

Maternal Diabetes Leads to Unphysiological High Lipid Accumulation in Rabbit Preimplantation Embryos

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According to the “developmental origin of health and disease” hypothesis, the metabolic set points of glucose and lipid metabolism are determined prenatally. In the case of a diabetic pregnancy, the embryo is exposed to higher glucose and lipid concentrations as early as during preimplantation development. We used the rabbit to study the effect of maternal diabetes type 1 on lipid accumulation and expression of lipogenic markers in preimplantation blastocysts. Accompanied by elevated triglyceride and glucose levels in the maternal blood, embryos from diabetic rabbits showed a massive intracellular lipid accumulation and increased expression of fatty acid transporter 4, fatty acid-binding protein 4, perilipin/adipophilin, and maturation of sterol-regulated element binding protein. However, expression of fatty acid synthase, a key enzyme for de novo synthesis of fatty acids, was not altered in vivo. During a short time in vitro culture of rabbit blastocysts, the accumulation of lipid droplets and expression of lipogenic markers were directly correlated with increasing glucose concentration, indicating that hyperglycemia leads to increased lipogenesis in the preimplantation embryo. Our study shows the decisive effect of glucose as the determining factor for fatty acid metabolism and intracellular lipid accumulation in preimplantation embryos. (*Endocrinology* 155: 1498–1509, 2014)

The global rise in the prevalence of diabetes mellitus is paralleled by an alarming increase in diabetic pregnant women. Currently, in the western world 3% to 10% of pregnancies are affected (1). Diabetes mellitus is associated with abnormalities in lipid metabolism such as hypertriglyceridemia and low levels of high-density lipoprotein (2). Maternal metabolic disorders are well known risk factors affecting fertility and embryonic and fetal development.

Early embryos are able to store intracellular lipids in the form of lipid droplets (3). Lipids play an important role in energy metabolism during early embryo development by serving as an energy source and influencing properties and functions of biological membranes, cell-cell interactions, cell proliferation, and intercellular and intracellular trans-

port (for a review, see Ref. 4). The size and number of lipid droplets are considered as markers of embryo vitality (5, 6). Embryos cultured with serum abnormally accumulate cytoplasmic lipids, probably as a result of incorporation of lipoproteins from the serum (6–8). An increased amount of intracellular lipids reflects poor embryo quality and developmental potential. Pig and bovine embryos contain the highest amounts of lipids. This observation correlates with the lowest survival rate after cryopreservation (9–12). Mouse embryos with low amounts of lipid droplets are not as sensitive toward adverse effects of vitrification as embryos from other species (13).

Lipid droplets are coated by lipid droplet-associated proteins (PLIN1–4). They regulate the assembly, maintenance,

ISSN Print 0013-7227 ISSN Online 1945-7170

Printed in U.S.A.

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Received August 14, 2013. Accepted December 18, 2013.

First Published Online January 15, 2014

Abbreviations: cSREBP, cytoplasmic sterol-regulated element binding protein; explDD, experimental insulin-dependent diabetes; FABP, fatty acid-binding protein; FASN, fatty acid synthase; FATP, fatty acid transporter; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; nSREBP, nuclear sterol-regulated element binding protein; PLIN, lipid droplet-associated protein; pc, post coitum; PVA, polyvinyl alcohol; qPCR, quantitative PCR; SREBP, sterol-regulated element binding protein.

and composition of lipid droplets, as well as lipolysis and lipid efflux (14–16). The best known are perilipin and adipophilin, also referred as PLIN1 and PLIN2. Adipophilin expression has been documented previously from day 2 post coitum (pc) onward in rabbit preimplantation embryos (17).

The first step in long-chain fatty acid utilization is their uptake across plasma membranes. This process involves 2 components, passive diffusion through the lipid bilayer and protein-facilitated transfer. In early embryos, the fatty acid uptake is poorly understood. However, a facilitated transport is possible, as indicated by the expression of fatty acid transport protein (FATP) 4 and fatty acid-binding protein (FABP) 4 in mouse blastocysts (18) and in human trophoblast cells (19, 20).

The expression levels of FATP4 correlate with measures of obesity and insulin resistance (21). FABP4, also known as adipocyte fatty acid-binding protein (AFABP) or aP2, affects intracellular lipid metabolism by transporting fatty acids to the nucleus for transcriptional regulation, to mitochondria for β -oxidation, and to lipid droplets for storage (22, 23). New evidence from population studies and experimental animal models indicates that serum FABP4 is a powerful new risk marker for predicting obesity, insulin resistance, and dyslipidemia (24, 25). However, the exact secretion mechanism of FABP4 is still not clear.

A source of intracellular fatty acids is de novo synthesis. Fatty acid synthase (FASN), the key enzyme in de novo lipogenesis, coordinates together with acetyl-CoA carboxylase energy homeostasis by converting excess food intake into lipids for storage (26, 27). FASN^{-/-} null mutant mice die during preimplantation development, demonstrating the important role during early embryonic development (28). The promoter region of FASN contains binding sites for the transcription factor sterol-regulated element binding protein (SREBP) 1 (29). SREBPs are synthesized as precursor proteins that remain bound to the endoplasmic reticulum. After activation, SREBP is processed and translocated to the nucleus (30, 31). The mature nuclear SREBP activates the transcription of genes regulating synthesis and uptake of fatty acids, triglyceride, and cholesterol (31, 32). Currently, 3 different isoforms have been described: SREBP1a and SREBP1c, derived from a single gene by alternative transcription start sites, and SREBP2 (33). SREBP-overexpressing transgenic animals showed an accumulation of cholesterol and/or fatty acids in liver cells (32, 34, 35), indicating that the SREBP1 isoforms are more directed toward activation of fatty acid biosynthesis, whereas SREBP-2 is more specific for controlling cholesterol biosynthesis.

Despite the increasing incidence of metabolic disorders in women of reproductive age, few attempts have to be made to analyze the maternal influence on embryonic lipid metabolism (36–38). Therefore, we have investigated intracellular

lipid accumulation and expression of key lipogenic markers (such as perilipin, adipophilin, FATP4, FABP4, FASN, and SREBP1) in response to hyperglycemia in vivo caused by an induced maternal diabetes mellitus type 1 and hyperglycemia created in vitro in rabbit preimplantation embryos.

