RESEARCH

Hyperandrogenism and insulin resistance modulate gravid uterine and placental ferroptosis in PCOS-like rats

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Abstract

Women with polycystic ovary syndrome (PCOS) have hyperandrogenism and insulin resistance and a high risk of miscarriage during pregnancy. Similarly, in rats, maternal exposure to 5α-dihydrotestosterone (DHT) and insulin from gestational day 7.5 to 13.5 leads to hyperandrogenism and insulin resistance and subsequently increased fetal loss. A variety of hormonal and metabolic stimuli are able to trigger different types of regulated cell death under physiological and pathological conditions. These include ferroptosis, apoptosis and necroptosis. We hypothesized that, in rats, maternal hyperandrogenism and insulin-resistance-induced fetal loss is mediated, at least in part, by changes in the ferroptosis, apoptosis and necroptosis pathways in the gravid uterus and placenta. Compared with controls, we found that co-exposure to DHT and insulin led to decreased levels of glutathione peroxidase 4 (GPX4) and glutathione, increased glutathione + glutathione disulfide and malondialdehyde, aberrant expression of ferroptosis-associated genes (Acsl4, Tfrc, Slc7a11, and Gclc), increased iron deposition and activated ERK/p38/JNK phosphorylation in the gravid uterus. In addition, we observed shrunken mitochondria with electron-dense cristae, which are key features of ferroptosis-related mitochondrial morphology, as well as increased expression of Dpp4, a mitochondria-encoded gene responsible for ferroptosis induction in the uteri of rats co-exposed to DHT and insulin. However, in the placenta, DHT and insulin exposure only partially altered the expression of ferroptosis-related markers (e.g. region-dependent GPX4, glutathione + glutathione disulfide, malondialdehyde, Gls2 and Slc7a11 mRNAs, Key Words

- ferroptosis
- mitochondria
- gravid uterus
- placenta
- PCOS

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and phosphorylated p38 levels). Moreover, we found decreased expression of Dpp4 mRNA and increased expression of Cisd1 mRNA in placentas of rats co-exposed to DHT and insulin. Further, DHT + insulin-exposed pregnant rats exhibited decreased apoptosis in the uterus and increased necroptosis in the placenta. Our findings suggest that maternal hyperandrogenism and insulin resistance causes the activation of ferroptosis in the gravid uterus and placenta, although this is mediated via different mechanisms operating at the molecular and cellular levels. Our data also suggest that apoptosis and necroptosis may play a role in coordinating or compensating for hyperandrogenism and insulin-resistance-induced ferroptosis when the gravid uterus and placenta are dysfunctional.

**Introduction**

Polycystic ovary syndrome (PCOS) is a complex and heterogeneous hormone-imbalance gynecological disorder that is influenced by genetic, environmental, and metabolic factors (Azziz et al. 2016). This disorder affects approximately 4–21% of all adolescent and reproductive-aged women and has a significant impact on their reproduction (Lizneva et al. 2016). Women with PCOS often suffer from hyperandrogenism/androgen excess and insulin resistance (collectively termed; HAIR), and they are at high risk for miscarriage and obstetric complications during pregnancy (Bahri Khomami et al. 2019). Therapeutic interventions for different phenotypes and disease-related pregnancy complications in women with PCOS present a significant unmet medical need (Rosenfeld & Ehrmann 2016). Although it is thought that maternal, placental and fetal defects all contribute to the onset and progression of miscarriage in PCOS patients, the pathogenesis of the pregnancy loss induced by HAIR and its precise regulatory mechanisms are still significant issues to be solved.

Ferroptosis is a recently described, iron-dependent form of regulated necrosis induced by oxidative stress and it is distinct from other established forms of cell death, such as apoptosis and necroptosis (Choi et al. 2019), due to its unique morphological and biochemical features (Dixon et al. 2012, Tang et al. 2019). Growing evidence indicates that excessive or impaired ferroptosis plays a causative role in a variety of pathological conditions and diseases (Stockwell et al. 2017). It appears that the outcome of ferroptosis is programmed cell death, but which specific physiological processes or pathological conditions and disorders lead to ferroptosis activation remain poorly explored. The major molecular mechanisms and signaling pathways that are involved in the regulation of ferroptosis have been demonstrated in in vivo and in vitro studies (Li et al. 2020). For example, suppression of glutathione biosynthesis and subsequent inhibition or degradation of glutathione peroxidase 4 (GPX4) activity, disturbed balance of iron homeostasis and activation of the mitogen-activated protein kinase (MAPK) signaling pathways all contribute to the initiation and execution of ferroptosis (Xie et al. 2016). In addition to ferroptosis, the alterations of apoptosis and necroptosis-mediated signaling pathways have also been proposed as the critical etiological factors of several human diseases (Gudipaty et al. 2018). However, little is known about the role of ferroptosis (Ng et al. 2019) in comparison with other forms of programmed cell death such as apoptosis (Spencer et al. 1996) in female reproduction.

Using rats, we have recently demonstrated that HAIR-induced fetal loss is associated with uterine and placental defects (Hu et al. 2019b, Zhang et al. 2019b). In particular, we exposed pregnant rats to 5α-dihydrotestosterone (DHT) and insulin (INS) from gestational day (GD) 7.5 to 13.5 and found that this triggered many features of PCOS (including HAIR) and lead to fetal loss. The fetal loss was related to disrupted reactive oxygen species (ROS) production in the uterus and placenta of rat dams with induced HAIR. Maternal HAIR-induced fetal loss was also associated with the inactivation of antioxidative proteins in the gravid uterus and placenta, namely nuclear factor erythroid 2-related factor 2 (Nrf2) and superoxide dismutase 1 (Hu et al. 2019b, Zhang et al. 2019b), which play an inhibitory role in the ferroptosis pathway (Xie et al. 2016, Tang et al. 2019). Moreover, the mRNA expression of several other negative regulators of ferroptosis such as heme oxygenase 1 (Ho1) (Tang et al. 2019) and metallothionein 1G (Mt1g) (Sun et al. 2016) were downregulated in the gravid uterus after combined maternal exposure to DHT and INS (Hu et al. 2019b).
Increased circulating ROS levels have been observed in both non-pregnant and pregnant rodents in which PCOS features have been induced (Lai et al. 2018, Zhang et al. 2019b). Elevated ROS production and decreased anti-oxidative capacity has been observed in the ovarian granulosa cells and leukocytes of PCOS patients (Banuls et al. 2017, Lai et al. 2018), and oxidative stress is proposed to contribute to miscarriage and infertility in women with PCOS (Agarwal et al. 2012, Schoots et al. 2018). It is, therefore, likely that the promotion of pathologic oxidative stress and activation of ferroptosis in the gravid uterus and placenta contribute to HAIR-induced fetal loss in both animal models and humans.

