Effects of palmitic acid and eicosapentaenoic acid on angiogenesis of porcine vascular endothelial cells

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Abstract
Restricted placental angiogenesis is an important cause of intrauterine growth retardation in piglets. During pregnancy, sow obesity can result in an increase in placental lipid deposition, subsequently inhibiting placental angiogenesis and fetal development. However, the effect of different types of fatty acids on placental angiogenesis is still unclear. Trophoblast cells and vascular endothelial cells constitute two important types of placental tissue. In this study, we used palmitic acid (C16:0) and eicosapentaenoic acid (C20:5, n-3), respectively, to treat porcine trophoblast cells (pTr2) and porcine iliac artery endothelial cells (PIEC) to study the effects of saturated fatty acids and n-3 polyunsaturated fatty acids (PUFAs) on placental angiogenesis in vitro. We found that C16:0 caused significant cytotoxicity in pTr2 and PIEC (p < 0.01) and inhibited the proliferation and migration of PIEC (p < 0.01), whereas C20:5 treatment exhibited very low cytotoxicity and minimal inhibition of cellular proliferation. Meanwhile, a low concentration of C16:0 had no effect on the tube formation in PIEC, whereas C20:5 significantly promoted tube formation of PIEC (p < 0.01). These results suggested that saturated fatty acids and n-3 PUFAs had different effects on placental angiogenesis. As essential functional fatty acid, n-3 PUFA might be effective measure in alleviating the placental lipotoxicity caused by sow obesity during pregnancy.

KEYWORDS
eicosapentaenoic acid, palmitic acid, placental angiogenesis, sow

1 INTRODUCTION

The placenta is a highly vascularized tissue that provides all the nutrients necessary for fetal growth (Meher et al., 2015, Saben et al., 2014). During pregnancy, placental angiogenesis is critical to enable the effective exchange of nutrients and waste between mother and fetus (Peng et al., 2019, Peng et al., 2019). Similar to other tissues, placental angiogenesis could be divided into the following steps: endothelial cell proliferation and migration, tube formation, and vessel elongation and maturation (Chen et al., 2017, Kaufmann et al., 2004). Recent studies have shown that sow obesity during pregnancy promotes ectopic deposition of lipids into the placenta, which results in increased placental lipotoxicity and inhibition of placental angiogenesis and fetal development (Calabuig-Navarro et al., 2017, Hastie & Lappas, 2014). Sows possess higher levels of triglycerides (TG) and nonesterified fatty acids (NEFA) in the placenta with high backfat during pregnancy compared with normal backfat thickness (Zhou et al., 2018). Moreover, backfat thickness and placental lipid concentration are known to be positively...
correlated with the placental inflammation and oxidative stress but negatively correlated with birth weight, litter birth weight, and weaned piglet weight (Zhou et al., 2018, Zhou et al., 2019). Therefore, reducing lipotoxicity might be an effective means to promote placental angiogenesis and fetal development.

Saturated fatty acids are known to be a critical source of lipotoxicity because they can increase the production of lipotoxic products, such as ceramide and diacylglycerol, causing mitochondrial dysfunction, oxidative stress, inflammation, and apoptosis (Chaurasia & Summers, 2015, Law et al., 2017, Senkal et al., 2017). Palmitic acid (C16:0) is the saturated fatty acids with the highest content in land animal and vegetable oils (Glencross, 2009). Meanwhile, C16:0 is also the most common saturated fatty acid accounting for 20%–30% of total fatty acids in the human body (Carta et al., 2017). However, in presence of obesity or other conditions, the mechanisms to maintain a steady state of C16:0 concentration may be disrupted leading to an over accumulation of tissue C16:0 resulting in dyslipidemia, hyperglycemia, increased ectopic fat accumulation, and increased inflammatory tone (Carta et al., 2017). Previous studies in humans have found that C16:0 induced mitochondrial damage and suppressed catepsarin activity, thus inhibiting angiogenesis (Yuan et al., 2017, Zhang et al., 2017).

n-3 polyunsaturated fatty acids (n-3 PUFAs) are essential fatty acids for mammal and can be selectively transferred to placental tissue. Because the first unsaturated bond appears in the third position of the methyl end of the carbon chain, n-3 PUFAs are also called ω-3 PUFAs (Greenberg et al., 2008, Larqué et al., 2011, Rosero et al., 2016). Recent studies showed that eicosapentaenoic acid (C20:5, n-3) and docosahexaenoic acid (C22:6, n-3) derived from deep-sea fish or seaweed oils promoted angiogenic factors in the human early pregnancy placental villi trophectoderm cell line (HTR8/SVneo) as well as mice placental angiogenesis (Basak & Duttaroy, 2013, Peng et al., 2019). These studies suggested that saturated fatty acids and n-3 PUFAs may have different effects on sow placental angiogenesis. Thus, here, we used C16:0 and C20:5, respectively, to treat porcine trophectoderm cells (pTr2) and porcine iliac artery endothelial cells (PIEC) to study the effects of saturated fatty acids and n-3 PUFAs on placental angiogenesis in vitro.