Materials and Methods

Alloxan treatment

Experimental insulin-dependent diabetes (expIDD) was induced in mature 18- to 20-week-old female nonpregnant rabbits by alloxan treatment (Sigma-Aldrich) as described before (39). Rabbits were held in the diabetic condition with permanent blood glucose concentrations of >14 mmol/L by regular insulin supplementation 4 times/d (Insuman Rapid and Lantus; sanofi aventis), started on the second day after alloxan treatment. The blood glucose level was monitored with a MediSense Precision Xceed Diabetes Management System (Abbott) 2 times/d. For that, fresh blood was collected at 10:00 AM and 6:00 PM by puncturing the vena auricularis lateralis and tested for glucose concentration by a commercial test strip.

All animal experiments were conducted in accordance with the principles of laboratory animal care, and the experimental protocol had been approved by the local ethics commission of the Landesverwaltungsamt Dessau (reference no. 42502-2-812).

Embryo recovery

Embryos were collected from sexually mature rabbits stimulated with 110 IU of pregnant mare serum gonadotropin sc (Intervet) 3 days before mating. After mating, 75 IU of human chorionic gonadotropin was injected iv (Intervet). Mating and embryo recovery were performed as described previously (40). On days 3, 4, and 6 pc embryos were flushed from oviducts or uteri, respectively, washed 3 times with PBS and randomly divided among the experimental groups. On day 6 pc, gastrulation stages can be reliably discriminated in the rabbit because implantation starts on day 6 18 hours, ie, half a day later (41). Only blastocysts from gastrulation stage 1 and 2 were used for further analyses.

Embryo in vitro culture

To study the effects of different glucose concentrations on perilipin/adipophilin, FABP4, FATP4, and FASN expression and intracellular lipid accumulation, day 6 blastocysts were cultured in groups of 6 to 10 at 37°C in a water-saturated atmosphere of 5% O₂, 5% CO₂, and 90% N₂. Blastocysts were cultured for 6 hours in serum- and insulin-free BSM II medium (42) with 0, 10 (standard culture medium), and 25 mM glucose (hyperglycemic conditions), respectively.

Quantification of triglycerides in blood samples

Triglyceride concentrations in blood samples were measured by a commercial kit (DiaSys Triglycerides FS; Diagnostic Systems). All procedures were performed according to the manufacturer's instructions. To measure the serum triglyceride concentration, blood samples were collected with the S-Monovette system (Sarstedt), left to coagulate for at least 30 minutes, and centrifuged for 10 minutes at 4°C and 1000 × g. The supernatant was stored at -80°C until use.

Oil Red O staining of rabbit blastocysts

Blastocysts were washed twice in ice-cold PBS and fixed in 4% (wt/vol) paraformaldehyde for 10 min. The fixed blastocysts were washed again in ice-cold PBS and transferred to 0.05% (wt/vol) polyvinyl alcohol (PVA)/PBS. Blastocyst coverings were removed mechanically. The embryonic disk was mechanically dissected with surgical forceps and scissors (Fine Science Tools GmbH) (39) and used immediately for Oil Red O staining. Oil Red O stock solution (0.5 mg of Red Oil O [Sigma-Aldrich] dissolved in 100 mL of isopropanol) was diluted 3:2 with distilled water. To remove the precipitate, the working solution was filtered using a filter with a pore diameter of 20 μm (BD Biosciences).

For Oil Red O staining, the embryonic disc was incubated in filtered Oil Red O solution for 2 hours. After staining the lipid droplets, the Oil Red O staining solution was removed by washing the embryonic disc for 3 hours in 0.05% (wt/vol) PVA/PBS. Subsequently, the embryonic disk was embedded on Superfrost slides (Menzel Gläser) using 4.8 g of Mowiol reagent (Calbiochem, Germany) dissolved in 12.0 g of glycerol (Merck). Embryonic disks were examined by light microscopy (BZ 8000; Keyence).

Preparation of a single-cell suspension

For the separation of blastocysts to single-cell suspensions, the gentleMACS-m-neural-tissue kit (Neural Tissue Dissociation Kit; Miltenyi Biotec) was used. Blastocyst coverings were removed mechanically, and blastocysts were transferred in a 1.5-mL tube. The preheated papain enzyme mix 1 provided with the kit was given directly to the blastocysts and incubated according to the manufacturer's protocol, except that the incubation step was performed at room temperature. Enzyme mix 2, prepared according to the manufacturer's protocol, was added directly to the sample. The specimen was incubated for 15 minutes at room temperature on a rotator.

To remove cell aggregates from the cell suspension, a pre-separation filter with a pore diameter of 40 μm (BD Biosciences) was used. After a washing step, the cells were resuspended in ice-cold 0.05% (wt/vol) PVA/PBS. Nile Red stock solution (100 $\mu\text{g}/\text{mL}$; Sigma-Aldrich) was prepared in dimethyl sulfoxide and stored in the dark. For Nile Red staining, the dye was added at a final concentration of 0.1 $\mu\text{g}/\text{mL}$. The cells were then incubated on ice for 5 minutes, centrifuged, and washed with 0.05% (wt/vol) PVA/PBS. Afterward they were resuspended in a volume of 0.05% (wt/vol) PVA/PBS and kept on ice in the dark until flow cytometry analysis.

Flow cytometry

All measurements were performed on a BD FACS Vantage (BD Biosciences) equipped with 3 lasers (excitation wavelengths: 488, 633, and 351 nm) as described previously (43). Cell debris, doublets, and aggregates were excluded from analysis. Blastocyst cells without Nile red staining served as a negative control for flow cytometry analysis. Measurements and data analysis were performed with FACSDiva (version 5.0.3; BD Biosciences). Nile Red specifically stains intracellular lipid droplets, excites at 485 nm, and emits at 525 nm. For sorting, a 90- μm nozzle was used, and the sorting rate was no more than 1000 events per second.

The visualization by dot plot and histogram shows 2 different cell populations R1 (P1) and R2 (P2). To prove that region R1 contains embryoblast and R2 contains trophoblast cells, we sorted both cell populations and used them subsequently for

RT-quantitative PCR (qPCR) analyses for cytokeratin 18 and *cdx2*, 2 trophoblast markers (44). Cytokeratin 18 and *cdx2* were highly expressed in the R2 cell population, demonstrating that these cells were almost exclusively trophoblast cells (Figure 1I).

The analysis of the percentage of cells located in R3 (cell debris or putative dead cells) revealed no significant differences between the normohyperglycemic and hyperglycemic groups. This result suggests that in our experiments increased cell death in the hyperglycemic group compared with that in the control group can be excluded.

RNA isolation and cDNA synthesis

mRNA of single blastocysts was extracted with Dynabeads oligo(dT)₂₅ (Invitrogen) and subsequently used for cDNA synthesis. All protocol procedures were performed according to the manufacturer's instructions, with modifications described previously (45).

