Advanced glycation end-products (AGE) accumulation compromises embryonic development and achievement of pregnancy by assisted reproductive technology

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Introduction

Advanced glycation end-products (AGE) are reactive cross-linked molecules formed by non-enzymatic reactions of reducing sugars with amino groups of proteins, lipids or nucleic acids (Thomas et al., 2005). Formation and accumulation of AGE occurs during normal aging but accelerates with hyperglycemia, insulin resistance, dyslipidemia, oxidative stress and/or renin–angiotensin system activation in diabetes (Ulrich and Cerami, 2001; Thomas et al., 2005; Unoki and Yamagishi, 2008). AGE, in turn, worsens these detrimental states (Thomas et al., 2005; Unoki and Yamagishi, 2008). AGE cause tissue and intracellular damage directly by macromolecular trapping and cross-linking and also indirectly by binding to specific AGE receptors (RAGE) on cell surfaces (Ulrich and Cerami, 2001; Thomas et al., 2005).

Insulin resistance is pivotal in the pathogenesis of polycystic ovary syndrome (PCOS) (Dunaif, 1997), the most common cause of infertility. Moreover, insulin resistance can reflect factors such as aging, stress, anxiety, depression, obesity and sedentary lifestyle (Rosenthal et al., 1983; Eck et al., 1996; Paolisso et al., 1999; Bjorntorp and Rosmond, 2000; VanItallie, 2002), all of which can be common among infertile patients. Thus, insulin resistance appears prevalent even in infertile patients without PCOS. It has in fact been shown that the prevalence of insulin resistance is significantly higher among...
Materials and Methods

Patients and study design

A consecutive series of 157 IVF/ICSI cycles in 157 infertile women were included in this study. Uterine infertility was excluded. Causes of infertility included ovarian dysfunction (52 women), tubal factors (19 women), endometriosis (7 women), male factors (3 women), ovarian dysfunction plus male factors (49 women), tubal plus male factors (18 women) or unexplained (9 women). PCOS was diagnosed in 71 women according to the 2003 Rotterdam criteria (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004). Age, BMI and numbers of previous failed IVF/ICSI attempts in the subjects were 39.0 ± 0.38 years (mean ± SEM), 21.9 ± 0.2 kg/m² and 2.5 ± 0.3 failures, respectively.

On Day 3 of a spontaneous menstrual cycle, clinical examinations—including BMI, blood pressure (BP), blood sampling, hormone measurements and a 75-g oral glucose tolerance test (OGTT)—were carried out between 9 and 12 a.m. after 12 h of fasting. Within 3 months of these pre-examinations, patients underwent IVF/ICSI using the long protocol for ovarian stimulation by GnRH agonist and hMG administration. At oocyte retrieval, blood was drawn, and fluid from the follicles of 16-mm diameter or larger was pooled. We froze the specimens (−70°C) and later measured the concentrations of Ne-carboxymethyl lysine (CML) and pentosidine (Pent) in plasma and FF and those of glyceraldehyde-derived AGE, i.e., toxic AGE (TAGE) in serum and FF. Informed consent was obtained from all subjects. The study was approved by the Women’s Clinic Jinno Ethics Committee.

Measurements of AGE

Pent concentrations in plasma and follicular fluid were measured using a competitive enzyme-linked immunosorbent assay (ELISA) kit (FSK Pentosidine; Pharmaceutical FUSHIMI, Kagawa, Japan) as previously described (Sanaka et al., 2002). In brief, pronase was added to plasma or follicular fluid to expose Pent molecules bound to proteins, and an incubation was carried out at 55°C for 1.5 h. The mixture was then heated in boiling water for 15 min to inactivate the enzyme. Pent antibody and pre-treated sample or Pent standard solution were added to each well of a microtiter plate and incubated at 37°C for 1 h. After washing, peroxidase-labeled goat anti-rabbit IgG polyclonal antibodies were added and incubated for 1 h at room temperature. A color development reagent containing 3,3′,5′-tetramethylbenzidine (TMB) was then added to each well. The reaction was stopped 10 min later by adding TMB stop buffer. Absorbance was measured within 10 min at 450 and 630 nm (main and reference wavelength, respectively). Sensitivity and intra- and inter-assay coefficients of variation were 2 ng/ml, 8.0 and 4.2%, respectively.