Mitochondria play a protective role in the regulation of glutathione-induced ferroptosis (Gao et al. 2019). In women with PCOS and miscarriage, as well as in pregnant PCOS-like rodents with fetal loss, there is mounting evidence for mitochondrial abnormalities and oxidative damage. For instance, decreased mitochondrial DNA copy number is associated with the development and severity of PCOS and several mitochondria-tRNA mutations are seen in PCOS patients (Agarwal et al. 2012, Zhang et al. 2019a). In addition, aberrant expression of mitochondrial biogenesis genes, oxidative phosphorylation and anti-oxidative proteins are found in PCOS patients who have recurrent miscarriage (Agarwal et al. 2012, Zhang et al. 2019a), as well as in PCOS-like rodents (Ding et al. 2019, Hu et al. 2019a,b, Zhang et al. 2019b).

On the basis of these preclinical and clinical studies, we hypothesized that maternal HAIR triggers impairments in GPX4/glutathione-regulated lipid peroxidation and iron-associated and mitochondria-mediated ferroptosis in the gravid uterus and placenta resulting in increased fetal loss during pregnancy.

The aim of this study was to determine whether exposure to DHT and INS in pregnant rats (which induces HAIR/PCOS (Hu et al. 2019b, Zhang et al. 2019b)) leads to activation of the ferroptosis cascade, elevated malondialdehyde (MDA, a marker of oxidative stress), iron accumulation and perturbed mitochondrial function in the uterus and placenta. Further, we conducted a parallel analysis of the expression of genes and proteins that are involved in necroptosis and apoptosis, two other programmed cell death pathways that might contribute to defects in the gravid uterus and the placenta. This study is the first to report an association between HAIR and different forms of regulated cell death in the gravid uterus and placenta in vivo. Our findings indicate that ferroptosis is one of the potential mechanisms by which maternal HAIR leads to uterine and placental dysfunction and at least partially explains the resultant fetal loss observed.

Materials and methods

Ethics approval

All experiments were conducted in compliance with all relevant local ethical regulations. Animal experiments were approved and authorized by the Animal Care and Use Committee of the Heilongjiang University of Chinese Medicine, China (HUCM 2015-0112), and followed the National Institutes of Health guidelines on the care and use of laboratory animals.

Animals, experimental setting and tissue collection

Adult Sprague–Dawley female (n=39) and male (n=21) rats were obtained from the Laboratory Animal Centre of Harbin Medical University, Harbin, China. All animals were health checked daily throughout the experiment and were maintained in an environmentally controlled and pathogen-free barrier facility on a standard 12 h light:12 h darkness cycle at 22 ± 2 °C and 55–65% humidity and with free access to normal diet and water. Before the experiment, female rats (n=9/group) were allowed to acclimatize for a minimum of 7 days and then were monitored daily by vaginal lavage to determine the stage of the estrous cycle (Zhang et al. 2016). Pregnancy was achieved by housing female rats on the night of proestrus with fertile males of the same strain at a 2:1 ratio. Confirmation of mating was defined by the presence of a vaginal plug and this was considered as GD 0.5. Body weight of the rats was recorded daily and rats were killed between 08:00 and 09:00 h on GD 14.5. All animal procedures in this study were performed as described in our previous publications (Hu et al. 2019b, Zhang et al. 2019b).

To induce HAIR, pregnant rats were randomly assigned to be intraperitoneally injected with DHT (1.66 mg/kg/day, suspended in sesame oil, Sigma-Aldrich) and/or human recombinant INS (6.0 IU/day, diluted in sterile saline, Eli Lilly Pharmaceuticals) or an equal volume of saline and sesame oil as controls on GD 7.5 as previously described (Hu et al. 2019b, Zhang et al. 2019b). This therefore generated the following four study groups: Control, DHT+INS, DHT, and INS. All animals were treated for 7 consecutive days. The dose of DHT used in our rats was chosen to mimic the hyperandrogenic state in PCOS patients who have approximately...
1.7-fold higher circulating DHT concentrations compared to healthy controls (Fassnacht et al. 2003, Silfen et al. 2003). The dose of INS was chosen because it induces metabolic disturbances including peripheral and uterine insulin resistance in rats (Zhang et al. 2016, 2018). We have previously shown that rats co-exposed to DHT and INS during pregnancy had metabolic and endocrine aberrations (HAIR) at GD 14.5 (Hu et al. 2019b, Zhang et al. 2019b) that replicate the changes observed in pregnant PCOS patients (Sir-Petermann et al. 2002, Maliqueo et al. 2013, Glinborg et al. 2018). The current investigation used gravid uterine and placental tissues collected from the same rats exposed to DHT and/or INS used in our previous study (Zhang et al. 2019b), in which circulating levels of androgens (testosterone, androstenedione, dehydroepiandrosterone, and DHT), glucose tolerance and fasting insulin, as well as fetal viability (litter size and fetal loss per litter) were reported. Briefly these data showed that rats co-exposed to DHT and INS had increased androgen levels (testosterone, androstenedione, and DHT) and worse insulin sensitivity, as well as decreased litter size, with a corresponding increase in the percentage of litters showing fetal loss. In addition, there was no effect of DHT and/or insulin on maternal body weight gain (Supplementary Fig. 1, see section on supplementary materials given at the end of this article). On GD 14.5, tissues, including the maternal uterus and placenta, as well as fetuses were dissected. These were then either fixed for morphological and immunohistochemical analyses or immediately frozen in liquid nitrogen and stored at −70 °C for quantitative real-time PCR (qPCR) and Western blot analyses. Only viable conceptuses (fetuses and placentas) were analyzed further.

