA and D-glucose, the fluorescence spectrum of AGEs-BSA showed that the excitation spectrum peak and emission spectrum peak were at 360 and 446 nm, respectively, in accordance with the characteristics of AGEs. However, that of the control BSA group had no such feature (Fig. 1).

The influence of BSA/AGE-BSA concentration on growth of SH-SY5Y. When the concentration of AGE-BSA exceeded 40 μg/ml, the growth of SH-SY5Y cells was influenced in a concentration-dependence manner, which led to decreased MTT metabolic rate, increased LDH release and a shortening in the axon length. It was suggested that AGE-BSA had evidently damaged neurons, which was in line with the studies of Wolter *et al* (16) and Takeuchi *et al* (17).

Compared with the control group, there was no significant difference in MMT metabolic rate in the BSA treated groups. AGE-BSA at 40 μg/ml reduced the MMT metabolic rate significantly ($P<0.05$) and further decreased in an increasing concentration-dependent manner until reaching the lowest

Table I. Concentration-dependent effect of BSA/AGE-BSA at 48 h on the MTT metabolic rate of SH-SY5Y cells.

	MTT metabolic rate	
Groups (μg/ml)	BSA group	AGE-BSA group
Control	1±0	1±0
20	1.0019±0.0213	0.9902±0.0143
40	1.0001±0.0222	0.9346±0.0209[a,b]
80	0.9952±0.0222	0.8783±0.0188[a,b]
160	0.9922±0.0213	0.6434±0.0178[c,d]
320	0.9928±0.0256	0.3406±0.0124[c,d]

Data are presented as the means ± SD. vs. Control, [a]P<0.05; vs BSA, [b]P<0.05, vs. control, [c]P<0.01, vs. BSA, [d]P<0.01. BSA, bovine serum albumin; AGE, advanced glycation end-product.

Table II. Concentration-dependent effect of BSA/AGE-BSA on LDH release by SH-SY5Y cells.

	LDH leakage rate (μ/l)	
Groups	BSA	AGE-BSA
Control	431.15±65.19	431.15±65.19
20	438.21±13.06	483.53±15.02
40	451.83±10.26	739.57±67.02[a,b]
80	453.90±17.85	1066.35±300.96[a,b]
160	447.80±24.61	1608.22±141.3[c,d]
320	441.21±19.32	2500.45±146.48[c,d]

Data are presented as the means ± SD. vs. Control, [a]P<0.05; vs. BSA group, [b]P<0.05; vs. control, [c]P<0.01, vs. BSA, [d]P<0.01. BSA, bovine serum albumin; LDH, lactate dehydrogenase; AGE, advanced glycation end-product; SD, standard deviation.

level at 320 μg/ml (P<0.01). A comparison of the AGE-BSA concentration group and the BSA alone group at the same concentration, demonstrated that the rate was significantly reduced at 40, 80, 160, 320 μg/ml (P<0.05). Although the group treated by 20 μg/ml was lower than the control group, it was not statistically significant (Table I).

Compared with the control group, there was no significant difference on LDH release in the BSA treated groups. AGE-BSA at a concentration of 40 μg/ml increased the release of LDH significantly (P<0.05) and reached the highest level at 320 μg/ml (P<0.01). The comparison of the AGE-BSA concentration group and the BSA alone group at the same concentrations demonstrated that the rate was significantly reduced at 40, 80, 160 and 320 μg/ml (P<0.05). Although the group treated with 20 μg/ml was lower than the control group, it was not statistically significant (Table II).

Compared with the control group, there was no significant difference in cell axon length in the BSA treated groups. In addition, 40 μg/ml AGE-BSA shortened the length significantly (P<0.05) and shortened gradually as concentration increased until reaching a peak at 320 μg/ml (P<0.01). The comparison of the AGE-BSA concentration group and the BSA alone group at the same concentrations demonstrated that the length was significantly short at 40, 80, 160 and 320 μg/ml (P<0.05). Although the group treated with 20 μg/ml was shorter than that of the control group, the observation was not significantly different (Table III).

Table III. Effect of different concentrations of BSA/AGE-BSA on axonal length of SH-SY5Y cells.

