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In vitro fertilization alters phospholipid profiles in mouse placenta

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Abstract

Purpose Studies on humans and rodents have clearly shown that in vitro fertilization (IVF) is associated with abnormal placenta formation and function. Currently, dysregulated placental lipid metabolism is one of the emerging pathogenetic pathways implicated in adverse pregnancy outcomes. The purpose of this study was to identify the effects of IVF on lipid metabolism in the mouse placenta.

Methods Two groups of mouse placentas, composed of control and IVF, were collected at embryonic day 18.5. Placental lipid profiles were measured using liquid chromatography coupled with mass spectrometry. The relative levels of individual lipid were examined and compared. The proteins and enzymes that regulate the phospholipid biosynthesis were also compared by western blot.

Results A significant increase in levels of phosphatidylcholines, phosphatidylethanolamines, phosphatidylinositols, phosphatidylglycerols, lysophosphatidylcholines, and mitochondrial cardiolipin were found in the IVF placenta. In addition, proteins and enzymes that regulate the phospholipid biosynthesis were also altered in IVF placentas.

Conclusions After lipidomic analysis, we present the first detailed overview of the effect of IVF on lipid metabolism, especially phospholipid profiles in the placenta in a mouse model. The widespread lipidomic shifts identified in this study might explicate some of the placental dysfunction observed after IVF, thereby illustrating that phospholipids serve as early warning biomarkers of health risks in IVF offspring.

Keywords In vitro fertilization (IVF) · Placenta · Lipidomic · Phospholipids

Introduction

Placenta is an organ that mediates maternal and fetal transport of gas, nutrients, and waste during pregnancy [1]. It plays an essential role in fetal development and maternal health, and the dysfunction increases the incidence of preeclampsia, gestational hypertension, and eclampsia in pregnant women [1, 2]. In addition, placental dysfunction affects the fetus, causing premature birth, fetal growth retardation, and neurodevelopmental abnormalities [3, 4]. Moreover, a healthy placenta is essential for the lifelong health of the mother and the offspring [5].

In vitro fertilization (IVF) is an effective therapy for infertility; however, previous studies have shown that IVF is associated with abnormal placenta formation and function. Increased placental weight, placental/fetal ratio, abnormal umbilical cord insertion, and abnormal placental shape have been observed in term singletons after IVF [6, 7]. IVF induced a thick placental barrier, decreased the apical microvilli, and increased multiple vacuoles in humans [8]. Several studies showed that IVF increases the term placental weight in mice [9, 10] thereby affecting the placental nutrient transport, resulting in lower fetal weight in mice [9]. Our previous study in mice also found that the expression levels of a majority of placental nutrient transporters were significantly downregulated in IVF-conceived placentas at different gestation ages [11]. IVF-caused abnormalities of the placenta could serve as a major factor contributing to adverse health outcomes in the mother and the offspring; however, the precise mechanisms underlying the placental dysfunction in IVF are yet to be clarified.

Phospholipids constitute a major component of the plasma membrane and the membranes of subcellular organelles in the cells. The alterations in these phospholipids influence the flexibility and fluidity and modify the functionality of transporter

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proteins in the membrane [12–14]. Studies have shown that the transport activity of transporters and channels is regulated by membrane phospholipid composition [12, 13]. The plasma membrane of the placental cells forms the barrier between the maternal and fetal blood that regulates the maternal-fetal exchange of material and organisms [15]. Phospholipids in the placental cell membranes play critical roles in regulating the protein structure and activity, especially specific transporters [16, 17]. Phospholipids also compose the physical properties of mitochondrial membranes and play major roles in several mitochondrial functions [18]. The dysregulated placental phospholipid metabolism is emerging as one of the key pathogenetic pathways implicated in adverse pregnancy outcomes [17, 19–21]. However, whether IVF affects the placental function by altering the membrane phospholipid composition is yet to be elucidated.

Thus, we hypothesized that IVF might perturb the phospholipid composition of the placental membrane, which predisposes the placental functions. In the present study, we evaluated the effect of IVF on membrane phospholipidome in the placentas of mice at E18.5. In contrast to the control placentas, IVF treatment resulted in remarkable alterations in individual phospholipids. Also, the proteins that regulate the phospholipid biosynthesis were affected; this phenomenon might be associated with placental dysfunction.

Materials and methods

Animals

Virgin 6- to 8-week-old Kunming female mice, adult Kunming males, and Kunming vasectomized males were used. All animals were provided with nesting material and housed in cages that were maintained under a constant 12-h light/dark cycle at 21–23 °C with free access to standard chow and tap water. The present study was reviewed and approved by the Ethics Committee of Animal and Medicine of the Tangdu Hospital of The Fourth Military Medical University (approval identification: TDLL-2013051) and was conducted in accordance with the guidelines from the Committee on the Use of Live Animals in Teaching and Research of the Tangdu Hospital of The Fourth Military Medical University. All efforts were made to minimize the number of animals used in the experiment and their suffering throughout the study.

Experimental design

Female mice were assigned to the IVF and control groups. The control group consisted of blastocysts that were generated by normal fertilization, flushed from the uterus, and immediately transferred into pseudopregnant females. In the IVF group, the blastocysts were obtained after in vitro fertilization and development, followed by transfer into pseudopregnant females.

Superovulation

Females were superovulated by intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin (PMSG, ProSpec-Tany TechnoGene Ltd., Ness-Ziona, Israel). After 48 h, the mice were injected with 5.0 IU human chorionic gonadotropin (hCG, Millipore, Billerica, MA, USA).