Detailed description of the methods including the primers (Table 1) and qPCR analysis, Western blot analysis, GPX4 immunostaining, Perls’ histochemical reaction, transmission electron microscopy (TEM), and quantification of glutathione, MDA and mitochondrial open reading frame of the 12S rRNA-c (MOTS-c) used in this study are provided in Supplementary files.

Data processing, statistical analysis, and graphical representations

No statistical methods were used to pre-determine the sample size. Data are presented as the mean±s.e.m., and the sample size (n) is listed in the figure legends and indicates the number of animals in each experiment. Statistical analyses were performed using SPSS version 24.0 for Windows (SPSS Inc.). The normal distribution of the data was tested with the Shapiro–Wilks test. Differences between groups were analyzed by one-way ANOVA followed by Tukey’s post-hoc test for normally distributed data or the Kruskal–Wallis test for skewed data (Supplementary Table 1). Body weight data were analyzed by one-way ANOVA with repeated measures. Data were not corrected for multiple testing. All P-values less than 0.05 were considered statistically significant.

Results

Because we were most interested in how HAIR induces changes in ferroptosis as opposed to apoptosis and necroptosis in gravid uterine and placental tissues, we have mainly described the observations in DHT+INS-exposed pregnant rats vs control pregnant rats subsequently.

Differential regulation of GPX4 in the gravid uterus and placenta exposed to DHT and INS

GPX4 is present in the cytoplasm, mitochondria and nucleus of mammalian cells (Conrad et al. 2007). Hence, we initially performed Western blot and immunohistochemical analyses to characterize the tissue and intracellular localization of GPX4 protein in rat uterine and placental tissues. In the Western blot analysis, the ~20-kDa band represents the cytosolic and mitochondrial GPX4 protein in the rat testis, epididymis, and ovary (Supplementary Fig. 2), as well as non-pregnant and pregnant uteri (Supplementary Fig. 3A and Fig. 1A), whereas the ~34-kDa band represents the nuclear GPX4 protein in the testis (Supplementary Fig. 2). Further immunohistochemical studies showed that, while positive immunostaining for cytosolic GPX4 was mainly observed in luminal and glandular epithelial cells, GPX4 immunostaining was additionally localized to the nucleus of stromal cells and myometrial smooth muscle cells in non-pregnant rats (Supplementary Fig. 3B). In control pregnant rats, GPX4 was localized to both the cytosol and nucleus of different cells within the decidua, myometrium and placenta (Fig. 1B1, B2, B3, and B4).

Although the significance of mitochondrial and nuclear GPX4 remains to be determined (Forcina & Dixon 2019), cytosolic GPX4 has been identified as a central regulator of ferroptosis (Stockwell et al. 2017). We thus evaluated GPX4 expression (Fig. 1A) and localization (Fig. 1B, C, D, E, and F) in the gravid uterus and placenta in rats exposed to DHT and INS. The Western blot analysis revealed a significant decrease in uterine GPX4.
abundance in DHT+INS-exposed pregnant rats (Fig. 1A). Consistent with this, there appeared to be weaker immunoreactivity of GPX4 in the cytosolic compartments of decidualized stromal and smooth muscle cells in the uterus of DHT+INS-exposed pregnant rats (Fig. 1C1 and C2). Although there was no significant difference in uterine GPX4 abundance by Western blot analysis in pregnant rats treated alone with DHT or INS (Fig. 1A), the number of cytosolic and/or nuclear GPX4-positive uterine cells was decreased when compared to controls using immunostaining (Fig. 1D1, D2 and E2). Similarly, while GPX4 protein abundance was unchanged in the placenta of DHT+INS-exposed pregnant rats by Western blot analysis (Fig. 1A), cytosolic GPX4 immunoreactivity appeared to be lower in the junctional and labyrinth zones in DHT+INS-exposed pregnant rats when examined by immunohistochemistry (Fig. 1C3 and C4). In particular, GPX4 immunostaining was no longer localized to the nuclei of spongiotrophoblast, glycogen, cytotrophoblast and syncytiotrophoblast cells of DHT+INS-exposed