	Axonal length (Pixel total points)	
Groups (μg/ml)	BSA (μg/ml)	AGE-BSA (μg/ml)
Control	42.00±12.78	42.15±10.00
20	40.45±11.48	41.52±11.40
40	41.94±10.88	35.44±9.19[a,b]
80	40.23±11.34	28.69±6.88[a,b]
160	40.82±11.45	21.13±5.61[c,d]
320	41.91±9.01	12.47±5.56[c,d]

Data are presented as the means ± SD. vs. Control, [a]P<0.05; vs. BSA, [b]P<0.05; vs. control, [c]P<0.01, vs. BSA, [d]P<0.01. BSA, bovine serum albumin; AGE, advanced glycation end-product; SD, standard deviation.

Influence of BSA/AGE-BSA concentration on Aβ protein expression in SH-SY5Y cells. The color of positive Aβ protein expression was brownish red. In the control group, Aβ protein expression was decreased in the cytoplasm. As compared with control group, there was no significant change in terms of Aβ protein expression in the cytoplasm of cells that were dose-dependently treated by BSA. However, those treated by different AGE-BSA concentrations showed a significant difference (P<0.05). Compared with the corresponding BSA concentration group, Aβ protein expression in the cytoplasm was significantly increased following treatment with AGE-BSA (P<0.05; Table IV).

Influence of AGE-BSA concentrations on APP mRNA expression in SH-SY5Y cells. The group with the least influence on the state of cell growth was chosen. Compared with the control group, there was no significant difference on APP mRNA expression following treatment with AGE-BSA, or the BSA groups (Table V).

Influence of BSA/AGE-BSA concentration on RAGE mRNA expression in SH-SY5Y cells. Compared with control group, there was no significant difference on RAGE mRNA expression following treatment with BSA alone. In the AGE-BSA group, the RAGE mRNA expression was significantly increased at 20 μg/ml (P<0.05). Moreover, at 40 μg/ml and 80 μg/ml, RAGE mRNA expression showed a significant difference as compared with the control group (RAGE mRNA). As compared with the corresponding BSA concentration group, RAGE mRNA expression was significantly increased following treatment with AGE-BSA (P<0.05; Table VI and Fig. 2).

Table IV. Dose-dependent effect of different concentrations of BSA/AGE-BSA on expression of Aβ protein in SH -SY5Y cells.

Groups (μg/ml)	Aβ protein (OD value) BSA group	Aβ protein (OD value) AGE-BSA group
Control	0.129±0.025	0.129±0.025
20	0.126±0.017	0.167±0.029[a,b]
40	0.125±0.022	0.226±0.076[a,b]
80	0.128±0.018	0.309±0.073[c,d]

vs. control, [a]P<0.05; vs. group BSA, [b]P<0.05; vs. control, [c]P<0.01, vs. BSA, [d]P<0.01. BSA, bovine serum albumin; AGE, advanced glycation end-product; SD, standard deviation; OD, optical density; Aβ, β-amyloid.

Table V. Dose-dependent effect of BSA/AGE-BSA on the expression of APP mRNA in SH-SY5Y cells.

Groups (μg/ml)	APP mRNA/actin mRNA BSA group	APP mRNA/actin mRNA AGE-BSA group
0	0.284±0.033	0.284±0.033
20	0.290±0.032	0.283±0.025
40	0.289±0.027	0.281±0.026
80	0.288±0.019	0.286±0.020

Data are presented as the means ± SD. BSA, bovine serum albumin; AGE, advanced glycation end-product; SD, standard deviation; APP, amyloid precursor protein.

Table VI. Dose-dependent effect of BSA/AGE-BSA on mRNA expression of RAGE in SH-SY5Y cells.

Groups (μg/ml)	RAGE mRNA/actin mRNA BSA group	RAGE mRNA/actin mRNA AGE-BSA group
0	0.491±0.017	0.491±0.017
20	0.494±0.027	0.522±0.031[a,b]
40	0.507±0.013	0.542±0.026[a,b]
80	0.486±0.010	0.591±0.040[a,b]

Data are presented as the means ± SD. vs. Control, [a]P<0.05; vs. BSA group, [b]P<0.05. BSA, bovine serum albumin; AGE, advanced glycation end-product; RAGE, receptor for AGE; SD, standard deviation.

Table VII. Levels of MTT metabolic rate, LDH release rate and axonal length in SH-SY5Y cells following intervention by LA before treatment with 160 μg/ml AGE-BSA.