2 | MATERIALS AND METHODS

2.1 | Cell culture

PIEC (a mature commercial cell, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, China; No: GNO15) was derived spontaneously from a porcine iliac artery endothelial cell culture (Zhang et al., 2018). PIEC were cultured in RPMI 1640 medium (Gibco, 12633012, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, 26170043, USA) and 1% penicillin/streptomycin solution (TransGen Biotech, FG101-01, China) at 37°C in 5% CO₂ incubator (Peng et al., 2020, Zhang et al., 2016). The porcine trophectoderm cells (pTr2) were gifted by the Guangdong Academy of Agricultural Sciences and cultured in DMEM-F12 (Gibco, 31331093, USA) supplemented with 5% FBS, 2 mm glutamine (Sigma, G8540, China), 0.1 U/ml bovine insulin (Sigma, I-035, China), and 1% penicillin/streptomycin solution (Bazer & Johnson, 2014, Kong et al., 2012).

2.2 | Fatty acids solution

The C16:0 (derived from vegetable) (purity ≥ 99%; Sigma, P5585, China) and C20:5 (derived from fish oil) (purity ≥ 98.5%; Sigma, 44864, China) solutions were prepared following the method of Elsner et al. (Elsner et al., 2011, Plotz et al., 2016). Briefly, a 50 mM stock solution was prepared for both C16:0 and C20:5 using 90% ethanol, and heated at 60°C for 5 mins. Then, fatty acids were bound to 2% fatty acid-free bovine serum albumin (BSA) (Aladdin, B265986, China) in RPMI 1640 culture medium supplemented with 1% FBS. The final BSA:fatty acids ratio was 2% BSA:1 mM fatty acids. Before treatment with pTr2 and PIEC, 1 mM fatty acids were diluted to the respective concentrations in serum-free basic RPMI 1640 medium.

2.3 | Cytotoxicity assay

Cellular cytotoxicity was detected using the LDH cytotoxicity assay kit (Beyotime, C0017, China). Both PIEC and pTr2 were seeded at a density of 10⁵ cells/well in 96-well plates (n = 5/group). After reaching 70% confluence, PIEC and pTr2 were treated with 150 μl of different concentrations of C16:0 and C20:5 (0, 25, 50, 100, 200, and 400 μM) for 24 h. Then, the cell culture supernatant (120 μl) was aspirated for the assay. The absorbance was measured at 490 nm using a plate reader.

2.4 | Cell proliferation assay

The cell viability of the PIEC after treatment with C16:0 and C20:5 was tested using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Beyotime, ST316, China). PIEC and pTr2 were seeded at a density of 10⁴ cells/well in 96-well plates (n = 5/group). After reaching 50% confluence, PIEC and pTr2 were treated with 100 μl of different concentrations of C16:0 and C20:5 (0, 25, 50, 100, 200, and 400 μM) for 24 h. Next, 10 μl of the MTT reagent (5 mg/ml in phosphate-buffered saline) was added to each well and incubated for another 4 h. After discarding the culture media, 150 μl of DMSO was added to each well and vibrated for 10 min to facilitate dissolution. The absorbance was measured at 490 nm using a plate reader.

2.5 | Wound-healing assay

The wound-healing assay in PIEC was performed following the method of Wang et al. (2017). Briefly, 80% confluent PIEC were placed in 12-well plates with serum-free basic medium for 24 h, followed by treatment with different concentrations of C16:0 or C20:5 (0, 12.5, and 25 μM) for 24 h (n = 6/group). Next, the confluent cell layer was
scratched with a 10-μl pipette tip, washed with PBS, and treated with serum-free basic RPMI 1640 medium for another 24 h. Images were recorded both before and after the experimental treatment, and the migration area was quantified using the Adobe Photoshop CS3 software.

2.6 Tube formation assay

The angiogenic potential of the PIEC was assayed based on their tube-forming ability (Gonzalez-King et al., 2017). Corning® atrigel® Basement Membrane Matrix (Corning, 354234, USA) was added into ice-cold 96-well plates (50 μl/well) and allowed to polymerize at 37°C for 30 min. After treatment with either C16:0 or C20:5 for 24 h, the PIEC were digested and seeded into 96-well plates at a density of 2 × 10^4 cells/well (n = 5/group). Tube formation images were captured at 12 h after the treatment, and the total number of tubes was analyzed using the Image J software.

