Different response of embryos originating from control and obese mice to insulin in vitro

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Abstract. The aim of the present work was to investigate the impact of maternal obesity on DNA methylation in ovulated oocytes, and to compare the response of in vitro-developing preimplantation embryos originating from control and obese mice to insulin. An intergenerational, diet-induced obesity model was used to produce outbred mice with an increased body weight and body fat. Two-cell and eight-cell embryos recovered from obese and control mice were cultured in a medium supplemented with 1 or 10 ng/ml insulin until blastocyst formation. In the derived blastocysts, cell proliferation, differentiation, and death rates were determined. The results of immunohistochemical visualization of 5-methylcytosine indicated a slightly higher DNA methylation in ovulated metaphase II oocytes recovered from obese females, however, the difference between groups did not reach statistical significance. Expanded blastocysts developed from embryos provided by control dams showed increased mean cell numbers (two and eight-cell embryos exposed to 10 ng/ml), an increased inner-cell-mass/trophectoderm ratio (two-cell embryos exposed to 1 ng/ml and eight-cell embryos exposed to 10 ng/ml), and a reduced level of apoptosis (two and eight-cell embryos exposed to 10 ng/ml). In contrast, embryos originating from obese mice were significantly less sensitive to insulin; indeed, no difference was recorded in any tested variable between the embryos exposed to insulin and those cultured in insulin-free medium. Real-time RT-PCR analysis showed a significant increase in the amount of insulin receptor transcripts in blastocysts recovered from obese dams. These results suggest that maternal obesity might modulate the mitogenic and antiapoptotic responses of preimplantation embryos to insulin.

Key Words: Apoptosis, DNA methylation, Insulin, Maternal obesity, Preimplantation embryo

Studies on gene expression and epigenetic regulation have suggested several molecular mechanisms that mediate the negative effect of female obesity on oocytes [1–4]. Moreover, it has been hypothesized that alterations in the epigenetic reprogramming of germ cells might negatively impact the development of the conceptus [5, 6]. However, only limited functional tests examining the impact of identified (epi) genetic changes on the development of the conceptus during the preimplantation period have been performed [7].

In a previous study, our laboratory standardized an alternative model for inducing maternal obesity based on the over-nutrition of experimental animals during intruterine and early postnatal development [8]. Outbred mice raised following this intergenerational model develop a somatic condition characterized by having a significantly greater body weight, more body fat, and increased plasma glucose, insulin, and leptin levels at the age of 35–39 days, which coincided with the period of greatest likelihood of conception in this species [8, 10, 11]. Further, blastocysts recovered from obese females showed an up-regulation of the insulin-responsive glucose transporter, Slc2a4 (Solute Carrier Family 2 Member 4) [7]. In a subsequent in vitro study, our group reported that preimplantation embryos originating from obese mice responded to the presence of leptin in the culture medium in a manner that was different from that in control mice. In the latter study, it was hypothesized that alterations in the developmental capacities and changes in the incidence of cell death in such embryos result from processes involved in the adaptation of the germ or early embryonic cells to the altered maternal environment [7].

Insulin plays a key role in the regulation of embryonic growth and differentiation [12]. It binds with high affinity to the insulin receptor (IR), shows a low affinity for insulin-like growth factor 1 receptors (IGF-1R), and does not bind to hybrid receptors [13]. The expression of insulin receptors in preimplantation embryos is species-specific. In mouse preimplantation embryos, the presence of receptors has been confirmed in two-cell (IGF-1R) and four-cell embryos (IR), in morulae, and in blastocysts (both types of receptors) [14, 15]. The expression of Insr in human preimplantation embryos is consistent with that seen in mice. Indeed, it has been detected in oocytes, four-cell embryos, and higher-stage embryos, while the expression of Igf1r...
Animals and experimental design

Using real-time RT-PCR, the involvement of insulin in the regulation of protein metabolism in mouse blastocysts has also been demonstrated [19].

The aim of the present study was to test the hypothesis that maternal obesity influences the methylation pattern of in vivo-developing germ cells, and that preimplantation embryos originating from control and obese females show different responses to insulin. Comparisons of the overall methylation status in the nucleoplasm of ovulated oocytes recovered from control and intergenerational-model-developed obese mouse females were made by visualizing 5-methylcytosine. The sensitivity of in vitro-developing preimplantation embryos from both types of females to insulin was then examined. The latter embryos were recovered at the two-cell stage, since this is the first stage in which the expression of insulin receptors has been documented [15], and at the eight-cell stage, since this is when the demand for glucose is increasing [20]. Two-cell embryos recovered from obese and control mice were cultured for 72 h in a medium supplemented with insulin at two different concentrations: 1 ng/ml, reflecting the blood levels of insulin in mouse females during early pregnancy [9], and 10 ng/ml, which had a significant mitogenic effect on mouse embryos in a previous in vitro study [12]. Eight-cell embryos were cultured for 48 h in a medium supplemented with insulin at 10 ng/ml. In the derived blastocysts, the cell proliferation, differentiation and death rates were determined using morphological fluorescence staining. To evaluate the possible mechanisms of different responses to insulin, mRNA expression of insulin receptor, insulin-like growth factor I receptor, and selected genes representing two main branches of insulin receptor signaling (phosphatidylinositol 3-kinases [PI3Ks] and mitogen-activated protein kinases [MAPKs] [21–23]) were analyzed in the blastocysts recovered from control and obese dams using real-time RT-PCR.

