Accumulation of advanced glycation end products in the rabbit blastocyst under maternal diabetes

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Abstract

Diabetes mellitus (DM) during pregnancy is one of the leading causes of perinatal morbidity and birth defects. The mechanism by which maternal hyperglycemia, the major teratogenic factor, induces embryonic malformations remains unclear. Advanced glycation end products (AGEs) are known to accumulate during the course of DM and contribute to the development of diabetic complications. Employing a diabetic rabbit model, we investigated the influence of maternal hyperglycemia during the preimplantation period on AGE formation (pentosidine, argpyrimidine, and N^ε-carboxymethyllysine (CML)) in the reproductive tract and the embryo itself. As a consequence of type 1 DM, the AGE levels in blood plasma increased up to 50%, correlating closely with an AGE accumulation in the endometrium of diabetic females. Embryos from diabetic mothers had increased protein-bound CML levels and showed enhanced fluorescent signals for AGE-specific fluorescence in the blastocyst cavity fluid (BCF). The quantification of CML by HPLC–mass spectrometry (MS/MS) showed a higher amount of soluble CML in the BCF of blastocysts from diabetic rabbits ($0.26 \pm 0.05 \mu\text{mol/l}$) compared with controls ($0.18 \pm 0.02 \mu\text{mol/l}$). The high amount of AGEs in blastocysts from diabetic mothers correlates positively with an increased *AGER* (receptor for AGE (*RAGE*)) mRNA expression. Our study gives alarming insights into the consequences of poorly controlled maternal diabetes for AGE formation in the embryo. Maternal hyperglycemia during the preimplantation period is correlated with an increase in AGE formation in the uterine environment and the embryo itself. This may influence the development of the embryo through increased AGE-mediated cellular stress by RAGEs.

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Introduction

Approximately 7% of pregnancies are complicated due to diabetes mellitus (DM; [American Diabetes Association 2013](#)). Increasing obesity rates are a serious risk factor for type 2 DM and gestational DM ([American Diabetes Association 2013](#)). DM during pregnancy is of great concern as it is a major cause of perinatal morbidity and mortality ([Combs & Kitzmiller 1991](#), [Greene 1999](#)). Although our understanding and management of DM have improved during the last decades, diabetic pregnancies are still reported to have numerous adverse effects ([Combs & Kitzmiller 1991](#), [Aberg *et al.* 2001](#), [Eriksson *et al.* 2003](#), [Corrigan *et al.* 2009](#)). Hyperglycemia is considered as a major teratogenic factor for congenital malformation, although other associated factors such as ketone bodies, branched amino acids, and triglycerides have also been shown to exert adverse effects on the developing embryo ([Eriksson *et al.* 2000](#)). However, it is not yet clear in which way maternal hyperglycemia affects prenatal embryo development.

There is upcoming evidence that advanced glycation end products (AGEs) might play a critical role in diabetic pregnancies.

AGEs are a complex group of compounds formed via non-enzymatic reactions between reducing sugars and N-terminal amino groups on proteins, lipids, and nucleic acids. End-stage products of the protein glycation can be divided into fluorescent AGEs (such as argpyrimidine), non-fluorescent AGEs (such as N^ε-carboxymethyllysine (CML)), and cross-linking compounds (such as pentosidine). Owing to the intrinsic fluorescence of some AGEs, plasma and tissue fluorescence can be used as markers for AGE accumulation ([Goh & Cooper 2008](#), [Bos *et al.* 2011](#)). Formation and accumulation of AGEs are related to aging as well as to prolonged hyperglycemia and oxidative stress resulting from DM ([Sell *et al.* 1991](#), [Lee & Cerami 1992](#), [Dyer *et al.* 1993](#), [Brownlee 1995](#)).