Concentrations of CML in plasma and follicular fluid were measured using a competitive ELISA kit (Pharmaceutical FUSHIMI). Methods were similar to those for Pent. Sensitivity and intra- and inter-assay coefficients of variation were 0.4 µg/ml, 5.0 and 11.4%, respectively.

Concentrations of TAGE (glyceraldehyde-derived AGE or glyceraldehyde-AGE) in serum and follicular fluid were measured with a competitive ELISA using immunopurified glyceraldehyde-AGE antibody (Takeuchi et al., 2000). Briefly, 96-well microtiter plates were coated with 1 µg/ml glyceraldehyde-AGE per well and kept overnight in a cold room. Wells were washed three times with 0.3 ml of phosphate-buffered saline (PBS)-TWEEN-20. Wells were then blocked by incubation for 1 h with 0.2 ml of PBS containing 1% bovine serum albumin (BSA). After washing with PBS-TWEEN-20, test samples (50 µl) were added to each well as a competitor for 50 µl of glyceraldehyde-AGE antibody (1:1000), followed by incubation for 2 h at room temperature with gentle shaking by a horizontal rotary shaker. Wells then were washed with PBS-TWEEN-20 and developed with an alkaline-phosphatase-linked anti-rabbit IgG utilizing p-nitrophenyl phosphate as a colorimetric substrate. Results are expressed as glyceraldehyde-AGE units (U) per milliliter of serum or follicular fluid, with 1 U corresponding to 1 µg of glyceraldehyde-AGE-BSA standard, as described previously (Takeuchi et al., 2000). Sensitivity and intra- and inter-assay coefficients of variation were 0.02 U/ml, 6.2 and 8.8%, respectively.

Ovarian stimulation, IVF and ICSI

Follicular development was stimulated with the ‘long protocol’, involving GnRH agonist and hMG administration, as described previously (Jinno et al., 2010). Briefly, buserelin acetate (Suprecur; Hoechst, Tokyo, Japan), at 900 µg per day, was administered nasally from Day 4 of the luteal phase preceding the IVF/ICSI cycle until administration of hCG. Daily administration of hMG (hMG-Ferring; Ferring Pharmaceuticals, Tokyo, Japan) was begun between Days 3 and 10 of the follicular phase in the IVF/ICSI cycle. hCG (Gonotropin, Teikokuzouki, Tokyo, Japan) at 10,000 IU was administered when one or more follicles were at least 18 mm in diameter and the serum 17β-estradiol concentration exceeded 300 pg/ml (a conversion factor to SI units: 3.671).

Oocytes were collected transvaginally 36 h after hCG administration. Semen was diluted 2-fold with human tubal fluid medium (no. 9962; Irvine Scientific, Irvine, CA, USA) containing 10% patient serum (Jinno, 1986). Diluted semen was centrifuged directly if semen analysis results were normal, or after placement upon two layers of Sil-Select solutions (FertiPro NV, Beemem, Belgium) if the analysis results were abnormal.
Semen analyses were performed according to World Health Organization criteria (World Health Organization, 1987). The sperm pellet was resuspended in the medium and then centrifuged, after which motile spermatozoa were collected by a swim-up technique. Harvested oocytes were inseminated within 2–6 h by exposing to 80 000 motile spermatozoa per ml. ICSI was performed when the male partner had severe infertility with a sperm count <5 × 10⁶ per ml and/or motility in fewer than 20% of sperm. Gardner’s G-III sequential medium (G-FERT, G-1 version 3 and G-2 version 3; Vitrolife, Gotteborg, Sweden) supplemented with human serum albumin (HSA-solution; Vitrolife) was used for insemination and embryo culture.

Oocytes were considered fertilized when two pronuclei were observed at 17–19 h following insemination or ICSI. At 2, 3 or 5 days after oocyte retrieval, two or three embryos were transferred to the uterus according to the number and quality of developing embryos in each patient. Morphologically high-quality embryos at 2 days after oocyte retrieval were defined as Grades 1 and 2 according to Veeck’s criteria (Scott et al., 1991). Briefly, Grade 1 or 2 is defined as an embryo with equal size of cells accompanied with no or little fragmentation, respectively. Grade 3 is an embryo with different sizes of cells. Grade 4 or 5 is an embryo with moderate or severe fragmentation, respectively.