Materials and Methods

Animals and experimental design

All experiments were performed using mice (Mus musculus) of the outbred CD-1 strain (Velaz, Prague, Czech Republic). An intergenerational diet-induced obesity model was used to produce mice with an increased body weight and body fat in early adulthood [8]. Female mice of the parental generation (30–35-day-old) were subjected to estrus synchronization using a modified superovulatory treatment using pregnant mare serum gonadotropin (5 IU intraperitoneal eCG [Folligon]; Intervet International, Boxmeer, Holland), followed by human chorionic gonadotropin (4 IU intraperitoneal hCG [Pregnyl]; Organon, Oss, Holland) 47 h later, and were mated with males of the same strain overnight. Unmated females (without a vaginal plug, C, n = 27, Ob, n = 28) were subjected to superovulation treatment with eCG and hCG (see above) and to oocyte isolation on Day 1 of pregnancy (12 h post hCG), in order to recover the embryos at the two-cell stage (Table 1; C, n = 48, Ob, n = 38), or on Day 3 of pregnancy (approx. 56 h after presumed ovulation) to recover the embryos at the eight-cell stage (Table 4; C, n = 31, Ob, n = 30), or on Day 4 of pregnancy (approximately 96 h after presumed ovulation) to recover the embryos at the blastocyst stage (Table 4; C, n = 21, Ob, n = 20). Preimplantation embryos were recovered by flushing the oviducts using a flushing-holding medium [25] containing 1% BSA (bovine serum albumin), and then classified under a Nikon SMZ 745T stereomicroscope (Nikon, Tokyo, Japan). Early embryo collection was performed in the sextuplet. Blastocyst collection was performed in duplicate.

Unmated females (without a vaginal plug, C, n = 27, Ob, n = 28) were subjected to superovulation treatment with eCG and hCG (see above) and to oocyte isolation on Day 1 of pregnancy (12 h post hCG), in order to recover metaphase II (MII) oocytes (Table 1). Cumulus-oocyte complexes were recovered by puncturing the ampulla tubae uterinae using sterile needles; they were then completely denuded by exposure to 0.3% hyaluronidase (Sigma Aldrich, St. Louis, MO, USA) in a flushing-holding medium containing 1% bovine serum albumin (BSA) (Sigma Aldrich) [25]. Oocyte collection was performed in triplicate.

Additionally, on Day 2 of pregnancy, 10 randomly selected females from each group were sacrificed via decapitation and blood samples were collected. The serum prepared from these samples was stored at −80°C until use. Insulin concentrations were quantified using a commercial Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem; Downers Grove, IL, USA; #90080), according to the manufacturer’s instructions.

All animal experiments were approved by the Ethical Committee for Animal Experimentation at the Institute of Animal Physiology and the State Veterinary and Food Administration of the Slovak Republic, and were performed in accordance with the Slovakian legislation based on European Commission Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes.
5-methylcytosine density in MII oocytes

MII oocytes that were freshly isolated from control and obese females were tested for the overall methylation density using 5-methylcytosine (5mC) immunochemical staining, followed by fluorescence microscopy. The oocytes were fixed for 30 min at room temperature in 4% paraformaldehyde (Merck, Darmstadt, Germany) in phosphate buffered saline (PBS) (Gibco, Invitrogen Corporation, Paisley, UK) and stored in 1% paraformaldehyde in PBS at 4°C for up to one week. In further processing, fixed oocytes were washed for 30 min in PBS with 0.05% Tween 20 before incubation for 10 min in 100 mM TRIS/HCl buffer (pH 8.5) (Sigma Aldrich) to denature the DNA. Subsequently, they were neutralized with 0.1% polyvinylpyrrolidone (PVP) (Sigma Aldrich) in 0.05% Tween 20 in PBS and subjected to 2 M hydrochloric acid for 30 min at room temperature. They were then washed extensively and permeabilized using 0.3% Triton X-100 (Sigma Aldrich) in PBS with 0.05% Tween 20 (Applichem Panreac, Darmstadt, Germany) in 4% paraformaldehyde in phosphate buffered saline (PBS) (Gibco, Invitrogen Corporation, Paisley, UK) and stored in 1% paraformaldehyde in PBS at 4°C for up to one day. In further processing, fixed oocytes were washed for 30 min in PBS with 0.05% Tween 20 in PBS and subjected to 2 M hydrochloric acid for 30 min at room temperature. They were then washed extensively and permeabilized using 0.3% Triton X-100 (Sigma Aldrich) in PBS with 0.05% Tween 20 (Applichem Panreac, Darmstadt, Germany) in 4% paraformaldehyde in phosphate buffered saline (PBS) (Gibco, Invitrogen Corporation, Paisley, UK) and stored in 1% paraformaldehyde in PBS at 4°C for up to one week. In further processing, fixed oocytes were washed for 30 min in PBS with 0.05% Tween 20 before incubation in a blocking solution (2% bovine serum albumin fraction V [Sigma Aldrich] in PBS) overnight at 4°C. The methylated DNA was then visualized using a mouse monoclonal antibody against 5mC (dilution 1:400 in blocking solution) (Calbiochem, La Jolla, CA, USA). Incubation with the primary antibody was performed for 60 min at 37°C. After extensive washes in blocking solution, the oocytes were incubated with the secondary polyclonal antibody (goat anti-mouse immunoglobulin G conjugated with Texas Red; dilution 1:200; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature, and then mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) on glass slides. Antibody staining controls involved the omission of the primary and/or secondary antibody (which resulted in the complete absence of immunofluorescence staining).