AGEs are identified to play a role in the development of diabetic complications such as diabetic nephropathy, cardiomyopathy, atherosclerotic disease, peripheral neuropathy, and ocular disease ([Ahmed & Thornalley 2007](#),

Nass *et al.* 2007). The post-translational modification of proteins by reducing sugars alters their biological structure and function and leads not only to a loss of molecular function but also to a reduced degradation of these damaged proteins. An additional proposed mechanism of AGE-induced damage is the release of reactive oxygen species, particularly superoxide and hydrogen peroxide by AGEs (Carubelli *et al.* 1995, Ortwerth *et al.* 1998). AGEs are able to activate intracellular cascades by binding specific receptors, for example, the receptor for AGEs (RAGEs). AGE–RAGE interactions induce a broad spectrum of signaling mechanisms such as p21ras, Erk1/2 MAP kinases (MAPKs), p38 and SAPK/JNK MAPKs, Rho GTPases, PI3K, and the JAK/STAT pathway (Bierhaus *et al.* 2005, Rouhiainen *et al.* 2013). Downstream consequence is the activation of nuclear factor κ B (NF κ B) that results in the release of pro-inflammatory mediators such as free radicals and cytokines (Berbaum *et al.* 2008). AGER (RAGE) has been shown to be expressed in the rabbit blastocyst, at mRNA and protein levels (Ott *et al.* 2014).

Women with gestational DM have significantly higher serum AGE levels compared with healthy controls, whereas women with types 1 and 2 DM, in good medical supervision, show normal AGE levels (Buongiorno *et al.* 1997). There is a strong relationship between mothers and neonates regarding AGEs. In women with gestational DM, high levels of AGEs and advanced oxidation protein products (AOPPs) are also detectable in the umbilical blood of their neonates. Both, diabetic mother and neonate, showed higher AGE and AOPP levels compared with healthy controls (Boutzios *et al.* 2013). Elevated AGE levels in women with gestational DM are associated with pregnancy complications such as birth asphyxia, congenital malformations, or stillbirth (Guosheng *et al.* 2009). The harmful effects of AGEs after implantation and placentation are likely to threaten the embryo/fetus too, as maternal hyperglycemic blood is connected to the blood system of the embryo. However, this study demonstrates that the preimplantation period is also of great importance on AGE formation, especially in mothers with poorly controlled preexisting DM.

The preimplantation period is a critical ontogenetic stage in embryo development and highly vulnerable for teratogenesis. At this period, the embryo is most sensitive to its surrounding milieu, especially to deregulations by external stimuli (Watkins *et al.* 2008). In this study, we investigated the influence of a poorly controlled maternal type 1 DM on AGE formation in preimplantation embryos and in the reproductive tract employing a rabbit model. As the rabbit blastocyst implants at day 6.8 post coitum (p.c.), we recovered the blastocysts before, i.e. at day 6.0 p.c. At this time, the rabbit blastocyst is covered by an extraembryonic mucin layer (Fischer *et al.* 1991, Herrler *et al.* 2002). However, this layer does not interfere with glucose uptake and metabolism *in vivo* and *in vitro* (Fischer *et al.* 2010, Ramin *et al.* 2010,

Schindler *et al.* 2013). We show that DM not only is critical for maternal metabolism but also affects the AGE accumulation and AGER mRNA levels in the developing embryo even before implantation.

Materials and methods

Alloxan treatment and allocation of samples

All animal experiments were performed in accordance with the principles of laboratory animal care and the experimental protocol was approved by the Local Ethical Commission of the 'Landesverwaltungsamt Dessau' (reference number: 42502-2-812).

Experimental type 1 DM was induced by alloxan (Sigma–Aldrich) treatment as described previously (Ramin *et al.* 2010). Rabbits were allowed to eat *ad libitum*. The blood glucose level of females with type 1 DM increased 1 day after alloxan administration and was kept in a range between 20 and 30 mmol/l by insulin supplementation (Huminsulin, basal (NPI), Lilly, Gießen, Germany; three times per day). The duration for the poorly controlled type 1 DM was ~10 days before mating and during the 6 days of pregnancy. On average, diabetic rabbits had a 4.5-fold higher blood glucose concentration in comparison to the normoglycemic reference group (27.6 ± 0.5 and 6.2 ± 0.1 mmol/l, $P < 0.001$; Fig. 1).

Follicle growth was stimulated by s.c. injection of 110 IU pregnant mare's serum gonadotropin (Intervet, Unterschleißheim, Germany) and ovulation was ensured by i.v. injection of 75 IU human chorionic gonadotropin (Intervet) after mating with fertile males. Samples from diabetic and normoglycemic rabbits were obtained 6 days after mating (p.c.). Rabbits were killed by an overdose of pentobarbital (Sigma–Aldrich) and exsanguination. Later, we obtained maternal blood, maternal tissues, and the blastocysts. The blastocysts were flushed out of the uteri and washed three times with PBS to avoid contamination of blastocyst samples with uterine tissue.