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primer sequences used for qPCR measurement.</th>
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<tbody>
<tr>
<td><strong>Gene</strong></td>
<td><strong>Primer sequence (5'-3')</strong></td>
</tr>
<tr>
<td>Slc1a5</td>
<td>Forward: TCGGGACCTCTTCTAGCTCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGAACCGCTGATGTTGTTTG</td>
</tr>
<tr>
<td>Acsl4</td>
<td>Forward: CTCCTGCTTTATCACTGGCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACAATACCTCCCTGCTCCTCCTCCT</td>
</tr>
<tr>
<td>Gls2</td>
<td>Forward: GGCCAAGTCAACCAAGATC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TAGTGGTGCTGCTAGTGTC</td>
</tr>
<tr>
<td>Cs</td>
<td>Forward: AGTGCGAAGAATCAGTAACTCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTGAGACCAAGAGACGCTGT</td>
</tr>
<tr>
<td>Gclc</td>
<td>Forward: AAGGATGTTGCTTGTTTGGT</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGGAGGTGTTGCTTGTTG</td>
</tr>
<tr>
<td>Gss</td>
<td>Forward: ATGCCGTGGCTGCTAGTATT</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGATGAGGACACCAATGGCA</td>
</tr>
<tr>
<td>Tfrc</td>
<td>Forward: AGGTCTTGTAGGGTATGTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGATGAGGACACCAATGGCA</td>
</tr>
<tr>
<td>Ireb2</td>
<td>Forward: GTTTGAAGAAGCAGCACTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACTCCCCACCCACAGATTC</td>
</tr>
<tr>
<td>Slc7a11</td>
<td>Forward: GTGCCGGATGTCATCCTTTT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTAAGGAAGACGCAACAAAG</td>
</tr>
<tr>
<td>Cisd1</td>
<td>Forward: GGCAATACACGCGCTTATC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAGCTGTGGCAGGAGAGTATTA</td>
</tr>
<tr>
<td>Dpp4</td>
<td>Forward: GACCTGTTGCTGTCTTCTAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAACTCAAAAGACGCCACACATC</td>
</tr>
<tr>
<td>Bcl2</td>
<td>Forward: TTCGAGATGTCCAGTCA</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAGCTGTGGCAGGAGAGTATTA</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>Forward: GTGTTGTTACCTCCTCTTTAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCCCTCTTCTGCTGGTTTGGT</td>
</tr>
<tr>
<td>Box</td>
<td>Forward: GATGCCCTCCTCCTCTTAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTCCTCTCCTCCTCCTCTTAC</td>
</tr>
<tr>
<td>Bak</td>
<td>Forward: GATGCCCTCCTCCTCCTTAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTCCTCTCCTCCTCCTTAC</td>
</tr>
<tr>
<td>Casp3</td>
<td>Forward: GACTGGAAAGCCGAAACTCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTCATATCATGTCAGTCCC</td>
</tr>
<tr>
<td>Mlkl</td>
<td>Forward: GGAACGTGGAGATAGAGACAAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTGATGTCTTCCGAGGAGT</td>
</tr>
<tr>
<td>Ripk1</td>
<td>Forward: CAGGATACGAGGTTTGGTATG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGTATGGCATGGTGGGTATG</td>
</tr>
<tr>
<td>Ripk3</td>
<td>Forward: ACTGAGAGAGGAAAGGGAAAGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTGGAGGGTAGAGGTGTGG</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Forward: TCTCTGCTCCCTGGTTTCTCTA</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTGAGACCAAGAGACG</td>
</tr>
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</table>

Acsl4, acyl-CoA synthetase long-chain family member 4; Bak, bcl-2 homologous antagonist killer; Bax, bcl-2-like protein 4; Bcl2, b-cell lymphoma 2; Bcl-xl, b-cell lymphoma-extra large; Casp3, caspase 3; Cisd1, CDSSh iron sulfur domain 1; Cs, citrate synthase; Dpp4, dipeptidylpeptidase 4; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Gclc, glutamate-cysteine ligase catalytic subunit; Gls2, glutaminase 2; Gss, glutathione synthetase; Ireb2, iron responsive element binding protein 2; Mlkl, mixed lineage kinase domain like pseudokinase; Ripk1, receptor interacting serine/threonine kinase 1; Slc1a5, solute carrier family 1 member 5; Slc7a11, solute carrier family 7 member 11; Tfrc, transferrin receptor.
pregnant rats compared to controls (Fig. 1C3 and C4). While cytosolic GPX4 immunoreactivity was decreased in both spongiotrophoblasts and glycogen cells, nuclear GPX4 immunoreactivity was absent in spongiotrophoblasts cells in pregnant rats treated with DHT alone (Fig. 1D3) and INS alone (Fig. 1E3). Similar to that of controls (Fig. 1B4), GPX4 immunoreactivity was found in the placental labyrinth zone in pregnant rat placentas exposed to DHT or INS alone (Fig. 1D4 and F4). No obvious GPX4 immunostaining was evident in the uterine and placentical tissue sections using the same concentration of isotype-matched rabbit IgG instead of the primary GPX4 antibody (Fig. 1F1, F2, F3, and F4).

**Differential regulation of glutathione content in the gravid uterus and placenta exposed to DHT and INS**

GPX4 uses glutathione as a substrate in its peroxidase reaction cycle (Conrad *et al.* 2007) and glutathione depletion is one of the key triggers for ferroptosis (Xie *et al.* 2016, Stockwell *et al.* 2017). Therefore, we measured the levels of glutathione and glutathione + glutathione disulfide in the gravid uterus and placenta in rats exposed to DHT and INS. As shown in Fig. 2A, co-exposure of rats to DHT and INS decreased glutathione levels in the gravid uterus, but not in the placenta, while increased glutathione + glutathione disulfide levels were detected in both tissues.
Alterations in glutathione and glutathione + glutathione disulfide levels were found in the gravid uterus in pregnant rats exposed to INS alone and levels of glutathione were lower in both the uterus and placenta in pregnant rats treated with DHT alone (Fig. 2A).

**Figure 2**
Alteration of glutathione, glutathione + glutathione disulfide, ferroptosis-related gene expression, and MDA in pregnant rats exposed to DHT and/or INS at GD 14.5. ELISA analysis of glutathione (the reduced state), glutathione + glutathione disulfide, and MDA in the uterus and placenta (A, n = 8/group). qPCR analysis of uterine and placental genes involved in modulating ferroptosis (B, n = 7–8/group). In all plots, values are expressed as means ± s.e.m. Statistical P values for selected comparisons are indicated as *P < 0.05, **P < 0.01, and ***P < 0.001. DHT, 5α-dihydrotestosterone; INS, insulin.

**Alterations in ferroptosis-related gene expression in the gravid uterus and placenta with DHT and INS**

Next, we examined whether maternal exposure to DHT and INS alters the expression of pro-ferroptosis (Slc1a5, Acsl4, Glis2, Cs, Tfrc and Ireb2) or anti-ferroptosis (Slc7a11, Gclc, and Gss) genes (Dai et al. 2020) in the uterus and placenta. In pregnant DHT+INS-exposed rats, uterine Acsl4, Slc7a11 and Gclc mRNAs were decreased, while Tfrc mRNA was increased (Fig. 2B). In comparison with the control uterus, maternal exposure to DHT alone decreased Cs, Ireb2, Slc7a11, Gclc and Gss mRNA expression, whereas exposure to INS alone increased Glis2 and Tfrc mRNAs and decreased Slc7a11 mRNA expression (Fig. 2B). qPCR analysis also showed that Glis2 mRNA expression was increased and Slc7a11 mRNA expression was decreased in the placenta after maternal co-exposure to DHT and INS. In comparison with the control placenta, exposure to DHT alone decreased Cs, Slc7a11 and Gss mRNA expression, whereas exposure to INS alone decreased Gclc and Gss mRNAs in parallel to increased Tfrc and Slc7a11 mRNA expression (Fig. 2B).

