

Acta Medica Okayama

Volume 62, Issue 2

2008

Article 4

APRIL 2008

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Abstract

We studied the effects of advanced glycation end products (AGEs), which are known to accumulate in patients with diabetes, autoimmune diseases, or those who smoke, on embryonal development. Pronuclear (PN) embryos were obtained by flushing the fallopian tubes of rats after superovulation and mating. The cleavage rate and blastocyst yield were evaluated at 24, 72, 96, and 120 h of culture. Glyoxal, an AGE-forming aldehyde, suppressed embryonal development at every stage from PN to blastocyst in a concentration-dependent manner. The cleavage rate of the embryo was also significantly decreased by treatment with glyoxal at concentrations of 1 mM or higher. The blastocyst yield was significantly decreased by treatment with glyoxal at concentrations of 0.5 mM or higher. N-acetyl-L-cysteine (L-NAC) at 1 mM significantly suppressed the glyoxal-induced embryonal toxicity. BSA-AGEs at 5 microg/ml or higher concentration significantly reduced the cleavage rate and blastocyst yield compared to those for BSA-treated embryos. L-NAC at 1 mM significantly suppressed BSAAGE-induced embryonal toxicity. Because AGEs are embryo-toxic, AGE contamination may influence the pregnancy rate of in vitro fertilization and embryo transfer. AGEs, which are increased in women under pathological conditions, may also be involved in their infertility.

KEYWORDS: advanced glycation end products, blastocyst, embryo, in vitro fertilization, N-acetyl-L-cysteine

Original Article

Adverse Effects of Advanced Glycation End Products on Embryonal Development

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We studied the effects of advanced glycation end products (AGEs), which are known to accumulate in patients with diabetes, autoimmune diseases, or those who smoke, on embryonal development. Pronuclear (PN) embryos were obtained by flushing the fallopian tubes of rats after superovulation and mating. The cleavage rate and blastocyst yield were evaluated at 24, 72, 96, and 120 h of culture. Glyoxal, an AGE-forming aldehyde, suppressed embryonal development at every stage from PN to blastocyst in a concentration-dependent manner. The cleavage rate of the embryo was also significantly decreased by treatment with glyoxal at concentrations of 1 mM or higher. The blastocyst yield was significantly decreased by treatment with glyoxal at concentrations of 0.5 mM or higher. N-acetyl-L-cysteine (L-NAC) at 1 mM significantly suppressed the glyoxal-induced embryonal toxicity. BSA-AGEs at 5 µg/ml or higher concentration significantly reduced the cleavage rate and blastocyst yield compared to those for BSA-treated embryos. L-NAC at 1 mM significantly suppressed BSA-AGE-induced embryonal toxicity. Because AGEs are embryo-toxic, AGE contamination may influence the pregnancy rate of *in vitro* fertilization and embryo transfer. AGEs, which are increased in women under pathological conditions, may also be involved in their infertility.

Key words: advanced glycation end products, blastocyst, embryo, *in vitro* fertilization, N-acetyl-L-cysteine

Advanced glycation end products (AGEs) include reactive, cross-linking molecules that are formed from the nonenzymatical reaction (also called the Maillard reaction) of reducing sugars and the amino groups of proteins, lipids, and nucleic acids. Plasma or serum AGEs levels are high in patients with hyperglycemia and/or oxidative stress such as

diabetes, rheumatoid arthritis, systemic lupus erythematosus (SLE), renal insufficiency, or cigarette smoking [1-8].

Glyoxal, a reactive aldehyde, arises from enzymatic and nonenzymatic degradation of glucose, lipid and protein catabolism, and lipid peroxidation. Plasma glyoxal levels are elevated even in young, complication-free patients with Type 1 diabetes. This aldehyde forms AGEs that react with proteins that are implicated in diabetic complications [9, 10]. Proteins reacting with glyoxals such as glyoxal and methylgly-

Received November 12, 2007; accepted November 28, 2007.

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oxal are indicative of the formation of reactive oxygen species (ROS).