Protein preparation of blastocysts and AGE detection by slot blot analyses

For protein extraction, a group of eight to ten blastocysts from at least three mothers were randomly pooled, dissolved in RIPA

Figure 1 (A) Blood glucose levels after alloxan injection (day 0) in rabbits. The blood glucose level was monitored using commercially available blood glucose test strips three times a day. Data are shown as an average of nine animals with three measured data each per day (mean $\pm$ S.E.M., *** $P < 0.001$).

lysis buffer (PBS, 1% vol/vol NP-40, 0.5% wt/vol sodium deoxycholate, and 0.1% wt/vol SDS), and homogenized with a syringe (Omnifix 40 Duo, Braun, Melsungen, Germany). After incubation on ice for 30 min, the samples were centrifuged at 13 000 *g* for 20 min. The supernatant was stored at -80°C until use for slot blot analysis.

Slot blot analyses were performed with 25 μg protein. Denatured protein samples (heated for 10 min at 80°C) were spotted onto a nylon membrane (GE Healthcare, München, Germany) using a slot blot apparatus (Biostep, Rabenau, Germany). The protein load was determined by Ponceau S staining. After blocking with 5% milk/TBS-T for 1 h, the membrane was incubated with monoclonal mouse antibodies against CML, argpyrimidine, or pentosidine (1:100, Biologo, Kassel, Germany), respectively, overnight at 4°C . Samples were rinsed three times with TBS-T for 5 min and subsequently incubated with a secondary goat anti-mouse IgG for 1 h (Dianova, Hamburg, Germany). The immunoreactive signal was visualized by ECL detection (Millipore, Schwalbach, Germany) and quantified by Fusion FX7 and the corresponding software Fusion 15.18. Protein modification rate was calculated as the ratio of protein load (Ponceau) and slot intensity by antibody reaction.

Protein preparation of maternal tissues and AGE detection by slot blot analyses

After flushing out the blastocysts, we dissected the uterine tissue. Protein isolation was carried out either with the entire uteri or with separated endometria. For the latter, the endometrium was scraped mechanically from the myometrium using sharp scalpels. Grinded uteri and scraped endometria were mixed with RIPA and homogenized with precellys (Peglab, Erlangen, Germany). Slot blot analyses were performed in the same way as for blastocysts. AGE accumulation in blood was determined using EDTA-plasma. Slot blot analysis with uterine proteins was performed in the same way as for blastocysts.

Immunohistochemical localization of AGEs in blastocysts

The immunohistochemical analysis was performed with single blastocysts as described previously (Schindler *et al.* 2014). Embryonic discs were incubated with mouse MABs against CML, argpyrimidine, and pentosidine (1:100, Biologo). The nuclei were counterstained with hematoxylin. Embryonic discs were assessed using a light microscope (BZ 8100E, Keyence, Neu-Isenburg, Germany). The specificity of immunostaining of the secondary antibody reaction was proven by the absence of signals in sections processed after omission of the primary antibody.

Immunohistochemical localization of AGEs in ovary and uterus

Ovarian and uterine tissues were fixed in Bouin's solution, embedded in paraffin, and sectioned at $5\text{ }\mu\text{m}$. Sections were mounted on silanized slides and deparaffinized at 60°C overnight. Later, sections were rehydrated through a series of graded alcohols. Endogenous peroxidases were inhibited by

incubating the slides with 3% H_2O_2 in methanol. After blocking in 10% goat serum for at least 1 h, the sections were incubated with the mouse MABs against CML, argpyrimidine, or pentosidine (1:100, Biologo) at 4°C overnight. Samples were washed with TBS-T and incubated with the HRP-conjugated secondary goat anti-mouse IgG (Dako, Hamburg, Germany). The AGE-modified proteins were visualized by the peroxidase–diaminobenzidine reaction. The nuclei were counterstained with hematoxylin. Analysis was performed as described for blastocysts.

Quantification of AGER mRNA in the rabbit blastocyst

mRNA of single blastocysts was extracted using Dynabeads Oligo (dT) 25 (Invitrogen) and subsequently used for cDNA synthesis. The nucleotide sequence for rabbit AGER was determined using human primers for amplification of rabbit lung cDNA. The obtained rabbit AGER primers are as follows: sense, GCTACTGC-TCCACCTTCTGG and antisense, GCAGTCAGAGCTGATGGTGA (ref. LOC100343142). The amount of AGER transcripts was determined by real-time quantitative PCR (RT-qPCR) using the Applied Biosystems StepOnePlus System (Applied Biosystems). The entire protocol for mRNA quantification and RT-qPCR has been described previously (Schindler *et al.* 2013).