2.7 Statistics analysis

Data in all figures were expressed as mean ± SEM. One-way ANOVA was performed using SAS 9.2 (SAS Inst. Inc., NC, USA). Tukey’s post hoc multiple comparison test was performed to compare significant variations. A p-value < 0.05 was considered to indicate a statistically significant difference.

2.8 Ethical approval

Because no animals were used in context of this manuscript, no ethical permit was required; all control wells received the same amount of solvent and BSA.

3 RESULTS

3.1 Effect of C16:0 and C20:5 on cytotoxicity and proliferation of pTr2 and PIEC

The pTr2 and PIEC were treated with different concentrations of C16:0 and C20:5 (0, 25, 50, 100, 200, and 400 μM) for 24 h to determine the appropriate treatment concentration of C16:0 and C20:5. The results showed that 50 μM C16:0 and 200 μM C20:5 caused significant cytotoxicity in the pTr2 (Figure 1a). Moreover, with an increase in C16:0 concentration (from 25 to 400 μM), significant inhibition of cellular proliferation until 400 μM was observed (Figure 1b). However, C20:5 treatment exhibited very low inhibition of cellular proliferation until 400 μM (Figure 1b). Similarly, in PIEC, 25 μM C16:0 caused significant cytotoxicity and inhibited PIEC...
cell proliferation (Figures 2a and 2b). On the contrary, C20:5 treatment exhibited very low levels of cytotoxicity, as well as minimal inhibition of cellular proliferation in PIEC (Figures 2a and 2b). Therefore, we chose 50 μM as the maximum concentration in the follow-up experiments.

3.2 | Effect of C16:0 and C20:5 on PIEC migratory capabilities

Next, a wound-healing assay was conducted to study the effect of C16:0 and C20:5 on the migration behavior of PIEC. We found that with an increase in the concentration of C16:0 (from 12.5 to 50 μM), a significant reduction in the migratory area of PIEC was observed (Figures 3a and 3b). However, 50 μM of C20:5 had no effect on the migratory capacity of PIEC (Figures 3a and 3c).

3.3 | Effect of C16:0 and C20:5 on PIEC tube formation

The PIEC were treated with 0, 12.5, 25, and 50 μM C16:0 and C20:5 for 24 h, followed by estimation of their angiogenic potential based on their tube-forming ability to further confirm the effect of C16:0 and C20:5 on PIEC angiogenesis in vitro. We found that a low concentration of C16:0 (from 12.5 to 50 μM) has no effect on the tube-forming ability of PIEC (Figures 4a and 4b). On the contrary, 12.5 μM of C20:5 significantly promoted tube formation in PIEC (Figures 4a and 4c).

4 | DISCUSSION

In sows, a certain amount of body fat deposition during pregnancy is known to be beneficial to achieve the best lactation performance and reduce the weight loss of the sow during lactation (Kim et al., 2013). Recent studies have shown that sows that were overweight during pregnancy exhibited an increased incidence of retarded intrauterine growth and decreased feed intake during lactation and piglet weight at weaning (Kim et al., 2015, Kongsted, 2005). Under overweight conditions, large amounts of lipids get deposited ectopically in the placenta, which results in increased placental lipotoxicity and subsequent inhibition of placental angiogenesis and fetal development (Calabuig-Navarro et al., 2017, Hastie & Lappas, 2014). However, the effect of different types of fatty acids on placental angiogenesis is still unclear.

A previous study has found that in maternal and umbilical cord plasma, NEFA concentrations linearly increased as the backfat thickness at 109 days of gestation increased (about 1.3 times) (Zhou et al., 2018). Saturated fatty acids in NEFA are generally considered to be the main source of lipotoxicity; however, no accurate concentration was detected in sow so far (Chaurasia & Summers, 2015, Law et al., 2017, Senkal et al., 2017). We summarized several studies and found that the
plasma NEFA concentration is between 125 and 350 μM during sow pregnancy (Table 1) (Farmer et al., 2012b, He et al., 2019, Tan et al., 2018). Based on this, we calculated that the NEFA concentration may be 163–455 μM in the plasma of obese sows. C16:0 is the most common saturated fatty acid accounting for 20%–30% of total fatty acids (Carta et al., 2017). Therefore, 0–400 μM C16:0 and C20:5 should be close to the physiological obesity concentration. We found that C16:0 treatment caused significant cytotoxicity in pTr2 and PIEC and inhibited cellular proliferation and migration in PIEC, whereas C20:5 treatment exhibited very low cytotoxicity and minimal inhibition of cellular proliferation. Moreover, a low concentration of C16:0 had no effect on the tube formation in PIEC, whereas C20:5 significantly promoted tube formation in PIEC. These results suggested that saturated fatty acids and n-3 PUFA had different effects on placental angiogenesis.