In vitro fertilization

Conventional IVF was performed using human tubal fluid (HTF, Millipore, Billerica, MA, USA) medium as described previously [11]. Briefly, the collected sperm from the cauda epididymis of adult male Kunming mice was suspended in HTF medium for at least 30 s and then placed in an incubator at 37 °C under 5.0% CO_2 and 95% humidity for 1–2 h for capacitation. The preincubated and capacitated sperm suspension was added to the freshly ovulated cumulus-oocyte complexes to obtain a concentration of $1–2 \times 10^6$/mL of motile sperm, as determined using a hemocytometer. Sperm and oocytes were co-cultured for 8 h in an insemination medium.

Embryo culture

In vitro fertilized eggs (22–23 h after hCG), as determined by the presence of two pronuclei, were cultured under optimized culture conditions (KSOM+AA; Millipore) and assessed for developmental efficiency. The embryo was cultured to blastocyst stage under mineral oil at 37 °C with 20% O_2 and 5% CO_2.

Embryo transfer recipients

Naturally ovulating Kunming 6-week-old female mice were mated with vasectomized Kunming males 3.5 days before embryo transfer. The morning after mating, the recipients were checked for the presence of a vaginal plug. The day of plugging was considered to be day 0.5 of the pseudopregnancy. Then, the blastocysts were transferred to the uterine horns ($n = 8$ per horn) of the pseudopregnant females on day 3.5 of the pseudopregnancy according to standard procedures.

Placenta dissection

Pregnant mice were euthanized by CO_2 inhalation, followed by cervical dislocation at E18.5, and the fetuses and placentas were harvested. After dissection, the placental wet weights were recorded. Subsequently, each placenta was immediately frozen in liquid nitrogen and stored at − 80 °C until lipid extraction and protein detection.

Lipid extraction

A total of five placental samples were used for lipid extraction, and to avoid the confounding of litters and selection bias, placentas were selected from five litters in each group, and the samples were selected randomly. Lipids were extracted from the whole placenta using a modified version of the Bligh and Dyer's method as described previously [22]. Briefly, the placenta was incubated in 750 μL of chloroform:methanol 1:2 (*v*/*v*) with 10% deionized water for 30 min. Subsequently, 350 μL of deionized water and 250 μL of chloroform were added. Then, the samples were centrifuged; the lower organic phase containing lipids was extracted and dried in the Speed Vac under OH mode. The samples were stored at − 80 °C until further analysis.

Lipid analyses

Normal phase LC/MS Polar lipids were analyzed using an Exion UPLC system coupled with a triple quadrupole/ion trap mass spectrometer (QTRAP 6500 Plus; SCIEX) as described previously [23]. The individual lipid classes of polar lipids were separated by normal phase (NP)-HPLC that was carried out using a Phenomenex Luna 3 μm silica column (internal diameter 150 × 2.0 mm^2) with the following conditions: mobile phase A (chloroform:methanol:ammonium hydroxide, 89.5:10:0.5) and mobile phase B (chloroform:methanol:ammonium hydroxide:water, 55:39:0.5:5.5). MRM transitions were set up for comparative analysis of various polar lipids. Individual lipid species were quantified using spiked internal standards as references. PC-14:0/14:0, PE-14:0/14:0, PS34:1-d31, PA-17:0/17:0, PG-14:0/14:0, Cer d18:1/17:0, SM d18:1/12:0, GluCer d18:1/8:0, LacCer d18:1/8:0, and Sph d17:1 were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Dioctanoyl phosphatidylinositol (PI) (16:0-PI) was obtained from Echelon Biosciences. Gb3-C17:0 was obtained from Matreya LCC, and GM3 d18:1/17:0 was synthesized in-house.

Reverse-phase LC/MS Glycerol lipids (DAG, TAG) were analyzed using a modified version of reverse-phase HPLC/ESI/MS/MS as described previously [24]. Briefly, the lipids were separated on a Phenomenex Kinetex 2.6 μm C18 column (i.d. 4.6 × 100 mm^2) using an isocratic mobile phase chloroform:methanol:0.1 M ammonium acetate (100:100:4) at a flow rate of 160 μL/min for 22 min. Using neutral loss-based MS/MS techniques, the levels of TAG were calculated as relative content to the spiked d5-TAG 42:0, d5-TAG 48:0, and d5-TAG 54:0 internal standards (CDN isotopes), while DAG species were quantified using d5-DAG (16:0/16:0) and d5-DAG (18:1/18:1) as internal standards (Avanti Polar Lipids).

Atmospheric pressure chemical ionization Free cholesterols and total cholesteryl esters were analyzed using HPLC/APCI/MS/MS as described previously; d6-Cho and d6-CE-18:1 (CDN isotopes) were used as internal standards [25].

Western blot analysis

Three placentas from three different pregnant mice from each experimental group were harvested ($n = 9$ per group), homogenized, and suspended in cold RIPA lysis and extraction buffer (Thermo Scientific). An equivalent of 10 μg protein extract was resolved by SDS-PAGE (12% gel) and transferred to polyvinylidene difluoride membrane (PVDF). Primary antibodies, such as anti-CTα (Abcam, ab109263), anti-CLS (Abcam, ab156882), anti-tafazzin (Abcam, ab105104), anti-SREBP1 (Abcam, ab28481), anti-SREBP2 (Abcam, ab30682), anti-pan-Akt (Abcam, ab8805), and anti-pan-Akt (phospho T308) (Abcam, ab38449), were used to determine the expression levels of the selected proteins in placental tissue. The immunoreactive signal was detected using the ECL western blotting substrate (Pierce™, 32,106) followed by autoradiography, and densitometric analysis was conducted using Image J software (http://rsbweb.nih.gov/ij/).