AGE fluorescence in the blastocyst cavity fluid

To obtain the blastocyst cavity fluid (BCF), single blastocysts were washed twice with ice-cold PBS and placed on a Petri dish. The remaining PBS was removed and the blastocyst was punctured using a syringe. The escaping BCF was taken in an Eppendorf tube and stored at -80°C . The AGE fluorescence was determined in BCF using a Synergy MX 200 microplate reader (BioTek, Bad Friedrichshall, Germany). Then, 3 μl of the undiluted BCF were analyzed in a black Take 3 Micro-Volume Plate (BioTek). PBS was used as a control. Fluorescence emission spectra were recorded at excitation wavelengths of 330 and 360 nm. The maximum emission for the tested excitation wavelengths were found at 405 and 440 nm respectively.

CML quantification in the BCF by HPLC/mass spectrometry

CML was synthesized according to the literature (Glomb & Monnier 1995). The identity of the reference compound was verified by nuclear magnetic resonance experiments. Furthermore, the elemental composition was confirmed by accurate mass determination. Up to seven BCF of at least two animals were pooled. The pooled BCF was diluted 1:10 with ultrapure water. The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a pump (PU-2080 Plus) with a degasser (LG-2080-02) and a quaternary gradient mixer (LG-2080-04), a column oven (Jasco Jetstream II), and an Autosampler (AS-2057 Plus). Chromatographic separations were performed on a stainless steel column packed with RP-18 material (VYDAC CRT, no. 218TP54, $250 \times 4.6\text{ mm}$, RP 18, $5\text{ }\mu\text{m}$, Hesperia, CA, USA) using a flow rate of 1.0 ml/min. The mobile phase used was water (solvent A) and methanol/water (7:3 (v/v), solvent B). To both solvents (A and B), 1.2 ml/l heptafluorobutyric acid was added. Analysis was

performed at 35 °C column temperature using isocratic elution at 98% of A/2% of solvent B. Mass spectrometric detection was conducted on a API 4000 Q Trap LC/mass spectrometry (MS/MS) system (AB Sciex, Darmstadt, Germany) equipped with a turbo ionspray source using electrospray ionization in the positive mode: sprayer capillary voltage, 2.5 kV; nebulizing gas flow, 50 ml/min; heating gas, 60 ml/min at 550 °C; and curtain gas, 40 ml/min. The multiple reaction monitoring mode was used, utilizing collision-induced dissociation of the protonated molecule with compound-specific orifice potential (50 V) and fragment-specific collision energies (CEs). CML (m/z 205.1 → 130.2 (CE 17), 84.1 (CE 46), and 159.1 (CE 15)) was detected at the retention time of t_R = 6.0 min.

Quantification was performed by the standard addition method. Briefly, increasing concentrations of an authentic reference compound by factors of 0.5, 1, 2, and 3× the concentration of the analyte in the sample were added to separate aliquots of the sample after workup procedure. The aliquots were analyzed, and a regression of response vs concentration was used to determine the concentration of the analyte in the sample. Calibration using this method resolves potential matrix interferences.

All samples were analyzed in a single batch to exclude inter-assay variations. Intra-assay coefficient of variation values (CV=9%) were determined by repeated analyses of a BCF sample ($n=5$). In addition, the limit of detection (LOD=4.8 pmol/ml) and the limit of quantification (LOQ=14.5 pmol/ml) with all steps of the analysis included were estimated according to the German standard method DIN 32645: 2008-11 ($n=5$, confidence level $P=0.95$, and $k=3$).