Morphology of blastocysts was assessed according to Gardner’s criteria (Gardner and Schoolcraft, 1999). Briefly, blastocysts were given a numerical score ranging from 1 to 6: 1, an early blastocyst with a blastocoel smaller than half of the embryo; 2, a blastocyst with a blastocoel larger than half of the embryo; 3, a full blastocyst with a blastocoel completely filling the embryo; 4, an expanded blastocyst; 5, a hatching blastocyst; and 6, a hatched blastocyst.

Embryologists who evaluated fertilization and embryonic development were blinded to information about AGE results. A 25-mg dose of progesterone was administered daily throughout the luteal phase after embryo transfer.

Clinical pre-examinations before IVF/ICSI attempts

Between 9 and 12 a.m. after at least 12 h of fasting, all patients underwent various examinations on Day 3 of a spontaneous menstrual cycle within 3 months before IVF/ICSI attempts. Physical examinations included BMI and systolic (S) and diastolic (D) BP. Glucose metabolism assessments included 75-g OGTT, homeostasis model assessment-insulin resistance index [HOMA-R, defined as fasting plasma glucose (FPG) × fasting immunoreactive insulin (FIRI)/405] and hemoglobin (Hb) A1c. Lipid metabolism assessments included total cholesterol (TC), low-density lipoprotein (LDL) cholesterol and triglyceride (TG). Endocrinologic assessments included LH, FSH, PRL, free testosterone, dehydroepiandrosterone sulfate (DHEA-S), 17β-estradiol, leptin and adiponectin.

Serum insulin and plasma glucose were measured by enzyme immunoassay (EIA, Lumipulse Insulin; Fuji Rebio, Tokyo, Japan) and amperometry using a GOD-fixation enzyme membrane and a peroxidizing electrode, respectively. Hb A1c was measured using rapid liquid chromatography. Sensitivities (with intra- and inter-assay coefficients of variation in parentheses) were 0.05 μU/ml (2.1%, 1.8%) for insulin; 0.1 mg/dl (1.0%, 1.0%) for glucose and 3.5% (0.81%, 0.82%) for Hb A1c.

Serum concentrations of 17β-estradiol, free testosterone, DHEA-S, FSH and PRL were measured with radioimmunocassay (RIA) kits from Diagnostic Products, Los Angeles, CA, except for FSH and PRL (Spacc-K kits; Daichi Radioisotope, Tokyo, Japan). Serum leptin was measured by RIA (Human Leptin RIA Kit; Linco Research, MO), and serum LH and adiponectin were measured by ELISA (Immulyze LH; Diagnostic Products and Human Adiponectin ELISA Kit; Ootsuka Seiyaku, Tokyo, Japan). Sensitivities and intra- and inter-assay coefficients of variation were 10 pg/ml (5.6%, 6.8%) for 17β-estradiol; 0.6 pg/ml (3.1%, 7.7%) for free testosterone; 20 ng/ml (3.9%, 4.3%) for DHEA-S; 0.5 IU/l (1.5%, 2.7%) for FSH; 1.0 ng/ml (6.3%, 6.9%) for PRL; 0.7 IU/l (6.2%, 5.5%) for LH; 0.3 ng/ml (6.3%, 6.3%) for leptin and 1.9 μg/ml (4.1%, 4.7%) for adiponectin.

Statistical analysis

Data were analyzed using Student’s t-test, the χ² test, Fisher’s exact test, analysis of variance (ANOVA), Fisher’s protected least significant difference (PLSD) test, receiver-operating characteristics (ROCs) curve analysis, multiple logistic regression analysis, multiple regression analysis, Pearson analysis or discriminant analysis as appropriate. P-values of <0.05 were considered to indicate significance. Results are presented as the mean ± SEM unless otherwise stated.

Results

In 157 women overall, the mean number of follicles larger than 12 mm in diameter, and serum concentrations of 17β-estradiol on the day of hCG administration were 13.3 ± 1.1 and 3051 ± 201 pg/ml, respectively. The mean numbers of retrieved oocytes, fertilized oocytes, Day-2 embryos and morphologically superior Day-2 embryos were 11.8 ± 0.8, 7.0 ± 0.5, 6.2 ± 0.5 and 3.6 ± 0.3, respectively.