An epifluorescence microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan) was used to excite the fluorescent staining (excitation, 580 nm/emission, 604 nm) and to capture digital photographs of the oocyte using a × 40 objective (Fig. 1). An identical microscope filter was used for all samples to ensure consistent lighting conditions. Oocytes were then analyzed using a × 40 objective (Nikon Eclipse 80i; Nikon, Tokyo, Japan) with an Axios cam ID 5.0MP camera (Carl Zeiss, Jena, Germany) connected to a computer (Microsoft Windows, version 7). Images were captured using the AxioVision 4.8 software (Carl Zeiss, Jena, Germany).

Table 1. Characteristics of control and obese female mice used for recovery of oocytes on Day 1 and for recovery of embryos at the two-cell stage on Day 2 of pregnancy

|                      | Control mice | Obese mice |
|----------------------|--------------|------------|
| No. of female F1 mice subjected to mating | 87           | 92         |
| Body weight (g)      | 19.56 ± 0.20 | 22.71 ± 0.22 a |
| Body fat (%)         | 7.36 (0.53)  | 11.73 (1.22) a |
| No. of unmated mice used for recovery of oocytes | 27           | 28         |
| No. of evaluated MII oocytes | 130         | 74         |
| Median grey level of 5mC labelling in MII oocytes | 74 (42)      | 101 (108) a |
| Integrated density of 5mC labelling in MII oocytes | 674,523 ± 26.83 | 743,226 ± 36.38 |
| No. of mated mice used for recovery of embryos at the two-cell stage | 48           | 38         |
| Blood insulin assessed in fertilized mice (ng/ml) | 0.86 ± 0.12  | 1.23 ± 0.18 |
| No. of recovered embryos at the two-cell stage | 427          | 370        |

Results are expressed as means ± S.E.M. or medians with interquartile range. Different superscript letters indicate significant differences. Statistical analysis: body weight, Student’s t-test (P < 0.001); body fat, Mann-Whitney (P < 0.001); median grey level of 5mC (5-methylcytosine) labelling, Mann-Whitney test (P < 0.001); integrated density of 5mC labeling, Student’s t-test (P = 0.062); blood insulin, Mann-Whitney test (P = 0.098).

Table 2. Developmental capacity of two-cell embryos isolated from control and obese dams and cultured for 72 h in vitro with insulin

| Concentration of insulin in culture medium | Control mice | Obese mice |
|-------------------------------------------|--------------|------------|
|                                           | 0 ng/ml | 1 ng/ml | 10 ng/ml | 0 ng/ml | 1 ng/ml | 10 ng/ml |
| No. of cultured two-cell embryos           | 113      | 112     | 97       | 92       | 92       | 84       | 78       |
| Blastocoele formation (% of embryos)       | 90.27    | 89.29   | 96.91    | 88.04    | 91.67    | 96.15    |
| No. of blastocysts with ≥ 32 cells          | 97       | 98      | 84       | 74       | 72       | 73       |
| No. of cells per blastocysts               | 68.13 ± 1.76 | 63.82 ± 1.52 | 68.56 ± 2.62 | 69.80 ± 1.98 | 66.65 ± 2.18 | 63.03 ± 2.01 |
| No. of cells in ICM                       | 20.13 ± 0.91 a | 22.58 ± 0.80 ab | 20.14 ± 0.89 ab | 23.74 ± 0.89 ab | 23.83 ± 0.89 b | 23.48 ± 1.01 ab |
| No. of cells in TE                         | 48.00 ± 1.37 a | 41.23 ± 1.21 b | 48.42 ± 1.15 a | 46.05 ± 1.80 ab | 42.88 ± 1.70 b | 39.55 ± 1.59 b |
| ICM/TE ratio                              | 0.43 (0.32) a | 0.58 (0.39) b | 0.42 (0.36) a | 0.49 (0.34) b | 0.54 (0.34) b | 0.66 (0.39) b |
| Blastocysts with dead cells (%)            | 97.93    | 97.95   | 96.42    | 100      | 91.66    | 100      |
| Incidence of dead cells in blastocysts (%) | 4.51 (2.89) a | 4.48 (4.18) a | 4.12 (3.25) a | 6.02 (3.79) b | 5.35 (5.89) ab | 5.13 (3.49) ab |

Results are expressed as means ± S.E.M. or medians with interquartile range. Different superscript letters indicate significant differences. Statistical analysis: blastocoele formation, Chi-squared test (P = 0.062); body weight, Student’s t-test (P < 0.001); body fat, Mann-Whitney (P < 0.001); median grey level of 5mC (5-methylcytosine) labelling, Mann-Whitney test (P < 0.001); integrated density of 5mC labeling, Student’s t-test (P = 0.062); blood insulin, Mann-Whitney test (P = 0.098).
Table 4. Characteristics of control and obese female mice used for recovery of embryos at the eight-cell stage on Day 3 and for recovery of embryos at the blastocyst stage on Day 4 of pregnancy

|                              | Control mice | Obese mice |
|------------------------------|--------------|------------|
| No. of female F1 mice subjected to mating | 34           | 33         |
| Body weight (g)              | 19.70 ± 0.35 | 22.91 ± 0.26 a |
| Body fat (%)                 | 7.38 (0.51)  | 11.73 (1.35) |
| Mean no. of mated mice used for recovery of embryos at the eight-cell stage | 31           | 30         |
| No. of collected embryos     | 236          | 273        |
| No. of recovered embryos at the ≥ eight-cell stage | 150          | 194        |
| No. of female F1 mice subjected to mating | 24           | 30         |
| Body weight (g)              | 21.63 ± 0.41 | 27.48 ± 0.38 a |
| Body fat (%)                 | 7.80 (0.60)  | 12.46 (3.33) a |
| Mean no. of mated mice used for recovery of blastocysts | 21           | 20         |
| No. of recovered embryos at the blastocyst stage | 148          | 145        |

Results are expressed as mean ± S.E.M. or medians with interquartile range. Different superscript letters indicate significant differences. Statistical analysis: body weight, Student’s t-test (P < 0.001 for all cases); body fat, Mann-Whitney (P < 0.001 for all cases).

