RESEARCH ARTICLE

Advanced glycation end products induce neural tube defects through elevating oxidative stress in mice

Ru-Lin Li*, **Wei-Wei Zhao, Bing-Yan Gao**
Laboratory for Development, College of Life Sciences, Northwest University, Xi'an, Shaanxi Province, China

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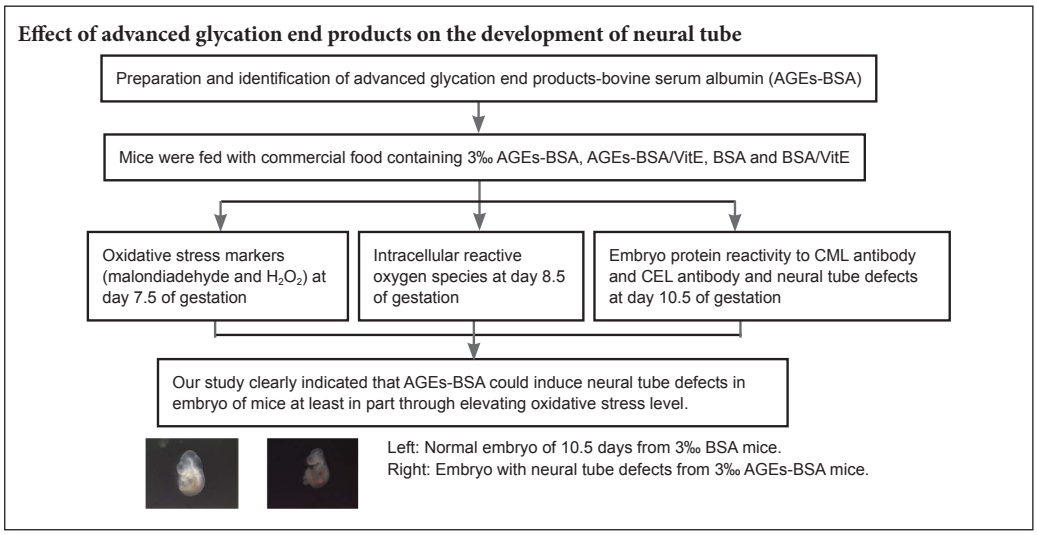
Graphical Abstract



***Correspondence to:**
Ru-lin Li, M.D., rulinli@nwu.edu.cn.

orcid:
0000-0003-0965-2518 (Ru-Lin Li)

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Abstract

Our previous study showed an association between advanced glycation end products (AGEs) and neural tube defects (NTDs). To understand the molecular mechanisms underlying the effect of AGEs on neural tube development, C57BL/6 female mice were fed for 4 weeks with commercial food containing 3% advanced glycation end product bovine serum albumin (AGE-BSA) or 3% bovine serum albumin (BSA) as a control. After mating mice, oxidative stress markers including malondialdehyde and H_2O_2 were measured at embryonic day 7.5 (E7.5) of gestation, and the level of intracellular reactive oxygen species (ROS) in embryonic cells was determined at E8.5. In addition to evaluating NTDs, an enzyme-linked immunosorbent assay was used to determine the effect of embryonic protein administration on the N-(carboxymethyl) lysine reactivity of acid and carboxyethyl lysine antibodies at E10.5. The results showed a remarkable increase in the incidence of NTDs at E10.5 in embryos of mice fed with AGE-BSA (no hyperglycemia) compared with control mice. Moreover, embryonic protein administration resulted in a noticeable increase in the reactivity of N-(carboxymethyl) lysine and N(ε)-(carboxyethyl) lysine antibodies. Malondialdehyde and H_2O_2 levels in embryonic cells were increased at E7.5, followed by increased intracellular ROS levels at E8.5. Vitamin E supplementation could partially recover these phenomena. Collectively, these results suggest that AGE-BSA could induce NTDs in the absence of hyperglycemia by an underlying mechanism that is at least partially associated with its capacity to increase embryonic oxidative stress levels.

Key Words: *nerve regeneration; neural tube defects; advanced glycation end products; diabetic embryopathy; oxidative stress; N-(carboxymethyl) lysine; malondiadehyde; N(ε)-(carboxyethyl) lysine; embryo; H_2O_2; bovine serum albumin; neural regeneration*

Introduction

Neural tube defects (NTDs) are severe birth defects of the central nervous system. The prevalence of NTDs varies widely between 1 and 10 per 1000 births, depending on geographic region and ethnic grouping (Au et al., 2010; Machín et al., 2015). Both genetic and non-genetic factors are involved in the etiology of NTDs (Copp and Greene, 2010; Hall and Solehdin, 2015). Although interventions such as folic acid supplementation and glucose level control have been recommended to prevent NTDs, a high incidence of NTDs still occurs in rats (Fleming and Copp, 1998; Suhonen, 2000; Loffredo et al., 2001; Liu et al., 2017).