Glyoxals and AGEs are also formed in various foodstuffs during processing, especially at high temperatures. A diet rich in AGEs is known to induce significant increases in inflammatory and endothelial dysfunction markers and as well as a more pronounced acute impairment of vascular function in type 2 diabetes mellitus [11]. Heat sterilization of glucose peritoneal dialysis fluid is known to generate glucose degradation products, which accelerate the formation of AGEs [12]. Formation and accumulation of AGEs in the peritoneum causes a deterioration of peritoneal permeability in patients treated using peritoneal dialysis [13].

AGEs cause tissue injury both directly through phenomena such as trapping and cross-linking and indirectly by binding to specific receptors for AGEs (RAGEs) on the surface of various cells. AGEs are known to generate ROS, which include superoxide anion and hydrogen peroxide, which cause tissue injury [14].

There have been some reports regarding the involvement of AGEs in reproduction. We have reported that AGEs significantly induces apoptosis in human first-trimester trophoblasts and reduce secretion of human chorionic gonadotropin [15]. These AGE-mediated damages in trophoblasts may lead to an impairment of implantation and placentation. We have also observed that the levels of serum AGEs and accumulation of AGEs in term placenta are significantly increased in preeclamptic women compared to healthy pregnant women [16]. These observations indicate the involvement of AGEs in the oxidative stress of trophoblasts complicated by implantation failure and placental dysfunction.

Although AGEs adversely affect various reproductive processes during pregnancy, the effects of AGEs on embryonal development have not yet been fully elucidated. In the present study, we investigated the effects of AGEs on the development of rat embryos.

Materials and Methods

Culture medium. The basic medium used for culture of rat pronuclear (PN) embryos was mR1ECM [17]. The mR1ECM was composed of 76.7 mM NaCl, 3.2 mM KCl, 2.0 mM CaCl₂, 0.5 mM MgCl₂,

25.0 mM NaHCO₃, 10.0 mM sodium lactate, 0.5 mM sodium pyruvate, 7.5 mM glucose, 1.0 mg/ml polyvinylalcohol, 2% (v/v) minimal essential medium (MEM) essential amino acid solution (Gibco Laboratories, Grand Island, NY, USA), 0.1 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA), and 1% (v/v) MEM nonessential amino acid solution (Gibco) [17]. Prior to use, the culture medium was covered with paraffin oil in a polystyrene culture dish and equilibrated with the gas phase and temperature (5% CO₂ in air at 37 °C) in a CO₂ incubator for 3–5 h.

Collection of rat embryos. Mature female Sprague-Dawley rats (2–4 months old) at proestrus, as assessed from vaginal smears, were placed overnight with mature males. At 14:00 h on the following day, the females were examined for mating by the presence of a vaginal plug or spermatozoa in the vagina. The oviducts were isolated and placed in a dish containing paraffin oil, and the culture media was supplemented with 0.1% (w/v) hyaluronidase. The cumulus-oocyte complexes were dissected out of the oviducts, placed in each medium, and freed from cumulus cells by repeated passage through a fine pipette. The denuded oocytes were washed 3 times with the culture media without hyaluronidase and observed for evidence of PN formation by means of a phase-contrast microscope. Only PN-formed oocytes were transferred into 100 μl of the same culture medium and cultured under 5% CO₂ in air at 37 °C.

The experiment was carried out in accordance with institutional Policies and Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize the suffering of the rats.

Culture of embryos. Cleavage and blastocyst rates were evaluated at 24, 72, 96, and 120 h after the start of culture for their development to the 2-cell, 4-cell, morula, and blastocyst stages, respectively, using a phase-contrast microscope. Embryos showing blastocoele cavity formation were classified as blastocysts.

Preparation of BSA-AGEs. BSA-AGEs were prepared as previously described [15]. AGEs were produced by incubation of BSA (Fraction V, fatty acid free, low endotoxin, Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 30 mg/ml with 0.5 M glucose in 0.2 M phosphate-buffered saline (PBS) containing 0.5 mM EDTA, pH 7.4, at

37 °C for up to 8 weeks. Unbound glucose was removed by extensive dialysis against PBS. BSA-AGEs were lyophilized and resuspended in PBS. The density of brown color, which is the typical physical appearance of AGEs, was quantified by measuring the optical density at 405 nm. The optical density at 405 nm of BSA, which was prepared under the same conditions without glucose, was confirmed to be very low (less than 0.1) at a concentration of 3% (w/v). The optical density at 405 nm of each batch of BSA-AGEs was between 1.8 and 2.1 at the same concentration. BSA-AGEs were resuspended in the culture medium before use.

