Maternal obesity in mice not only affects fresh embryo quality but also aggravates injury due to vitrification

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Abstract

Purpose The aims of the present study are to identify the mechanism(s) whereby obesity impairs fresh embryos and to clarify the effects of vitrification on lipid droplet content within embryos from maternally obese mice.

Methods The diet-induced obesity mouse model was established, and the zygotes were captured and cultured to day 3. The eight-cell embryos were selected and divided into fresh and vitrified groups. The blastocysts derived from fresh embryos were used as a control. The expression profiles of endoplasmic reticulum (ER) stress genes (*Atf4*, *Grp78*, and *Hsp70*) and other genes (*MnSOD*, *p53*, *Gadd45g*, *caspase-3*, *IGF-II*, *ZO-1*, and *E-cadherin*) on day-3 fresh and post-warming eight-cell embryos from obese and control groups were determined. For day-5 fresh blastocysts and blastocysts previously vitrified on day 3, the expression profiles for all of the above genes were also determined.

Results For the fresh group, obesity significantly upregulated *Hsp70*, *p53*, *IGF-II*, and *ZO-1* expression in embryos on day 3 and notably upregulated *Atf4*, *MnSOD*, *Gadd45g*, *caspase-3*, *ZO-1*, and *E-cadherin* expression in blastocysts on day 5. For vitrified ones, obesity significantly upregulated *Atf4*, *MnSOD*,

and *Gadd45g* expression in embryos on day 3 and notably upregulated *Hsp70* expression and downregulated *MnSOD* in day 5 blastocysts previously vitrified on day 3.

Conclusions Obesity impairs fresh embryos and aggravates embryonic vitrification injury at a molecular level.

Keywords Obesity · Embryo · Blastocyst · Vitrification injury

Introduction

Human obesity is an important health concern, and many studies concerning assisted reproductive technologies (ART) have shown that obesity is associated with detrimental reproductive outcomes [1–7]. However, there are few reports on the influence of human obesity on embryonic vitrification.

Many studies have shown that increased lipid accumulation aggravates vitrification injury in porcine and bovine embryos and that delipidation by micromanipulation or the use of chemicals can significantly reduce embryonic sensitivity to chilling or cryopreservation [8–13]. However, the lipid contents of bovine and porcine oocytes are very high (63 and 161 ng/oocyte, respectively), and we cannot adequately evaluate the effects of lipid content in other species [14, 15]. Almost nothing is known about lipids in the human oocyte, except that these cells are anecdotally known to be relatively poor in lipid content. In this regard, human oocytes are considered to be similar to those of mice, which are estimated to have a total lipid content of 4 ng/oocyte [16]. It has been found that the lipid content of oocytes in mice with diet-induced obesity (DIO) was significantly higher than in a control group [17], and in our previous work, we reported that the embryos of DIO mice manifested significantly higher lipid content [18]. We can now therefore infer that human embryos would also possess higher lipid

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Capsule Obesity impairs fresh embryos and aggravates embryonic vitrification injury at a molecular level.

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contents in obese compared with normal-weight individuals and that this might aggravate cryopreservation injury.

We previously found significantly reduced developmental competence and elevated apoptosis rate in DIO mouse embryos compared to the control group [18], which is consistent with previous reports [19, 20]. However, we also demonstrated that the embryonic survival and developmental rate differences between obese and control mice were eliminated after embryos were vitrified and thawed, in contrast to the results in domestic animals [18]. A high-fat diet significantly reduced mitochondrial membrane potential in mature oocytes [17], with significant consequences for developmental competence [21–23]. Cumulus–oocyte complexes (COCs) from mice fed a high-fat diet showed increased expression of the endoplasmic reticulum (ER) stress marker genes ATF4 and GRP78, and increased apoptotic rates were observed in granulosa and cumulus cells of mice fed a high-fat diet [17]. However, the molecular changes in embryos from maternally obese mice have not been completely elucidated. We only evaluated these changes at a gross morphologic level and need to further study them at a molecular level so as to identify the mechanism(s) whereby obesity impairs fresh embryos and to clarify the differential effects on vitrification injury of lipid drop-let content within embryos from maternally obese mice.

A key feature of lipotoxicity is the perturbation of ER homeostasis known as ER stress that triggers the unfolded protein response (UPR) and disrupts protein secretion pathways. The UPR is characterized as the elevation in transcription factor 4 (Atf4), which regulates the promoters of several genes such as the ER chaperone glucose-regulated protein 78 (Grp78), heat shock protein 70 (Hsp70), and others implicated in the UPR [24, 25]. Extensive and prolonged exposure to high lipid eventually overwhelms these compensatory cascades, ultimately leading to increased expression of apoptosis-related genes such as transformation-related protein (TRP53, p53), caspase 3, and growth arrest and DNA-damage-inducible 45 gamma (Gadd45g), resulting in apoptotic cell death [26].