Determination of glyoxal and methylglyoxal in plasma and blastocysts

The quantification of glyoxal (GO) and methylglyoxal (MGO) was achieved mainly as described by Espinosa-Mansilla *et al.* (2007). Standard curves of GO and MGO (40% aqueous solution, Sigma-Aldrich) were obtained by preparing serial dilution of GO and MGO (2700, 900, 300, 100, and 0 nM) in HPLC-grade water (Millipore). The rabbit plasma proteins and cell lysate proteins of the embryos were precipitated using trifluoroacetic acid (1/10 of the sample volume). After 10 min of incubation on ice, the samples were centrifuged at 13 000 *g* for 10 min. To 100 µl of the supernatant, HPLC-grade water was added (final volume, 1 ml) followed by the addition of 0.125 ml ammonium chloride (0.5 M; pH 10.0) and 2.5 ml of 5,6-diamino-2,4-hydroxypyrimidine sulfate (0.75 mM). The mixture was incubated for 90 min at 60 °C with constant shaking. Subsequently, citrate buffer (10 mM; pH 6.0) was added to a final volume of 25 ml. For the analysis, 10 µl of the samples were injected (ZORBAX, Eclipse XDB-C18 4.6×150 mm 5-micron column; Agilent, Oberhaching, Germany) and then separated by gradient elution with solvents A (3% acetonitrile) and B (97% citrate buffer) at a flow rate of 0.8 ml/min.

Statistical analysis

To obtain statistically funded data, we repeated the animal experiment at least three times ($n=3$). In each animal

experiment, we had nine diabetic and six healthy rabbits. We pooled tissue samples and blastocysts from each individual experiment from at least three animals. Levels of significance between groups were calculated using Student's *t*-test after proving normal distribution (SigmaPlot v. 11.0). The Mann-Whitney *U* rank sum test was used when a normal distribution was not guaranteed. Data are expressed as mean ± S.E.M. The levels of statistical significance were * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

Results

Determination of α -dicarbonyls and AGEs in the plasma of female rabbits with type 1 DM

As the majority of AGEs *in vivo* appear to be formed from α -dicarbonyls (Brownlee 1995, Rabbani & Thornalley 2012), we measured the plasma level of GO and MGO by HPLC. The AGE precursor MGO was not altered, whereas GO (control 398 ± 31 nM and diabetic 522 ± 59 nM) was tendentially enhanced (Fig. 2A). To determine the AGE status of the diabetic rabbits, we investigated various AGEs by slot blot analysis with specific antibodies against pentosidine, CML, and argpyrimidine respectively. All of the analyzed protein modifications showed a considerable increase in the plasma probes of diabetic rabbits (Fig. 2B and C).

Determination of AGE modifications in the reproductive tract of female rabbits with type 1 DM

The constitution of the uterus tissue is crucial for the course of pregnancy. The endometrium, a dynamic mucosa adjacent to the myometrium of the uterus, is important for the implantation process. Argpyrimidine,

Figure 2 (A) Concentration of glyoxal (GO) in diabetic and non-diabetic rabbits. GO was quantified by HPLC (mean ± S.E.M.; $N=3$, $n=3$; P =as indicated). (B) Relative amount of protein-bound pentosidine, N^{ϵ} -carboxymethyllysine (CML), and argpyrimidine in the plasma of rabbits after being diabetic for ~2 weeks compared with the non-diabetic control (set 100%). The quantification was performed by slot blot analysis (mean ± S.E.M.; $N=3$, $n=9$; * $P<0.05$ and ** $P<0.01$). The AGE amount is related to the protein load (Ponceau S staining). (C) A representative slot blot is shown for pentosidine.

Figure 3 (A) Immunohistochemical analysis of argpyrimidine, N^{ϵ} -carboxymethyllysine (CML), and pentosidine in the pregnant uterus of rabbits with type 1 diabetes mellitus and in healthy controls. The AGE-modified proteins were visualized by the peroxidase–diaminobenzidine reaction (brown color). The nucleus was counterstained with hematoxylin (blue colour). The negative control is the control reaction of the HRP-conjugated secondary goat anti-mouse IgG without a primary antibody. CML and argpyrimidine show an exclusive cytosolic staining, whereas pentosidine is localized to both the cytosol and the nucleus. The epithelial endometrium (E) of diabetic rabbits shows a stronger staining, whereas the staining of the myometrium (M) seems to be unchanged. (B) Relative amounts of protein-bound argpyrimidine, CML, and pentosidine in the entire uterus and the separated endometrium from diabetic and healthy rabbits measured by slot blot analysis (mean $\pm$ s.e.m.; $N=3$, $n=9$). The amounts of AGE are related to the protein load (Ponceau S staining).