Competitive enzyme-linked immunosorbent assay (ELISA) for AGEs. A competitive ELISA was carried out as previously described [16]. Each well of a 96-well microtiter assay plate (Immulon IB, Thermo Lab Systems, Franklin, MA, USA) was coated with AGEs-modified bovine serum albumin (BSA-AGEs) in dilution buffer (10-fold diluted solution of Block Ace[®], Dai Nippon Pharmaceutical Laboratories, Osaka, Japan) at 4 °C overnight. Triplicate washing with washing buffer (10-fold diluted solution of Block Ace[®] containing 0.05% Tween 20) was then carried out. Each well was blocked with blocking buffer (fourfold diluted solution of Block Ace[®]) at 37 °C for 1 h.

The mR1ECM was incubated with an equal volume of antibody solution (0.2 µg/ml, in final, anti-AGEs monoclonal antibody, an antibody against an N^ε-carboxymethyllysine-protein (CML), TransGenic, Kumamoto, Japan) at 37 °C for 1 h. This mixture (100 µl) was transferred to an assay plate and incubated at 37 °C for 1.5 h. The wells were then washed 3 times with washing buffer. After incubation at room temperature for 1 h with the horseradish peroxidase-

conjugated secondary antibody (sheep anti-mouse IgG, Jackson ImmunoResearch Laboratories, West Grove, PA, USA, 5000-fold dilution), the wells were again washed 3 times with washing buffer. The binding of coated AGEs and anti-AGEs antibodies was visualized with o-phenylenediamine dihydrochloride (Nakalai Tesque, Kyoto, Japan). After the reaction was stopped by the addition of 100 µl of sulfuric acid (1 M), absorbance at 490 nm was read on a microplate reader (Model 680, Bio-Rad, Osaka, Japan).

Statistical analysis. Statistical significance was determined by Fisher's exact probability test. A *p* value < 0.05 was considered statistically significant.

Results

Glyoxal, in a concentration-dependent manner, suppressed cleavage of embryos at every stage from PN to blastocyst (Table 1). Although 0.1 mM glyoxal reduced the cleavage rate and blastocyst yield compared to untreated embryos, there were no significant differences. The blastocyst yield was significantly decreased by treatment with glyoxal at 0.5 mM or higher concentrations. The cleavage rate of embryos is also significantly decreased by treatment with glyoxal at concentrations of 1 mM or higher. Morphological changes referred to as fragmentation were observed in some embryos, which were treated with glyoxal, and stopped from developing (Fig. 1).

N-acetyl-L-cysteine (L-NAC) at 1 mM significantly suppressed glyoxal-induced embryonal toxicity (Table 2). However, 0.1 mM L-NAC did not show any such protective effects.

BSA-AGEs, in a concentration-dependent manner, suppressed the cleavage of embryos at every stage form PN to blastocyst (Table 3). BSA-AGEs at 5

Table 1 Effects of glyoxal on rat embryonal development

| Culture (hours) | Embryonal development | Untreated (n = 24) | Glyoxal (mM) | | | | |
|-----------------|-----------------------|--------------------|--------------|---------------|-------------|------------|-------------|
| | | | 0.1 (n = 27) | 0.5 (n = 29) | 1 (n = 20) | 5 (n = 20) | 10 (n = 12) |
| 24 | 2-cell | 23 (95.8%) | 25 (92.6%) | 27 (93.1%) | 17 (85.0%) | 3 (15.0%)* | 0* |
| 72 | 4-cell | 21 (87.5%) | 21 (77.8%) | 23 (79.3%) | 9 (45.0%)** | 0* | 0* |
| 96 | Morula | 17 (70.8%) | 16 (59.3%) | 16 (55.2%) | 2 (10.0%)* | 0* | 0* |
| 120 | Blastocyst | 16 (66.7%) | 14 (51.9%) | 6 (20.7%)* ** | 0* | 0* | 0* |