Glycation is the nonenzymatic reaction of glucose or other reducing sugars with primary amino groups of proteins. Major products of glycation and oxidation of proteins and lipids include advanced glycation end products (AGEs) such as fluorescent pentosidine (Bhat et al., 2014; Siddiqui et al., 2015), nonfluorescent Nε-(carboxymethyl) lysine (CML), and other species (Ramasamy et al., 2005). AGEs are generated in vivo as a normal consequence of metabolism, but their formation is accelerated under conditions of hyperglycemia. Chronic accumulation of AGEs is associated with diabetes, rheumatoid arthritis, systemic lupus crythematosus, renal insufficiency, inflammation, and aging (Cerami, 1997; Nicholl

and Bucala, 1998; Rodriguez-Garcia, 1998; Mohamed et al., 1999; Schmidt et al., 2000; Singh, 2001). However, the effects of AGEs on embryonic development remain unclear.

The results of a study suggest that AGEs could play an important role in diabetic embryopathy (Li and Chen, 2014). As AGEs can cause single-strand breaks in genomic DNA, they can have serious teratogenic effects. While the incidence of congenital abnormalities is not increased in short-term, pregnancy-induced hyperglycemia, it is increased if long-term poor glycemic control predates conception, further implicating a role for AGEs in diabetic embryopathy (Millis, 1988; Eriksson and Wentzel, 2016). Our recent study indicated serum AGEs level to be an important risk factor for NTD occurrence (Li et al., 2014; Daglar et al., 2016). These findings support the notion that AGEs could be an important factor in the mechanism underlying increased incidence of NTDs in diabetic or non-diabetic mothers.

An abundance of evidence supports the involvement of AGEs in a vicious cycle of inflammation, oxidative stress, amplified AGEs production, followed by more inflammation and more oxidative stress production (Yan, 1997; Obrosova, 2002; Jiang, 2004). Increased generation of reactive oxygen species (ROS) in response to AGEs could occur through multiple mechanisms, for example the ligation of receptor for AGEs (RAGE) and catalase (Ramasamy et al., 2005; Lin et al., 2016). As RAGE is expressed in early embryos (Yan, 1997; Wang et al., 2008), AGEs might exert their effects on embryonic development through RAGE or other mechanisms to induce oxidative stress.

The present study sought to determine if exogenous AGE-modified proteins could induce NTDs in mice and, if so, the molecular mechanism underlying this phenomenon.

Materials and Methods

Animals

One hundred and ninety-two 4–6-week-old (40–60 g) specific pathogen-free C57BL/6 mice (48 males, 144 females) were obtained from the Animal Center of Xi'an Jiaotong University of China [SCXK (Shaan) 2017-003]. All experimental procedures were approved by the Animal Ethics Committee of Northwest University of China (Approval No. NWU-AWC-20170605M).

Preparation of AGEs

AGE bovine serum albumin (AGE-BSA) was produced by incubating BSA (Sigma, St. Louis, MO) at a concentration of 50 g/L with 1 M D-glucose (Sangon Bio, SHH, China) in 0.1 M phosphate-buffered saline (PBS, pH 7.4) at 37°C for up to 8 weeks. Unbound glucose and low molecular weight reactants were removed through dialysis against PBS. AGE-BSA was lyophilized and resuspended in PBS. The products were quantified by measuring optical density (OD) at 405 nm.

Induction of diabetes

Diabetes was induced in 4–6-week-old female C57BL/6 mice with 100 mg/kg streptozotocin (Sigma) dissolved in 10 mM sodium citrate (pH 4.5). Normal blood glucose levels were maintained in streptozotocin-diabetic mice by subcutaneously implanting these animals with insulin pellets (Sigma) before pregnancy, until development of hyperglycemia was induced at E4.5 (D group), thereby exposing the embryo to hyperglycemia during the entire post-implantation period (Li et al., 2005). Mice in the control group were only administered PBS. Afterwards, female and male mice were placed in the same cage. On the day of copulation, hyperglycemic mice were fed with commercial food containing 0.125% (w/w) vitamin E (VitE) (D/VitE group).