Groups (g/l)	MTT metabolic rate	LDH leakage rate (μ/l)	Axon length (Pixel total points)
Control	0.643±0.018	1608.22±141.37	21.13±5.61
0.01	0.684±0.022	1538.40±102.07	22.33±4.81
0.1	0.840±0.068[a]	1151.70±146.47[a]	29.11±7.56[a]
1	0.630±0.029	1662.52±110.82	20.89±3.68

Data are presented as the means ± SD. vs control, [a]P<0.05. BSA, bovine serum albumin; LDH, lactate dehydrogenase; AGE, advanced glycation end-product; SD, standard deviation; LA, lipoic acid.

Table VIII. Levels of Aβ protein expression in SH-SY5Y cells that were intervened by LA before being treated with 80 μg/ml AGE-BSA.

Groups	Aβ protein (OD value)
80 μg/ml AGE-BSA	0.309±0.073
0.1 g/l LA	0.213±0.046[a]
Control	0.129±0.025

Data are presented as the means ± SD. vs. 80 μg/ml AGE-BSA group, [a]P<0.01. BSA, bovine serum albumin; AGE, advanced glycation end-product; SD, standard deviation; LA, lipoic acid; Aβ, β-amyloid; OD, optical density.

Observation of cell growth state following dose-dependent intervention with α-LA. According to the above results, the cells in the AGE-BSA 160 μg/ml) group were chosen. In the α-LA (0.1 g/l) intervention group, the MMT metabolic rate significantly increased, LDH release significantly decreased, and axonal length significantly increased as compared with that of the control group (P<0.05). Compared with the AGE-BSA (160 μg/ml) group, it was noted that the α-LA (0.01 g/l) intervention group showed an increased MMT metabolic rate, a decreased LDH release rate and increased axonal length as compared that of the control group - although this was not significantly different. It is interesting to note that the protective effect in the α-LA (1 g/l) intervention group disappeared. Compared with the control group, there was no evident changes in MTT metabolic rate or axonal length, and yet LDH release slightly increased (P>0.05; Table VII).

According to the above described results, it was found that α-LA (0.1 g/l) was protective for SH-SY5Y cells under treatment by 160 μg/ml AGE-BSA. Thus, we selected the cells in the AGE-BSA (80 μg/ml) group, and used α-LA at 0.1 g/l to intervene in the cells. Compared with the AGE-BSA (80 μg/ml) group, the Aβ expression significantly decreased in the intervention group (P<0.01; Table VIII).

The cells in the AGE-BSA (80 μg/ml) group were chosen. In the α-LA (0.1 g/l) intervention group, there were no evident changes in APP mRNA as compared with the control group (Table IX and Fig. 3).

Changes in RAGE mRNA expression in cells intervened by α-LA (0.1 g/l). The cells in the AGE-BSA (8 μg/ml) group were chosen. In the α-LA (0.1 g/l) intervention group, there