Mitochondrial copy number

Mitochondria DNA copy number was quantified by real-time quantitative RT-PCR as previously described [26]. Cytochrome b (Cytb) and β-actin are markers of mitochondrial and genomic DNA, respectively. Placental genomic DNA was isolated using DNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Both markers were quantified by real-time PCR using the SYBR Green PCR master mix (Takara Bio) with gene specific primers.

Cytb left primer: 5′-gcaaccttgacccgattcttcgc-3′, the right primer: 5′-tgaacgattgctagg gccgcg-3′, β-actin left primer: 5′-ggactcctatgtgggtgacg-3′, the right primer: 5′-aggtgtggtgccagat cttc-3′.

Statistical analyses

Data were expressed as mean ± SEM, and D'Agostino and Pearson omnibus normality test revealed the normal distribution of the data. For the fetal weight and placental weight data, we reanalyzed the associating data in unit of the litter; statistics was calculated using the mean value for each foster mother, and therefore, "*n*" represents the number of litters [27]. The differences between the controls and IVF groups were determined statistically using Student's *t* test. All the analyses were performed using SPSS. The least significant difference (LSD) post hoc test examined any significant difference between the groups. Results were considered to be statistically significant if $P < 0.05$. For all analyses, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

Individual membrane phospholipids were altered significantly in IVF placentas

In this study, 696 pronuclei were collected in the IVF group; the 2-cell rate was 92.28 ± 2.71%, the 4-cell rate was 86.88 ± 1.11%, and the 8-cell rate was 84.42 ± 0.73%, and the blastocyst rate was 79.67 ± 0.69%. We did not found any differences in embryo implantation rate between the two groups (0.713 ± 0.027 vs. 0.700 ± 0.071, $P > 0.05$; five litters, respectively) as we described in previous study [11]. In addition, the fetal development rate after implantation (the number of viable fetuses/the total number of implantation sites) was significantly higher in the control group than in the IVF group (0.841 ± 0.077 vs. 0.685 ± 0.091, $P < 0.05$; five litters, respectively), suggesting that a large number of embryos died at an early stage after implantation in the IVF group [11]. The fetal weight in the IVF groups (1517 ± 130 mg, $n = 39$, five litters) was significantly lower ($P < 0.01$) than that in the control group (1664 ± 90 mg, $n = 48$, five litters) at E18.5 (Supplemental Table 1); however, the weight of the IVF placentas (158 ± 27 mg, $n = 39$, five litters) was significantly heavier ($P < 0.01$) than those of the control placentas (117 ± 20 mg, $n = 48$, five litters; Supplemental Table 1) which were consistent with the previous study [11].

A total of 320 lipid species from 18 individual lipid classes were detected and analyzed by lipidomics (Table 1). Cholesteryl esters (CE), triacylglycerols (TAG), and diacylglycerols (DAG) are neutral lipids used for energy storage. Cholesterols (Cho), glycerophospholipids, and sphingolipids are the primary structural components of the cellular plasma membrane and the intracellular membranes of organelles. In this study, the glycerophospholipids that were detected and analyzed included phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylinositols (PI), phosphatidylglycerols (PG), phosphatidic acids (PA), lysobisphosphatidic acids (LBPA), lysophosphatidylcholine (LPC), lysophosphatidylinositols (LPI), and cardiolipins (CL), while the sphingolipids included sphingomyelins (SM), ceramides (Cer), glucosylceramides (GluCers), and monosialodihexosyl gangliosides (GM3).

Furthermore, the levels of the individual classes of membrane lipids were compared in the control and IVF placentas. The IVF-related changes in the 18 individual lipid classes were examined and summarized in Table 1. The levels of two major neutral lipids, TAG and CE, were significantly reduced in the IVF placenta ($P < 0.001$ and $P < 0.001$, respectively); however, a significant increase in the level of several classes of glycerophospholipids, including PE ($P < 0.001$), PC ($P < 0.01$), PG ($P < 0.01$), PI ($P < 0.001$), LysoPC ($P < 0.05$), and CL ($P < 0.001$) was observed in the IVF placentas (Table 1). The relative levels of total LPI and total LBPA were decreased significantly ($P < 0.05$) in the IVF group as compared to the control group. In the case of sphingolipids, the levels of SM, Cer, GluCer, and GM3 were not altered in the IVF placentas than those in the control group.

Table 1 Relative levels of individual membrane lipid classes in placentas

Main class (common name)	Control ($n = 5$) μmol/g fresh mass	IVF ($n = 5$) μmol/g fresh mass
Cho	5.102 ± 0.485	4.995 ± 0.119
CE	0.895 ± 0.017	0.799 ± 0.026***
TAG	0.899 ± 0.096	0.630 ± 0.027***
DAG	0.412 ± 0.109	0.418 ± 0.099
PC	6.074 ± 0.475	7.370 ± 0.459**
PE	2.207 ± 0.148	2.654 ± 0.078***
PS	0.635 ± 0.046	0.623 ± 0.037
PI	0.487 ± 0.087	0.786 ± 0.064***
PG	0.060 ± 0.002	0.065 ± 0.002**
PA	0.022 ± 0.002	0.022 ± 0.002
LBPA	0.031 ± 0.003	0.027 ± 0.002*
LysoPC	0.940 ± 0.102	1.200 ± 0.146*
LPI	0.069 ± 0.006	0.052 ± 0.007**
CL	0.048 ± 0.006	0.069 ± 0.005***
SM	1.455 ± 0.223	1.236 ± 0.112
Cer	0.114 ± 0.011	0.105 ± 0.001
GluCer	0.114 ± 0.024	0.084 ± 0.018
GM3	0.005 ± 0.001	0.004 ± 0.000