RT-PCR

RT-PCR amplification was performed with 0.5 μL of cDNA from single blastocysts in a 25- μL volume containing 200 μM concentrations of each dNTP, 2.5 U of Taq polymerases, and specific oligonucleotides for adipophilin, FABP4, FATP4, FASN, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (primers are listed in Supplemental Table 1 published on The Endocrine Society's Journals Online web site at <http://end.endojournals.org>). The nucleotide sequence for rabbit FASN was determined using human primers for amplification of rabbit liver cDNA. The amplification was done for 40 cycles (94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 60 seconds). The resulting PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. The PCR products were sequenced and analyzed as described previously (46). Sequence homology was proven by using the alignment BLASTN tool.

RT-qPCR

Amounts of FAS, FATP4, FABP4, adipophilin, and GAPDH cDNA were determined by real-time qPCR with SYBR Green detection by using the StepOnePlus System (Applied Biosystems) with a no template control for each primer set (described in Ref. 47). The nucleotide sequences of the primers used in this study are listed in Supplemental Table 1. The amount of cDNA per sample was normalized by referring to the GAPDH gene. GAPDH was shown to be unaffected by the treatment because no variation was observed between the absolute GAPDH levels and GAPDH RNA levels in blastocysts from healthy rabbits (47). qPCRs for target and reference genes were always run in duplicate from the same cDNA dilution taken from the same RT reaction.

The absolute amount of the transcripts was calculated along with a standard (calibrator) sample. As standards we used defined concentrations of plasmid standards constructed for the gene of interest and GAPDH. For all genes investigated a partial sequence was amplified from rabbit tissues. Purified PCR products were ligated into pGEM-T with subsequent transformation in XL1Blue competent bacteria (described in Ref. 47).

Results are given in molecules of the target gene per GAPDH molecule. Statistical analysis was performed on the amounts of molecules. Fold changes were calculated when the results are expressed as percentages.

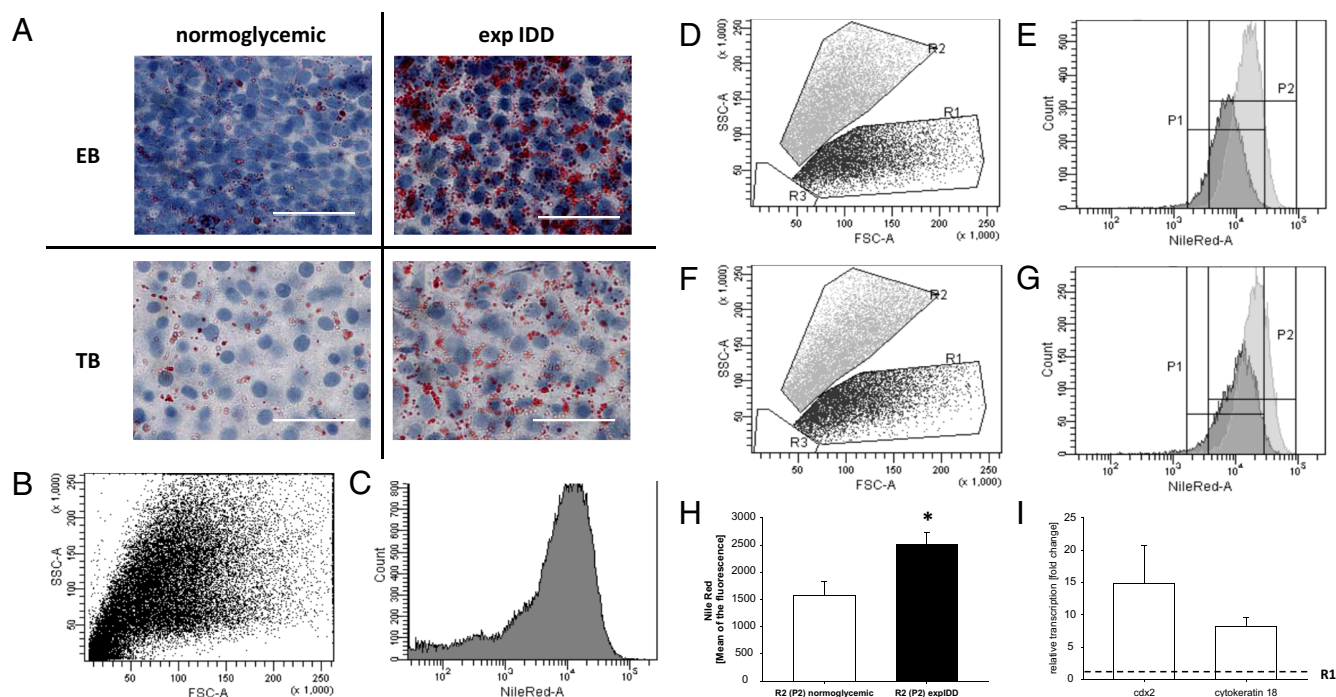


Figure 1. Oil Red O staining and Nile Red flow cytometry analyses of blastocyst cells from diabetic rabbits. A, Blastocysts derived from healthy and diabetic (expIDD) rabbits were stained with Oil Red O, marking intracellular lipid droplets red in trophoblast (TB) and embryoblast (EB) cells (scale bar corresponds to 50 μ m). Nuclei were counterstained with hematoxylin in blue. B–G, For flow cytometry analysis blastocyst cells were stained with Nile Red. A representative analysis is shown. Blastocyst cells were visualized on a dot plot of forward scatter (FSC) vs side scatter (SSC) (B, D, and F) or in a Nile Red histogram (C, E, and G). The visualization by dot plot, using the parameters granularity and cell size, indicated 2 different cell populations, region R1 with embryoblast cells and R2 with trophoblast cells, respectively. For further analyses, blastocyst cells were gated in region R1 and R2. Cell debris and death cells were excluded (region R3). Blastocyst cells were gated in region R1 and R2 from normoglycemic (D) and diabetic (F) rabbits. Nile Red absorbance of blastocyst cells from regions R1 and R2 is shown for normoglycemic (E) and for diabetic (G) rabbits in P1 and P2, respectively. H, Nile Red mean fluorescence in R2 (P2) of normoglycemic and diabetic cells determined in 3 independent experiments is shown. Blastocyst cells were sorted in region R1 and R2 and subsequently used for RT-qPCR analysis of trophoblast markers *cdx2* and cytokeratin18. I, Results are shown as relative amounts and fold change of R1 (means \pm SEM; N = 4, n \geq 18). R2 sorted cells were identified as trophoblast cells with 10- and 5-fold higher *cdx2* and cytokeratin 18 RNA amounts, respectively, than those in the R1 cells.