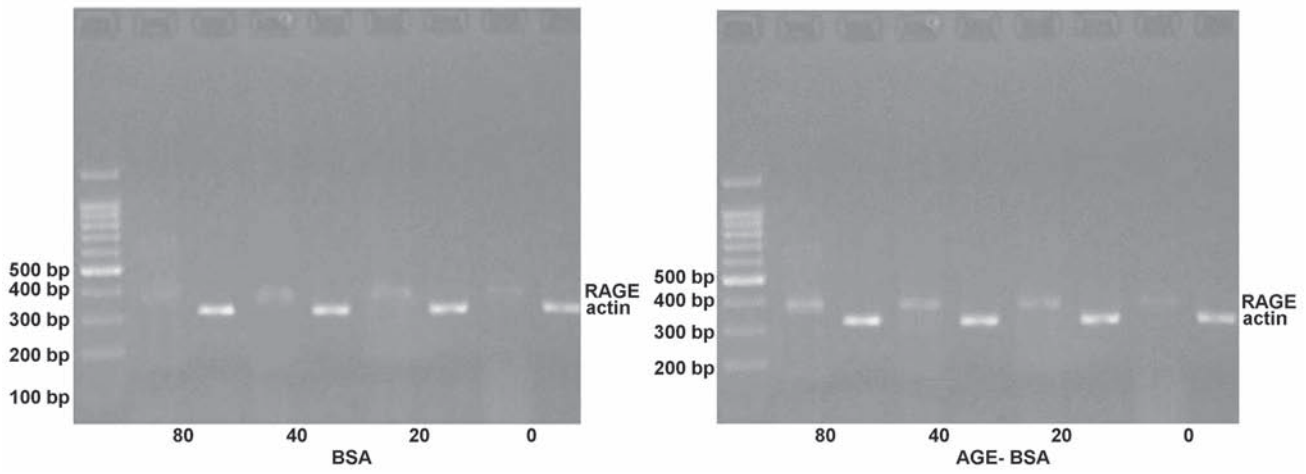

Figure 2. Expression of RAGE mRNA in SH-SY5Y cells treated by different concentrations of BSA and AGE-BSA. BSA, bovine serum albumin; AGE, advanced glycation end-product; RAGE, receptor for AGE.

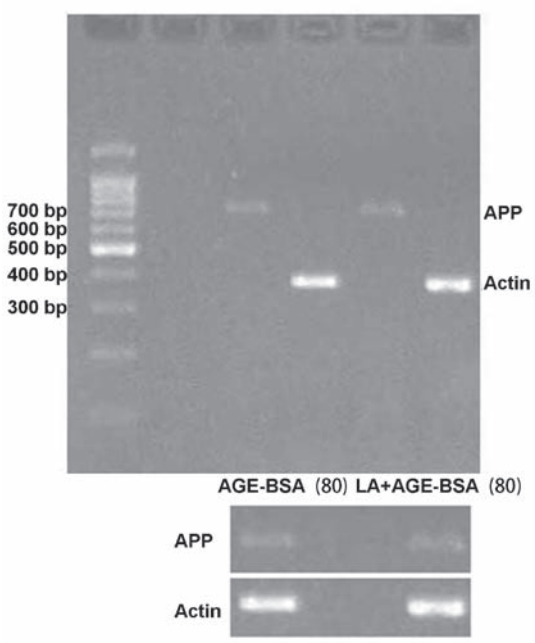

Figure 3. Levels of APP mRNA expression in SH-SY5Y cells that were intervened by LA before treatment with 80 μg/ml AGE-BSA. BSA, bovine serum albumin; AGE, advanced glycation end-product; APP, amyloid precursor protein; LA, lipoic acid.

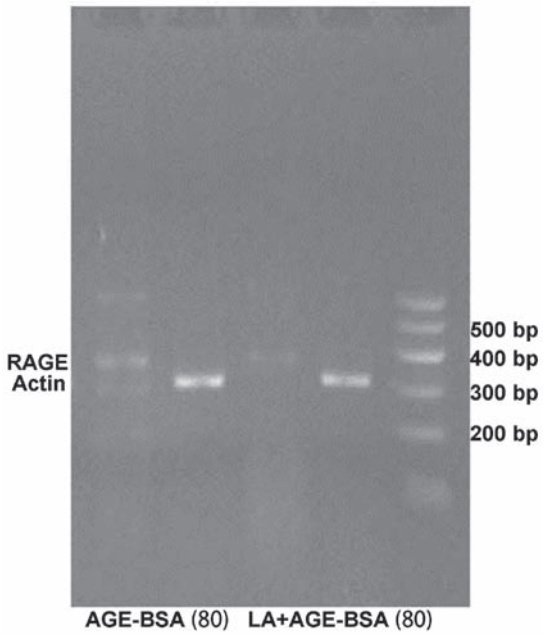

Figure 4. Levels of RAGE mRNA expression in SH-SY5Y cells that were intervened by LA before treatment with 80 μg/ml AGE-BSA. BSA, bovine serum albumin; AGE, advanced glycation end-product; RAGE, receptor for AGE; LA, lipoic acid.

was no evidence of any changes in APP mRNA expression as compared with the control group (Table X and Fig. 4).

Discussion

Recent studies showed that AGEs play a very important role in the pathogenic process of neurodegenerative diseases, and such studies mainly focused on the direct toxic effect of AGEs on neurons. Sasaki *et al* found that there were many AGEs deposited in SP, NFTS, microglial cells and astrocytes in the brain tissue of AD patients (18). It was also indicated that AGE precursors such as methylglyoxal, glyoxal and HNE displayed cytotoxicity independently of Aβ, and further promoted Aβ aggregation (16). Takeuchi *et al* employed chicken egg albumin and BSA-AGEs to treat SH-SY5Y cells, primary astrocytes and N9 cells, which led to cell death (17).