Data are presented as mean ± SD

Cho free cholesterols, *CE* cholesteryl esters, *TAG* triacylglycerols, *DAG* diacylglycerols, *PC* phosphatidylcholines, *PE* phosphatidylethanolamines, *PS* phosphatidylserines, *PI* phosphatidylinositols, *PG* phosphatidylglycerols, *PA* phosphatidic acids, *CL* cardiolipins, *LBPA* lysobisphosphatidic acids, *LysoPC* lysophosphatidylcholine, *LPI* lysophosphatidylinositols, *SM* sphingomyelins, *Cer* ceramides, *GluCer* glucosylceramides, *GM3* monosialodihexosyl gangliosides

Significant at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (by Student's *t* test)

Overall increase in phosphatidylcholine species in the mouse placentas generated by IVF

PC is a class of the major glycerophospholipid components of eukaryotic cellular membranes. It plays a major role as a precursor of signaling molecules and as a key element of lipoproteins. A total of 35 molecular species of PC were detected; of these, the level of 23 increased significantly in the IVF group as compared to that in the control group ($P < 0.05$) (Fig. 1). Only two PC species, PC34:0p and PC40:7 were significantly decreased ($P < 0.05$) in the IVF group than in the control group (Fig. 1).

Enhanced phosphatidylethanolamines levels in the mouse placentas generated by IVF

In this study, 31 molecular PE species were detected, and our analysis demonstrated that IVF could significantly alter the PE

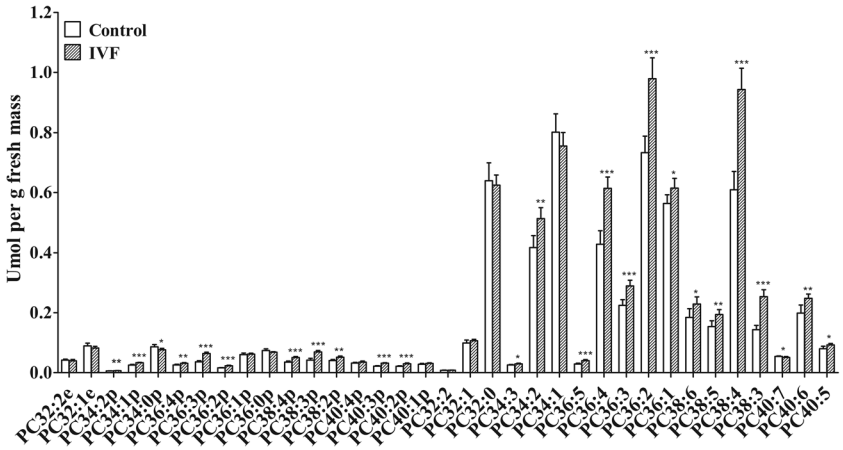

Fig. 1 Relative levels of individual phosphatidylcholine species in the in vitro fertilization (IVF) and control placentas. Significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (by *t* test). Error bars show standard deviation

levels in the placenta as compared to that in the control group. 15/31 PE species were at higher concentrations in the IVF group than that in the control group ($P < 0.05$) (Fig. 2). However, the level of PE42:1p, the only PE species, was lower in IVF than that in the control group (Fig. 2) ($P < 0.05$).

Increased placental mitochondrial glycerophospholipids in the IVF mice

CL is found in the inner mitochondrial membrane, constituting about 20% of the total lipid composition [28, 29]. In this study, we demonstrated a significant increase ($P < 0.001$) in the total amount of CL in the IVF placentas than that in the control group. The concentrations of individual CL species in the placentas of the IVF and control groups were compared, and the amounts of all seven CL species were found to be significantly increased in the IVF group as compared to that in the control group ($P < 0.05$) (Fig. 3a).

PG is a precursor of CL biosynthesis. Moreover, PG species also play a vital role in the maintenance of mitochondrial structure and function. The current study demonstrated significantly increased levels of PG34:1, PG36:2, and PG36:1 species in the IVF group as compared to that in the control group ($P < 0.05$) (Fig. 3b); however, the level of PG38:5 was significantly reduced in the IVF group ($P < 0.05$).

Furthermore, the increase in the total amount of CL in the IVF placentas might be attributed to the increase in the mitochondria copy in the placental cell or higher CL content in the mitochondria. Next, we evaluated the number of mitochondrial DNA vs. nuclear genome number ratio and measured mitochondrial DNA content in placentas as Mt/N. However, the Mt/N ratio was not altered by IVF ($P > 0.05$) (Fig. 3c), thereby suggesting that the number of mitochondria copies in the placental cell may not be affected by IVF.

Altered levels of other glycerophospholipid classes in the IVF placentas

In addition, we also determined the amounts of other glycerophospholipids classes (PA, PI, and PS). Similar to PC, these glycerophospholipids play an essential role in membrane architecture, cell division, and function and serve as the depot of lipid second messengers.