vs. Untreated: **p* < 0.0001, ***p* < 0.005, ****p* < 0.001

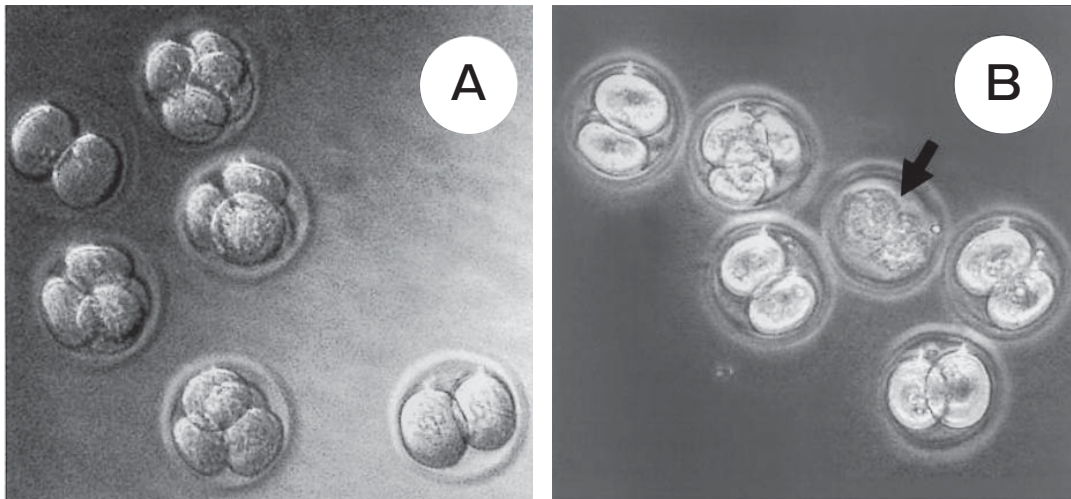


Fig. 1 Cytoplasmic fragmentation observed in embryos treated with glyoxal. A, Untreated embryos; B, Embryos treated with glyoxal. Arrow indicates cytosolic fragmentation.

Table 2 Effects of N-acetyl-L-cysteine on glyoxal-Induced embryonal toxicity

| Culture (hours) | Embryonal development | Untreated (n=17) | Glyoxal 1 mM | | |
|-----------------|-----------------------|------------------|--------------|--------------------------|--------------|
| | | | (n = 18) | N-acetyl-L-cysteine (mM) | |
| | | | | 0.1 (n = 20) | 1 (n = 21) |
| 24 | 2-cell | 16 (94.1%) | 16 (88.9%) | 17 (85.0%) | 21 (100%) |
| 72 | 4-cell | 12 (70.6%) | 7 (38.9%) | 7 (35.0%) | 16 (76.2%)* |
| 96 | Morula | 12 (70.6%) | 2 (11.1%) | 1 (5.0%) | 10 (47.6%)** |
| 120 | Blastocyst | 9 (52.9%) | 0 | 0 | 8 (38.1%)*** |

vs. Glyoxal 1 mM without N-acetyl-L-cysteine: * $p < 0.03$, ** $p < 0.02$, *** $p < 0.005$

Table 3 Effects of BSA-AGEs on rat embryonal development

| Culture (hours) | Embryonal development | BSA 5 μ g/ml (n = 27) | BSA-AGEs (μ g/ml) | | |
|-----------------|-----------------------|---------------------------|------------------------|-------------|-------------|
| | | | 1 (n = 23) | 5 (n = 26) | 10 (n = 24) |
| 24 | 2-cell | 26 (96.3%) | 22 (95.7%) | 24 (92.3%) | 22 (91.7%) |
| 72 | 4-cell | 26 (96.3%) | 19 (82.6%) | 14 (53.8%)* | 4 (16.3%)** |
| 96 | Morula | 22 (81.5%) | 13 (56.5%)*** | 4 (15.4%)** | 0** |
| 120 | Blastocyst | 16 (59.3%) | 12 (52.2%) | 3 (11.5%)* | 0** |

vs. Untreated: * $p < 0.0005$, ** $p < 0.0001$, *** $p < 0.07$

$\mu\text{g/ml}$ or higher concentration significantly reduced the cleavage rate and blastocyst rate compared to those for BSA-treated embryos.