Concentrations of Pent, CML and TAGE in follicular fluid (FF-Pent, FF-CML and FF-TAGE, respectively) and those of TAGE in serum (S-TAGE) had significant negative correlations (Pearson analysis) with follicular growth in terms of number of follicles larger than 12 mm in diameter (FF-Pent, R = −0.32, P < 0.0001; FF-CML, R = −0.32, <0.0001; FF-TAGE, R = −0.24, <0.01; and S-TAGE, R = −0.22, <0.05, respectively) and serum concentrations of 17β-estradiol (FF-Pent, R = −0.29, <0.001; FF-CML, R = −0.31, <0.0001; FF-TAGE, R = −0.26, <0.01; and S-TAGE, R = −0.25, <0.01) on the day of hCG administration. Concentrations of Pent and CML in plasma (P-Pent and P-CML) and skin AGE estimates had no significant correlations with these parameters.

Similarly, FF-Pent, FF-CML, FF-TAGE and S-TAGE had significant negative correlations (Pearson analysis) with number of oocytes retrieved (FF-Pent, R = −0.34, P < 0.0001; FF-CML, R = −0.34, <0.0001; FF-TAGE, R = −0.26, <0.01; and S-TAGE, R = −0.23, <0.01) and number of fertilized oocytes (FF-Pent, R = −0.23, <0.01; FF-CML, R = −0.29, <0.001; FF-TAGE, R = −0.19, <0.05; and S-TAGE, R = −0.25, <0.01), but P-Pent, P-CML or skin AGE estimates did not have significant correlations with either numbers of oocytes retrieved or numbers of oocytes fertilized. Numbers of Day-2 embryos and morphologically superior Day-2 embryos correlated negatively and significantly (Pearson analysis) with FF-Pent (R = −0.22, P < 0.01 and −0.22, <0.01, respectively), FF-CML (R = −0.23, <0.01 and −0.16, <0.05) and S-TAGE (R = −0.21, <0.05 and −0.26, <0.01).

FF-Pent was significantly lower in patients whose IVF/ICSI resulted in ongoing pregnancy than in no pregnancies or spontaneous abortion (P < 0.05, ANOVA). S-TAGE was significantly lower in patients whose IVF/ICSI resulted in ongoing or lost pregnancy than in no pregnancy (P < 0.01, ANOVA; Fig. 1). Higher skin AGE estimates tended to be associated with better IVF/ICSI outcomes (P = 0.06, ANOVA).

Associations of 12 factors (age, BMI, Day-3 FSH, numbers of previous IVF/ICSI attempts, presence of tubal infertility, presence of ovarian dysfunction, P-Pent, P-CML, S-TAGE, FF-Pent, FF-CML and FF-TAGE) with achievement of ongoing pregnancies by IVF/ICSI.
were analyzed by a forward stepwise (Wald) logistic regression analysis. Among these factors, only age, FF-Pent and S-TAGE significantly predicted the achievement of ongoing pregnancy (Table I).

ROC curve analyses were performed concerning age, FF-Pent, S-TAGE and Day-3 FSH for prediction of IVF/ICSI outcome other than ongoing pregnancy, i.e. no pregnancy or clinical abortion (Fig. 2). Areas under the curves (AUC) for age (0.709, \( P = 0.002 \)), FF-Pent (0.686, \( P = 0.006 \)) and S-TAGE (0.667, \( P = 0.02 \)) but not for Day-3 FSH (0.592, \( P = 0.18 \)) were significantly greater than for the reference line (0.5). Cutoff values of 16.25 ng/ml and 7.24 U/ml for FF-Pent and S-TAGE, respectively, corresponding to the point nearest the coordinates on the ROC curve (1 – specificity = 0, sensitivity = 1), were chosen as optimal cutoff values for further analyses.