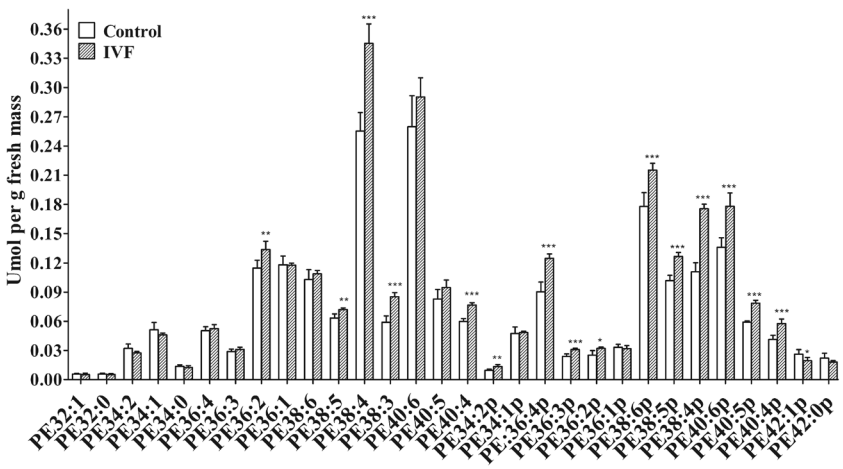

Fig. 2 Relative levels of individual phosphatidylethanolamine species in the in vitro fertilization (IVF) and control placentas. Significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (by *t* test). Error bars show standard deviation

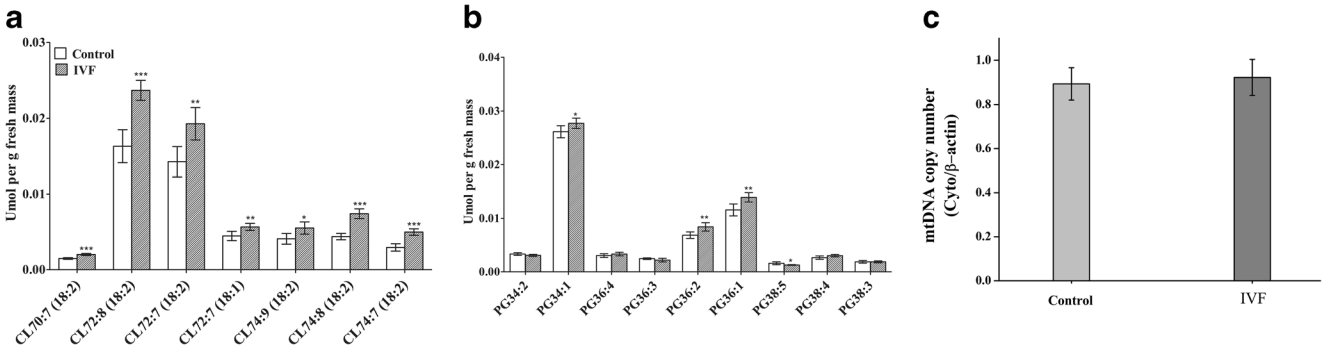

Fig. 3 Relative levels of individual cardiolipins and phosphatidylglycerol species in the in vitro fertilization (IVF) and control placentas. **a** Altered levels of individual cardiolipin species. **b** Altered levels of each phosphatidylglycerol species. Significance level was indicated at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (by *t* test). Error bars showed standard deviation

Contrary to the overall increase in the phospholipid classes, the levels of all PA species in the IVF group were not altered as compared to those in the control group ($P \geq 0.05$); however, IVF could lead to significant alterations in the levels of some PA species in the IVF placenta as compared to those in the control group. The levels of PA36:2 and PA36:1 were significantly increased in the placentas of the IVF group as compared to those in the control group ($P < 0.05$) (Fig. 4a), while that of PA32:0, PA34:2, and PA34:1 decreased significantly ($P < 0.05$) (Fig. 4a).

Although the concentrations of total PS did not alter in the samples from the IVF placentas, the levels of PS34:1, PS36:1, PS38:5, PS40:7, and PS40:5 were reduced significantly in the placentas of the IVF group as compared to those of the control group ($P < 0.05$) (Fig. 4b); however, the levels of PS36:2 and PS38:3 increased significantly in the IVF group ($P < 0.01$) (Fig. 4b).

Furthermore, different groups revealed different levels of placental PI species. Specifically, the analysis demonstrated a predominant PI38:4 species, which constituted approximately 50% of total PI in the placental samples. The levels of 7/13 PI species were elevated; however, that of only two species were reduced in the IVF placentas ($P < 0.05$) (Fig. 4c). Notably, PI38:4 was also predominantly elevated in the IVF placentas with 1.87-fold increase relative to the control placentas (Fig. 4c).

Changes in lysophospholipids in the IVF placentas

Lysophospholipids are derived from phospholipids by the selective loss of one fatty acyl residue induced by enzymes and/or reactive oxygen species. Lysophosphatidylcholine (LysoPC), lysobisphosphatidic acid (LBPA), and lysophosphatidylinositol (LPI) are the most prominent lysoglycerophospholipids and speculated to play a major role in health and diseases. Notably, individual species of LysoPC, LBPA, and LPI in the placentas were altered by IVF. The level of LysoPC was increased significantly, while the levels of LBPA and LPI were reduced significantly in the IVF placentas as compared to those in control. The levels of 3/8 LysoPC species were increased significantly in the IVF placentas than that in the