Animal intervention

A total of 192 C57BL/6 mice (48 males, 144 females) were randomly assigned to three groups: an AGE-BSA group (n = 72) consisting of 54 females and 18 males fed with commercial food containing 3% AGE-BSA, a BSA group (n = 48) consisting of 36 females and 12 males fed with commercial food containing 3% BSA, and a control group (n = 72) consisting of 54 females and 18 males fed with common commercial food.

Four weeks after feeding, the AGE-BSA group was subdivided into three subgroups: a D + AGEs group consisting of six females and two males with induced diabetes fed with commercial food after embryonic day 15 (E0.5); an AGE-BSA/VitE group of six females and two males fed with commercial food containing 0.125% (w/w) VitE; and an AGE-BSA group with six females and two males fed with commercial food after E0.5.

The BSA group was subdivided into two groups: a BSA/VitE group of six female and two male mice fed with commercial food containing 0.125% (w/w) VitE, and group of six female mice and two male mice fed with commercial food after E0.5.

The control group was subdivided into three groups: a D/VitE group of six females and two males in which diabetes was induced, and whom were fed with commercial food containing 0.125% (w/w) VitE after E0.5; a D group of six females and two males in which diabetes was induced, and whom were fed with commercial food after at E0.5; and a control group of six females and two males fed with commercial food after E0.5.

Male mice in the experiment were used only for breeding and were not ultimately included in the final analysis.

Detection of serum AGEs levels in pregnant mice

Blood samples were collected from the tails of pregnant mice after 4 weeks of feeding with 3% AGE-BSA commercial food or 3% BSA commercial food. Blood was centrifuged at 1500 × g for 10 minutes. Serum was collected in 1.5-mL tubes for AGEs assay. Determination of AGEs was based on spectrofluorimetric detection modified from a previously published method (Kalousova et al., 2002). Briefly, serum was diluted at 1:50 with PBS (pH 7.4) and fluorescence intensity was measured at the emission maximum (440 nm) upon excitation at 365 nm with a microplate reader (Tecan, Shanghai, China).

Preparation of CML-BSA and N(ε)-(carboxyethyl) lysine (CEL)-BSA

CML-BSA and CEL-BSA were prepared as previously de-

scribed (Koito et al., 2004). Briefly, for CML-BSA, 50 g/L BSA was incubated at 37°C for 24 hours with 45 mM glyoxylic acid (Sigma) and 150 mM sodium cyanoborohydride ($NaCNBH_3$) (Sigma) in 2 mL of 0.2 M phosphate buffer (pH 7.4), followed by dialysis against 0.01 M PBS (pH 7.4). For CEL-BSA, 50 g/L BSA was incubated at 37°C for 24 hours with 45 mM pyruvic acid (Sigma) and 150 mM NaCNBH3 in 2 mL of 0.2 M phosphate buffer (pH 7.4), followed by dialysis against 0.01 M PBS (pH 7.4).

Preparation of polyclonal anti-CML-BSA and anti-CEL-BSA antibodies

One mg of each immunogen was emulsified in 50% Freund's complete adjuvant (Boao, Beijing, China) and intradermally injected into rabbits. This was followed by four booster injections of 0.5 mg immunogen in 50% Freund's incomplete adjuvant (Boao) at 14, 28, and 42 days. Obtained serum was subjected to further affinity purification. Antibodies to CML-BSA and CEL-BSA were purified by affinity chromatography.

Preparation and immunochemical reactivity of embryonic protein

Embryos were collected from mice in each of the five groups (AGE-BSA, BSA, AGE-BSA/VitE, BSA/VitE, and control). The tissue was homogenized using a pestle for 20 strokes in lysis buffer and incubated on ice for 20 minutes. Samples were then centrifuged at 880,000 × *g* for 10 minutes. After centrifugation, the supernatant was transferred into a new tube. Protein concentration was measured using a Bradford assay. Each well was coated with mouse embryonic proteins (10 μg/mL) and then blocked with 1% non-fat milk. Following three washes with PBS containing Tween-20, each well was incubated with purified mouse polyclonal anti-CML and -CEL antibodies (1:4000) and washed three times. Horseradish peroxidase-conjugated anti-rabbit IgG (1:3000; Sangon, Shanghai, China) was added and incubated for 1 hour at 37°C. After washing three times, TMP solution was added and OD values were measured at 490 nm with a microplate reader (Tecan, Shanghai, China).