The neurotoxic mechanism of AGEs remains unclear. It was reported that AGEs could indirectly modify Aβ in SP and tau protein in NFTS, and that the protein modified by AGEs were cytotoxic, a property that increased with the extent of modification (19). However, the inflammatory response generated by glycosylated Aβ simulating the microglial cell was much more severe than that of non-glycosylated Aβ (20). *In vitro*, Aβ formed a core with AGEs, which provoked additional aggregation by soluble Aβ. The unmodified Aβ as signal combined with the receptor for AGEs induced gene expression and aggravated the glycosylated process.

The modified tau protein could cause abnormal phosphorylation and cross-linking and significantly decreased

Table IX. Levels of APP mRNA expression in SH-SY5Y cells that were intervened by LA before treatment with 80 μg/ml AGE-BSA.

Groups (g/l)	APP mRNA/actin mRNA
80 μg/ml AGE-BSA	0.278±0.030
0.1 g/lα-LA	0.281±0.027[a]
Control	0.284±0.033

Data are presented as the means ± SD. vs. control, [a]P>0.05. BSA, bovine serum albumin; AGE, advanced glycation end-product; SD, standard deviation; APP, amyloid precursor protein; LA, lipoic acid.

Table X. Levels of RAGE mRNA expression in SH-SY5Y cells intervened by LA before treatment with 80 μg/ml AGE-BSA (mean ± SD).

Groups (g/l)	RAGE mRNA/actin mRNA
80 μg/ml AGE-BSA	0.591±0.040
0.1 g/l α-LA	0.543±0.030[a]
Control	0.49±0.017

Data are presented as the means ± SD. vs. control, [a]P<0.05. BSA, bovine serum albumin; AGE, advanced glycation end-product; SD, standard deviation; LA, lipoic acid.

microtubule assembly, further promoting pathologic changes commonly seen in AD. It was reported that AGE-modified MAP-tau would result in increased APP expression and increased formation of Aβ when introduced into the neuron. However, in our study, it was demonstrated that AGE-BSA also directly improved Aβ formation in a concentration-dependent manner, suggesting increased formation of Aβ is involved in the cytotoxicity of AGEs. Moreover, it was also revealed that there was no clear alteration of APP mRNA expression in SH-SY5Y cells at 48 h after being treated by different protein concentrations (20, 40 and 80 μg/ml) in the RT-PCR test. It was speculated that the increased formation of Aβ was not caused by an increase in APP expression as a result of increased APP mRNA expression. It was also reported that the toxic effect of AGEs were predominantly mediated by oxidative stress (21). Maczurek *et al* (22) found that there was AGE-specific receptor on the surface of neurogliocytes. It was combined with AGEs and further activated intracellular signal transduction pathway, which resulted in the generation of many cytokines and oxidative stress. Misonou *et al* (23) used H_2O_2 to study the effect of oxidative stress on SH-SY5Y cells, and found that it decreased COOH-terminal fragments in the β-secretase pathway. Cho *et al* (24) proved that the RAGE signaling pathway could enhance BACEI expression by activating NFAJI both *in vitro* and *in vivo*, further causing increases in Aβ formation. Furthermore, AGE-RAGE could play a part by inducing CTGF expression, and not necessarily by oxidative stress (25,26).

In addition, we also confirmed that AGE-BSA induced increases in RAGE mRNA expression in SH-SY5Y cells. RAGE is the receptor of Aβ, and its expression would be increased in the abundant Aβ environment (27). It was also combined with Aβ at the nanomole level and amplified the neuronal damage that was mediated by Aβ (28). In the *in vitro* study, the combination of Aβ and RAGE could activate NF-κB and simulate secretion of macrophage colony stimulating factor (M-CSF) and IL-6, suggesting that Aβ-RAGE played a very important role in the neuronal inflammatory response (29). A prior study found that in a transgenic rat model that expressed both neuronal RAGE and APP showed evident cognitive disorder and decreased axonal conduction ability as compared the rat with normal expression of neuronal RAGE or APP in three to four month-old rats. At this time, the rat showed no characteristic pathological changes consistent with AD, indicating that the activation of RAGE by Aβ could involve neuronal damage in the early stages of AD (6).