CML, and pentosidine are present in the endometrium and myometrium. AGEs are localized to smooth muscle cells, not only in the myometrium but also in the endothelium of vessels. In the endometrium, CML and pentosidine are exclusively present in the epithelium; argpyrimidine is also slightly present in the stroma. CML and argpyrimidine were mainly localized to the cytoplasm. Pentosidine showed a cytosolic staining and stained nuclei. CML, pentosidine, and argpyrimidine showed a strongly stained endometrial epithelium in diabetic rabbits (Fig. 3A).

Slot blot analyses revealed no differences in protein-bound CML, pentosidine, and argpyrimidine between diabetic and normoglycemic rabbits for the entire uterus and for the endometrium in particular (Fig. 3B).

Immunohistochemical staining of the ovary revealed no differences between normoglycemic and diabetic females. Figure 4 shows the AGE distribution in the ovary from normoglycemic controls. All determined AGEs were detectable in the ovary. CML and argpyrimidine showed an exclusive cytosolic staining, whereas pentosidine was localized to both the cytoplasm and nuclei. The oocyte showed positive staining for CML, pentosidine, and argpyrimidine in the cytoplasm, but not in the nucleus in the investigated follicle stages (primary, secondary, and tertiary follicle).

Determination of AGE modifications in 6-day-old rabbit blastocysts developed under diabetic conditions

Immunohistochemical detection of pentosidine, CML, and argpyrimidine showed strong staining in the embryoblast (EB) and well-stained trophoblast (TB) cells (Fig. 5A). All detected AGEs were exclusively present in the cytoplasm. Slot blot analysis revealed a significantly higher level of protein-bound CML in blastocysts from diabetic mothers (Fig. 5B). Similarly, argpyrimidine level was tendentially increased. The detection of the reactive α -dicarbonyls MGO showed no differences between blastocysts from diabetic and normoglycemic rabbits (Fig. 5C). GO was under the detection limit.

The BCF is known to be an important reservoir for nutrients during preimplantation. We used the fluorescent properties of AGEs to determine AGE accumulation in the BCF. Specific peaks for the known AGE fluorescence with excitation and emission at wavelengths of 330/405 and 360/440 nm, respectively, were detectable. Both were significantly increased in the BCF of blastocysts developed under diabetic conditions (Fig. 6A). The protein content of the BCF was equal in both groups with 0.43 ± 0.012 $\mu\text{g}/\mu\text{l}$. Besides fluorescent AGEs, the non-fluorescent CML as a free adduct was identified by HPLC/MS in the BCF. The quantification of

Figure 4 Immunohistochemical analysis of N^{ϵ} -carboxymethyllysine (A), pentosidine (B), and argpyrimidine (C) in the ovary of healthy rabbits. Follicle growth was stimulated by s.c. injection of pregnant mare's serum gonadotropin. The AGE-modified proteins were visualized by the peroxidase–diaminobenzidine reaction (brown color). The nuclei were counterstained with hematoxylin (blue color). The negative control is the control reaction of the HRP-conjugated secondary goat anti-mouse IgG without a primary antibody. CML and argpyrimidine show an exclusive cytosolic staining, whereas pentosidine was localized to both the cytoplasm and the nuclei, except for nuclei of the oocytes*.

CML showed a higher amount of soluble CML in the BCF of blastocysts from diabetic mothers with $0.26 \pm 0.05 \mu\text{mol/l}$ compared with controls with $0.18 \pm 0.02 \mu\text{mol/l}$ (Fig. 6B).

AGER mRNA amount in rabbit blastocysts

AGER mRNA was detectable from the early blastocyst stage (day 4 p.c.) onwards (Ott *et al.* 2014). At day 6 p.c., the amount of AGER mRNA was significantly increased under diabetic conditions (Fig. 7).

Discussion

An intrauterine exposure to hyperglycemia has been shown to cause alterations in pre- and postnatal growth patterns. The underlying metabolic disorder may predispose offspring to develop metabolic diseases in later life (Silveira *et al.* 2007). Several animal studies demonstrated a developmental delay for embryos recovered from diabetic mothers (Giavini *et al.* 1986, Moley *et al.* 1991, Ramin *et al.* 2010). Our study provides new insights into the effects of a maternal DM during the preimplantation period by analyzing the AGE formation in both, the mother and the developing embryo. Thus far, AGE formation is mostly associated with aging and diseases. By contrast, Ling *et al.* (2001) had already reported AGE modifications in fetal rats from day 10 p.c. onwards. A recently published study has revealed a high rate of glycosylated and oxidized proteins in undifferentiated mouse embryonic stem cells (ESCs) and in mouse blastocysts at day 3.5 p.c. HSPA8 (HSC70) was identified as the major protein modified by CML in undifferentiated mouse ESCs (Hernebring *et al.* 2006). Similar to the

findings of our study on day 6 rabbit blastocysts, AGE-modified proteins were mainly present in the EB, and strongly stained cells were also found in the TB (Fig. 5A). The protein modifications by pentosidine, CML, and argpyrimidine were almost exclusively observed in the cytoplasm.