Preparation of embryonic cell suspensions and assessment of intracellular ROS production

E8.5 embryos were dissected from uteri in PBS, and extraembryonic structures were placed in Dulbecco's Modified Eagle's Medium (DMEM). Whole embryos were minced and passed through a cell strainer with 40-μM nylon mesh (BD Falcon-TM, Shanghai, China), to generate single-cell suspensions. The suspension was prepared at a density of 2×10^7 cells/mL. Embryonic cells were cultured in DMEM under a range of AGE-BSA concentrations for 24 hours: 0, 200, 400, and 800 μg/mL in DMEM. Cells were subsequently washed twice with PBS. Intracellular ROS production was measured with 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) (Molecular Probes, Shanghai, China). Embryonic cells were incubated with DCF-DA (5 μM) in serum-free medium at 37°C for 30 minutes and then washed with PBS. DCF fluorescence was excited at 488 nm, and emission at 530 nm was measured on a Gemini EM Reader (Molecular Devices, Shanghai, China). Fluorescence intensity values are presented as the percentage of control values after subtraction of background fluorescence.

Measurement of malondialdehyde (MDA) and H_2O_2

E7.5 embryos were collected for to assay MDA and H_2O_2. MDA was assayed using an ultraviolet-visible spectrophotometer and photometer (Agilent, Beijing China) using the thiobarbituric acid test (Ohya et al., 1993). H_2O_2 was assayed using a kit from Cayman Chemicals (Shanghai, China) according to the manufacturer's instructions. Results were normalized to protein concentrations in cell extracts.

Statistical analysis

Data are expressed as the mean ± SD and were analyzed by SPSS software (SPSS 19.0, IBM, Armonk, NY). One-way analysis of variance followed by a least significant difference test was used to examine differences between treatment groups. *P* values less than 0.05 were considered statistically significant.

Results

Verification of AGE-BSA

The OD at 405 nm of AGE-BSA gradually increased from 0 to 2.4, while that of control BSA prepared under the same conditions without glucose was lower than 0.15 (**Figure 1**).

Serum AGEs level in AGE-BSA feeding pregnant mice

Serum levels of AGEs increased significantly ($P < 0.01$) in AGE-BSA pregnant mice compared with BSA control pregnant mice. VitE supplementation did not affect levels of AGEs in AGE-BSA or BSA control pregnant mice ($P > 0.05$; **Figure 2**).

Immunochemical reactivity of embryonic proteins

Reactivity of CML-BSA and CEL-BSA antibodies, which recognize embryonic proteins, increased significantly in embryos of AGE-BSA mice compared with control mice and BSA mice, indicating that mice fed with AGE-BSA food could increase embryonic CEL and CML contents ($P < 0.01$; **Figure 3**). VitE supplementation did not change embryo protein reactivity to CML and CEL (data not shown).

AGE-BSA induces NTDs in mice *in vivo*

As shown in **Figure 4**, the incidence of NTDs was significantly increased in AGE-BSA mice at E10.5, similar to the effect of hyperglycemia (**Figure 5**). Incidence of NTDs was also significantly increased in diabetic AGE-BSA mice compared with diabetic mice or AGE-BSA mice ($P < 0.05$). In contrast, there was no effect of BSA or saline control administration on embryonic developmental malformation. The increased incidence of NTDs induced by AGE-BSA administration in mice could be partially blocked by VitE supplementation (**Figure 4** & **Table 1**).

Serum levels of MDA and H_2O_2 in embryos of mice fed with AGE-BSA

To further test whether increased incidence of NTDs is the result of oxidative stress induced by AGE-BSA, oxidative stress