Protein preparation and immunoblotting

Protein preparation, quantification, and Western blotting were performed with 8 to 10 blastocysts as described previously (48). For Western blot analysis, 25 μ g of total protein lysates was subjected to SDS-polyacrylamide electrophoresis and electrotransferred to nitrocellulose membranes. For detection of FASN, SREBP1, and β -actin, membranes were blocked in Tris-buffered saline containing 0.1% (vol/vol) Triton X-100 (0.1% TBST) with 3% (wt/vol) nonfat dry milk at room temperature for at least 1 hour. For perilipin detection, membranes were blocked in 0.1% TBST with 3% (wt/vol) BSA for 2 hours. The primary antibody was incubated at 4°C overnight. The following antibodies were used as follows perilipin (1:1000; Sigma-Aldrich), FASN (1:1000; Santa Cruz Biotechnology), SREBP1 (1:1000; Active Motif), β -actin (1:40 000; Sigma-Aldrich), anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:15 000; DAKO Cytomation), and anti-mouse IgG conjugated to HRP (1:45 000; Dianova). The protein amount was calculated as the ratio of band intensities (perilipin protein or FASN protein vs β -actin protein, respectively) in the same blot to correct for differences in protein loading.

Immunohistochemical localization of FABP4 and FASN

Blastocysts were washed twice in ice-cold PBS and fixed in 4% (wt/vol) paraformaldehyde at 4°C overnight. The sample was prepared, and the immunohistochemical protocol was per-

formed as described previously (48). Antibodies for FABP4 and FASN (both Santa Cruz Technology) were diluted 1:100 and 1:500 in 3% (wt/vol) BSA/PBS, respectively. The secondary antibody Dako EnVision+ System HRP-labeled polymer anti-mouse (1:1 in PBS) and diaminobenzidine (WAK-Chemie Medikal) were used for detection.

All steps were performed within the same experiment, and samples were examined microscopically during the same session, using identical microscope and camera settings (BZ 8000; Keyence).

FABP4 ELISA

FABP4 concentrations in blastocyst fluids were measured by ELISA (MyBioSource). All protocol procedures were performed

Table 1. Mean Nile Red Fluorescence Intensity From Normoglycemic and Diabetic (expIDD) Blastocyst Cells in Region R1 (P1) and Region R2 (P2)

	Nile Red (Mean \pm SEM)	P Value
R1 (P1) normoglycemic	951 \pm 280	.6
R1 (P1) expIDD	1093 \pm 202	
R2 (P2) normoglycemic	1566 \pm 267	.03
R2 (P2) expIDD	2503 \pm 233	

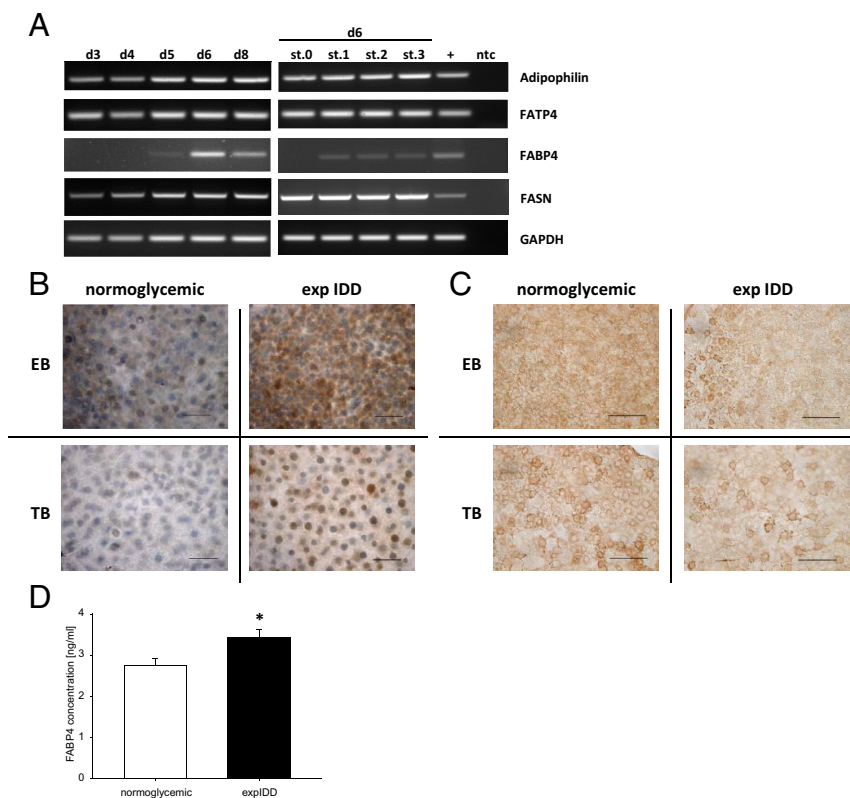


Figure 2. Expression patterns of the lipogenic markers adipophilin, FATP4, FABP4, and FASN in rabbit preimplantation embryos. A, RT-PCR was performed with specific primers for adipophilin, FATP4, FABP4, and FASN on rabbit morulae at day (d) 3, on blastocysts at days 4, 5, 6, and 8 pc, and on 6-day-old blastocysts of different gastrulation stages with stage (st) 0, 1, 2, and 3. Transcripts of adipophilin, FATP4, and FASN were detected in all analyzed developmental stages. FABP4 transcription was measurable from gastrulation stage 1 (st 1) onward. A probe without cDNA was used as negative control (ntc). As a positive control, a liver cDNA probe was used (+). The internal control was the expression of GAPDH in all probes. The FABP4 and FASN proteins were detected by immunohistochemical analysis and visualized by a peroxidase-diaminobenzidine reaction (brown color) (scale bar corresponds to 50 μ m). B, In blastocysts from healthy controls (normoglycemic), FABP4 was localized in the cytosol and nucleus of embryoblast cells (EB). The trophoblast cells (TB) were not or less stained for FABP4. In blastocysts from diabetic rabbits (expIDD), the embryoblast and trophoblast cells showed more intense staining for FABP4, especially in the nuclei of both cell lineages. Nuclei were counterstained in blue with hematoxylin. C, Immunohistochemical localization revealed FASN in the cytosol of embryoblast and trophoblast cells. No difference in staining and localization was found between blastocysts from diabetic and normoglycemic rabbits. D, FABP4 concentrations were analyzed by ELISA in blastocyst fluids of single blastocysts from normoglycemic and expIDD rabbits (means \pm SEM; N = 3; n \geq 9; *, $P < .05$).

according to the manufacturer's instructions. Flushed blastocysts were washed 2 times with ice-cold PBS. Blastocyst stage and size were determined. Blastocysts were then placed on a dry watch glass. Extracellular coverings were removed mechanically, blastocysts were punctured, and the effluent cavity fluid was collected. Blastocyst fluids were stored at -80°C until use. FABP4 concentrations were quantified in duplicate and compared with an internal FABP4 standard (0.625–50 ng/mL).