In the present study, the expression profile of ER stress genes (*Atf4*, *Grp78*, and *Hsp70*) and other stress-responsive, apoptotic, and developmental genes (*MnSOD*, *p53*, *Gadd45g*, *caspase-3*, *IGF-II*, *ZO-1*, and *E-cadherin*) in fresh and 3-h post-warming eight-cell embryos and in day-5 fresh blastocysts and blastocysts previously vitrified on day 3 were determined to identify the molecular changes associated with embryos exposed to large amounts of lipids.

Materials and methods

All experimental procedures were approved by the Ethics Committee of Sun Yat-sen University. All chemicals were from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA) unless specified otherwise.

All diet and all culture media used in this study and the protocol used for embryo vitrification and warming are described in our previous publication concerning embryonic vitrification injury [18].

Mice, dietary intervention, collection of physiologic data, zygote collection, and culture

The experimental design that was followed is illustrated in Fig. 1. From 5 weeks of age, female C57BL/6 mice (Laboratory Animal Services, Sun Yat-sen University, Guangzhou, China) were fed one of two diets for 7 weeks. The high-fat diet (obese group) provided 45 % fat, 20 % protein, and 35 % carbohydrate (D12451; Research Diets, Inc., New Brunswick, NJ, USA), and the matched control diet (control group) provided 10 % fat, 20 % protein, and 70 % carbohydrate (D12450B; Research Diets, Inc.). All mice were provided with food and water ad libitum. Body weight was recorded every week throughout the feeding period. Blood samples were taken from a subset of females that were fasted for 8 h ($n = 10$ per diet group) via cardiac puncture under CO₂ anesthesia. Blood was allowed to clot at room temperature and centrifuged at 4000 rpm for 10 min, and serum was removed.

Reagents necessary for the measurement of samples, method of ovulation induction, zygote acquisition, and embryo culture have been described in our previous publication [18].

On day 3, eight-cell embryos (with an even or uneven blastomere number, fragmentation ≤ 10 %, and intact zona pellucida) in each group were selected and divided into the fresh and the vitrified groups. Half of the fresh embryos were pooled separately for RNA isolation; the others were continuously cultured to blastocyst stage. The vitrified embryos were thawed 5 days later and were divided in the same way as for the fresh (non-vitrified) group. On day 5 (post-warming, 2 days later for the vitrified group), the expanded blastocysts were selected for analysis of gene expression.

Gene expression analysis by RT-PCR

First-strand complementary DNAs (cDNAs) were synthesized directly from the day-3 eight-cell embryos and day-5 expanded blastocysts using the TaqMan Gene Expression Cells-to-CT Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Each group contained 25 embryos or 15 blastocysts. The first-strand cDNA amplification was performed using the TaqMan PreAmp Master Mix Kit (Ambion, Austin, TX, USA). Quantitative real-time PCR was performed using commercially available TaqMan probes (Ambion, Austin, TX, USA) (presented in Table 1) under the following conditions: denaturation for 15 s at 95 °C, annealing, and primer template extension for 60 s at 60 °C, repeated 40 times. GAPDH was used as a validated internal control for every sample. All primers were

Fig. 1 A schematic of the experimental design

shown to have comparable amplification efficiencies against the internal control.

Relative gene expression was calculated using the $\Delta\Delta CT$ [27] method. The CT of the internal control (GAPDH) in each run was subtracted from the CT of the respective gene, followed by subtraction of the mean ΔCT value of a control sample in each run, providing the $\Delta\Delta CT$. Relative gene expression

was calculated with the formula $2^{(-\Delta\Delta CT)}$ and expressed as the fold change relative to a control sample. Three replicates for each sample were created, and the means were used for statistical analysis.

Statistical analyses

Statistical analysis was performed with SPSS 13.0 software (Cary, NC, USA). Normality was tested using a Kolmogorov–Smirnov test. Differences between dietary treatments in body weight and serum metabolic indices were determined using independent *t* tests. The values for mRNA expression were analyzed with one-way ANOVA and Tukey post hoc tests. For all analyses, $P < 0.05$ was defined as statistically significant.