Table 5. Developmental capacity of eight-cell embryos isolated from control and obese mice and cultured 48 h in vitro with insulin

| Concentration of insulin in culture medium | Control mice | Obese mice |
|-------------------------------------------|--------------|------------|
|                                          | 0 ng/ml | 10 ng/ml | 0 ng/ml | 10 ng/ml |
| No. of cultured eight-cell embryos       | 57      | 57       | 62      | 62       |
| Blastocoele formation (% of embryos)     | 100.00  | 100.00   | 98.24   | 94.11    |
| No. of evaluated blastocysts              | 57      | 57       | 62      | 62       |
| No. of cells per blastocyst               | 98.29 ± 3.14 | 116.04 ± 3.78 a | 96.51 ± 2.40 | 87.45 ± 2.82 a |
| No. of cells in ICM                       | 35.72 ± 1.65 | 48.40 ± 1.52 a | 40.27 ± 1.52 | 31.57 ± 1.50 a |
| No. of cells in TE                        | 62.58 ± 2.25 ab | 67.64 ± 2.37 a | 56.24 ± 1.91 b | 55.88 ± 1.97 b |
| ICM/TE ratio                              | 0.58 (0.32) a | 0.70 (0.32) ab | 0.72 (0.45) b | 0.61 (0.28) a |
| Blastocysts with dead cells (%)           | 98.24    | 98.38    | 98.38   | 98.38    |
| Incidence of dead cells in blastocyst (%) | 2.92 (2.28) ab | 2.35 (1.40) a | 2.99 (2.73) ab | 3.30 (3.85) ab |

Results are expressed as mean ± S.E.M. or medians with interquartile range. Different superscript letters indicate significant differences. Statistical analysis: blastocoele formation, Chi-squared test with one degree of freedom (P > 0.05 for all cases); mean number of cells per blastocyst, ICM, and TE, ANOVA (P > 0.001, P < 0.0001, and P < 0.0003 for whole blastocysts, ICM and TE, respectively), followed by Tukey’s test; ICM/TE ratio, Kruskal–Wallis test (P = 0.0015), followed by Dunn’s test; proportion of blastocysts with dead cells, Chi-squared test with one degree of freedom (P > 0.05 for all cases); incidence of dead cells in blastocysts, Kruskal–Wallis test (P = 0.013), followed by Dunn’s test.
setting and exposure time were used for all stained objects. The intensity of the 5mC fluorescent signals was evaluated using Ellipse software (ViDiTo, Košice, Slovak Republic). First, the RGB image was split into three individual 8-bit images, each representing one color channel, and the red channel image was selected for assessment. Then, the fluorescence intensity from 0 to 255 shades for each pixel (0, no fluorescence; 255, maximum fluorescence) was measured in the nuclear area of the captured oocytes. The fluorescence units were regularly adjusted by considering the background in which no oocyte was present as zero, to compensate for the possible variations in the fluorescence lamp intensity. Finally, the median gray level and the integrated density of the 5mC fluorescence signal were calculated using the software. The median gray level represented the median value of the fluorescence units of all the pixels in the selection. The integrated density was derived from the mean gray level (the mean value of fluorescence units of all the pixels in the selection) in the selected area (the number of pixels inside the closed contour object).

**Culture of mouse preimplantation embryos with insulin**

The embryos recovered from control and obese females were cultured in vitro under standard conditions (humidified atmosphere, 5% CO₂, 37°C) with or without recombinant human insulin expressed in active yeast, suitable for cell culture (Sigma-Aldrich; #I3536). The final concentrations of insulin in the culture medium were 0 ng/ml (control), 1 g/ml and 10 ng/ml. Preimplantation embryos were cultured with regards to their developmental needs, that is, one embryo per 1 μl (at least) of synthetic oviduct medium [EmbryoMax® KSOM (potassium simplex optimized medium) with amino acids and D-Glucose (Millipore, Darmstadt, Germany)] supplemented with 0.1% embryo culture tested BSA (Sigma-Aldrich). Drops of medium were placed in plastic cell-culture dishes, covered with mineral oil (Zenith Biotech, Calgary, Canada), and pre-incubated overnight under standard conditions. Two-cell stage embryos were cultured for 72 h and exposed to 1 and 10 ng/ml insulin. Eight-cell stage embryos were cultured for 48 h and exposed to 10 ng/ml insulin. On the final day of culture, the number of embryos reaching the blastocyst stage (i.e., showing the formation of the blastocoele) was assessed using stereomicroscopy.