Statistics

All data are expressed as means \pm SEM. Levels of significance between groups were calculated by factorial ANOVA with Bonferroni adjustment. A value of $P < .05$ was considered statistically significant. All experiments were conducted at least 3 times.

Results

In previous studies, we demonstrated that the increases in maternal blood glucose concentrations correlate with increased glucose levels in the uterine secretions (39). Here, we focused on the lipid content and lipogenic markers to test whether a diabetic disorder during early pregnancy would affect the embryonic fatty acid metabolism.

Elevated triglyceride levels in female rabbits with expIDD at day 6 pc

In blood samples of diabetic (expIDD) pregnant rabbits, triglyceride levels were increased approximately 6-fold (3.55 ± 0.43 mM; mean \pm SEM) compared with that in the healthy controls (0.57 ± 0.05 mM) [number of independent experiments used for blastocysts (N) = 3; total number of blastocysts used for analysis (n) = 9; $P < .001$], respectively.

Accumulation of intracellular lipid droplets in normoglycemic and diabetic (expIDD) blastocysts

The effect of alloxan-induced maternal diabetes mellitus type 1 on intracellular lipid accumulation was examined by Oil Red O staining. Blastocysts from diabetic rabbits showed a higher accumulation of intracellular lipids in the embryoblast and trophoblast, indicated by the higher amount of red-stained lipid droplets (Figure 1A). To further support these data, we used a quantitative method to determine the cellular lipid content based on Nile Red staining and flow cytometry analyses. In Figure 1, B to G, the results of a representative flow cytometry analysis are illustrated. Blastocyst cells were visualized by dot plot and histogram. A higher Nile Red fluorescence intensity was found in blastocyst cells from diabetic rabbits in both cell populations R1 (P1) and R2 (P2). However, in contrast to the R2 (Figure 1I and Table 1), the difference in the Nile Red fluorescence mean in the R1 between normoglycemic and diabetic blastocysts was not significant (data not shown). The mean Nile Red absorbance is given in Table 1.

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Table 2. Relative Protein Amount of Lipogenic Markers in Blastocysts from Normoglycemic and Diabetic (expIDD) Rabbits

Protein	Normoglycemic	expIDD	P Value
Perilipin	100.0 ± 3.5	136.8 ± 10.5	.048
FASN	100.0 ± 6.8	105.3 ± 1.2	.473
nSREBP1	100.0 ± 7.2	107.2 ± 5.2	.437
cSREBP1	100.0 ± 12.7	76.9 ± 5.8	.138
Ratio: nSREBP1/ cSREBP1	0.51 ± 0.03	0.70 ± 0.03	.005

Results are given relative to protein amounts in blastocysts from healthy rabbits (normoglycemic) (means ± SEM; N = 3; n = ≥10).

Adipophilin and perilipin expression in preimplantation embryos from diabetic rabbits

Intracellular lipid droplets are coated by the lipid droplet-associated proteins perilipin and adipophilin. The transcription of adipophilin was detectable in the rabbit preimplantation embryos from day 3 old morulae onward in all analyzed days and stages (Figure 2A). Transcription levels in blastocysts (day 6 pc stage 1) from diabetic rabbits (expIDD) were significantly increased compared with those from normoglycemic rabbits (Table 2). Quantification of adipophilin in the embryoblast and trophoblast revealed that the increase in the adipophilin amount resulted from transcriptional up-regulation, mainly in embryoblast cells (Table 4). In addition, the perilipin protein amount was significantly increased in blastocysts from diabetic rabbits (Table 3).

FATP4 and FABP4 expression in preimplantation embryos from diabetic rabbits

FATP4 and FABP4 are expressed in rabbit blastocysts. Whereas FATP4 transcription was detectable in all investigated embryonic stages, starting on day 3 pc (Figure 2A), FABP4 expression was only measurable in expanded and gastrulating blastocysts on day 6 pc, from stage 1 onward and on day 8 pc (Figure 2A). Analysis of FABP4 transcription, however, revealed a cell lineage-specific FABP4 distribution pattern with the main expression in the embryoblast (Figure 2B). Only a few cells stained positive for FABP4 in the trophoblast.

In diabetic conditions, the transcription levels for both FATP4 and FABP4 were increased in blastocysts (Table 2).

FATP4 mRNA was increased in the embryoblast and trophoblast (Table 4). For FABP4, the RNA amount was increased only in the embryoblast (Table 4). Compared with embryos from healthy rabbits, blastocysts from diabetic mothers showed more intense FABP4 staining in both cell lineages, with the most prominent localization in the nuclei (Figure 2B).

Because FABP4 can be secreted from cells and is present in the circulation, we analyzed the FABP4 concentration in blastocyst fluid. Analysis by ELISA showed that FABP4 is present in blastocyst fluid and that the amount is increased in the cavity fluid from diabetic blastocysts (Figure 2D).

FASN expression in preimplantation embryos from diabetic rabbits

The key enzyme of de novo lipogenesis, FASN, was detectable from day 3 old morulae (d3) onward in all analyzed stages (Figure 2A). Neither FASN transcription (Table 2) nor protein (Table 3) amounts were significantly different in blastocysts from diabetic rabbits. In addition, no alterations of FASN transcription levels in embryoblasts and trophoblasts from normoglycemic and diabetic rabbits were detectable. However, a cell lineage-specific transcription was observed (Table 4), with higher transcripts in the embryoblast. FASN protein was localized in both embryoblast and trophoblast cells with a cytoplasmic localization (Figure 2C). In blastocysts from diabetic rabbits, no differences in staining intensity or in cellular localization were observed.

SREBP1 expression in preimplantation embryos from diabetic rabbits

Under hyperglycemic conditions, no changes in total SREBP1 protein amounts were observed (Table 2). However, the ratio of cytoplasmic (cSREBP1) and mature, nuclear SREBP1 (nSREBP1) was influenced. Blastocysts from diabetic rabbits had relatively more SREBP1 located in the nucleus (based on the total SREBP1 protein amount and the ratio of nSREBP1 to cSREBP1) compared with that of blastocysts from normoglycemic rabbits (Table 2), indicating a higher transcriptional regulation by SREBP1 in blastocysts from diabetic rabbits.