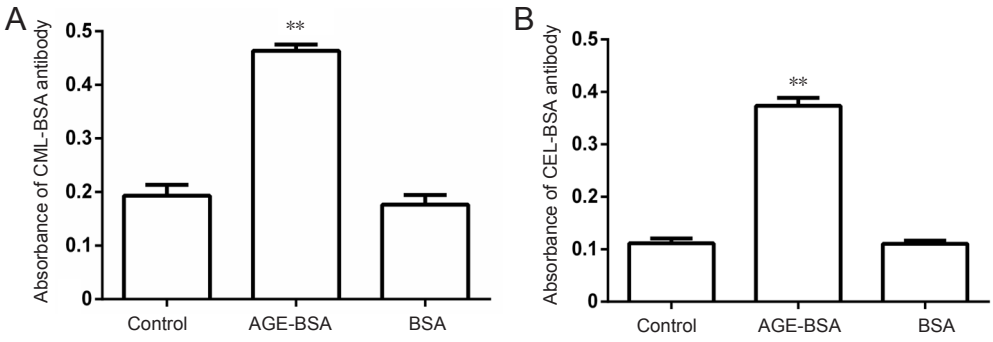

Figure 3 Immunochemical reactivity of antibody against CML-BSA (A) and CEL-BSA (B) with embryo proteins.
***P* < 0.01, *vs.* BSA group. Data are expressed as the mean ± SD (*n* = 6; one-way analysis of variance followed by the least significant difference test). Control group: Mice were fed with normal commercial food. AGE-BSA group: Female mice were fed with commercial food containing 3% AGE-BSA. BSA group: Female mice were fed with commercial food containing 3% BSA. AGEs: Advanced glycation end products; BSA: bovine serum albumin; AGE-BSA: advanced glycation end product bovine serum albumin; CML-BSA: Nε-(carboxymethyl) lysine-bovine serum albumin; CEL-BSA: N(epsilon)-(carboxyethyl) lysine-bovine serum albumin.

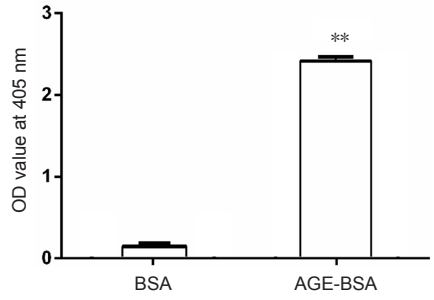

Figure 1 Optical density (OD) value at 405 nm of advanced glycation end product bovine serum albumin (AGE-BSA) and bovine serum albumin (BSA) control at the end of the 8-week incubation.
***P* < 0.01, *vs.* BSA group. Data are expressed as the mean ± SD (*n* = 6; one-way analysis of variance followed by the least significant difference test).

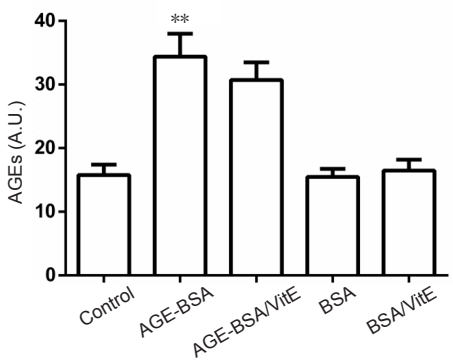

Figure 2 Serum levels of AGEs in mice fed with AGE-BSA or BSA.
***P* < 0.01, *vs.* BSA group. Data are expressed as the mean ± SD (*n* = 6; one-way analysis of variance followed by least significant difference test). Control group: Mice were fed with normal commercial food. AGE-BSA group: Female mice were fed with commercial food containing 3% AGE-BSA. AGE-BSA/VitE group: Female mice were fed with 3% AGE-BSA for 4 weeks and then fed with 0.125% (w/w) VitE at day 0.5 of gestation. BSA group: Female mice were fed with commercial food containing 3% BSA. BSA/VitE group: Female mice were fed with 3% BSA for 4 weeks and then fed with 0.125% (w/w) VitE at day 0.5 of gestation. AGEs: Advanced glycation end products; BSA: bovine serum albumin; AGE-BSA: advanced glycation end product bovine serum albumin; VitE: vitamin E; AU: arbitrary units.