After the culture of two-cell embryos, the insulin concentration of the spent culture medium was determined using the Human Insulin stereomicroscopy. (i.e., showing the formation of the blastocoele) was assessed using

**Real-time RT-PCR analysis of the selected transcripts in in vitro-obtained blastocysts**

Total RNA was extracted from 4 batches of approximately 35 mouse blastocysts using TRizol Reagent (Invitrogen Life Technologies, Karlsruhe, Germany), according to the manufacturer’s instructions. Contaminating DNA in the RNA preparations was digested using amplification-grade DNase I (Invitrogen Life Technologies). For the quantitative analysis, 0.08 pg of luciferase mRNA (Promega, Madison, WI, USA) per blastocyst were added to the TRizol lysis reagent, prior to RNA extraction, to correct for differences in RNA recovery and loading of RT-PCRs. Four separate batches of blastocysts were used for RNA isolation and cDNA synthesis in each experimental group. The RNA was reverse transcribed using Superscript™ III RTase. H− Reverse Transcriptase (Invitrogen Life Technologies) and a mixture of anchored oligo-dT primers and random hexamers (Thermo Fisher Scientific, Waltham, MA, USA). To detect the presence of genomic DNA contamination in the RNA preparations, reverse transcriptase negative controls (no reverse transcriptase in the reaction) were performed in parallel, using part of each RNA sample. The cDNA preparations were subsequently cleaned via ethanol precipitation, and the cDNA pellets were diluted in an appropriate amount of 10 mM Tris (pH 8.3), so that 1 μl of the cDNA corresponded to 2.5 blastocyst (and 0.2 pg Luc mRNA) equivalents. PCR amplifications were performed using the LightCycler 480 real-time PCR system (Roche Diagnostics, Mannheim, Germany). The reactions were performed in 25 μl of reaction volume containing 1 μl of cDNA, 1 x RT2 qPCR SYBR Green Master Mix (Qiagen, Valencia, CA, USA), and 0.4 μM of specific primers (Table 6). An initial denaturation step at 95°C for 10 min was followed by 45 cycles at 95°C for 15 sec, annealing at the primer-specific temperature for 30 sec and elongation at 72°C for 20 sec (Table 6). For the luciferase amplification, we used primers that were designed in a previous study [26]. Fluorescence
was measured after the elongation step at a temperature of 3°C – 4°C below the melting temperature ($T_{acq}$ “acquiring temperature”, Table 6). Amplification specificity was assessed using melting curve analysis. The relative standard curve method was used to analyze the fluorescence data, and the expression of the target genes was normalized to the luciferase mRNA [27].

**Statistical analysis**

Statistical analysis was performed using PRISM v.5.01@2007 (GraphPad Software, La Jolla, CA, USA). To determine if data sets were well-modeled by a normal distribution, data were analyzed using the D’Agostino–Pearson normality test.

The differences between data showing a normal Gaussian distribution were assessed using the unpaired Student’s t-test or ANOVA, followed by Tukey’s post-hoc test. This concerns differences between control and obese females regarding body weight, blood insulin concentration, and integrated density of 5mC labelling in the recovered oocytes, and differences between control- and obese-female-derived embryos exposed to insulin in terms of the mean number of cells per blastocyst, the mean number of cells in the inner cell mass (ICM), and the mean number of cells in the trophectoderm (TE). In such cases, the results are expressed as mean ± standard error of the mean (S.E.M.).

The differences between data that did not pass the normality tests were assessed using the Mann-Whitney test or the Kruskal-Wallis test, followed by Dunn’s post-hoc test. This concerns differences between control and obese females in terms of body fat and median grey level of 5mC in recovered oocytes, and the differences between control- and obese-female-derived embryos exposed to insulin in terms of the ICM/TE ratio in blastocysts, the proportion of dead cells in blastocysts, the relative quantity of transcripts, and residual

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**Fig. 1.** Illustrative micrographs of mouse oocytes obtained *in vivo* from control (A) and obese (B) females. The methylation density of the nuclear material (N) was visualized using 5-methylcytosine immunofluorescent labeling (red). C: Negative staining control. Original magnification: × 400. Scale bar: 100 µm.

**Fig. 2.** Illustrative fluorescence micrographs of mouse blastocysts obtained *in vitro*. Blastocysts were obtained after 72 h of culture of two-cell embryos recovered from control (A–C) and obese mice (D–F) and after 48 h of culture of eight-cell embryos recovered from control (G–I) and obese mice (J–L). A, D, G, J: The nuclear morphology was visualized using chromatin staining by using Hoechst 33342 (blue). B, E, H, K: The trophectodermal (TE) cell lineage was visualized via immunohistochemical labeling of CDX2 protein (red). C, F, I, L: The specific DNA degradation in the nucleoplasm was visualized using transferase dUTP nick-end labeling (TUNEL) (green). White arrowheads show apoptotic cells with a fragmented nuclear morphology and TUNEL-positive nucleoplasm. Yellow arrowhead shows apoptotic cells with a fragmented nuclear morphology and TUNEL-negative nucleoplasm. Asterisk shows TUNEL-positive spermhead. Original magnification: × 400. Scale bar: 100 µm.
concentrations of insulin in the culture medium. In such cases, results
are expressed as medians with interquartile ranges.

For the assessment of differences between score-type data, standard
chi-square tests with one degree of freedom were used. This concerns
differences between control- and obese-female-derived embryos
exposed to insulin in terms of blastocoele formation and the proportion
of blastocysts with dead cells.

Differences with a P < 0.05 were considered statistically significant.

Results

The obese mice used to produce oocytes, two-cell embryos, eight-
cell embryos, and blastocysts showed an increased body weight (P
< 0.001) and had more body fat than control females (P < 0.001)
(Table 3). The serum insulin levels assessed in randomly
selected females on Day 2 of pregnancy were slightly increased in
the group of obese females, but not significantly (P = 0.098, Table 1).

5- methylcytosine density in MII oocytes

The nuclear material of MII oocytes obtained from obese mice showed
a significantly higher median grey level of 5-methylcytosine
labelling than that of MII oocytes obtained from control mice (P <
0.001, Table 1). However, when variabiliy in the size of the nuclear
area was considered and the integrated density of 5-methylcytosine
signal was computed, the difference between groups did not reach
statistical significance (P = 0.062).

