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Chronic fetal hypoxia disrupts the peri-conceptual environment in next-generation adult female rats

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Key points:

- Exposure to chronic hypoxia during gestation influences long-term health and development, including reproductive capacity, across generations.
- If the peri-conceptual environment, in the developing oviduct, is affected by gestational hypoxia, then this could have implications for later fertility and the health of future generations.
- In this study, we show that the oviducts of female rats who were exposed to chronic hypoxia whilst *in utero*, have reduced telomere length, decreased mitochondrial DNA biogenesis, and increased oxidative stress
- Our results show that exposure to chronic gestational hypoxia leads to accelerated ageing of the oviduct in early adulthood, and help us understand how exposure to hypoxia during development could influence reproductive health across generations.

Abstract (245):

Exposure to chronic hypoxia during fetal development has important effects on immediate and long-term outcomes in offspring. Adverse impacts in adult offspring include impairment of cardiovascular function, metabolic derangement, and accelerated ovarian ageing. However, it is not known whether other aspects of the female reproductive system may be similarly affected. In this study, we examine the impact of chronic gestational hypoxia on the developing oviduct. Wistar rat dams were randomized to either normoxia (21%) or hypoxia (13%) from day 6 post-mating until delivery. Post-delivery female offspring were maintained in normoxia until 4 months of age. Oviductal gene expression was assayed at the RNA (q-rtPCR) and protein (Western blotting) levels. Oviductal telomere length was assayed using Southern blotting. Oviductal telomere length was reduced in the gestational hypoxia-exposed animals compared to the normoxic controls ($p<0.01$). This was associated with a specific post-transcriptional reduction in the KU70 subunit of DNA-pk in the gestational hypoxia-exposed group ($p<0.05$). Gestational hypoxia-exposed oviducts also showed evidence of decreased mitochondrial DNA biogenesis; reduced mtDNA copy number ($p<0.05$), and reduced gene expression of *Tfam* ($p<0.05$) and *Pgc1α* ($p<0.05$). In the hypoxia-exposed oviducts there was up-regulation of mitochondrial-specific antioxidant defense enzymes (MnSOD; $p<0.01$). Exposure to chronic gestational hypoxia leads to accelerated ageing of the oviduct in adulthood. The oviduct plays a central role in early development as the site of gamete transport, syngamy, and early development, hence accelerated ageing of the oviductal environment could have important implications for fertility and the health of future generations.

Introduction

Many human fetuses are exposed to chronic gestational hypoxia, either via factors intrinsic to the pregnancy, for example impaired utero-placental blood flow (Kuzmina *et al.*, 2005), or factors arising from the maternal environment, for example pregnancy at high altitude (Ducsay, 1998; Postigo *et al.*, 2009; Giussani *et al.*, 2016). The immediate effects of gestational hypoxia have been characterized in both human pregnancies and animal models, and include adverse outcomes such as IUGR, low birth weight and stillbirth (Giussani *et al.*, 2001; Keyes *et al.*, 2003; Richter *et al.*, 2012; Gonzalez-Candia *et al.*, 2016). The long-term outcomes for the adult offspring of chronic gestational hypoxia are generally less well understood, but some aspects, for example the increased risk of later cardiovascular dysfunction, have been well described in animal models (Giussani *et al.*, 2012; Giussani & Davidge, 2013). Furthermore, there is evidence from animal models that exposure to chronic gestational hypoxia can adversely impact brain development (Phillips *et al.*, 2017), renal ageing (Gonzalez-Rodriguez *et al.*, 2013), and insulin resistance (Camm *et al.*, 2011).

The link between exposure to various suboptimal intrauterine environments and subsequent impairment of reproductive function has been demonstrated in a number of animal models (Aiken *et al.*, 2013; Aiken *et al.*, 2016). These studies have mainly been performed in rodents, and have focused primarily on alterations to maternal diet (Chan *et al.*, 2015b). It has been shown that accelerated ageing of the somatic ovarian tissue, with a concomitant decrease in ovarian reserve in early-mid reproductive life, is a consequence of a maternal low protein diet (Aiken *et al.*, 2013), obesogenic maternal diet (Aiken *et al.*, 2016), and maternal caloric restriction (Bernal *et al.*, 2010) in various rodent models.

The primary outcome of most studies that have demonstrated a link between the early life environment and impairment of female fertility has been ovarian reserve (Chan *et al.*, 2015b; Ho *et al.*, 2017). As a key determinant of future reproductive potential (Depmann *et al.*, 2015; Pelosi *et al.*, 2015), ovarian reserve is a useful and specific marker of fertility potential, but reproduction depends on a wide range of factors beyond the availability of gametes. In the female, successful pregnancy depends not only on a viable oocyte, but also on a suitable reproductive tract environment. The oviduct has several vital roles in successful reproduction, including gamete transport (Wang & Larina, 2018), syngamy (Parada-Bustamante *et al.*, 2016), and early embryonic development (Robertson *et al.*, 2015). Oviductal problems are a major cause of infertility in human populations, accounting for approximately 25-35% of all female infertility (Practice Committee of the American Society for Reproductive, 2015). Such problems

can range from complete blockage of the oviduct, which impairs gamete transport and prevents conception, to sub-clinical oviductal damage, for example through smoking, which alters the tubal epithelium and increases the risk of ectopic pregnancy (Horne *et al.*, 2014; Nio-Kobayashi *et al.*, 2016). Impact on the oviductal environment of the adult offspring is thus an important consideration in investigating the effect of developmental programming on female reproductive potential.