L-NAC at 1 mM significantly suppressed BSA-AGEs-induced embryonal toxicity (Table 4). L-NAC at 1 mM also suppressed the adverse effects of 5 $\mu\text{g/ml}$ BSA-AGEs on blastocyst formation.

An enzyme-linked immunosorbent assay using an antibody against an N^ε-carboxymethyllysine-protein and BSA-AGEs (0.1–10 $\mu\text{g/ml}$) as a standard revealed that the AGEs concentrations in mR1ECM were undetectable.

Discussion

In the present study, we showed the adverse effects of glyoxal and AGEs on embryonal development from the PN stage to blastocyst. Glyoxal and methylglyoxal are both reactive molecules that form AGEs reacting with proteins. It has been reported that methylglyoxal interferes with the development from morula to blastocyst by induction of apoptosis in a culture system [18]. Apoptosis is induced in first-trimester human trophoblasts by treatment with BSA-AGEs [15].

Apoptotic morphological changes are known to be observed in 70–80% of all *in vivo* or *in vitro*-produced blastocysts from mice or humans, and in practically all *in vivo* or *in vitro*-produced blastocysts from cattle [19]. In the present study, we observed cytoplasmic fragmentation in some embryos that showed arrested development. These embryos contain blastomeres of unequal size and multiple cellular fragments, and they will often show arrested development with subsequent

degeneration. Fragmented embryos reach the blastocyst stage less frequently and are associated with decreased pregnancy rates [20]. Apoptosis is known to be responsible for fragmentation and impairment of early embryonic development in IVF and may result from suboptimal culture conditions [20]. Fragmentation has also been observed in human embryos *in vivo*, indicating that fragmentation may also reduce the quality of the embryos and cause implantation failure under pathological conditions such as diabetes and endometriosis [21].

Many animal and human studies have elucidated the adverse effects of ROS on embryonal quality [22], although a group of investigators has reported that women who become pregnant by IVF have higher levels of ROS than those who do not [23]. High levels of ROS including superoxide anion and the hydroxyl radical, with hydrogen peroxide as an intermediary, generated by embryos or constituents in medium during *in-vitro* embryo development can cause damage in embryos such as a rise in lipid peroxides [24] and an increase in protein oxidation and DNA strand breaks [25]. A significant relationship is known to be observed between increasing concentrations of hydrogen peroxide production and elevated numbers of fragmented embryos, suggesting that ROS may induce apoptosis in embryos [26]. ROS generated from AGEs [14] are likely to cause embryonal fragmentation, as observed in the present study. RAGE is known to be expressed during physiological embryonic development [15]. The binding of AGEs to RAGE results in the generation of intracellular oxidative stress [15]. AGEs may also interfere with the possible physiological role of RAGE.

Table 4 Effects of N-acetyl-L-cysteine on BSA-AGEs-induced embryonal toxicity

| Culture (hours) | Embryonal development | BSA | BSA-AGEs | BSA-AGEs | BSA |
|-----------------|-----------------------|---------------------|---------------------|---------------------|---------------------|
| | | 5 $\mu\text{g/ml}$ | 5 $\mu\text{g/ml}$ | 5 $\mu\text{g/ml}$ | 5 $\mu\text{g/ml}$ |
| | | N-acetyl-L-cysteine | N-acetyl-L-cysteine | N-acetyl-L-cysteine | N-acetyl-L-cysteine |
| | | 0 | 0 | 1 mM | 1 mM |
| | | (n=26) | (n=23) | (n=24) | (n=21) |
| 24 | 2-cell | 25 (96.2%) | 20 (87.0%) | 22 (91.7%) | 20 (95.2%) |
| 72 | 4-cell | 24 (92.3%) | 9 (39.1%) | 13 (54.2%) | 20 (95.2%) |
| 96 | Morula | 21 (80.8%) | 5 (21.7%) | 10 (41.7%) | 18 (85.7%) |
| 120 | Blastocyst | 15 (57.7%) | 2 (8.7%) | 9 (37.5%)* | 14 (66.7%) |