Cell number, cell differentiation, and cell death in in vitro-
obtained blastocysts

The presence of insulin in the culture medium did not affect the
overall ability of two-cell or eight-cell embryos to reach the blastocyst
stage (P > 0.05, Table 3). For the control dams, the supplementation of the culture medium
with 10 ng/ml insulin resulted in a significant increase in the number of cells per blastocyst in the expanded blastocyst subgroup obtained
after the in vitro culture of two-cell (P < 0.05, Table 3) and eight-cell
embryos (P < 0.001, Table 5). In the case of obese dams, insulin had
no effect on blastocyst cell numbers, either in the blastocysts obtained
after the culture of two-cell (P > 0.05, Tables 2 and 3) or eight-cell
embryos (P > 0.05, Table 5).

In the control mice, the blastocysts obtained in vitro from two-cell embryos cultured in medium supplemented with 1 ng/ml insulin
showed an increased ICM/TE ratio (P < 0.01, Table 2), significantly
so for the subgroup of early blastocysts (P < 0.05, Table 3). A similar
(though insignificant) trend was observed for the blastocysts obtained
from eight-cell embryos. However, blastocysts obtained from eight-cell
embryos cultured in medium supplemented with 10 ng/ml insulin
showed a significant increase in the number of cells in the ICM cell
line (P < 0.001, Table 5). For the obese mice, no altered ICM/TE
ratio was seen in the blastocysts derived from the two-cell embryos
cultured with insulin (P > 0.05). Interestingly, in all groups with
10 ng/ml of insulin but one (8-cell embryos cultured with 10 ng/
ml of insulin), the blastocysts originating from obese mice showed
a higher ICM/TE ratio than those originating from control mice
(Tables 2, 3, and 5).

For the control dams, the presence of 10 ng/ml insulin in the culture
medium significantly reduced the proportion of dead cells in the expanded blastocysts obtained from two-cell embryos (P < 0.05,
Table 3). The same result was observed in the blastocysts obtained
from eight-cell embryos exposed to 10 ng/ml insulin (P < 0.05, Table
5). In the case of obese dams, no effect was seen in terms of the
proportion of dead cells in blastocysts derived from insulin-treated
and insulin-un-treated two-cell and eight-cell embryos (P > 0.05,
Tables 2, 3, and 5).

Residual insulin concentration after in vitro culture of two-cell
embryos

The spent medium left after 72 h of in vitro culture of the two-cell
embryos obtained from control and obese dams showed no differences

Table 6. Relative quantity of selected transcripts in in vivo-developed blastocysts obtained from control and obese mice

| Gene symbol | GenBank accession no. | Primers | Amplicon size | Tac  | Tacq | Control mouse mRNA quantity | Obese mouse mRNA quantity | P value |
|-------------|----------------------|---------|---------------|------|------|-----------------------------|---------------------------|--------|
| Insr        | NM_010568.2          | PPM05115E | 108 bp       | 60   | 79   | 4.48 (0.74)                 | 5.61 (0.59) *             | 0.028  |
| Igf1r       | NM_010513.2          | PPM04714F | 124 bp       | 68   | 81   | 5.56 (3.13)                 | 5.75 (2.60)               | 0.63   |
| Mapk1 (ERK2)| NM_011949.3          | PPM03571E | 87 bp        | 60   | 75   | 12.79 (2.51)                | 11.82 (1.74)              | 0.14   |
| Mapk3 (ERK1)| NM_011952.2          | PPM03585E | 86 bp        | 60   | 79   | 9.52 (2.08)                 | 10.04 (2.06)              | 0.88   |
| Pik3ca      | NM_008839.2          | PPM05112A | 105 bp       | 60   | 77   | 8.81 (1.37)                 | 9.43 (1.57)               | 0.89   |
| Pik3cb      | NM_029094.3          | PPM05089A | 123 bp       | 60   | 80   | 2.73 (0.81)                 | 2.78 (0.63)               | 0.89   |
| Pik3cd      | NM_008840.3          | PPM05083A | 136 bp       | 60   | 82   | 4.41 (2.03)                 | 3.63 (0.51)               | 0.34   |
| Slc2a8 (GLUT8)| NM_019488.4         | PPM04165A | 180 bp       | 65   | 78   | 11.60 (1.93)                | 10.45 (1.68)              | 0.20   |

Primers (catalog numbers of RT2 qPCR Primer Assays, Qiagen), size of amplicons (in base pairs, bp), and annealing and acquiring temperatures (Tac and Tacq, respectively) used in PCR are shown. Results (relative mRNA quantities) are expressed as medians with interquartile ranges. Different superscript letters indicate significant differences. Statistical analysis: Mann-Whitney test. Insr, Insulin receptor; Igf1r, Insulin-like growth factor I receptor; Mapk1 (synonym ERK2), Mitogen-activated protein kinase 1; Mapk3 (synonym ERK1), Mitogen-activated protein kinase 3; Pik3ca, Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide; Pik3cb, Phosphatidylinositol 3-kinase, catalytic, beta polypeptide; Pik3cd, Phosphatidylinositol 3-kinase, catalytic, delta polypeptide; Slc2a8 (synonym GLUT8), Solute carrier family 2, (facilitated glucose transporter), member 8.
in terms of residual insulin concentration \(0.86 \pm 0.10\) vs. \(0.72 \pm 0.23\) ng/ml after exposure to 1 ng/ml insulin, and \(3.41 \pm 0.94\) vs. \(5.48 \pm 1.18\) after exposure to 10 ng/ml insulin; \(P > 0.05\) for both).