Results

Metabolic index of mice fed on a high-fat diet is significantly elevated

After 7 weeks on the diets, control mice weighed 22.63 g, compared with mice on the high-fat diet, which averaged

Table 1 Assay IDs and GenBank sequence accession numbers for expression assays used for real-time RT-PCR

Gene	Assay ID ^b	GenBank sequence accession number
<i>Atf4</i>	Mm00515325_g1	NM_009716.2
<i>Grp78</i>	Mm00517690_g1	NM_001163434.1
<i>Hsp70</i>	Mm03038954_s1	NM_010478.2
<i>MnSOD</i>	Mm01313000_m1	NM_013671.3
<i>p53</i>	Mm01731290_g1	NM_001127233.1
<i>Gadd45g</i>	Mm01352550_g1	NM_011817.2
<i>caspase-3</i>	Mm01195085_m1	NM_009810.2
<i>IGF-II</i>	Mm00439564_m1	NM_001122736.1
<i>ZO-1</i>	Mm00493699_m1	NM_001122736.1
<i>E-cadherin</i>	Mm01247357_m1	NM_009864.2
<i>GAPDH</i> ^a	Mm99999915_g1	NM_008084.2

^a GAPDH was used as an internal control

^b Primer sets were TaqMan® gene expression assays

30.09 g ($P < 0.001$). Fasting serum insulin ($P < 0.001$), triglycerides ($P < 0.005$), cholesterol ($P < 0.05$), and glucose ($P < 0.005$) were all significantly increased in the obese group compared with the control group (Table 2).

Obesity impairs the ER function and elevates expression of apoptosis and developmentally related genes in fresh embryos

Fresh day-3 embryos There were no significant differences in the expression of *Atf4* and *Grp78* between the control and obese groups, except for *Hsp70*, which was significantly elevated in embryos from the obese mice. *p53*, *IGF-II*, and *ZO-1* were all significantly upregulated in the obese group compared to controls, without notable differences in *MnSOD*, *caspase-3*, *Gadd45g*, or *E-cadherin* expression (Fig. 2).

Fresh day-5 blastocysts The expression of *Atf4* was significantly upregulated in the obese group, with no changes in *Grp78* and *Hsp70* expression. *MnSOD*, *Gadd45g*, *caspase-3*, *ZO-1*, and *E-cadherin* were all significantly upregulated in the obese group compared to controls, with no significant differences in *p53* or *IGF-II* expression (Fig. 3).

Vitrification impairs the function of ER and elevates expression of apoptosis and developmentally related genes in embryos

Day-3 control embryos The majority of genes (*Atf4*, *Grp78*, *Hsp70*, *Gadd45g*, *IGF-II*, *ZO-1*, and *E-cadherin*) were significantly upregulated, with similar expression of *MnSOD*, *p53*, and *caspase-3* in the vitrified embryos compared to fresh embryos on day 3 (Fig. 2). Day-5 blastocysts: *Grp78* and *E-cadherin* genes were significantly upregulated, and *Hsp70*, *Gadd45g*, and *ZO-1* were downregulated extensively, with similar expression among *Atf4*, *MnSOD*, *p53*, *caspase-3*, and *IGF-II* in blastocysts previously vitrified compared to fresh blastocysts (Fig. 3).

Table 2 Average body weight and metabolite concentrations in control and obese female mice

	Controls	Obese	<i>P</i> value
Final body weight (g)	22.63 ± 1.47	30.09 ± 5.68	<0.001
Glucose (mmol/L)	4.62 ± 1.02	6.29 ± 1.25	<0.005
Insulin (ng/ml)	0.33 ± 0.12	0.55 ± 0.10	<0.001
Triglycerides (mmol/L)	1.13 ± 0.19	1.57 ± 0.33	<0.005
Cholesterol (mmol/L)	3.42 ± 0.81	4.96 ± 1.80	<0.05

Values are means ± SD. Body weight, $n = 45$ mice/group; plasma metabolite concentrations, $n = 10$ mice/group; independent *t* test

Day-3 embryos in the obese group For thawed embryos, *Atf4*, *Grp78*, *Hsp70*, *MnSOD*, *Gadd45g*, *IGF-II*, *ZO-1*, and *E-cadherin* genes were all significantly upregulated, with similar expression for *p53* and *caspase-3* in the vitrified embryos compared to fresh embryos on day 3 (Fig. 2). Day-5 blastocysts: *Grp78* and *Hsp70* were significantly upregulated, and *MnSOD*, *Gadd45g*, and *ZO-1* were greatly downregulated; with similar expression among *Atf4*, *p53*, *caspase-3*, and *IGF-II* in blastocysts previously vitrified compared to fresh blastocysts, *E-cadherin* showed a tendency toward an increase but did not reach significance (Fig. 3).