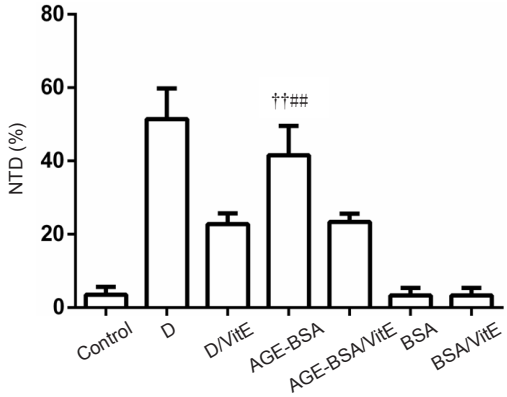

Figure 4 Effects of AGE-BSA on incidence of NTDs in C57BL/6 mice.
After feeding for 4 weeks with food containing AGE-BSA, C57BL/6 mice displayed significantly higher incidence of NTDs compared with control (fed with normal commercial food) mice (††*P* < 0.01, *vs.* control). VitE could decrease, but not recover all, NTDs in diabetic and AGE-BSA mice (##*P* < 0.01, *vs.* AGE-BSA/VitE). Data are expressed as the mean ± SD (*n* = 6; one-way analysis of variance followed by least significant difference test). Control group: Female mice were fed with normal commercial food; D group: Diabetes was induced in female mice. D/VitE group: Diabetic female mice were fed with 0.125% (w/w) VitE at day 0.5 of gestation. AGE-BSA group: Female mice were fed with commercial food containing 3% AGE-BSA. AGE-BSA/VitE group: Female mice were fed with 3% AGE-BSA for 4 weeks and then with 0.125% (w w) VitE at day 0.5 of gestation. BSA group: Female mice were fed with commercial food containing 3% BSA. BSA/VitE group: Female mice were fed with 3% BSA for 4 weeks and then with 0.125% (w/w) VitE at day 0.5 of gestation. AGEs: Advanced glycation end products; BSA: bovine serum albumin; AGE-BSA: advanced glycation end product bovine serum albumin; VitE: vitamin E; NTDs: neural tube defects.

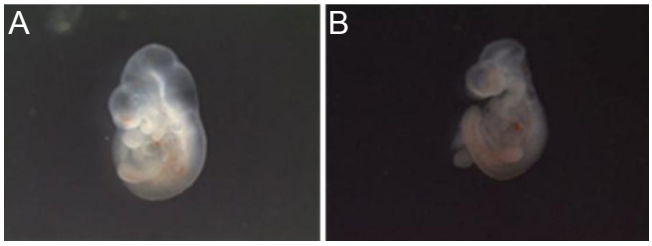

Figure 5 Embryos with normal neural tube formation and neural tube defects.
(A)Normal embryo at embryonic day 10.5 (E10.5) from mother fed with 3% bovine serum albumin. (B) E10.5 embryo exhibiting neural tube defects from mother fed with 3% advanced glycation end product bovine serum albumin.

Table 1 Morphology of embryonic day 10.5 mouse embryos from different groups

Group	Total	Normal	Exencephaly (single)	Exencephaly (combined)	Spina bifida
Control	59	55	4	0	0
D	52	26	18	6	2
D+AGEs	56	23	19	10	4
AGEs-BSA	54	22	26	6	0
BSA	60	56	2	0	2

Date are expressed as number (*n*). Exencephaly (single): Only one malformation located on forebrain, midbrain, or hindbrain. Exencephaly (combined): More than two malformations in one embryo. D: Diabetes; AGEs: advanced glycation end products; BSA: bovine serum albumin; AGE-BSA: advanced glycation end product bovine serum albumin.

markers MDA and H_2O_2 were assayed in embryos. Significant increases in MDA levels ($P < 0.01$; **Figure 6**) and H_2O_2 ($P < 0.01$) were observed in embryos of AGE-BSA mice compared with BSA control mice. Moreover, MDA and H_2O_2 levels were significantly higher in diabetic AGE-BSA mice compared with diabetic mice or AGE-BSA mice. VitE significantly decreased MDA and H_2O_2 levels ($P < 0.01$) in embryos of AGE-BSA mice compared with BSA control mice (**Figure 6**).

Intracellular ROS in embryonic cells

In situ ROS production by embryonic cells at E8.5, as measured by DCF fluorescence, indicated significantly increased ROS levels after 24-hour incubation with 800 μg/mL AGE-BSA. Intracellular ROS production increased by 150.40 ± 29.17% in embryonic cells cultured in 800 μg/mL AGE-BSA medium compared with control cells cultured in 800 μg/mL BSA medium (100%; $P < 0.01$; **Figure 7**).

Discussion

Contributions and limitations of previous studies

AGEs, a heterogenous group of molecules formed by the nonenzymatic reaction of glucose with protein, are critical pathogenic mediators of diabetes complications and other associated diseases such as aging, atherosclerosis, and Alzheimer's disease. The formation of AGEs occurs at a constant and slow rate in normal conditions, but is markedly accelerated in hyperglycemia because of increased availability of glucose, and significantly increased by eating habits or lifestyle choices such as smoking. However, the role of AGEs in embryonic development has remained in question.