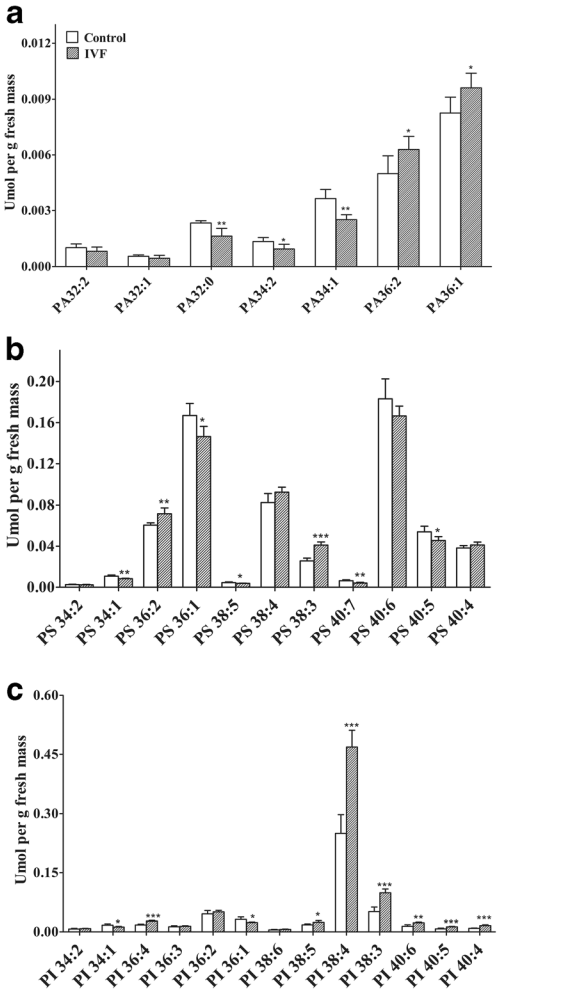

Fig. 4 Relative levels of individual phosphatidic acid, phosphatidylserine, and phosphatidylinositol species in the in vitro fertilization (IVF) and control placentas. Altered levels of phosphatidic acids (**a**), phosphatidylserines (**b**), and phosphatidylinositols (**c**) between the two groups were examined. Significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (by *t* test). Error bars show standard deviation

control group ($P < 0.05$) (Fig. 5a). On the other hand, the IVF placentas displayed a remarkable reduction in the levels of LBPA34:1, LBPA36:3, LBPA36:2, and LBPA36:1 ($P < 0.05$) (Fig. 5b). In addition, the levels of LPI16:0 and LPI18:0 were reduced significantly in the IVF group as compared to those in the control group ($P < 0.05$) (Fig. 5c).

IVF reduced the expression levels of proteins that regulate phospholipid biosynthesis

The family of sterol regulatory element-binding proteins (SREBPs) binds to sterol regulatory element (SRE) and E box sequences that are detected in the promoter regions of genes involved in cholesterol and fatty acid biosynthesis [30]. Previous studies have shown that SREBP1 preferentially regulates the genes involved in fatty acid biosynthesis, while SREBP2 primarily regulates the genes in the cholesterol pathway [30]. Interestingly, the levels of SREBP1 protein were increased significantly in the IVF group as compared to the control group ($P < 0.05$) (Fig. 6a, b). However, the expression level of SREBP2 was reduced significantly in the IVF group than in the control group ($P < 0.05$) (Fig. 6a, b). The serine-threonine kinase Akt (PKB/Akt) is a downstream effector of phosphatidylinositol signaling and has been found to be involved in cell proliferation and growth [31]. Akt activation serves as a switch for cellular signaling pathways by generating a multitude of intracellular responses via downstream targets and interacting partners [31]. In the present study, we found that IVF resulted in a significant increase in the phosphorylation levels of Akt (P-Akt) ($P < 0.05$); however, the levels of Akt proteins were not altered (Fig. 6a, b). These results indicated that the Akt signaling pathway in placentas after IVF was activated abnormally.

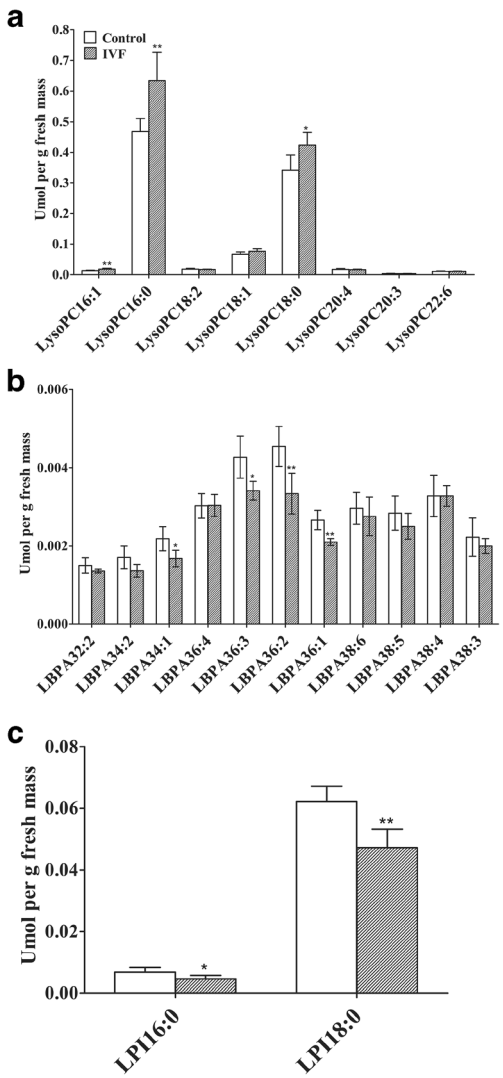

Fig. 5 Relative levels of individual lysophospholipid species in the in vitro fertilization (IVF) and control placentas. Altered levels of lysophosphatidylcholines (**a**), lysobisphosphatidic acids (**b**), and lysophosphatidylinositols (**c**) between the two groups were examined. Significance was indicated at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (by *t* test). Error bars show standard deviation

CTP, phosphocholine cytidylyltransferase (CTα), is a key enzyme in the CDP-choline or Kennedy pathway for de novo phosphatidylcholine biosynthesis [32]. Herein, we found that the expression level of CTα was increased significantly in the IVF placentas ($P < 0.05$) (Fig. 6c, d) and may account for the significant increase in the phosphatidylcholines levels in the IVF placentas. CL might be synthesized on the inner mitochondrial membrane by cardiolipin synthase (CLS) and tafazzin [33]. Consequently, we found that the levels of CLS and tafazzin protein were significantly increased in the IVF group as compared to the control group ($P < 0.05$) (Fig. 6c, d), which might account for the significant increase in CL levels in the IVF placentas.