Obesity aggravates the vitrification damage to embryos at a molecular level

Day-3 embryos The similarities or differences in *Grp78*, *Hsp70*, *p53*, *caspase-3*, *IGF-II*, *ZO-1*, and *E-cadherin* expression between control and obese groups remained unchanged after the embryos were vitrified and warmed. Obesity upregulated the expression of *Atf4*, *MnSOD*, and *Gadd45g* significantly in the 3-h post-warming embryos on day 3 (Fig. 2). Day-5 blastocysts: The similarities or differences in the expression of *Atf4*, *Grp78*, *p53*, *Gadd45g*, *caspase-3*, *IGF-II*, and *ZO-1* between control and obese groups in fresh blastocysts remained unchanged when compared with the previously vitrified blastocysts (Fig. 3). Obesity upregulated the expression of *Hsp70* and downregulated the expression of *MnSOD* significantly in the day-5 blastocysts previously vitrified on day 3. In addition, obesity upregulated the expression of *E-cadherin*, but not as markedly as in the control group (Fig. 3).

Discussion

The effect of obesity on mouse embryos

The negative impact of obesity on reproductive success is well documented [28] and is associated with the induction of an altered ER stress [19] responses to lipotoxicity in cumulus-oocyte complexes (COCs)/oocytes/zygotes. We previously reported that embryonic developmental potential was reduced and apoptotic rate was elevated in maternal diet-induced mouse obesity. The present study now elucidates cellular mechanisms by which obesity contributes to poor embryo quality. Specifically, obesity in mice induced ER stress and promoted higher expression of stress-related genes.

The ER plays essential roles in multiple cellular processes, including the synthesis and folding of proteins that are then trafficked to the secretory pathway. ER stress activates the UPR [29], which in turn reduces protein secretion and promotes the degradation of misfolded proteins, thereby impairing embryonic developmental potential. ER stress caused by obesity is thought to be induced by an augmented

D3 Embryos

Fig. 2 Relative mRNA expression in day-3 embryos and day-5 blastocysts. Relative mRNA expression as determined by RT-PCR of various gene transcripts in day-3 fresh and 3-h post-warming eight-cell embryos. Values are mean $\pm$ SD expressed as fold change compared with

the (fresh) control group. $n = 3$ pools of embryos per group. Different superscript letters indicate significant differences by one-way ANOVA, followed by Tukey's post hoc test; $P < 0.05$

demand for protein synthesis under nutrient excess and by elevated levels of saturated free fatty acids [29], which have been shown to cause ER stress and to activate the UPR [19] along with increased expression of *Atf4*, *Grp78*, or *Hsp70* to increase ER folding capacity and expand ER membrane surface area [30].

The ER lumen stores a 1000-fold excess of Ca^{2+} compared with cytosol and, when stressed, releases Ca^{2+} , thereby evoking Ca^{2+} signaling, which then critically affects mitochondrial function [31]. ER Ca^{2+} release and subsequent Ca^{2+} uptake by mitochondria and excessive fuel (carbohydrates and fatty acids) within the obese reproductive environment result in

D5 Blastocysts

Fig. 3 Relative mRNA expression in day-5 blastocysts. Relative mRNA expression as determined by RT-PCR of various gene transcripts in fresh day-5 blastocysts and blastocysts that were previously vitrified on day 3 from control and obese mice. Values are mean $\pm$ SD expressed as fold

change compared with the (fresh) control group. $n = 3$ pools of embryos per group. Different superscript letters indicate significant differences by one-way ANOVA, followed by Tukey's post hoc test; $P < 0.05$

increasing production of reactive oxygen species, uncoupling of oxidative phosphorylation, matrix swelling, and subsequent release of various apoptotic factors, including cytochrome C and effector caspases that lead to cellular apoptosis [26, 31]. In embryos from obese mice, we found that significantly higher *MnSOD* (in day-5 blastocysts) expression levels exist in

mitochondria for the conversion of superoxide to hydrogen peroxide.

Damage to ER and mitochondria eventually induces apoptosis, upregulating the expression of *p53* (in day-3 embryos) and *Gadd45g* (in day-5 blastocysts), which are well recognized as modulators of cell cycle arrest and apoptosis.

Lastly, the expression of *caspase-3* (in day-5 blastocysts) was increased (our results), manifested by markedly delayed embryonic development and a higher apoptosis rate (our previous observations [18]).