The numbers of retrieved oocytes (14.6 ± 1.3 versus 8.4 ± 0.9, \( P = 0.0004 \), unpaired t-test), fertilized oocytes (9.0 ± 0.9 versus 4.7 ± 0.5, \( P = 0.0001 \)), Day-2 embryos (8.0 ± 0.8 versus 4.3 ± 0.5, \( P = 0.0004 \)) morphologically superior Day-2 embryos (5.0 ± 0.6 versus 2.2 ± 0.3, \( P = 0.0002 \)), as well as ongoing pregnancy rates per cycle [23% (17/75 cycles) versus 3.4% (2/59 cycles), \( P = 0.0015 \)] were significantly higher in 75 women with S-TAGE below 7.24 U/ml than in 59 women with S-TAGE above 7.24 U/ml. Similarly, the numbers of retrieved oocytes (16.0 ± 1.5 versus 9.1 ± 0.8, \( P < 0.0001 \)), fertilized oocytes (9.0 ± 0.9 versus 5.8 ± 0.5, \( P = 0.002 \)), Day-2 embryos (8.0 ± 0.9 versus 5.1 ± 0.5, \( P = 0.002 \)) and morphologically superior Day-2 embryos (4.9 ± 0.6 versus 2.9 ± 0.3, \( P = 0.003 \)) were significantly higher in 64 women with FF-Pent below 16.25 ng/ml than in 91 women with FF-Pent above 16.25 ng/ml.

**Table I** Results of forward stepwise (Wald) logistic regression analysis on associations of 12 factors with achievement of ongoing pregnancy by IVF/ICSI.

<table>
<thead>
<tr>
<th>Variables (unit)</th>
<th>Significance</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>0.027</td>
<td>0.872</td>
<td>0.772–0.984</td>
</tr>
<tr>
<td>Pentosidine in follicular fluid (ng/ml)</td>
<td>0.027</td>
<td>0.890</td>
<td>0.803–0.987</td>
</tr>
<tr>
<td>Toxic AGEs in serum (U/ml)</td>
<td>0.044</td>
<td>0.740</td>
<td>0.552–0.991</td>
</tr>
</tbody>
</table>

*aAge, BMI, Day-3-FSH, numbers of previous IVF/ICSI attempts, presence/absence of tubal infertility, presence/absence of ovarian dysfunction, concentrations of Pent and CML in plasma, concentrations of TAGE in serum, and concentrations of Pent, CML and TAGE in follicular fluid.*

**Figure 1** Concentrations of pentosidine and Nε-carboxymethyl lysine (CML) in plasma (P-Pent and P-CML), those of toxic advanced glycation end-products in serum (S-TAGE), those of Pent, CML and TAGE in follicular fluid (FF-Pent, FF-CML and FF-TAGE), and skin AGE estimates by AGE-reader were compared among women whose IVF/ICSI resulted in no pregnancy, miscarriage and ongoing pregnancy.

**Figure 2** ROCs curves of age, follicular fluid pentosidine (FF-Pent), serum TAGE (S-TAGE) and Day-3-FSH, and a discriminant score (DS) calculated from these four variables for prediction of IVF/ICSI outcome other than ongoing pregnancy, i.e. no pregnancy or miscarriage.
When S-TAGE was below 7.24 U/ml, number of retrieved oocytes as well as rate of ongoing pregnancy per cycle were high for age < 40 but significantly lower for ages above 40 (Table II). When S-TAGE was above 7.24 U/ml, however, number of retrieved oocytes was significantly decreased early on at ages 35–40 and further at ages above 40. The rate of ongoing pregnancy was compromised even at ages below 35, worsening further for ages above 35. Consequently, the largest differences in number of oocytes retrieved and ongoing pregnancy rate between high and low S-TAGE groups were observed at ages 25 and 35 (Fisher’s PLSD); for age, 0.01; and for Day-3 FSH, 0.01 among three age-classes (ANOVA).

### Table II Numbers of retrieved oocytes and rates of ongoing pregnancy by age, Day-3-FSH levels and serum toxic advanced glycation end-products (S-TAGE) levels.