It is known that AGEs accumulate intracellularly (Goldin *et al.* 2006). Besides the physiological appearance of AGEs in preimplantation embryos, we observed pathological AGE accumulations in blastocysts from diabetic mothers. Blastocysts are obviously susceptible for AGE formation while growing up in a diabetic uterine milieu. A 6-day-old rabbit blastocyst developed in a diabetic uterine milieu showed tendentially more AGEs intracellularly and in the BCF compared with age- and stage-matched control blastocysts (Figs 5 and 6). Protein-bound CML and argpyrimidine levels were elevated, whereas protein-bound pentosidine was unchanged. CML and argpyrimidine are AGEs resulting from reactions of α -dicarbonyls (GO and MGO) and amino groups that are known to be highly reactive. Although glucose and fructose are present in significant concentrations in uterine secretions, the fact that they react slowly with proteins to form AGEs must be taken into consideration. Pentosidine is sourced mostly from ribose but also from glucose (Dyer *et al.* 1991, Grandhee & Monnier 1991). Previous investigations had demonstrated an unchanged glucose uptake in rabbit blastocysts developed under normoglycemic and diabetic conditions (Schindler *et al.* 2013). These findings might explain the unchanged protein-bound pentosidine concentration. The intracellular glucose concentration has not been determined so far. We found measurable, but unaltered, concentrations of MGO in blastocysts

Figure 5 (A) Immunohistochemical analysis of argpyrimidine, N^ε-carboxymethyllysine (CML), and pentosidine in embryoblast (EB) and trophoblast (TB) cells of 6-day-old rabbit blastocysts. The AGE-modified proteins were visualized by the peroxidase–diaminobenzidine reaction (brown color). The used antibodies show an exclusive cytosolic staining. The EB has tendentially more AGE modifications than the TB. The nucleus was counterstained with hematoxylin (blue colour). The negative control is the control reaction of the HRP-conjugated secondary goat anti-mouse IgG without a primary antibody. (B) Relative amounts of protein-bound argpyrimidine, CML, and pentosidine in 6-day-old rabbit blastocysts from diabetic and healthy (control) rabbits analyzed by slot blot analysis (mean $\pm$ s.e.m.; $N=6$, $n=8-10$; $*P<0.05$). The amount of AGE is related to the protein load (Ponceau S staining). (C) Methylglyoxal quantification in 6-day-old rabbit blastocyst by HPLC (mean $\pm$ s.e.m.; $N=3$, $n=8-10$).

from diabetic and normoglycemic rabbits (Fig. 5C). The quantification of GO in blastocysts failed at the detection limit of 300 nM. Recent estimates of the cellular concentrations are 1–5 μ M for MGO and 0.1–1 μ M for GO (Dobler *et al.* 2006). Formation of MGO arises from a decreased activity of the reductive pentose phosphate pathway and formation of GO arises from lipid peroxidation (Fu *et al.* 1996, Januszewski *et al.* 2003, Thornalley & Rabbani 2009). We have new evidence that the lipid metabolism is altered in the rabbit blastocyst in case of a maternal DM. Besides noticeable lipid accumulations, the transport protein for fatty acids and other lipophilic substances, fatty acid-binding protein, is upregulated (Schindler *et al.* 2014). The two pathways – altered fatty acid metabolism and AGE formation – may interact and contribute to specific aspects of maternal subfertility in DM. In the rabbit model, maternal DM is associated with a reduced ovulation rate and lower number of blastocysts. Blastocyst development is retarded (Ramin *et al.* 2010). A recently published study has demonstrated that in ART patients an increased AGE concentration in human follicular fluid is associated with diminished follicle growth, a lower fertilization rate, and delayed embryo development (Jinno *et al.* 2011).