Interestingly, we found that the expression of *IGF-II* and *ZO-1* in day-3 embryos and *ZO-1* and *E-cadherin* in day-5 blastocysts was markedly elevated in the obese group. In the preimplantation two-cell mouse embryo, IGF-II stimulates cell division and blastocyst formation [32], while ZO-1, one of the peripheral membrane proteins of tight junctions [33], is synthesized late in the four-cell stage [34] and is involved in blastocyst formation from morula by regulating accumulation of fluid and differentiation of nonpolar blastomeres to polar trophoblast cells [35]. E-cadherin, an integral membrane component of the adherens junction (first detected at early morula and blastocyst stages [36]), is necessary for the formation of the trophectoderm epithelium since E-cadherin null embryos undergo compaction but fail to form blastocysts containing trophoblast cells [37]. The upregulation of these genes is possibly activated so as to compensate for obesity-related stress that is similar to vitrification-induced stress to the embryo [38]. Although the expression of *IGF-II*, *ZO-1*, and *E-cadherin* was significantly upregulated in embryos from obese mice, their embryonic development was still notably delayed.

The effect of obesity on vitrification

We previously reported significantly reduced developmental competence in obese mouse embryos compared to controls [18]. However, we also demonstrated that the differences in embryonic survival and developmental rate between control and obese mice were eliminated after eight-cell embryos at day 3 were vitrified and thawed [18]. It appears that mouse obesity does not aggravate vitrification injury of embryos. However, in the present study, we additionally found that obesity significantly elevated the expression of *Atf4*, *MnSOD*, and *Gadd45g* in eight-cell embryos 3 h after warming, although the upregulation disappeared in day-5 blastocysts that were previously vitrified. Dramatically elevated *Hsp70* expression and downregulation of *MnSOD* were also observed.

ATF4 is one of the proteins upregulated by UPR [39]. ATF4 induces a set of genes involved in amino acid metabolism, antioxidant stress response, and protein secretion, all of which aid in homeostasis [40]. The *MnSOD* gene encodes manganese superoxide dismutase, which exists in mitochondria for the conversion of superoxide to hydrogen peroxide, and plays important roles in oxidative stress response [41]. The *Gadd45g* family of stress-inducible acidic nuclear proteins is implicated in apoptosis and DNA repair [42, 43], and the significant upregulation of the three gene members indicated that 3 h after warming, eight-cell embryos from obese mice bore more stress induced by obesity compared to their counterparts.

In the obese group, the expression of *Hsp70* was elevated nearly 17 times in day-5 blastocysts previously vitrified compared to fresh blastocysts, which was reduced almost 5.5 times in the control group. HSP70 plays an important role in the refolding of proteins that were damaged by environmental stress [44], and the induction of HSP70 protects cells from damage [45, 46]. In our study, the significant downregulation of *MnSOD* in previously vitrified day-5 blastocysts from obese mice may be caused by decompensation to stress. Superoxide dismutase (SOD) activity has been shown to diminish with increasing female age, and successful ART was associated with higher SOD activity [47]. This implies that day-5 blastocysts previously vitrified on day 3 in the obese group are still in a stressed state.

Therefore, although the embryonic survival and developmental rate differences between control and obese mice were eliminated after day-3 eight-cell embryos were vitrified and thawed [18], the obese condition itself induced organelle damage and gene expression changes; i.e., mouse obesity aggravated vitrification injury at molecular levels not reflected in morphologic changes.

The *Gadd45* family is implicated in cell cycle control and DNA repair [42, 43] and has been shown to regulate G2/M arrest in response to genotoxic stress and to maintain genomic stability. The elimination of developmental differences between embryos from control and obese groups after vitrification and warming is probably contingent upon the significant downregulation that we observed in *Gadd45g* in blastocysts that were previously vitrified. Although we did not find an increase in apoptosis-related genes such as p53 and caspase (which would decrease embryonic development), we found elevated expression in *Atf4* and *Hsp70*; the latter might be caused by a protective response due to UPR, since expression of these genes would have little effect on embryonic development. Accordingly, UPR does not always cause cell death; rather, only when the external stressor fails to withdraw is the handling capacity of the ERAD system overwhelmed by the accumulation of misfolded proteins in the ER lumen, initiating a pathologically stressful condition within the cells. In other words, after the embryos obtained from obese mice were vitrified and warmed, they could then manage the stressful condition caused by obesity.

Many studies have shown that in porcine and bovine embryos, increased lipid accumulation aggravated vitrification injury with respect to embryonic development and apoptosis [8–13], suggesting that high lipid content in embryos aggravated vitrification injury on morphologic parameters. The discordant results between mice and domestic animal species may be explained by the markedly higher lipid content in embryos from the latter, as the total lipid content per oocyte was 4 ng in mice [48], in contrast to 161 ng in pigs and 63 ng in cattle [49, 50]. Thus, lipid toxicity induced by high lipid content may be less pronounced in mouse embryos than in domestic animal embryos.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional, national, and/or international guidelines for the care and use of animals were followed.

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