Table 3. Relative mRNA Amount of Lipogenic Target Genes in Blastocysts from Normoglycemic and Diabetic (expIDD) Rabbits

mRNA	Normoglycemic	expIDD	%	P Value
Adipophilin	51.76 ± 15.28	167.78 ± 3.99	287.4 ± 29.9	.020
FABP4	4.45 ± 2.03	9.91 ± 2.27	222.6 ± 51.1	.041
FATP4	855.4 ± 124.1	1382.4 ± 179.6	188.9 ± 23.2	.038
FASN	0.66 ± 0.18	0.38 ± 0.06	58.7 ± 10.1	.195

Results are given as molecules and as a percentage of transcription levels in normoglycemic blastocysts (means ± SEM; N = 3; n = ≥10).

Table 4. Relative mRNA Amount of Lipogenic Target Genes in Embryoblast and Trophoblast From Normoglycemic and Diabetic (explDD) Rabbits

mRNA	Embryoblast		Trophoblast	
	Normoglycemic	explDD	Normoglycemic	explDD
Adipophilin	100.0 ± 8.3	131.3 ± 11.8 ^a	118.1 ± 6.6	125.1 ± 17.7
FABP4	100.0 ± 17.1	293.3 ± 71.2 ^a	14.0 ± 3.5 ^b	9.9 ± 1.8 ^b
FATP4	100.0 ± 6.4	147.2 ± 15.3 ^a	92.8 ± 10.4	148.6 ± 15.1 ^a
FASN	100.0 ± 23.5	91.4 ± 23.4	25.9 ± 6.7 ^b	15.8 ± 2.3 ^b

Results are given as a percentage of the amount in the embryoblast from healthy rabbits (normoglycemic) (means ± SEM; N = 3; n = ≥10).

^a Amount significantly different between control and explDD ($P < .05$).

^b Amount significantly different between embryoblast and trophoblast ($P < .05$).

Glucose-dependent expression of lipogenic genes

To clarify whether glucose can regulate the expression of lipogenic genes, we cultured blastocysts without glucose (0 mM) and with 10 mM (standard culture media) or 25 mM (hyperglycemia in vitro) for 6 hours in vitro. The quantification of adipophilin and FABP4 transcript levels and perilipin protein showed increased expression of all markers in blastocysts cultured under hyperglycemic conditions (Figure 3, A, B, C, and E). No significant differences were measured for the FATP4 transcript levels in response to the various glucose concentrations (Figure 3D). In contrast to diabetic conditions in vivo, the FASN protein amount was increased in blastocysts cultured with 10 and 25 mM compared with that in blastocysts cultured with 0 mM, respectively (Figure 3, F and G). Total protein amounts of SREBP1 were increased in a glucose-dependent manner with a higher accumulation of mature nSREBP1 in the nucleus (Figure 3, H–K).

Glucose-dependent accumulation of lipid droplets

Excess energy intake leads to an accumulation of intracellular lipids. To analyze whether a high concentration or the lack of glucose influences the amount of lipid droplets in embryos, we cultured blastocysts (day 6 pc) from normoglycemic and diabetic rabbits for 6 hours with either 0, 10, or 25 mM glucose and afterward stained intracellular lipid droplets with Oil Red O. Blastocyst cells from both normoglycemic and diabetic (Figure 4) rabbits showed a glucose-dependent accumulation of intracellular lipids. High glucose concentrations led to a higher amount of lipid droplets compared with 0 and 10 mM in blastocysts from normoglycemic rabbits. The lowest amount of lipid droplets was found in blastocysts cultured with 0 mM. A comparable glucose-dependent increase in lipid droplets was observed in blastocysts from diabetic rabbits. However, the amount of lipid droplets in each group (0–25 mM) was higher in diabetic blastocysts than in the corresponding controls.

Discussion

Since the initial observation by Mills in 1982 (49), a great number of studies have shown the determining effect of maternal hyperglycemia on pregnancy outcome, offspring malformation, and the risk of later life diseases (39, 50–55). Adverse effects of high glucose concentrations have been shown by in vitro and in vivo culture experiments, such as retarded embryo development and increased apoptosis (39, 55–63). The current study shows for the first time the direct influence of maternal diabetes in vivo and hyperglycemia in vitro on embryonic lipid storage. One central finding of this study is the highly increased amount of intracellular lipid droplets in blastocysts from diabetic rabbits. Embryos with higher amounts of cytoplasmic lipids are more sensitive to chilling and cryopreservation (64) and have a lower developmental efficiency (10, 11). Therefore, the increased amount of lipid droplets in blastocysts may contribute to the adverse effects of maternal diabetes on preimplantation embryo development.

The massive lipid accumulation in blastocysts from diabetic rabbits raises the question of whether the embryo produces lipids from excess maternal glucose supply or by an increased transport of fatty acids into the cell. Type 1 diabetes mellitus is not only characterized by high serum glucose concentrations but also by increased serum triglyceride concentrations. These phenomena are closely reflected by hyperglycemia and hypertriglyceridemia in the diabetic rabbit model. Diabetes mellitus is associated with maternal and fetal dyslipidemia, which manifests as high plasma triglyceride concentrations, elevated concentrations of nonesterified fatty acids, increased concentrations of low-density lipoprotein cholesterol, and decreased levels of high-density lipoprotein cholesterol (20, 65–68). An increase in fatty acids enhances the formation of lipid droplets and expression of adipophilin and FABP4 (20, 69–71). Therefore, it is likely that the diabetes-associated hyperlipidemia is a potent regulator of embryonic lipid metabolism. This view is supported by the observation