In our study, intracellular Aβ expression was evidently increased after being treated by AGE-BSA, suggesting increased formation of Aβ. RAGE mRNA expression was increased, which was seen after Aβ combined with neurons and RAGE expression being evident on the cytoplasmic membranes of oligodendroglia cells. Then, NF-κB was initiated resulting in a persistent inflammatory response, neuronal cytotoxicity and cell death (30). However, in the environment with abundant Aβ, the expression was persistently increased, which amplified the pathogenic effect that was induced by Aβ (31). Zlokovic (32) found that in the neurons of APP transgenic mice, the expression of RAGE accelerated the decrease in cognitive function and dysfunction of neurons that was induced by Aβ. RAGE on the surface of encephalic vascular endothelial cells could assist Aβ to pass through the blood-brain barrier in the peripheral circulation (33), upregulate CCR5 expression, and stimulate T cells that entered the endocardium, causing a systemic inflammatory response (34). Above all, as the ligand of RAGE, Aβ could mutually interact, and thereby play a major role in cytotoxicity of AGE-BSA.

Moreover, since RAGE is the specific receptor of AGEs, it can mediate toxic effect of AGEs in many chronic diabetic complications. It was found that the combination of AGEs-RAGE also activated the NF-κB signaling pathway in microglial cells and astrocytes (20), which was one of the promoters that regulated RAGE expression (35). The activation of site 1 and 2 upregulated RAGE expression, and further promoted the combination of AGEs and RAGE.

In addition, such combination also activated many pathways such as that of protein kinase C signaling, protein tyrosine kinases, and P21RAS, causing a coordinated cellular damage response (36). In short, AGEs possess evident toxicity against nerve cells, directly leading to increased formation of Aβ and neurodegeneration. In the context of a toxic effect, the Aβ-RAGE combination, AGEs-RAGE combination, Aβ and RAGE could all be involved. The increased formation of Aβ would not be realized by increased APP expression caused by increased APP mRNA expression, the detailed mechanism requires further exploration.

The α-LA with terminal concentrations of 0.01, 0.1 and 1 g/l was employed to pre-treat SH-SY5Y cells for 30 min to study the influence of α-LA on the toxic effects of nerve cells caused by AGEs. The results show that α-LA at a dose of