**Alterations in MDA levels in the gravid uterus and placenta with DHT and INS**

Given that one of the key consequences of ferroptosis is elevated lipid peroxidation (Dixon et al. 2012,
Tang et al. 2019), we next examined the impact of DHT and INS on the levels of MDA, a marker of lipid peroxidation (Gawel et al. 2004), in the gravid uterus and placenta. As shown in Fig. 2C, maternal co-exposure to DHT and INS resulted in increased MDA levels in both the gravid uterus and placenta. However, there were no significant changes in MDA levels between the DHT-exposed rats or the INS-exposed rats and control rats.

**Alterations in intracellular iron deposition in the gravid uterus and placenta with DHT and INS**

Because disturbed iron transport and impaired metabolism within cells/tissues results in ferroptosis (Galaris et al. 2019), whether chronic exposure to DHT and INS can modulate tissue iron deposition was also examined. Perls’ histochemical reaction showed specific cytoplasmic and granular iron storage in rat uterine epithelial and decidualized stromal cells on GD 6, which is prior to the induction of HAIR (Supplementary Fig. 3A1 and A2). As compared to control pregnant rats (Fig. 3A1, A2, A3 and Supplementary Fig. 4B1, B2), iron accumulation was increased in the external muscle layer, the mesometrial triangle, as well as in the decidua of DHT+INS-exposed pregnant rats (Fig. 3B1, B2, B3 and Supplementary Fig. 4B3, B4). Similarly, a significant increase in iron storage in the mesometrial triangle was also observed in DHT-exposed rat dams (Fig. 3C1 and C2). In the mesometrial triangle,

![Figure 3](https://joe.bioscientifica.com)
decidua, granular and cytoplasmic iron-positive staining was absent in the DHT-exposed rats (Fig. 3C3) but was barely detectable in the INS-exposed rats (Fig. 3C3 and D3). However, no iron-positive staining was found in the placental junctional zone in any of the experimental groups (Fig. 3A4, B4, C4, and D4), while intense iron-positive staining was consistently detected in immature erythrocytes within the placental labyrinth zone in all experimental groups (Fig. 3A5, B5, C5 and D5). These results indicate that the amount of deposited iron was elevated, especially in the gravid uterus, following exposure to DHT and/or INS.

**Alterations in the MAPK signaling pathway in the gravid uterus and placenta with DHT and INS**

Taking into consideration that the MAPK signaling pathway, including ERK, p38, and c-JUN NH2-terminal kinase (JNK), is involved in the execution of ferroptosis in other cells (Xie et al. 2016), we evaluated whether co-exposure to DHT and INS may be linked to activation of the MAPK signaling pathway in the gravid uterus and placenta. As shown in Fig. 4A, in the gravid uterus, maternal DHT + INS exposure resulted in an increased abundance of phosphorylated ERK1/2 (p-ERK1/2) and decreased total ERK1/2, which subsequently resulted in an increased p-ERK1/2:ERK1/2 ratio. Moreover, both p-JNK and total JNK protein abundance were increased, whereas the p-JNK:JNK ratio remained unchanged in the gravid uterus of DHT + INS-exposed rats (Fig. 4A). Additionally, a similar increase in p-p38 protein abundance and the p-p38:p38 ratio was observed in both the gravid uterus (Fig. 4A) and placenta (Fig. 4B) after maternal co-exposure to DHT and INS. These results indicate that both ERK1/2 and JNK signaling are only activated in the gravid uterus, whereas p38 signaling is activated in both the gravid uterus and placenta after maternal co-exposure to DHT and INS.
Changes in mitochondrial morphology are associated with changes in mitochondria-encoded gene and protein expression in the gravid uterus and placenta with DHT and INS

By TEM (Fig. 5 and Supplementary Fig. 5), we found shrunken mitochondria with numerous electron-dense cristae or absent cristae in the gravid uterus of DHT+INS-exposed rats (Fig. 5B1 arrows) compared to controls (Fig. 5A1). Further, mitochondria were swollen and collapsed with poorly defined tubular cristae in the gravid uterus of rat exposed to DHT and/or INS (Fig. 5B1, C1, and D1). Our TEM findings of the uterus in DHT+INS rats are consistent with ferroptosis-related mitochondrial morphology (Dixon et al. 2012, Xie et al. 2016, Li et al. 2020). Treatment with DHT or INS also reduced the number of mitochondrial cristae in the uterus (Fig. 5C1 and D1). Ultrastructural analysis of the placenta showed that mitochondria in the trophoblast of the junctional zone were significantly affected by maternal exposure to DHT and/or INS (Fig. 5A2, B2, C2, and D2). For instance, mitochondria showed blebbing, few or no tubular cristae and decreased electron density in all treatment groups (Fig. 5B2, C2, and D2). However, there was little mitochondrial damage observed in the trophoblast of the placental labyrinth zone in all treatment groups compared to controls (Fig. 5A3, B3, C3, and D3).

Based on these morphological observations, the expression of known mitochondria-encoded genes (Cisd1, an anti-ferroptosis gene and Dpp4, a pro-ferroptosis gene (Stockwell et al. 2017, Tang et al. 2019)) and protein (MOTS-c, an enhancer of insulin sensitivity (Kim et al. 2017)) were analyzed by qPCR and ELISA. In the pregnant rat uterus, DHT+INS-exposure decreased Cisd1 mRNA expression, increased Dpp4 mRNA expression and decreased the MOTS-c protein level (Fig. 5E and F upper panel). In contrast, we found significantly higher uterine Cisd1 and Dpp4 mRNA expression in INS-exposed pregnant rats (Fig. 5E upper panel), but unchanged uterine MOTS-c protein levels in DHT-exposed pregnant rats compared to controls (Fig. 5F upper panel). In the placenta, Cisd1 mRNA expression was increased and Dpp4 mRNA expression was decreased in DHT+INS-exposed pregnant rats compared to controls (Fig. 5E lower panel). A decrease in placental Dpp4 mRNA expression was also observed in INS-exposed pregnant rats (Fig. 5E lower panel). However, there was no significant difference in MOTS-c protein levels in the placenta between any of the experimental groups (Fig. 5F lower panel).