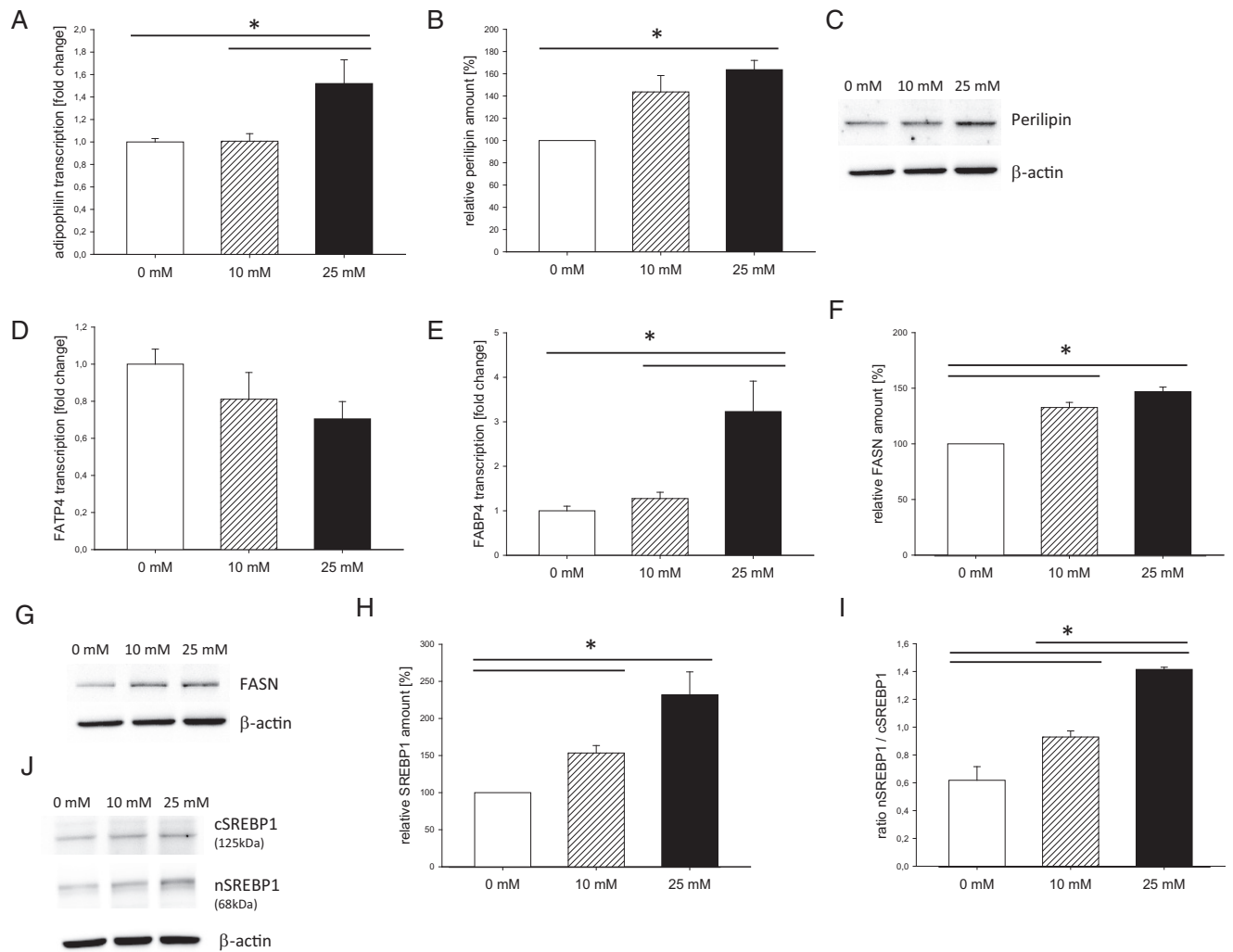


Figure 3. Relative expression of lipogenic genes in rabbit blastocysts cultured in vitro with 0, 10, or 25 mM glucose. A, In vitro culture of blastocysts for 6 hours with 0, 10, or 25 mM glucose led to a significantly higher adipophilin transcription level in blastocysts of the 25 mM group (*, $P < .05$). Blastocysts were cultured in groups of at least 5 blastocysts ($N = 3$; $n \geq 5$). mRNA levels were analyzed by RT-qPCR, and results are shown as means \pm SEM (*, $P < .05$). B and C, Relative perilipin amounts after 6 hours of cultivation without or with 10 or 25 mM glucose were analyzed by Western blotting with anti-perilipin antibody and set in relation to total β -actin. A representative Western blot for perilipin is shown in panel C. Results of 3 independent stimulation experiments ($N = 3$; $n \geq 9$; means \pm SEM) are shown in panel B. The group without glucose (0 mM) was set at 100%. D and E, RT-qPCR analysis of FATP4 (D) or FABP4 (E) after in vitro culture (for 6 hours) of blastocysts with 0, 10, or 25 mM shows a distinctly different expression pattern. In vitro culture with 10 or 25 mM or without glucose was performed in groups of at least 5 blastocysts in each treatment and repeated in 3 independent replicates ($N = 3$; $n \geq 5$). Results are shown as means \pm SEM (*, $P < .05$). F and G, FASN protein amount was analyzed by Western blot and the 0 mM group (control) was set at 100% (*, $P < .05$). Results (F) and a representative Western blot (G) are shown. H–K, Relative SREBP1 amounts after 6 hours of cultivation without or with 10 or 25 mM glucose were analyzed by Western blotting with anti-SREBP1 antibody, which specifically detects cSREBP1 (125 kDa) and nSREBP1 (68 kDa). Culture was performed in groups of at least 5 blastocysts in each treatment and repeated in 3 independent replicates ($N = 3$; $n \geq 5$). H, Relative SREBP1 protein amounts (nSREBP1 and cSREBP1) in relation to those in the 0 mM group, which was set at 100%. I, Ratio of nSREBP1 vs cSREBP1. J, Representative Western blot for SREBP1.

that both normoglycemic and diabetic blastocysts cultured for 6 hours in vitro showed a lower amount of lipid droplets than that of their in vivo counterparts, implying that metabolic factors other than glucose might play a role, too. Furthermore, FASN expression was not altered in blastocysts from diabetic rabbits. Because lipogenesis encompasses fatty acid and subsequent triglyceride synthesis, it is tempting to speculate that the unchanged FASN expression in vivo is the result of an increased lipid supply by the diabetic mother and

the increased expression of FABP4 and FATP4 in their blastocysts (Figure 5). On the other hand, it is also possible that the 3-fold increase in glucose in uterine secretions (20) is not high enough to regulate FASN expression. Finally, the increased FABP4 amount in the cavity fluid from diabetic blastocysts may play a pivotal role by trafficking free fatty acids to blastocyst cells.