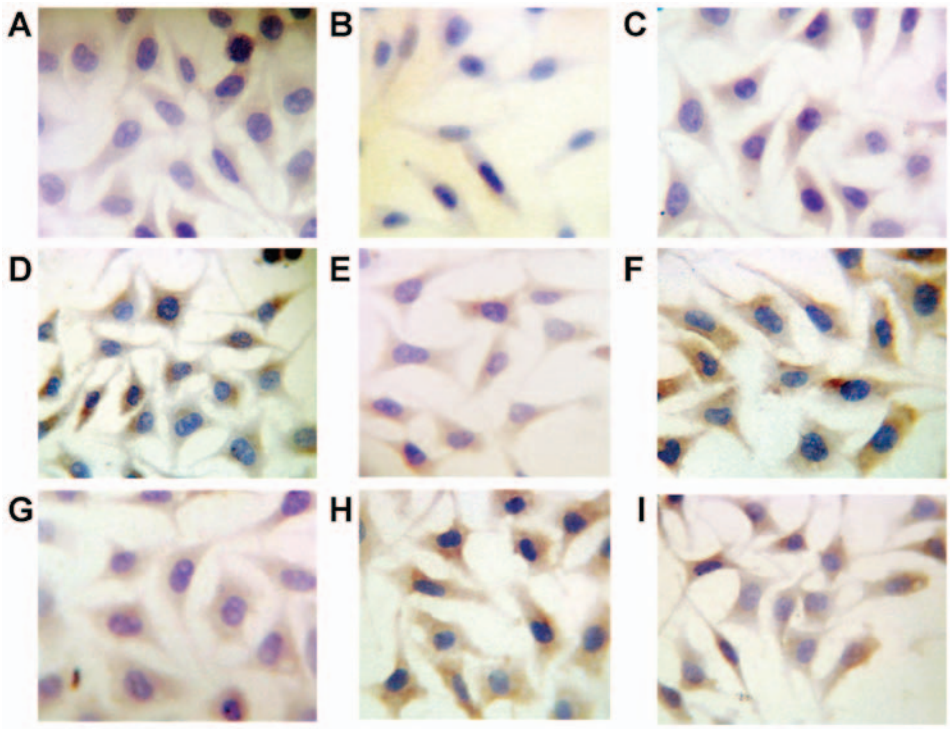

Figure 5. (A) 1-1 expression of Aβ in normal SH-SY5Y (10x40); (B) the SH-SY5Y of negative control group (10x40); (C) 1-2 expression of Aβ in SH-SY5Y treated by BSA(20 μg/ml) for 48 h (10x40); (D) 1-3 Expression of Aβ in SH-SY5Y treated by AGE-BSA(20 μg/ml) for 48 h (10x40); (E) 1-4 expression of Aβ in SH-SY5Y treated by BSA (40 μg/ml) for 48 h (10x40); (F) 1-5 expression of Aβ in SH-SY5Y treated by AGE-BSA (40 μg/ml) for 48 h (10x40); (G) 1-6 expression of Aβ in SH-SY5Y treated by BSA (80 μg/ml) for 48 h (10x40); (H) 1-7 expression of Aβ in SH-SY5Y treated by AGE-BSA (80 μg/ml) for 48 h (10x40); (I) photo 1-8 expression of Aβ in SH-SY5Y intervention by LA(0.1 g/l) 30 min before being treated by AGE-BSA(80 μg/ml) for 48 h (10x40).

0.1 g/l significantly resisted the toxic effect of AGEs-BSA to SH-SY5Y. Compared with the corresponding control group, the MTT metabolic rate was significantly increased, LDH release was significantly decreased and cell morphology was greatly improved. It suggested that α-LA had a protective effect for nerve cells.

In our study, we found that LA had a protective effect on nerve cells but not in a dose-dependent manner. Therefore, we deduced that LA entered cells by a certain carrier, and when the carrier was saturating, the effect would not be increased as the carrier supply. Moreover, increased LA concentration provoked altered osmotic pressure and pH, directly influenced the external environment of incubated cells, and further influenced the effect of LA.

We chose α-LA with an optimal concentration of 0.1 g/l to pre-treat cells in the AGE-BSA (80 μg/ml) group for 30 min and then tested the influence of α-LA on intracellular Aβ protein, APP mRNA and TAGE mRNA expression profiles. We found that as compared with the corresponding AGEs-BSA treatment group, the intracellular Aβ expression significantly decreased, which suggested that α-LA was partly related to a decreased expression in Aβ, at least in the protection of SH-SY5Y cells (Fig. 5).

There was no evident change in APP mRNA expression, which suggested that decreased Aβ was not realized by decreased APP expression that was caused by decreased APP mRNA expression. Furthermore, the expression of TAGE mRNA was significantly reduced. The results indicated that using an anti-RAGE antibody to prevent the combination of RAGE and ligand, significantly reduced the generation of inflammatory cytokines and nitric oxide by microglial cells (37).

The RAGE antagonist, sRAGE (soluble RAGE) was used to treat diabetic rats that lacked Apo-E. In this mode, treatment inhibited development of early angiosclerosis (38). High doses of sRAGE to mouse would isolate Aβ in the plasms, and would thus decrease intracranial Aβ levels (31). It was deduced that the weakened combination of Aβ-RAGE and AGEs-RAGE were also involved in the protective effect of LA for nerve cells. AGEs decreased the intake of glucose into SH-SY5Y cells, which was related to the inactivation of the glucose transporter, as previously reported by Keller *et al* **(ref.?)** However, after LA enters cells, pyruvate dehydrogenase would be activated, which would thus increase aerobic metabolism of glucose. Besides, it also made the glucose transporter (GLUT1, GLUT3, and GLUT4) translocate to the cytoplasmic membrane by activating phosphatidylinositol-3. Therefore, α-LA possibly enabled neuronal cells to resist damage that was caused by AGEs-BSA via improving glucose metabolism. The effect of decreased Aβ formation on improving glycometabolism still requires further exploration.

In conclusion, we found that Aβ is the key factor for AD pathological mechanisms, which is also an important target for AD treatment. In addition, α-LA could reduce Aβ generation and inhibit Aβ aggregation, and it also demonstrated utility in absorbing and passing through the blood-brain barrier, thus playing a very important role in the central nervous system degenerative diseases. Thus, we believe that Aβ should have wide developmental utility in the future.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GN and JG carried out the studies, participated in collecting data, and drafted the manuscript. YT and KZ performed the statistical analysis and participated in its design. JL was also involved in the conception of the study and helped to draft the manuscript. QX helped to design the studies and modified the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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