**Gene expression in in vivo-developed blastocysts isolated from control and obese dams**

The transcript level of insulin-like growth factor I receptor \(\text{I}g\text{f}1\text{r}\) gene did not change between the blastocysts isolated from obese and control dams. On the other hand, the transcript level of insulin receptor \(\text{I}n\text{s}r\) gene was significantly higher in the blastocysts isolated from obese dams (Table 6). Our results showed that the transcripts of all three isoforms of PI3K catalytic subunits (alpha, beta, and delta) are expressed in mouse blastocysts. However, the transcript levels of PI3K catalytic subunits, facilitated glucose transporter 8 (\(\text{S}l\text{c}2\text{a}8\), GLUT8), and mitogen-activated protein kinases ERK1 and ERK2 (\(\text{M}a\text{p}k3\) and \(\text{M}a\text{p}k1\)) did not change between the blastocysts isolated from obese and control dams (Table 6).

**Discussion**

**Impact of maternal obesity on DNA methylation in ovulated MII oocytes**

Gametogenesis in mammals is characterized by extensive epigenetic reprogramming events. These erase the pre-existing epigenetic profile of the somatic cells from which the germ cells are derived, and establish new epigenetic patterns in mature gametes [28]. Thus, by the time the primordial germ cells (PGCs) have migrated to the genital ridge, they are largely devoid of DNA methylation. In the mouse female germline, DNA methylation is re-established after birth and during oocyte growth [29].

A correlation between maternal obesity and changes in overall or single-gene methylation has been shown at various stages of germ cell development and early embryo development. For example, oocytes at the germinal vesicle (GV) stage recovered from female mice with high-fat-diet-induced obesity show reduced 5mC levels compared to controls, as do GV oocytes recovered from obese female mice with a mutation in the leptin gene \(\text{o}b\text{o}b\) [2]. Furthermore, the DNA methylation patterns of several metabolism-related genes are changed not only in the MII oocytes of high-fat-diet (HFD) obese mice, but also in the oocytes and livers of their offspring [1]. In a previous study, we also detected a significant delay in, and less demethylation of, the parental pronuclei of zygotes at the pronuclear stage 4 isolated from obese females [10]. In the present study, analysis of the overall genomic 5mC status indicated a slight difference in the degree of DNA methylation between ovulated MII oocytes derived from control and obese females as well. However, the significance of the obtained results was lower than in previous studies. One reason would be the use of superovulation treatment during the recovery of oocytes, which has been shown to modulate the epigenome of the germ cells per se [30].

Although obesity might be a cause of epigenetic modulation in different cell types, the mechanisms that might induce these changes remain largely unknown. In mammals, DNA methylation occurs via the action of several site-specific methyltransferases. DNA methyltransferase 1 (DNMT 1) is generally considered to be a maintenance enzyme during cell division [31]. It is active in all mouse preimplantation embryos, with its expression progressively increasing between the GV oocyte and MII oocyte stages, and again between the morula and blastocyst stages [32]. The enzymes DNMT 3A and DNMT 3B are essentially responsible for de novo methylation. DNMT 3A protein expression progressively increases between the GV oocyte and one-cell embryo stages, and gradually decreases between the one-cell and eight-cell stages. The expression profiles of DNMT 3B in oocytes and early embryos largely coincide with this pattern [32]. A recent report that female Japanese macaques exposed to a high-fat diet show an elevated fetal DNMT 1 expression [33] supports the idea that maternal obesity may regulate epigenetic modulations via methyltransferases.

**In vitro response of embryos derived from control dams to insulin**

Insulin increased the number of cells in blastocysts derived from control embryos. It also increased the ICM/TE ratio and reduced apoptosis. Similar effects have been reported in in vitro studies involving different species [12, 34–41].

To our knowledge, this is the first report of a physiological concentration of insulin (1 ng/ml) having a positive effect on the ICM/TE ratio of mouse blastocysts. However, since no other qualitative parameters have been influenced, we might assume that the biological impact of insulin at such concentration on preimplantation development is quite low.

A previous study indicated that the total number of cells in mouse blastocysts might be increased by 10 ng/ml insulin [12]. In agreement with the present results, the authors of the latter study hypothesized that the increase was predominantly due to the proliferation of ICMs. The present results also indicate an increase in the total cell numbers in blastocysts derived from control embryos exposed to 10 ng/ml insulin. Furthermore, as previously reported [12, 40], the exposure of mouse embryos to supra-physiological concentrations of insulin (up to 10 μg/ml) in vitro had a beneficial effect on the total number of cells per blastocyst. The results of similar studies involving the blastocysts of cows (insulin concentration up to 10 μg/ml[34, 38, 41]), goats (4–400 ng/ml [36]), and sheep (1–10 mg/ml [39]) also agree with these findings. In contrast to studies on mouse embryos, developing bovine preimplantation embryos have been reported to show an increased trophoderm, rather than ICM proliferation, when exposed to insulin (10 μg/ml [38]).

Previous studies on chickens and cows have shown anti-apoptotic effects for both physiological and supra-physiological concentrations of insulin during early embryonic development in vitro [35, 37, 38]. In the current study, a reduction in the incidence of apoptosis was also seen in expanded blastocysts derived from control mice after exposure of two- and eight-cell embryos to 10 ng/ml insulin. However, the results of most previous studies on mice are not consistent with these findings [42, 43], and report that the supplementation of bovine insulin at supra-physiological concentrations in vitro (35 ng/ml, 3, and 4 μg/ml) could lead to a reduced stimulation of glucose uptake and increased apoptosis in blastocysts. Apparently, in mice, the type of insulin, the concentration used, and the time of embryo exposure affect the ability of the hormone to act as an anti- or pro-apoptotic factor.

