Supplementary Materials and Methods

Gene expression analysis by qPCR

The isolation and quantification of the RNA and the qPCR assay were performed as previously described (Zhang et al. 2017; Zhang et al. 2016). The PCR amplifications were performed with SYBR green qPCR master mix (#K0252, Thermo Scientific, Rockford, IL). Total RNA was extracted from the frozen whole uterine and placental tissues with Invitrogen Trizol reagent (#15596026, Thermo Scientific), and single-stranded cDNA was synthesized from each sample (2 μg) with M-MLV reverse transcriptase (#0000113467, Promega Corporation, Fitchburg, WI) and RNase inhibitor (40 U) (#00314959, Thermo Scientific). Uterine and placental RNA purities (A260/A280 ratios) were evaluated with a NanoDrop 1000 spectrometer (Thermo Fisher Scientific). The integrity of the extracted RNA samples was additionally determined by using an Experion RNA StdSens Analysis Kit (Bio-Rad). Only samples presenting with a ratio greater than 1.8 and an RNA quality indicator value lower than 7 were kept for further analyses. Any samples showing poor RNA quality were also excluded from further analysis. cDNA (1 μl) was added to a reaction master mix (10 μl) containing 2× SYBR green qPCR reaction mix (Thermo Scientific) and gene-specific primers (5 μM of forward and reverse primers). All reactions were performed at least twice, and each reaction included a non-template control, and specific sample sizes are denoted in the figure legends. Several housekeeping genes, including Gapdh, Actb, and U87 were tested before analysis. However, only Gapdh was stably expressed between the groups and thus used as the reference gene for our analysis. Fold changes in mRNA expression were calculated by the ΔΔCT method using Gapdh and expressed after normalizing to the control group. The qPCR primers used in this study are listed in Table 1. All sets of primers were validated for qPCR prior to analysis. This involved determining that the efficiency of amplification using a standard curve of cDNA was above 85% and not different from the Gapdh reference gene, and there were no non-specific PCR products seen in a melt curve analysis immediately after the amplification or in parallel reactions with un-transcribed RNA or in reactions without templates (the negative controls). Further, in order to avoid introducing variability, all uterine and placental samples for a given target gene were analysed on a single plate.

Protein isolation and Western blot analysis

A detailed explanation of the tissue lysate preparation and the Western blot analysis protocol has been published (Zhang et al. 2017; Zhang et al. 2016). Tissue proteins were isolated by
homogenization in RIPA buffer (Sigma-Aldrich) supplemented with cOmplete Mini protease inhibitor
cocktail tablets (Roche Diagnostics, Mannheim, Germany) and PhosSTOP phosphatase inhibitor
cocktail tablets (Roche Diagnostics). After determining the total protein concentration by Bradford
protein assay (Thermo Fisher Scientific), equal amounts (30 μg) of protein were resolved on 4–20%
TGX stain-free gels (Bio-Rad Laboratories GmbH, Munich, Germany) and transferred onto PVDF
membranes. The membranes were probed with anti-Gpx4 antibody (ab125066, Abcam, Cambridge,
UK), anti-ERK1/2 antibody (#4695, Cell Signaling Technology, Danver, MA, USA), anti-phospho-
ERK1/2 antibody (#9911, Cell Signaling Technology), anti-p38 MAPK antibody (#8690, Cell Signaling
Technology), anti-phospho-p38 MAPK antibody (#4511, Cell Signaling Technology), anti-JNK antibody
(#9252, Cell Signaling Technology), anti-phospho-JNK antibody (#4668, Cell Signaling Technology),
and anti-cleaved caspase-3 antibody (#9664, Cell Signaling Technology) all diluted 1:1,000 in 0.01 M
Tris-buffered saline supplemented with Triton X-100 (TBST) containing 5% w/v non-fat dry milk
followed by anti-rabbit IgG HRP-conjugated goat secondary antibody (A0545, Sigma-Aldrich). Signal
was detected using the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher
Scientific) and captured using a ChemiDoc MP Imaging System (Bio-Rad). Initial experiments were
performed to verify the identification of cytosolic, mitochondrial and nuclear Gpx4 by Western blot
analysis using rat testis (which has high expression compared to other tissues (Maiorino et al.
2003; Tramer et al. 2002)), epididymis and ovary (Supplemental Fig. 2). For each Western blot, ultraviolet
activation of the Criterion stain-free gel was used to assess total protein loading for each sample
(Zhang et al. 2016). Band densitometry was performed using Image Laboratory (Version 5.0, Bio-Rad)
and the intensity of each protein band was normalized to the total protein in the individual sample. Due
to the number of samples per group, multiple gels were run per group, each containing three replicates
per group. For quantification and to ensure standardization across blots, the expression of the target
protein was normalized to the mean value for the control group on the blot and then all the normalized
values were statistically compared to assess the effect of the treatment groups. This ensured that we
could accurately compare protein abundance across groups with the one tissue.