Aberrant regulation of necroptosis-related and anti-/pro-apoptosis-related gene and protein expression in the gravid uterus and placenta with DHT and INS

Different types of cell death are seen in uterine and placental tissue during healthy and pathological pregnancy (Welsh 1993, Sharp et al. 2010). To extend our observations on the effect of maternal DHT and INS treatment on ferroptosis and mitochondrial impairment, we analyzed the expression of necroptosis (Mkk1, Ripk1 and Ripk3), anti-apoptosis (Bcl2 and Bcl-xl) and pro-apoptosis (Bax, Bak, Casp3 and cleaved caspase-3) mRNAs and proteins (Xie et al. 2016, Choi et al. 2019, Tang et al. 2019) in the gravid uterus and placenta. As shown in Fig. 6A, DHT+INS-exposure significantly decreased uterine Ripk1 mRNA expression, while uterine Mkk1 and Ripk3 mRNAs were increased by DHT and/or INS exposure when compared to control pregnant rats (Fig. 6A upper panel). Furthermore, co-exposure to DHT and INS increased Bcl-xl and Bax mRNA expression in the gravid uterus, with similar increases in these genes seen in DHT-exposed and/or INS-exposed pregnant rats compared to controls (Fig. 6B upper panel). Gravid uterine Bcl2 mRNA expression was not altered by co-exposure to DHT and INS; however, it was increased by DHT and decreased by INS when compared to control pregnant rats. In DHT+INS-exposed pregnant rats, Casp3 mRNA expression and cleaved caspase-3 protein abundance were decreased in the gravid uterus (Fig. 6B upper panel and C). In contrast, in the placenta we found that both Ripk1 and Ripk3 mRNAs were increased in DHT+INS-exposed pregnant rats compared to controls (Fig. 6A lower panel). Furthermore, maternal co-exposure to DHT and INS increased placental Bcl-xl, Bax and Bak mRNA expression (Fig. 6B lower panel). Of note, placental Bcl2 mRNA expression was also increased in DHT+INS-exposed rats and most significantly increased in the INS alone exposure. However there was no significant effect of the DHT alone exposure on placental Bcl2 mRNA level. There were, however, no changes in Casp3 mRNA expression or cleaved caspase-3 protein abundance in the placenta (Fig. 6B lower panel and C). Lastly, similar increases in placental Bcl-xl, Bax, Bak and Casp3 mRNAs were seen in DHT-exposed and/or INS-exposed pregnant rats compared to controls (Fig. 6B lower panel).

Discussion

Because PCOS patients frequently suffer from miscarriage and infertility (Bahri Khomami et al. 2019), it is important
Figure 5

Electron microscopy and mitochondria-mediated ferroptosis-related gene and protein expression in pregnant rats exposed to DHT and/or INS at GD 14.5. Mitochondrial ultrastructural defects in the uterus (A1, B1, C1, and D1, mesometrial decidua) and placenta (junctional (A2, B2, C2, and D2) and labyrinth zones (A3, B3, C3, and D3)). Images are representative of two tissue replicates. Md, mesometrial decidua; Jz, junctional zone (maternal side); Lz, labyrinth zone (fetal side). Red asterisks indicate mitochondria, and white arrows indicate shrunken mitochondria with electron-dense cristae. Scale bars (500 nm) are indicated in the photomicrographs. qPCR analysis of mitochondrial genes involved in modulating ferroptosis (E, n = 8/group). ELISA analysis of MOTS-c content (F, n = 8/group). In all plots, values are expressed as means ± s.e.m. Statistical P values for selected comparisons are indicated as *p < 0.05, **p < 0.01, and ***p < 0.001. DHT, 5α-dihydrotestosterone; INS, insulin.
to understand the molecular mechanisms through which HAIR affects tissues such as the gravid uterus and placenta. Until now, there have been no reports exploring the relationship between PCOS and regulated cell death in the uterus and placenta. Our results thus fill an important clinically relevant knowledge gap by experimentally demonstrating that maternal HAIR can cause the activation of ferroptosis in the gravid uterus and placenta, although this is mediated through different molecular and cellular mechanisms. We propose that alterations in the ferroptosis pathway in the uterus and placenta due to maternal HAIR likely contribute to impaired fetal survival seen in experimental animal models and future work is required to assess whether this is also the case in women with PCOS.