The majority of previous in vitro studies on mice have revealed the positive effects of insulin on the development of preimplantation...
embryos. Increased numbers of embryos have been reported to develop into blastocysts after exposure to a physiologically reachable insulin concentration, 10 ng/ml [12], concentrations between 5 and 50 ng/ml [40], supra-physiological insulin concentrations (75 ng/ml [19], 0.25 μg/ml [44]), and extremely high insulin concentrations (5 mg/ml [45]). In the present study, the two-cell and eight-cell embryos were amply able to reach the blastocyst stage, upon which insulin supplementation seemed to have no effect. When 10 ng/ml insulin was used, a trend towards the stimulation of blastocoele formation was observed, but significance was not reached (96.91% vs. 90.27%, P > 0.05). Apparently, the positive effects of insulin on embryo development are more evident when culture conditions are less than adequate [19].

**In vitro response to insulin of embryos derived from obese dams**

In contrast with the control embryos, the presence of insulin in the culture medium had no effect on cell numbers, the ICM/TE ratio (experiment with two-cell embryos), the ICM cell numbers (experiment with eight-cell embryos), or the apoptosis rates in blastocysts developed from embryos originating from obese dams. The lower sensitivity of such embryos suggests that they show some kind of “insulin resistance.” This might be explained by alterations in the signaling pathways that regulate cell proliferation, differentiation (the MAPK/ERK pathway), and apoptosis (the PI3K/Akt pathway) in embryos recovered from obese mice.

This lowered insulin sensitivity might also be one reason why apoptosis is increased in blastocysts recovered from obese mouse females obtained using the intergenerational model of obesity [11]. As hypothesized earlier, the increased incidence of apoptosis in embryos might be associated with embryo loss or implantation failure [46]. Furthermore, a lowered insulin sensitivity of early conceptus might have long-term consequences and negatively impact the health of the offspring. As documented in our recent study, maternal obesity during the periconceptional period was accompanied by lower weights in newborn pups and by several deviations from a normal behavior in juvenile mice [10]. However, the underlying mechanisms are unknown.

To evaluate the possible mechanisms behind the different responses of embryos from obese mice to insulin, the mRNA expression of selected genes connected with insulin signaling was analyzed in *in vitro*-derived blastocysts.

Our results showed increased levels of insulin receptor (*Insr*) transcripts in the blastocysts isolated from obese dams. Consistent with this elevation, increased levels of facilitated glucose transporter 4 (*Slc2a4*, GLUT4) transcripts in the same type of blastocysts were found in our previous work [7]. Likewise, significantly higher levels of insulin receptor and GLUT4 transcripts were found in omental adipose tissue samples obtained from insulin-resistant obese patients, in comparison with the samples from non-obese patients or insulin-sensitive obese patients [47]. As hypothesized in the latter study, an increased expression of these transcripts could represent a compensatory mechanism for impaired signaling from insulin receptor in tissues of obese individuals. Conversely, in blastocysts originating from mouse females with HFD-induced obesity, no significant change in insulin receptor transcripts was documented [48]. The discrepancy between the results may be caused by the use of different experimental models (intake of standard diet vs. high-fat diet during the time of follicular growth and early preimplantation period) or by differences in the timing of gene expression analysis: in older blastocysts (recovered on Day 4 vs. Day 3.5 after plug detection in current vs. previous study, respectively); the compensatory mechanism could be more advanced (and therefore detectable) than in earlier ones. As shown in various studies on overweight individuals who differed in insulin sensitivity, the expression of insulin receptor is dynamic and varies depending on time [49].

However, we failed to identify the signaling disorders behind the compensatory elevation of *Insr* expression in mouse blastocysts. Although there are data on decreased PI3K protein levels and reduced Akt phosphorylation in peri-implantation ovarian tissues of obese rats [50] and mice [51], our results showed no differences in the expression of selected phosphoinositide 3-kinases or mitogen-activated protein kinases between blastocysts isolated from control and obese dams. Yet, in our study, the expression of all three isoforms of PI3K catalytic subunits (alpha, beta, and delta) transducing signals from the insulin receptor (and other receptor tyrosine kinases) were documented for the first time.

Finally, our results showed no differences in the expression of insulin-like growth factor I receptor (*Igf1r*) and glucose transporter 8 (*Slc2a8*, GLUT8) between the blastocysts isolated from control dams and dams obtained using the intergenerational model of obesity. This observation appears to be in accordance with previous findings in blastocysts recovered from mouse females with HFD-induced obesity [48]. However, there are contrary reports on a decreased signal of IGF-1R protein visualized using immunohistochemistry [52] and a reduced expression of facilitated glucose transporter 1 (*Slc2a1*, GLUT1) transcripts in such types of blastocysts [48]. Thus, further studies will be necessary to elucidate the impact of maternal obesity on insulin signaling in embryonic cells.

In conclusion, this work shows that embryos obtained from control and obese mice appear to show different responses to insulin *in vitro*. Two- and eight-cell embryos originating from obese mice show a significantly lower sensitivity to insulin exposure, while expanded blastocysts developed from embryos provided by control dams show increased mean cell numbers, an increased ICM/TE ratio, and a reduced level of apoptosis. These results suggest that alterations in the maternal *milieu* might modulate the mitogenic and antiapoptotic responses of preimplantation embryos to insulin.

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