<table>
<thead>
<tr>
<th>S-TAGE &lt; 7.24 U/ml</th>
<th>S-TAGE ≥ 7.24 U/ml</th>
<th>Probability*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt; 35</td>
<td>17.5 ± 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.1 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>30% (6/20 cycles)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14% (1/7 cycles)</td>
</tr>
<tr>
<td>35 ≤ age &lt; 40</td>
<td>18.0 ± 2.8</td>
<td>10.6 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>38% (10/26 cycles)</td>
<td>4.8% (1/21 cycles)</td>
</tr>
<tr>
<td>Age ≥ 40</td>
<td>9.5 ± 1.5&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5.3 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>3.4%&lt;sup&gt;d&lt;/sup&gt; (1/29 cycles)</td>
<td>0% (0/31 cycles)</td>
</tr>
<tr>
<td>FSH &lt; 10</td>
<td>18.1 ± 1.6&lt;sup&gt;f&lt;/sup&gt;</td>
<td>11.9 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>25% (13/52 cycles)</td>
<td>6.1% (2/33 cycles)</td>
</tr>
<tr>
<td>10 ≤ FSH &lt; 15</td>
<td>9.0 ± 1.7</td>
<td>4.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>29% (4/14 cycles)</td>
<td>0% (0/16 cycles)</td>
</tr>
<tr>
<td>FSH ≥ 15</td>
<td>2.8 ± 0.8</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>0% (0/9 cycles)</td>
<td>0% (0/10 cycles)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data in the same row were compared between high and low S-TAGE by unpaired t-test, χ² test or Fisher’s exact test.

<sup>b</sup>Number of retrieved oocytes per stimulated cycle.

<sup>c</sup>Not significant.

<sup>d</sup>Ongoing pregnancy rate per stimulated cycle (No. of ongoing pregnancies/No. of stimulated cycles).

<sup>e</sup>P < 0.05 versus Age ≤ 35 (Fisher’s PLSD); P < 0.0001 among three age-classes (ANOVA).

<sup>f</sup>P < 0.05 versus Age ≤ 35 and P < 0.01 versus 35 ≤ age < 40 (Fisher’s PLSD); P < 0.01 among three age-classes (ANOVA).

<sup>g</sup>P < 0.01 versus Age ≤ 35 (Fisher’s exact test) and P < 0.01 versus 35 < age < 40 (χ² test).

<sup>h</sup>P < 0.01 versus 10 ≤ FSH < 15 and P < 0.0001 versus FSH ≥ 15 (Fisher’s PLSD); P < 0.0001 among three age-classes (ANOVA).

<sup>i</sup>P < 0.001 versus 10 ≤ FSH < 15 and P < 0.0001 versus FSH ≥ 15 (Fisher’s PLSD); P < 0.0001 among three age-classes (ANOVA).

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When S-TAGE was below 7.24 U/ml, number of retrieved oocytes as well as rate of ongoing pregnancy per cycle were high for age < 40 but significantly lower for ages above 40 (Table II). When S-TAGE was above 7.24 U/ml, however, number of retrieved oocytes was significantly decreased early on at ages 35–40 and further at ages above 40. The rate of ongoing pregnancy was compromised even at ages below 35, worsening further for ages above 35. Consequently, the largest differences in number of oocytes retrieved and ongoing pregnancy rate between high and low S-TAGE groups were observed at ages 35–40. Similarly, the rate of ongoing pregnancy was low irrespective of Day-3 FSH when S-TAGE was higher than 7.24 U/ml, but was high when Day-3 FSH was below 15 U/l with S-TAGE under 7.24 U/ml (Table II).

We then attempted to predict the achievement of ongoing pregnancy by discriminant analysis using four variables: age, FF-Pent, S-TAGE and Day-3 FSH. Canonical discriminant function coefficients of these parameters were determined (age, 0.725; FF-Pent, 0.617; S-TAGE, 0.524; Day-3 FSH, 0.307; Wilks’ lambda, 0.877; P = 0.002). The discriminant score (DS) was defined as the sum of each variable multiplied by its coefficient; when the DS was less than a cutoff value, achievement of ongoing pregnancy could be predicted.

The optimal cutoff value as determined by an ROC curve (AUC = 0.786, P < 0.001, Fig. 2) as described already, was demonstrated to be 46.0. The result of discriminant analysis using this optimal cutoff value is shown in Table III (sensitivity = 0.62 and specificity = 0.90; P < 0.001, χ² test).