Discussion

In this study, we investigated the effects of IVF on placental phospholipids at a molecular level in a mouse model. The levels of two major neutral lipids, TAG and CE, were downregulated and that of the phospholipids, including PC, PE, PI, PG, CL, and LysoPC and were increased in the placentas of mice at E18.5 as a consequence of IVF. In addition, the levels of proteins and enzymes that regulated the phospholipid biosynthesis were also altered in IVF placentas. Dysregulated placental lipid metabolism is emerging as one of the key pathogenetic pathways implicated in adverse pregnancy outcomes [17, 19].

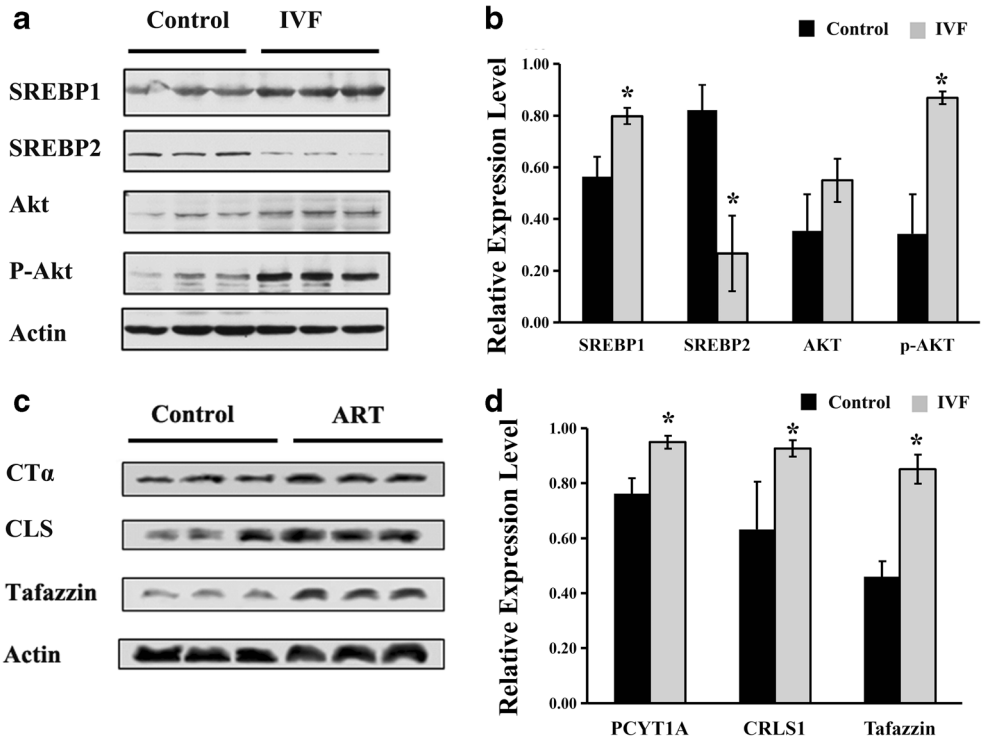

Fig. 6 ART reduced the expression levels of proteins that regulate phospholipid biosynthesis. **a**–**b** Effects of IVF on SREBP1, SREBP2, AKT, and phosphorylation of AKT protein levels were assessed in placentas. c–d Effects of IVF on CTα, CLS, and tafazzin protein levels were assessed in placentas

IVF manipulations might disrupt normal biological processes because the embryo is exposed to physical conditions that are not encountered in vivo [34]. Notably, studies in humans demonstrated an increased risk of pregnancy complications, including spontaneous abortion, premature birth, low birth weight, preeclampsia, placenta previa, and placental abruption, after IVF [35]. Accumulating evidence also suggested that IVF children might have an increased risk for developing metabolic and cardiovascular diseases [36, 37]. The placenta plays an essential role in fetal development and maternal health. Placental dysfunction not only increases the incidence of complications during pregnancy but also increases the risk to health in the mother and offspring throughout the life [5, 7]. Some studies reported that IVF led to morphologic, molecular, or functional changes in the placenta [7–9]. Our previous studies also found that IVF procedures increased the morphological abnormalities and epigenetic perturbations in the placenta [11].

The regulation of lipid metabolism is an essential component of reproduction [38]. Lipids are vital signaling molecules involved in the regulatory mechanisms underlying oocyte maturation and competence acquisition [39]. Fayezi et al. reported that the increased content of phospholipid in the follicular fluid was significantly and negatively correlated with the fertility rate [40]. In mammalian embryos, lipid metabolism also appears to be essential for the pre-implantation development [41]. Lipids also play a critical role in endometrial receptivity and embryo implantation [42]. The elevated levels of maternal lipids are associated with preeclampsia, preterm delivery, and large-for-gestational-age infants [43]. Lipid metabolism also regulated the placental function, and abnormalities in the lipid composition in the placentas were found in pathogenic placentas. Previous studies demonstrated an increase in the total phospholipid content as well as high neutral lipid content in placentas of pre-eclamptic women [21, 44]. The decline in the neutral lipid and an increase in the phospholipid content in placentas were associated with restricted fetal intrauterine growth [21]. In this study, we found reduced neutral lipids and increased levels of phospholipids in the IVF placentas. Interestingly, these results were similar to those wherein placentas were found to be associated with fetal intrauterine growth restriction. This phenomenon suggested that a decline in the placental neutral lipid/phospholipid ratio might affect the transfer of the nutrients to the fetus and resulted in low birth weight; these incidences are elevated in the case of conception by assisted reproductive technology [45]. Moessinger et al. demonstrated that the activity of the Kennedy pathway regulates the balance between phospholipids and neutral lipids [36]. In this study, we found that the expression level of CTα, the key enzyme in the Kennedy pathway, was significantly increased in the IVF placentas, which might account for the reduced neutral lipid/phospholipid ratio of IVF placentas.