In mammals, GPX4 plays a major role in antioxidant defense by regulating responses to oxidative stress. Furthermore, loss of function of GPX4 protein and depletion of GSH levels are the key mechanisms for triggering ferroptosis (Dixon et al. 2012, Stockwell et al. 2017). In vivo knockout studies have shown that mice lacking the entire GPX4 gene experience early embryonic lethality (Imai et al. 2003) and that GPX4-deficient male mice are infertile (Schneider et al. 2009). Although the presence of GPX4 has been shown in uteri from cows (Ramos et al. 2015, Baithalu et al. 2017) and pigs (Dalto et al. 2015), the localization and physiological role of GPX4 has not been demonstrated in human and rodent reproductive tissues, including the uterus. Here, we show that GPX4 is widely expressed in the non-pregnant and pregnant rat uteri, including decidualized stromal cells. The data presented showing the differential cellular GPX4 localization in the uterus is consistent with variations in the compartmentalization of GPX4 between the cytosol and nucleus in different cells (Conrad et al. 2007). Furthermore, GPX4 is down-regulated in the gravid uterus by maternal exposure to DHT and INS. Correspondingly, the levels of glutathione are decreased and glutathione+glutathione disulfide levels are increased in the uterus by DHT and INS co-exposure. Taken together, these results suggest that maternal HAIR disrupts the GPX4-glutathione regulatory axis and can result in the induction of ferroptosis in the uterus during pregnancy. The finding that glutathione levels in the uterus were lowest in the INS-only treated rats which also showed a non-significant reduction in GPX4 suggests that other signaling pathways and factors such as the transcription factors Nrf1 and Nrf2 (Lu 2009) might be altered by the treatments and contribute to the resultant changes in glutathione status and should be investigated for causality in the future (also in the placenta of DHT and/or INS-exposed dams). Indeed, we have previously found altered abundance of antioxidants in the uterus of pregnant rats exposed to DHT and/or INS (Hu et al. 2019b, Zhang et al. 2019b). Consistent with previous work on the human placenta (Mistry et al. 2008, 2010), the present study...
shows that the GPX4 protein is highly expressed in the rat placenta during pregnancy. Although analysis of whole placental homogenates showed no significant change in GPX4 levels, immunolocalization revealed a loss of GPX4 in specific cell types in the placenta (the glycogen and spongiosphoblast cells) in response to maternal co-exposure to DHT and INS. The more minor alterations in GPX4 abundance, combined with the high levels of glutathione + glutathione disulfide and absence of changes in glutathione levels in the placenta, suggest that maternal HAIR induces ferroptosis to a lesser extent in the placenta compared to the gravid uterus. GPX4 is known to protect cells/tissues against lipid peroxidation by inhibiting lipid-associated hydroperoxides (Conrad et al. 2007). In addition, genetically ablating or inducing decreased GPX4 expression leads to the activation of ferroptosis (Friedmann Angeli et al. 2014, Chen et al. 2015). Together, our data therefore suggest that HAIR-induced ferroptosis is mediated by both dysregulation of GPX4 expression and aberrant increases in lipid peroxidation. The induction of uterine and placental ferroptosis by maternal exposure to DHT and INS may be a novel mechanism contributing to the malfunction of those tissues and hence impaired fetal development during pregnancy. However, how maternal HAIR-mediated uterine and placental ferroptosis compromises the growth and development of the fetus is not clear at this time and should be the subject of future investigations. Moreover, future work should be employed to assess whether the activation of ferroptosis, lipid peroxidation and poor fetal outcomes by maternal HAIR may be preventable by antioxidant administration.

Iron can serve as an essential signaling molecule that modulates diverse physiological processes and iron homeostasis is required for the normal growth and development of the placenta and fetus during pregnancy (Cao & Fleming 2016, Ng et al. 2019). By Perls’ histochemical reaction, we found a considerable proportion of uterine epithelial and decidualized stromal cells stained positively for iron storage on GD 6. These data are consistent with a previous report showing the cellular expression of ferritin heavy chain, a component of the multi-subunit iron-binding protein ferritin, in the uterus during early pregnancy (Zhu et al. 1995). An extensive body of evidence indicates that, while iron deficiency is linked to abnormal pregnancy (Ng et al. 2019) and increased risk of fetal death (Guo et al. 2019), iron overload is associated with the manifestation of PCOS (Escobar-Morreale 2012). Previous findings by Kim and colleagues indicate that increased circulating iron levels are associated with metabolic abnormalities, including HAIR in PCOS patients (Kim et al. 2014). Several studies have demonstrated that, in addition to its antioxidative property, Ho1 is a critical regulator for mobilization of intracellular pools of free iron (Poss & Tonegawa 1997, Kovtunovych et al. 2010). More recently, we have demonstrated that maternal co-exposure to DHT and INS suppresses Ho1 mRNA expression in the gravid uterus, but not in the placenta (Hu et al. 2019b, Zhang et al. 2019b). While the uptake of transferrin-bound iron, a major maternal iron source for placental transfer, is mainly mediated through iron import proteins such as transferrin receptor 1 (TFR1, TFRc) (Cao & Fleming 2016), a specific ferroptosis marker (Feng et al. 2020), our results show that combined exposure to DHT and INS increases Tfrc mRNA expression in association with increased iron deposition in the gravid uterus. Further, we have provided ultrastructural evidence that shrunken mitochondria with numerous electron-dense cristae, a key feature of ferroptosis-related mitochondrial morphology, are present in the gravid uterus. However, the placentas of the same animals exhibited increased mRNA expression of Cisd1, a mitochondrial iron export factor, and no change in Tfrc mRNA or iron accumulation. Ferroptosis can be induced by excessive accumulation of free iron in tissues and cells (Galaris et al. 2019) and our findings support the notion that, in response to exposure to DHT and INS, aberrant iron accumulation and activation of ferroptosis occurs in the gravid uterus but not in the placenta. Given the fact that whether or not mitochondria are involved in ferroptosis is still under debate (Gao et al. 2019), further investigations are needed to determine which cellular compartments contribute to the defective utilization of iron and increased ferroptosis observed in the gravid uterus under conditions of HAIR.