Correlations between AGE and physical and laboratory parameters first were screened by Pearson analysis. S-TAGE correlated positively and significantly with leptin (R = 0.51, P < 0.001), BMI (R = 0.35, P < 0.0001), LDL (R = 0.37, P < 0.0001), TC (R = 0.27, P < 0.01), TG (R = 0.31, P < 0.001), FPG (R = 0.25, P < 0.01), PG after 60 min (R = 0.25, P < 0.01), HOMA-R (R = 0.21, P < 0.05), insulin (R = 0.19, P < 0.05) and age (R = 0.19, P < 0.05). Of these 10 parameters, only leptin (standardized coefficient β, 0.51; P < 0.001) correlated significantly with S-TAGE by stepwise multiple regression analysis (S-TAGE = 0.373 × leptin + 5.041; adjusted R<sup>2</sup> = 0.24, P < 0.001). FF-Pent correlated positively and significantly with age (R = 0.27, P < 0.001, Pearson analysis) and Day-3 FSH (R = 0.22, P < 0.01), and correlated negatively with Hb A1c (R = −0.17, P < 0.05). Stepwise multiple regression analysis using these three parameters as independent variables indicated that FF-Pent equaled 0.360 × age − 4.237 × HbA1c + 0.104 × Day-3-FSH + 24.306 (adjusted R<sup>2</sup> = 0.13; P < 0.001; for age, standardized coefficient β was 0.27, P < 0.01; for HbA1c, β was −0.21, P < 0.01; and for Day-3 FSH, β was 0.18, P < 0.05). Partial correlations between S-TAGE or FF-Pent and various parameters
controlled for age were similar to the results of Pearson analyses mentioned above (data not shown).

**Discussion**

This study presents the first direct clinical evidence based on human IVF/ICSI therapy for an important role of AGE accumulation in ovarian dysfunction and diminished fertility. Accumulations of Pent, CML and TAGE in follicular fluid and TAGE in serum correlated negatively and significantly with follicular growth, fertilization and embryonic development. Lower concentrations of Pent in follicular fluid and TAGE in serum were the most significant novel predictors for achievement of ongoing pregnancy, indicating the presence of conventional determinants such as age and Day-3 FSH. Elevation of serum TAGE above 7.24 U/ml appeared to indicate ovarian dysfunction causing diminished fertility, even with young age and normal Day-3 FSH. A DS calculated from four variables—age, follicular fluid Pent, serum TAGE and Day-3 FSH (DS = the sum of each variable multiplied by its coefficient: age, 0.725; FF-Pent, 0.617; S-TAGE, 0.524; or Day-3 FSH, 0.307)—correlated best with achievement of ongoing pregnancy.

Our observations are consistent with previous reports of AGE affecting reproduction. While immunohistochemical localization of AGE such as CML and Nε-carboxyethyl lysine was observed in normal and PCOS ovaries, staining was stronger in the granulosa cell layer and endothelial cells of PCOS ovaries (Diamanti-Kandarakis et al., 2007). Young normoglycemic women with PCOS had higher serum AGE than healthy women (Diamanti-Kandarakis et al., 2005). Positive signals for Pent, ubiquitin and activated caspase 12, as well as nick-end-labeling evidence of apoptosis were detected in human oocytes of primordial, primary and atretic follicles in premenopausal women without chronic diseases; these signals increased with age (Matsumine et al., 2008). Glucose-derived AGE induced secretion of chemokines and apoptosis in human first-trimester trophoblasts in vitro, suggesting that AGE could impair implantation and placental function (Konishi et al., 2004).

Accumulation of AGE in follicles and circulation may simply reflect the severity of AGE-forming states such as insulin resistance, dyslipidemia, oxidative stress and renin–angiotensin system activation (Ulrich and Cerami, 2001; Thomas et al., 2005; Unoki and Yamagishi, 2007), which might directly impair folliculogenesis (Dunaif, 1997; Tatone et al., 2008). More likely, however, AGE could impact folliculogenesis synergistically with these states. Accumulation of AGE in tissues and cells induce macromolecular trapping and cross-linking, causing molecules to malfunction and resist removal by proteolysis (Ulrich and Cerami, 2001; Thomas et al., 2005). Directly and indirectly through RAGE, AGE increases oxidative stress (Thomas et al., 2005), a major cause of macromolecular damage in follicles (Tatone et al., 2008) and other cells (Terman and Brunk, 2004) during aging. Interaction of TAGE with RAGE alters intracellular signaling and gene expression, releases pro-inflammatory molecules and produces oxidative stress, all of which contribute to diabetic vascular complications (Takeuchi and Yamagishi, 2009), which may be mirrored in the follicular vasculature.