In our in vitro model, we can create a hyperglycemic situation independent of any triglyceride influence. In the case

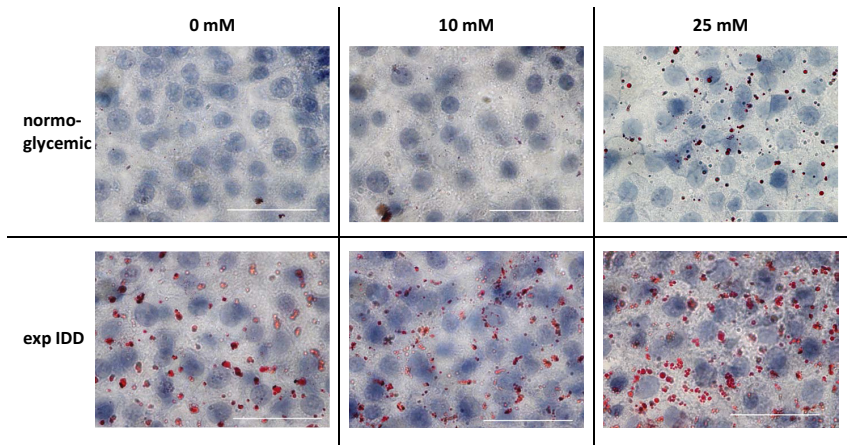


Figure 4. Intracellular lipid accumulation in blastocysts cultured in vitro with 0, 10, and 25 mM glucose. Six-day-old blastocysts were removed from healthy (normoglycemic) and diabetic (expIDD) rabbits and cultured in vitro with 0, 10, or 25 mM glucose for 6 hours. Then lipid droplets were stained red with Oil Red O (red dots). Nuclei were counterstained blue with hematoxylin. Representative pictures from trophoblasts are shown (scale bar corresponds to 50 μm). Culture was performed in groups of at least 4 blastocysts in each treatment and repeated in 3 independent replicates ($N = 3$; $n \geq 4$).

of an oversupply of glucose in vitro, the embryo shows a glucose-dependent regulation of lipogenic marker genes, including FASN (Figure 5). We interpret these findings as increased de novo lipogenesis, the first step of lipogenesis. However, FATP4 expression was not affected in vitro because the blastocysts were not supplied with lipids from the outside. Glucose can stimulate de novo lipogenesis via several mechanisms. First, glucose can be converted into lipids for storage (72). Furthermore, embryos cultured with high glucose concentrations show a slightly increased uptake of palmitic acid and an enhanced uptake of arachidonic acid (36). That high glucose and enhanced fatty acid uptake increase the accumulation of lipid droplets has also been shown in renal tubal epithelium and pancreatic β -cells (73–76). The up-regulation of FASN was SREBP1 dependent (74–76). We found a glucose-dependent accumulation of lipid droplets, a higher nuclear SREBP1 amount in vitro and an increased

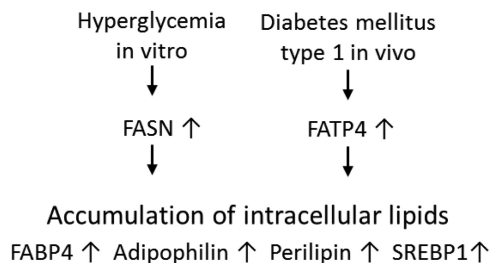


Figure 5. Schematic mechanism of intracellular lipid accumulation in rabbit blastocysts in diabetic conditions in vivo and hyperglycemia in vitro. In the preimplantation embryo, hyperglycemia leads to a nonphysiological lipid accumulation with increased expression of the adipogenic markers adipophilin, perilipin, FABP4, and SREBP1. In in vivo and in vitro conditions, the embryonic gene regulation differed only in FATP4 or FASN with increases in FATP4 in vivo and FASN in vitro.

nSREBP1/cSREBP1 ratio in vivo. Therefore, SREBP1 could belong to the connecting link between glucose and lipid storage in preimplantation embryos.

Several studies clearly show that glucose is a main source and key signal for SREBP1 induction and cleavage of SREBP1 precursor protein in a dose-dependent manner (77–79), as shown by our in vitro experiments, too. This effect is not due to osmotic properties, because glucose could not be replaced by the glucose analogs 2-deoxyglucose or 3-O-methyl glucopyranose (77, 79). SREBP1 activity is necessary not only for full induction of de novo fatty acid synthesis but also for intracellular lipid droplet accumulation by increasing

cholesterol biogenesis and activation of the low-density lipoprotein receptor (80–82), leading to increased lipogenesis.

The present results are particularly important with respect to possible short- and long-term consequences of in vitro biotechnologies such as assisted reproductive technologies, in which embryos are cultured for various times with high glucose concentrations before transfer. Experimental evidence suggests that culture conditions influence normal development and may contribute to prenatal and/or postnatal disorders (83–86). We show for the first time that high glucose concentrations in vitro lead to a higher lipid accumulation and an elevated expression of key lipogenic target genes, indicating that the glucose concentration is critical for metabolic set points of embryonic cells. Embryonic cells adapt to changes in glucose concentration in the surrounding milieu and may retain the information during later differentiation, as has been shown in embryonic stem cells (87).

However, we cannot rule out the possibility that factors other than hyperglycemia or hyperlipidemia may also influence intracellular lipid accumulation and lipogenic gene expression. Insulin and IGFs, which are known to be regulated by diabetic developmental conditions (39, 47), are potent regulators of FASN expression and enzyme activity (88–90), FABP4 expression (91, 92), and SREBP1 transcription and maturation (78, 93, 94). Therefore, the altered insulin/IGF system and/or a potential interplay between insulin/IGFs, hyperlipidemia, and hyperglycemia has to be kept in mind, when the underlying mechanism of aberrant embryonic lipid storage in diabetic blastocysts is discussed.

In conclusion, maternal diabetes alters the concentration of a variety of maternal nutrients, which in turn modifies the metabolic uterine environment for the developing embryo. Exposure to such an altered nutrient profile can disrupt normal development or, less dramatically, change embryonic metabolism. We demonstrated that embryonic lipid storage is altered under induced maternal diabetes mellitus in the rabbit. Intracellular lipid accumulation and expression of key genes for lipid storage (perilipin and adipophilin), fatty acid transport, and metabolism (FATP4 and FABP4) and lipogenesis (SREBP1) are increased. This effect can be explained in part by the fact that hyperglycemia in vitro increases the expression of lipogenic target genes and the amount of intracellular lipid droplets. Because preimplantation embryo development is one of the most critical periods in an individual's life, future health trajectories may be (mis)programmed with severe consequences later in life.

Acknowledgments

We thank Sabine Schrötter and Michaela Kirstein for excellent technical assistance.

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This work was supported by the German Research Council (DFG) (Grant NA 418/4-2), the European Union (FP-7 Epihealth 278418), and the Wilhelm Roux Programme of the Martin Luther University Faculty of Medicine.

Disclosure Summary: The authors have nothing to disclose.

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