**Histological processing and Gpx4 immunohistochemistry**

Histological processing and immunohistochemistry were performed according to previously
described methods (Hu et al. 2018; Zhang et al. 2016). Fresh tissues were dissected and immediately
fixed in 4% formaldehyde in neutral buffered solution at 4°C for 24 h and then embedded in paraffin.
Sections (5 μm) were deparaffinized and rehydrated in xylene and graded series of ethanol (99.99%, 80%, and 70% in distilled water, Sigma-Aldrich) for 10 min each. After incubation with the Gpx4 antibody (1:200 dilution, Abcam) overnight at 4°C in a humidified chamber, the sections were stained using the avidin-biotinylated-peroxidase ABC kit followed by a 5-min treatment with 3,3′-diaminobenzidine (DAB, SK-4100, Vector Laboratories). All sections were further counterstained with methyl green (H-3402, Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Stained observed and imaged on a Nikon E-1000 microscope (Japan) under bright-field optics and photomicrographed using Easy Image 1 (Bergström Instrument AB, Sweden).

**Perls’ histochemical reaction**

Iron deposition was detected using DAB-enhanced Perls’ staining as previously described (Meguro et al. 2007). After deparaffinization and rehydration, sections were immersed in a mixture of equal volumes of potassium ferrocyanide solution (HT201, Sigma-Aldrich) and hydrochloric acid solution (HT202, Sigma-Aldrich) for 1 h at room temperature. Sections were washed with PBS five times for 5 min each and incubated with DAB for 10 min and pararosaniline solution (HT203, Sigma-Aldrich) for 2 min. The placental nucleated erythrocytes stained with dark blue particles were used as positive controls. Images of excess iron deposits were captured on a Nikon E-1000 microscope (Japan) under bright-field optics and photomicrographed using Easy Image 1 (Bergström Instrument AB, Sweden).

**Mitochondria structure by transmission electron microscopy (TEM)**

TEM was performed according to a published method (Hu et al. 2019). Fresh uterine and placental tissues were fixed in 2.5% glutaraldehyde in phosphate buffered saline (PBS, pH 7.2–7.4) for 1 h at room temperature (RT) and further rinsed with 0.1 M PBS three times for 15 minutes each. Secondary fixation with 1% osmium tetroxide in PBS was performed for 1 h prior to sequential dehydration with an acetone gradient (50%, 70%, and 90% for 15 min each and 100% three times for 30 min each time at RT). Samples were finally embedded in Epon epoxy resin. Random areas from uterine and placental tissues were oriented for ultrastructural analysis. The blocks were cut in 50–60 nm sections using a Reichert ultramicrotome (Leica, Germany), collected on 300 mesh copper grids, and stained with 3% uranyl acetate and counterstained with lead citrate before visualization. The post-stained sections were examined and imaged with a transmission electron microscope (H-7650, Hitachi, Japan) equipped with an electron imaging spectrometer. Image collection and parameter settings were identical for each of the different tissues/regions analyzed (Supplemental Fig. 5).
Quantification of glutathione, MDA, and mitochondrial open reading frame of the 12S rRNA-c (MOTS-c)

The intracellular glutathione, MDA, and MOTS-c levels were assessed using a glutathione/glutathione+glutathione disulfide assay kit (ab239709, Abcam, Cambridge, UK), MDA assay kit (ab118970, Abcam), and MOTS-c ELISA kit (CEX132Ra, Cloud-Clone/USCNK, Oxfordshire, UK), respectively, according to the manufacturers’ protocols. The reproducibility (intra- and inter-assay coefficients of variation) of glutathione, MDA, and MOTS-c assays are 15%/15%, 15%/15%, and 10%/12%, respectively. A standard curve for glutathione, MDA, and MOTS-c concentration was generated and used for calculating their concentration in the samples. The concentration of glutathione, glutathione+glutathione disulfide, MDA, and MOTS-c in each group was normalized to the total tissue protein concentration as determined by the Bradford protein assay (Thermo Fisher Scientific).

References


## Supplemental Table 1

<table>
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<tr>
<th>Tissue</th>
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<th>Placenta</th>
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<tr>
<td>Protein (n = 9/group)</td>
<td>Glutathione, Glutathione+Glutathione disulfide (Fig. 2A)</td>
<td>Glutathione, Glutathione+Glutathione disulfide (Fig. 2A)</td>
<td>Gpx4 (Fig. 1A)</td>
<td>Gpx4 (Fig. 1A)</td>
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<td>MDA (Fig. 2C)</td>
<td>ERK1/2, p-ERK1/2 and the ratio (Fig. 4A)</td>
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<td>p38, p-p38, and the ratio (Fig. 4A)</td>
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<td>Cleaved Caspase-3 (Fig. 6C)</td>
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<td>Gene (n = 7–8/group)</td>
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<td>Slc7a11 (Fig. 2B)</td>
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<td>Slc1a5, Acs4, Gls2, Cs, Gclc, Gss, Tfr2, Ireb2 (Fig. 2B)</td>
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<td>Bcl-xl, Bax (Fig. 6B)</td>
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<td>Bcl2, Casp3 (Fig. 6B)</td>
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