Diabetic patients have higher S-TAGE than healthy subjects, while S-TAGE is related to cumulative hyperglycemic burden (Nakamura et al., 2007; Takeuchi and Yamagishi, 2009). Even in a non-diabetic general population, S-TAGE was related positively to FPG and LDL (Yamagishi et al., 2009). In our study, S-TAGE correlated positively with leptin, BMI, LDL, TC, TG, FPG, PG at 60 min, HOMA-R, insulin and age, all of which are related to obesity, dyslipidemia, hyperglycemia and insulin resistance. Of 10 parameters, leptin was the most significant determinant of S-TAGE, probably reflecting the induction of leptin resistance by hyperleptinemia associated with obesity. This exacerbates obesity and insulin resistance, leading to a vicious cycle of escalating metabolic derangement (Zhang and Scarpace, 2006).

Our study showed that women with S-TAGE of at least 7.24 U/ml had fewer retrieved oocytes and lower rates of ongoing pregnancy, even when they were younger than 40 years or had Day-3 FSH below 10 IU/l. This observation supports two important points. First, S-TAGE measurement may facilitate early detection of diminished female fertility when Day-3 FSH is not yet elevated, allowing successful treatment. Elevation of serum Day-3 FSH, which occurs when remaining primordial follicles have decreased to the extent seen in perimenopausal women, is an advanced sign of severely diminished ovarian reserve limiting success of treatment (Scott and Hofmann, 1995). Second, elevated TAGE appears to decrease follicular reserve or functionally suppress folliculogenesis.

Results of this study suggest a novel treatment strategy for ovarian dysfunction by decreasing AGE or AGE effects. Benfotiamine, a lipid-soluble thiamine derivative, is thought to inhibit AGE formation by multiple mechanisms including interactions with post-Amadori precursors of AGE, reduction of triose-phosphate generation in hyperglycemia by acting as a co-enzyme in mitochondrial energy production from carbohydrate metabolism and regulation of glucose metabolism (Thomas et al., 2005). Serum AGE concentrations and tissue AGE accumulation in the retina and kidney have been decreased by benfotiamine (Babaie-Jadidi et al., 2003; Hammes et al., 2003). We performed a pilot treatment with benfotiamine, 75 mg per day, for 3 months for seven non-pregnant patients and repeated assisted reproductive technology (ART) and AGE measurements (unpublished data). Concentrations of CML (from 4.1 ± 0.3 to 3.0 ± 0.2 μg/ml) and TAGE (from 3.1 ± 0.03 to 2.0 ± 0.27 U/ml) in follicular fluid were decreased significantly by benfotiamine, although significant effects on ART outcomes were not concluded because of a limited number of subjects. A larger prospective randomized study remains to be performed.

Other agents (such as pyridoxamine, carnosine, alpha-lipoic acid, ACE inhibitors, angiotensin receptor antagonists, metal chelators, metformin and peroxisome proliferator receptor agonists) are also potential inhibitors of AGE accumulation (Thomas et al., 2005). Interruption of adverse effects of AGE by soluble RAGE (Koyama et al., 2007) or a RAGE antagonist may be yet another approach applicable to the treatment of ovarian dysfunction.

**Authors’ roles**

M.J., MD, played a major role in formulation of study design, acquisition of data, interpretation of data, writing the manuscript and revising it critically and final approval of the version to be published. M.T., PhD, was involved in acquisition of TAGE data by measuring TAGE, (ii) writing a part of TAGE measurement and (iii) final approval of the version to be published. A.W. acquired clinical ART data, wrote a part of clinical ART procedures and was involved in final approval of the version to be published. K.T., MD, played a role.
in analysis and interpretation of data, wrote a part of data analysis and was involved in final approval of the version to be published. J.H., N.E. acquired the ART embryonic data, wrote a part of embryonic assessments and was involved in final approval of the version to be published. A.M. obtained the Pent and CML data by measuring them, wrote a part of Pent and CML measurements and was involved in final approval of the version to be published.

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