Preparation of AGE-BSA by incubation of BSA and glucose *in vitro* has been widely used to mimic the effects of AGEs (Schmidt et al., 1995). By incubating BSA and glucose *in vitro* for 8 weeks, an AGE-BSA mixture including intermediate and late AGEs is produced (Schmidt et al., 1995; Alam et al., 2016; Koch et al., 2017; Pietsch et al., 2017). AGE-specific fluorescence, which has been described in several publications (Monnier, 1981; Lo, 1994; Leclere, 2001), is widely used to detect AGEs *in vivo* and *in vitro* (Nagai, 2000; Valencia et al., 2004). One study showed that fluorescence detected at an excitation/emission of 365/440 nm is a good choice for glucose-modified human serum albumin (Schmitt et al., 2005). Indeed, 365/440 nm has been proven to be a very effective wavelength to detect specific AGEs in blood and tissue samples (Kalousova, 2002; Jing et al., 2009; Bohlooli et al., 2013). As such, 365/440 nm was used in this study to detect AGEs levels in mouse blood.

Apart from endogenous AGE formation, AGEs are also absorbed by the body from exogenous sources including cigarette smoke and highly heated processed foods, which contain high amounts of AGEs (Stirban and Tschöpe, 2015). Kinetic studies indicated that 10–30% of dietary AGEs consumed are intestinally absorbed. Thus, plasma concentrations of AGEs appear to be directly influenced by dietary AGE intake and the body's capacity for AGE elimination.

Distinguishing characteristics of the current study

In the present study, AGE-BSA was fed to mice to examine the effect of AGEs (Peppa et al., 2003; Chen et al., 2013). The results showed that serum levels of AGEs significantly increased in AGE-BSA mice compared with BSA control mice. Moreover, CML-BSA and CEL-BSA reactivity were remarkably increased in the embryos of AGE-BSA mice compared with control mice, indicating increased CEL and CML contents in embryos of mice fed with AGE-BSA food. Thus, feeding is a suitable method to administer AGEs to animals.

The results of this study demonstrated a significantly higher incidence of NTDs in AGE-BSA mice compared with control mice. In addition, a higher incidence of NTDs was observed in diabetic AGE-BSA mice compared with diabetic mice. This result is consistent with previous results indicating that AGEs is a risk factor for induction of NTDs in the absence of hyperglycemia (Carmichael, 2003; Suarez et al., 2012). As food is the major source of exogenous AGEs, it is reasonable to conclude that lifestyle, especially eating behaviors, could be an important factor for pregnant women. Notably, VitE supplementation could inhibit the increased incidence of NTDs caused by AGE-BSA compared with the non-VitE group, similar to its effect on diabetic mice. Therefore, the mechanism underlying the teratogenic effect of AGE-BSA on embryonic development may be similar to the mechanism induced by diabetes, at least with regard to increased oxidative stress.

While overproduction of oxidative stress is associated with embryonic dysmorphology in diabetic pregnancy, administration of antioxidants could attenuate this teratogenic effect in embryos subjected to a hyperglycemic environment both *in vivo* and *in vitro* (Loeken, 2005). For non-diabetic pregnant women, oxidative stress may also be a risk factor for inducing embryonic malformation. First, a high level of free radicals can directly damage DNA or inhibit Pax3 expression, which is crucial for neural tube development. Second, oxidative stress caused by AGE-RAGE interaction is the major mechanism underlying the biological effect of AGE. Although we did not observe significantly different serum MDA levels between NTDs-affected and unaffected control pregnant women in the presence of different AGEs levels (Li et al., 2014), we found significantly different MDA levels in embryos between AGE-BSA and control mice. One

explanation for this is that the materials used to assay MDA were different: one is for human serum, while the animal experiment assayed MDA levels of whole embryos. Second, as the background, diet, and other lifestyle habits of mice are identical, it is easy to observe the effect of AGEs on MDA levels to the exclusion of other factors. In contrast, many factors vary between individual humans, including different eating habits. Furthermore, we observed that the antioxidant VitE could decrease the incidence of NTDs caused by AGE-BSA feeding but could not totally block the teratogenic effect. Thus, increased oxidative stress is unlikely to be the only mechanism by which AGEs induce NTDs.

Limitations

The results of this study clearly indicated that AGE-BSA could induce NTDs in embryonic mice, at least in part by elevating oxidative stress levels. However, this mechanism is not necessarily the only cause of NTDs. Further studies will be important to demonstrate the detailed localization of CML and CEL in pathological embryonic tissues with specific antibodies, as well as changes in signaling pathways downstream of the oxidative stress caused by AGEs.