As a vital component of the cell membrane and the membranes of subcellular organelles, phospholipids play critical roles in regulating the structure and activity of the transporters and channels [13, 46]. PC and PE are the most abundant phospholipids in all mammalian cell membranes and play critical roles in energy metabolism. The changes in the levels of PC and/or PE in various tissues are implicated in metabolic

disorders, such as atherosclerosis, insulin resistance, and obesity [47]. In the current study, the relative levels of both PC and PE were found to be significantly increased in the IVF placentas. CL is localized in the inner mitochondrial membrane, and it is essential for optimal mitochondrial function [28]. Nonetheless, the alterations in the content and/or molecular species of CL are associated with mitochondrial dysfunction in multiple tissues and pathological conditions [28]. An increased level of CLs in the mitochondria results in reduced efficiency of ATP synthesis [29, 48]. Moreover, in the present study, the content of total and individual CL was increased in the IVF placentas, which might be attributed to the increase in the content of mitochondrial CLs or an increase in the copy of placental mitochondria. The relative number of mitochondrial DNA was not increased in IVF placentas, indicating that IVF might not affect the level of placental mitochondria. Emerging evidence demonstrates that ART affects the mitochondrial function of oocyte and pre-implantation embryo, including mitochondrial activity, mitochondrial copy number, membrane potential, and ATP content, thereby influencing the outcomes of IVF [49]. Whether the dysfunction of mitochondria caused by ART in the oocyte and pre-implantation embryo persisted in placentas until the late stage of pregnancy necessitates further exploration. Phosphatidylinositols play a vital role as precursors for phosphorylated derivatives [50]. The phosphatidylinositols can be phosphorylated to form phosphoinositides, which constitute about 10–15% of the membrane phospholipids [51]. The phosphoinositides interact specifically with proteins and are involved in the control of intracellular signaling, vesicular trafficking, and cytoskeleton dynamics [51]. Herein, the level of phosphatidylinositols was significantly increased in the IVF placentas, in turn, affecting the membrane trafficking and lipid signaling pathways in the placenta. Our previous study also showed that IVF affected the composition of phospholipids in E18.5 fetal livers [52]. Therefore, the changes in the levels of phospholipids in fetal liver may be caused by the distorted levels of placental phospholipids due to IVF.

SREBPs, including SREBP1 and SREBP2, can transcriptionally activate a cascade of enzymes required for endogenous cholesterol, triglyceride (TG), and phospholipids synthesis [53]. Thus, SREBPs are considered as master regulators of lipogenesis and cholesterogenesis [54]. Some studies have shown SREBP1 and SREBP2 have overlapping but distinct functions. SREBP1 preferentially regulates the genes involved in fatty acid biosynthesis, while SREBP2 regulates the genes in the cholesterol pathway [55, 56]. SREBP1 is activated in Akt signaling-dependent pathway, and the mammalian target of rapamycin (mTOR) kinase is the major Akt downstream effector [30]. Akt mainly regulates SERBP1 by two-way pathways: first, the activation of SREBP1 induces the expression of enzymes involved in lipid biosynthesis and second, SREBP1 is stabilized by inhibition of GSK3 [30]. Furthermore, the accumulation of intracellular lipids including phosphoglycerates has been found by the activated Akt-SREBP1 pathway [30]. In the present study, the levels of phosphorylated Akt (p-Akt) but not total Akt protein levels were increased significantly in the IVF placentas. In addition, the expression level of SREBP1 was increased significantly in the IVF placenta that might be ascribed to the IVF-increased levels of phospholipids, including PC, PE, PI, PG, CL, and LysoPC, in the placentas of mice at E18.5. However, the expression of SREBP2 was reduced significantly, which may account for the reduced levels of cholesteryl esters. PC is a major phospholipid component of the membranes, as well as the biosynthetic precursor to other membrane phospholipids, including sphingomyelin, PE, and PS [57]. The phosphocholine cytidylyltransferase (CT) is a key and rate-limiting enzyme in the Kennedy pathway for the synthesis of phosphatidylcholine [57]. Moreover, the expression of CTα was increased significantly in the IVF placentas, which might account for the significant increase in PC levels. Increased expression levels of CLS and tafazzin may account for the significant increase in de novo CL biosynthesis in the IVF placentas.

Nevertheless, after lipidomic analysis, we present the first detailed overview of the effect of IVF on phospholipid profiles in the placenta in a mouse model. The levels of PC, PE, PI, PG, CL, and lysophospholipids in placentas were significantly affected by IVF, with further functional implications. The dysregulated phospholipid metabolism might affect the biological pathways resulting in dysfunction in IVF placentas. The widespread lipidomic shifts identified in this study might explicate some of the placental dysfunction observed after IVF, thereby illustrating that phospholipids serve as early warning biomarkers of health risks in IVF offspring. Thus, further studies are essential for investigating the roles of differentially expressed phospholipids in the pathophysiology of adverse pregnancies associated with assisted reproduction.

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Compliance with ethical standards

The present study was reviewed and approved by the Ethics Committee of Animal and Medicine of the Tangdu Hospital of The Fourth Military Medical University (approval identification: TDLL-2013051) and was conducted in accordance with the guidelines from the Committee on the Use of Live Animals in Teaching and Research of the Tangdu Hospital of The Fourth Military Medical University.

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

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