vs. BSA-AGEs 5 $\mu\text{g/ml}$ without N-acetyl-L-cysteine: * $p < 0.04$

It has been reported that methylglyoxal triggers apoptosis in blastocysts via activation of mitochondrial processes and caspases, a route often observed during oxidative stress-mediated cell injury [18]. ROS generated in culture media may impact embryonal development, *i.e.*, the cleavage rate, blastocyst yield, and quality [27]. It has been reported that curcumin, which is a common dietary pigment having potent antioxidant effects, prevents methylglyoxal-induced oxidative stress and apoptosis in mouse blastocysts [18].

In the present study, we observed the protective effects of an antioxidant, L-NAC, on glyoxal- or AGEs-induced embryotoxicity at an early stage, from the PN stage. It has been reported that chronic exposure of pericytes to BSA-AGEs leads to an increase in apoptosis associated with an increase in cellular ceramide and diacylglycerol levels [28]. L-NAC inhibits diacylglycerol/ceramide production and protects pericytes from apoptosis [28].

Glyoxal is also known to cause glutathione (GSH) oxidation. Glyoxal markedly increases the cytotoxicity of hydrogen peroxide-induced hepatocytes and GSH depletion [29]. Synthesis of GSH during oocyte maturation has been reported in the oocytes of mouse, hamster, pig, and cattle [25]. This increase in GSH content provides oocytes with large glutathione stores available to protect embryos against ROS until the blastocyst stage [25]. Depletion of glutathione causes an increase in hydrogen peroxide.

L-NAC has been widely used in protection against the toxic effects produced by several chemicals because of its radical scavenger properties and because L-NAC is a precursor of intracellular cysteine and glutathione, which are important intracellular defenses against oxidants. L-NAC is known to have potential protective activities that are attributed to the property of a glutathione precursor against the well-known embryotoxicity induced by methyl mercuric chloride in mice [30].

Using embryo culture media supplemented with anti-oxidant precursors such as β -mercaptoethanol [31] and vitamin E [32] is known to be beneficial for embryonic inner cell mass (ICM) development. Pentoxifylline, an inhibitor of phagocytosis and generation of toxic ROS and proteolytic enzymes, reduces oxidative stress-induced embryotoxicity [33]. Cysteamine, a glutathione synthesis enhancer, has been shown to improve embryo development when

added during *in vitro* maturation of bovine, porcine, and ovine oocytes [34]. Various antioxidants including these compounds may also prevent glyoxal- or AGEs-induced embryotoxicity.

We also measured the concentrations of AGEs in various commercially available fertilization media and cleavage media for human *in vitro* fertilization and embryo transfer (IVF-ET). We detected AGEs in some media, although the concentrations of AGEs in the media varied from lot to lot and were very low (equivalent to 0.2 $\mu\text{g}/\text{ml}$ of BSA-AGEs or less). Bioincompatible glucose degradation products, which accelerate AGEs formation, are known to develop during heat sterilization of peritoneal dialysis fluids [12]. Trace amounts of redox-active metal ions in biological buffers could induce oxidative stress attributed to AGEs-proteins *in vitro*. Under suboptimal storage conditions, AGEs may be generated in various solutions, including culture media or constituents for the media. Although the concentrations of AGEs in the media were very low, AGEs at these concentrations may influence embryonal development. The serum concentrations of AGEs, which are evaluated based on the CML concentrations, are reported to be approximately 0.6 $\mu\text{g}/\text{ml}$ in diabetic patients [35]. The local concentrations of AGEs in the fallopian tubes or the peritoneal fluid may be higher under pathological conditions associated with oxidative stress such as endometriosis or infection.

Further study is necessary to evaluate the pathological effects of AGEs on human embryos *in vitro* and *in vivo*.

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