Significance

In conclusion, exogenous application of AGE-BSA to C57BL/6 mice resulted in an increased incidence of NTDs in the absence of hyperglycemia, thus replicating the effects of maternal diabetes on embryonic development. These findings both confirm and provide a new mechanistic basis for the role of AGEs in NTDs occurring in nondiabetic pregnancies and NTDs caused by maternal diabetes.

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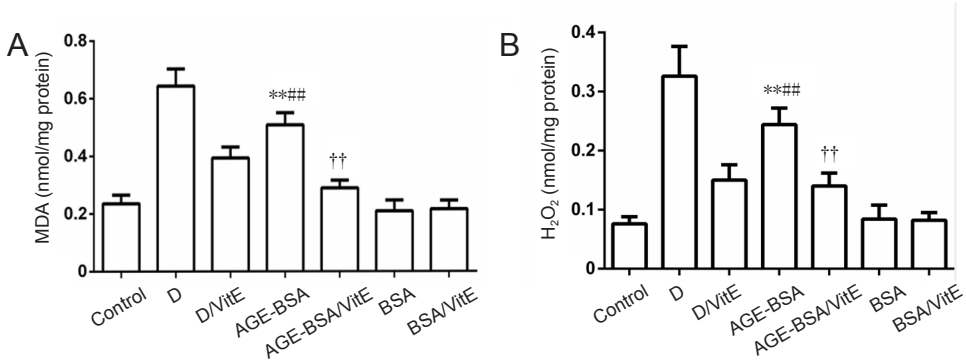

Figure 6 MDA and H_2O_2 levels in AGE-BSA and control mice with different treatments.

For MDA (A) and H_2O_2 (B) assays, $^{**}P < 0.01$, *vs*. BSA group; $^{\#\#}P < 0.01$, *vs*. AGE-BSA/VitE group; $^{\dagger\dagger}P < 0.01$, *vs*. control group. Data are expressed as mean ± SD ($n = 6$; one-way analysis of variance followed by least significant difference test). Control group: Female mice were fed with normal commercial food. D: Diabetes was induced in female mice. D/VitE group: Diabetic female mice were fed with 0.125% (w/w) VitE at day 0.5 of gestation. AGE-BSA: Female mice were fed with commercial food containing 3% AGE-BSA. AGE-BSA/VitE group: Female mice were fed with 3% AGE-BSA for 4 weeks and then with 0.125% (w/w) VitE at day 0.5 of gestation. BSA group: Female mice were fed with commercial food containing 3% BSA. BSA/VitE group: Female mice were fed with 3% BSA for 4 weeks and then with 0.125% (w/w) VitE at day 0.5 of gestation. AGEs: Advanced glycation end products; BSA: bovine serum albumin; AGE-BSA: advanced glycation end product bovine serum albumin; VitE: vitamin E; MDA: malondialdehyde.

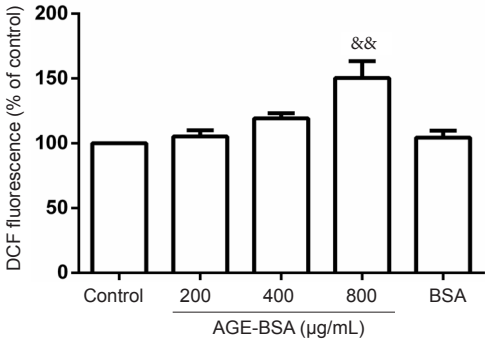

Figure 7 Effects of AGE-BSA on reactive oxygen species production in embryonic cells on embryonic day 8.5 (E8.5).

After culturing embryonic cells harvested at E8.5 in DMEM containing 800 μg/mL AGE-BSA for 24 hours, increased reactive oxygen species production was observed ($^{\&\&}P < 0.01$, *vs*. other 4 groups). Data are expressed as mean ± SD ($n = 6$; one-way analysis of variance followed by least significant difference test). Control group: E8.5 cells were cultured in DMEM. 200 μg/mL AGE-BSA group: E8.5 cells were cultured in DMEM containing 200 μg/mL AGE-BSA. 400 μg/mL AGE-BSA group: E8.5 cells were cultured in DMEM containing 400 μg/mL AGE-BSA. 800 μg/mL AGE-BSA group: E8.5 cells were cultured in DMEM containing 800 μg/mL AGE-BSA. BSA group: E8.5 cells were cultured in DMEM containing BSA. AGEs: Advanced glycation end products; BSA: bovine serum albumin; AGE-BSA: advanced glycation end product bovine serum albumin; DMEM: Dulbecco's Modified Eagle's Medium.

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