Trophectoderm cells are one of the most important cell types in the pig blastocyst, which is the first epithelium formed during embryonic development, leading predominantly to extraembryonic tissues, including the placenta (Houghton, 2010, Kong et al., 2012). In this study, we found that the cytotoxicity of pTr2 increased posttreatment with C16:0 in a dose-dependent manner. This result indicated that for

**Figure 3** Effect of C16:0 and C20:5 on PIEC migratory capabilities. (a) Representative photomicrographs of the wounded PIEC monolayer following 24 h of treatment with C16:0 and C20:5. (b) Relative migration after treatment with C16:0. (c) Relative migration after treatment with C20:5. n = 6/group, different letters denote significant differences, p < 0.01

**Table 1** The nonesterified fatty acids (NEFA) concentration in sow plasma during gestation

| Author          | Gestation                   | NEFA (μM) | Breed and parity                |
|-----------------|-----------------------------|-----------|---------------------------------|
| Farmer et al., 2012b | Day 28 of gestation         | 137       | Yorkshire × Landrace, first-parity |
|                 | Day 75 of gestation         | 125       |                                 |
|                 | Day 110 of gestation        | 342       |                                 |
| He et al., 2019  | Day 107 of gestation, thermoneutral | 328     | Landrace × Large White, 1 parity |
|                 | Day 107 of gestation, heat stress | 481     |                                 |
| Tan et al., 2018 | Day 106 of gestation        | 350       | Landrace, 2.2–5.8 parity        |
overweight sows, excessive C16:0 probably inhibited placental angiogenesis by inhibiting the function of trophoblast cells. Additionally, proliferation and migration of vascular endothelial cells are important steps in placental angiogenesis (Chen et al., 2017, Kaufmann et al., 2004). In this study, we found that the proliferation and migration of PIEC were significantly decreased posttreatment with C16:0. These results showed that C16:0 also had a direct inhibitory effect on the angiogenesis of PIEC.

*n*-3 PUFAs are essential for human and porcine and can only be obtained from the diet (Greenberg et al., 2008, Rosero et al., 2016). However, due to its unstable effect, there is no recommended amount of *n*-3 PUFA during sow pregnancy by Usa, N. C. (2012). Most studies showed that sow gestation diets—*n*-3 PUFA supplementation—have little effect on litter size (Farmer et al., 2010, Quelen et al., 2010, Rooke et al., 1998). But, Mateo et al. found that continuous feeding of *n*-3 PUFA-rich diets for two reproductive cycles can significantly increase the birth weight and weaning weight of piglets; the related mechanism is still unclear (Mateo et al., 2009). Recent studies show that *n*-3 PUFAs enhance the neovascularogenesis and cell migration of Human endothelial progenitor cells in vitro (Chiu et al., 2014). The mechanism of action included the upregulation of the c-kit protein and the phosphorylation of the ERK1/2, Akt, and endothelial nitric oxide synthase signaling molecules (Chiu et al., 2014). Studies on rodents also found that the consumption of *n*-3 PUFAs induced the formation of new blood vessels in the mice ischemia–reperfusion model and subsequent placental development (Chiu et al., 2014, Peng et al., 2019). Consistent with these studies, we found that a low dose of C20:5 significantly promoted tube formation in PIEC in vitro, which suggested that *n*-3 PUFAs might be effective fatty acid that could relieve placental lipotoxicity and promote angiogenesis in the placenta of sows. Further studies are required to elucidate the related mechanisms.

### 5 CONCLUSIONS

Our findings revealed that saturated fatty acids and *n*-3 PUFAs had different effects on sow placental angiogenesis. C16:0 could inhibit the proliferation of pTR2 and PIEC, whereas C20:5 promoted the angiogenesis of PIEC. As essential functional fatty acid, *n*-3 PUFAs might be an effective measure in alleviating the placental lipotoxicity caused by sow obesity during pregnancy.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

ETHICS
The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to. Because no animals were used in context of this manuscript, no ethical permit was required.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS
Jie Peng, Guoli Li, Xiu Zhang, Yanhua Huang, and Yimei Tang conceptualized the study. Jie Peng and Yimei Tang administered the project. Jie Peng and Yimei Tang wrote the original draft. Menglin Yang and Yimei Tang contributed in data curation and methodology. Guoli Li provided resources. Xiu Zhang reviewed and edited the manuscript. Yanhua Huang acquired funding.

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