Given that aberrant accumulation of intracellular iron induces oxidative stress (Galaris et al. 2019) and subsequently results in multiple modes of cell death (Lei et al. 2019), it is not surprising that, in addition to ferroptosis, apoptosis (a non-inflammatory form of cell death) and necroptosis (a pro-inflammatory form of cell death) may also be involved in HAIR-induced fetal loss in pregnant rats. Indeed, pregnant rats co-exposed to DHT and INS exhibited decreased Casp3 mRNA expression and cleaved caspase-3 protein abundance in the uterus, but not in the placenta, even though selectively increased expression of anti-apoptotic genes (Bcl-xl) and pro-apoptotic genes (Bax) was observed in both tissues. We suspect that suppression of apoptosis might serve as a compensatory mechanism to protect against increased ferroptosis in order to maintain homeostasis of the gravid
uterus after exposure to DHT and INS. This is supported by studies assessing the interaction and interplay of different cell death pathways in cancer research (Riegman et al. 2019). Ferroptosis and necroptosis are two different forms of regulated necrosis (Xie et al. 2016, Choi et al. 2019). Necroptosis requires mitochondrial ROS generation and is primarily regulated by the RIPK1, RIPK3 and MLKL proteins (Choi et al. 2019). We found that, in DHT+INS-exposed pregnant rats, the level of ROS (Zhang et al. 2019b) and expression of Ripk1 and Ripk3 mRNAs was increased in the placenta. Therefore, it is tempting to speculate that the activation of necroptosis in response to PCOS-related HAIR might serve to counteract the ferroptosis pathway in the placenta. Of note, we also found that maternal hyperandrogenism and insulin resistance resulted in decreased ROS concentration in the uterus ((Hu et al. 2019b) and this study). Given the time-dependent regulation of uterine ROS levels in normal rats during early-mid pregnancy (Hu et al. 2019b), it is possible that DHT+INS-induced ROS generation and accumulation might not be sustained in the gravid uterus on GD 14.5. Additionally, both ferroptosis and necroptosis might intersect and crosstalk with HAIR-induced oxidative damage and subsequently result in increased fetal loss. It remains to be determined whether HAIR-induced pregnancy loss is due to increased iron-mediated uterine ferroptosis or to necroptosis-related defects in the placenta, or both. There is evidence that different forms of programmed cell death, including ferroptosis, apoptosis, and necroptosis, may coexist under the physiological condition and disease state (Choi et al. 2019, Riegman et al. 2019). However, due to the limited commercial antibodies available for rat tissues, we are not able to extend the study to assess whether there is co-existence of ferroptosis and apoptosis in the gravid uterus and placenta in our experimental rats.

In this study, we found that some pro-ferroptosis genes such as Acsl4, Tfrc and Dpp4 were oppositely regulated in the uterus by co-exposure to DHT and INS. However, several anti-ferroptosis genes, including Slc7a11, Gcls, and Cisd1, were downregulated in the gravid uterus after co-exposure to DHT and INS. These results suggest that the suppression of anti-ferroptosis gene transcription might play a dominant role in promoting ferroptosis in this tissue under conditions of HAIR. Compared to the gravid uterus, the placenta showed a distinct profile of ferroptosis-related gene changes in response to the combined DHT and INS exposure. For example, the combined exposure increased Cisd1 mRNA expression and decreased Dpp4 mRNA expression in the placenta, which was opposite to that observed in the gravid uterus. Furthermore, we often observed contrasting expression patterns of pro- and anti-ferroptosis genes in the gravid uterus and placenta with exposure to DHT or INS alone compared to the combined exposure. We do not know the exact reason for these inconsistencies; however, we do know the ontogeny of changes we observed and how these relate to the development of HAIR as the expression of ferroptosis-related genes and proteins was only assessed at one gestational age when the pregnant rats already displayed HAIR (Hu et al. 2019b, Zhang et al. 2019b). In addition, components of HAIR may have acted synergistically or through separate pathways to bring about divergent effects on gene expression and signaling pathways and regulate the ferroptosis process in the gravid uterus and placenta. Overall, our findings demonstrate the complexity and challenges in establishing direct roles and patterns linking individual pro-/anti-ferroptosis genes to the ferroptosis pathway in the gravid uterus and placenta in response to maternal DHT and/or INS in vivo. Future work should therefore investigate the tissue-specific and time-dependent changes in ferroptosis-related gene expression in the uterus and placenta during the maternal hormonal manipulation.

In comparison to the single exposure groups (DHT or INS), specific changes within the maternal uterus and placenta appeared to be driven by hyperandrogenism, insulin resistance, or both (co-exposure to DHT and INS). Experiments utilizing gene and pathway inhibitors in decidual and trophoblast cells would be beneficial for exploring the causality of changes observed regarding ferroptosis and iron metabolism in the future. Work is also required to assess whether elevated ferroptosis in the uterus contributes to placental dysfunction in rat dams with HAIR due to DHT and INS, a key area that would additionally be aided by a time-course analysis. Moreover, it is also possible that HAIR may activate pathways within the uterus which serve to protect and block the propagation of ferroptosis in placental tissue, as opposed or in addition to intrinsic pathways operating within the placenta itself. The gravid uterus and placenta are composed of multiple cell types, each with their distinct gene/protein expression program and likely sensitivity to the DHT and INS treatment. Future work should additionally undertake analyses of the ferroptosis and apoptosis pathways in dissected placental zones or isolated cell types from the gravid uterus and placenta from rats treated with DHT and/or INS. Nonetheless, the concomitant presence of different forms of regulated cell...
death would be expected to disrupt uterine and placental function and play a role in the fetal loss observed in DHT+INS-exposed pregnant rats.

Recently, Zhang and colleagues reported that oxidative stress-induced ferroptosis contributes to the pathogenesis of preeclampsia (Zhang et al. 2020). In particular, they found decreased GPX4, glutathione, and SLC7A11 protein levels and increased MDA content in the preeclamptic placenta in humans and rats (Zhang et al. 2020). Together with our work, these findings support the notion that the ferroptosis pathway is involved in the pathogenesis of female reproductive disorders.

In summary, our findings suggest maternal co-exposure to DHT and INS alters the ferroptosis pathway in the gravid uterus and placenta; however, this occurs via different regulatory mechanisms and signaling pathways. For instance, in contrast to the placenta, increased ferroptosis in the gravid uterus in response to DHT and INS was related to decreased GPX4 and glutathione abundance, altered expression of ferroptosis-associated genes (Acsl4, Tfrc, Slc7a11, and Gclc), increased MDA and iron deposition, upregulation of the ERK/p38/JNK pathway and mitochondrial Dpp4 expression, as well as the appearance of typical ferroptosis-related mitochondrial morphology. In addition, DHT and INS were associated with reduced activation of apoptosis in the uterus and increased necroptosis in the placenta. Both the maternal uterus and placenta play essential roles in embryo implantation and support fetal growth and development during pregnancy (Schatz et al. 2016, Sharma et al. 2016). Therefore, while the present study improves our understanding of the impact of HAIR on regulated cell death in specific tissues during pregnancy, more preclinical and clinical studies are needed to further investigate the molecular and functional connectivity between the maternal decidua, placenta and fetus in PCOS.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-20-0155.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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