A major reason for AGE-mediated damage is the activation of RAGEs (Bierhaus *et al.* 2005, Nass *et al.* 2007, Berbaum *et al.* 2008, Rouhiainen *et al.* 2013). RAGE–ligand interaction leads to an increase in the expression of AGER. This type of positive feedback loop results in prolonged NF κ B activation (Bierhaus & Nawroth 2009, Fritz 2011). In our study, we found a similar positive correlation between AGE accumulation and an increased *AGER* mRNA expression in blastocysts from diabetic mothers, confirming the view that AGE–RAGE interaction leads to an upregulation of RAGEs. This finding could be a proof for an active AGE–RAGE system in the blastocyst, which may negatively affect the embryo quality in diabetic mothers.

From which source the observed AGEs in the early embryo originate is uncertain. As AGE formation, especially due to glucose reactions, is an exceedingly

Figure 6 (A) Relative AGE fluorescence in the blastocyst cavity fluid (BCF) of blastocysts from diabetic and control rabbits (mean $\pm$ s.e.m., $N=3$, n as indicated; $*P<0.05$ and $***P<0.001$). (B) The free AGE adduct N^ε-carboxymethyllysine (CML) in the BCF was quantified by HPLC/mass spectrometry ($N=4$, $n=15-18$).

Figure 7 RAGE mRNA amount in 6-day-old blastocysts from diabetic and control rabbits. RAGE mRNA was related to the amount of GAPDH mRNA molecules per blastocyst (mean $\pm$ S.E.M.; $N=6$, n =as indicated, $^{**}P<0.01$).

slow process and the degradation of AGEs *in vivo* is negligible, it can be assumed that AGEs may descent from germ cells. Matsumine *et al.* (2008) have demonstrated increased levels of pentosidine in the primordial, primary, and atretic follicles in premenopausal women. The reduced fertility and reduction in the follicle quality during aging are supposed to be, besides other reasons, due to AGE accumulation (Tatone & Amicarelli 2013). Our rabbit model confirms the presence of AGEs in oocytes (Fig. 4). As the experimentally induced type 1 DM had no effect on the AGE concentration in oocytes, it is unlikely that the accumulated AGEs in diabetic blastocysts are of oocyte origin in our model. However, it is likely that AGEs found in the oocyte might be still present in the developing embryo. This view may be relevant to women of older age, as AGE accumulation takes place for a longer term in this case, and to women with a long-term poorly controlled DM.

The increased CML concentration in the diabetic blastocyst (Fig. 5B) was additionally reflected by increased CML concentrations in the BCF (Fig. 6B). Furthermore, diabetic mothers showed increased amounts of CML in the plasma (Fig. 2B) and, through immunohistochemical detection, in the endometrium (Fig. 3A). The disparity between the results of immunohistochemistry and slot blot analysis in uterine tissue (Fig. 3) may be caused by the broad spectrum of AGE modification in tissues. The used antibodies are able to detect, besides protein-bound modifications, free AGEs. It is known that the non-enzymatic reaction between reducing sugars and amino groups also affects lipids and nucleic acids. The immunohistochemical method might capture more AGE modifications than slot blot analysis that detects only protein-bound AGEs. As CML level is increases in both, mother and blastocyst, it is possible that the maternally formed CML are transferred to the embryo. Little is known about the transport mechanism of AGEs. Experiments with Caco-2 cells showed a low transepithelial flux of CML. However, there was no measurable

active transport for CML across the epithelial monolayer, neither via PEPT1 (SLC15A1) nor by carriers for neutral amino acids. The observation led to the conclusion that the transport is based on simple diffusion (Grunwald *et al.* 2006). Further studies are necessary to clarify this hypothesis.

For the first time, we demonstrate that one consequence of maternal DM is AGE formation in preimplantation embryos. AGEs do accumulate in blastocysts if the maternal DM is poorly controlled. Although our results do not provide a causative mechanism between embryo toxicity and DM, it is likely that AGEs play a role as stimuli for activating intracellular stress pathways and, additionally, do affect the molecular function of intracellular proteins. It is known that even moderate changes in the preimplantation environment can adversely affect the pre- and postnatal phenotypes (Fleming *et al.* 2004, Sinclair & Singh 2007). A clear consequence of these findings is the necessity for a strict control of maternal blood glucose levels during pregnancy from the day of conception onwards.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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