A limited number of studies have previously reported on the impact of an adverse intrauterine environment on the developing oviduct. Wister rat offspring exposed to a maternal low-protein diet during gestation, followed by postnatal catch-up growth, showed evidence of reduced telomere length and increased oxidative stress in the oviduct in early adulthood (Aiken *et al.*, 2013). We hypothesise that exposure to chronic gestational hypoxia may also adversely affect the oviduct, and hence the peri-conceptual environment, in a similar way.

Using an established model of hypoxic pregnancy in rats, we investigated the impact of exposure to a 40% reduction in environmental oxygen (13% versus 21% ambient oxygen from day 6 of pregnancy) on the oviduct of the adult female offspring. A reduction in the environmental oxygen tension by 40% reflects the difference in oxygen availability between pregnancies occurring at sea level compared to 3500-4000m altitude (Postigo *et al.*, 2009). Hence our rat model of gestational hypoxia is highly relevant to human pregnancy at these altitudes, where it is estimated that ~40,000 babies are born each year in Bolivia alone (Roost *et al.*, 2009). The aim of this study was therefore to evaluate whether there is evidence of accelerated ageing in the oviducts of young adult female rats exposed to chronic gestational hypoxia.

Materials and Methods

Ethical approval

All animal experiments were approved by the University of Cambridge Animal Welfare and Ethical Review Board (ref. no. PC6CEFE59). All animal experiments were conducted in accordance with the British Animals (Scientific Procedures) Act (1986) and were compliant with EU Directive 2010/63/EU. Animals underwent euthanasia by CO_2 inhalation and cervical dislocation.

Study design

Wistar rat dams at 10-12 weeks of age (Charles River Ltd., Margate, UK; wild-type RRID: RGD_13508588) were housed in individually ventilated cages (21% oxygen, 70-80 air changes/hour) under standard conditions, with a regular 12-hour light/dark cycle. All animals were fed a standard laboratory chow diet (20% protein) and fed *ad libitum* with free access to water. After initial acclimatization (10 days) they were mated with fertile male Wistar rats, and pregnancy confirmed through the observation of a vaginal plug. The day of the plug was designated day 0 of pregnancy (full term 21-22 days). Upon confirmation of pregnancy, dams were weighed and housed individually. On day 6 of pregnancy, dams were randomly divided into two groups; control (21%) and hypoxic (13%) pregnancy (n=8 per group). Pregnant rats assigned to the hypoxia group were placed inside a chamber that could hold 9 rat cages, which combined a PVC isolator with a nitrogen generator, as previously described (Giussani *et al.*, 2012; Herrera *et al.*, 2012). The hypoxia model did not alter maternal food intake or gestational length. Pregnancies undergoing hypoxia were maintained at a constant inspired fraction of oxygen of 13% from day 6 to 20 of gestation. All dams delivered under normoxic conditions, and normoxia (21%) was maintained for all animals during lactation, weaning and thereafter. Following determination of birth weight, litters were culled to 4 males and 4 females to standardise nutritional access and maternal care (Herrera *et al.*, 2012). All pups were suckled by their own mothers. At four months of age, adult female pups underwent euthanasia by CO_2 inhalation and cervical dislocation. At postmortem, the reproductive tract tissues were harvested immediately after dissection. The oviducts were snap-frozen in liquid nitrogen until used for analysis. No sample was refrozen after the initial thaw.

Telomere length analysis

High-molecular weight DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quantity and purity was determined using a Nanodrop spectrophotometer (Nanodrop Technologies (Thermo Fisher, Scientific, Hemel Hempstead, UK). Agarose gels were run to ensure all DNA samples were of high-molecular weight. DNA (1.2μg) was digested with *Hinf*I and *Rsa*I restriction enzymes for 2h at 37°C. The restricted samples were quenched with 5x SDS loading buffer (Roche Diagnostics, Mannheim, Germany) and loaded onto agarose gels containing SYBR safe stain (Invitrogen, Paisley, Scotland, UK). After pulsed field gel electrophoresis, the gels were checked for non-specific degradation of an undigested DNA control and complete digestion of the enzyme-restricted DNA by visualizing the stained gels under UV light (Syngene, Cambridge, UK). The

separated DNA fragments were transferred to nylon membrane (Roche Diagnostics, Mannheim, Germany) by Southern blotting, and telomeric repeat length was determined using a commercial method of chemiluminescent detection as described previously (Tarry-Adkins *et al.*, 2006). Molecular weight markers on each gel were a mid-range pulsed-field gel marker (New England Biolabs, Ipswich, MA, USA) and dioxygenin (DIG; low range) molecular-weight marker (Roche Diagnostics, Mannheim, Germany). Standard undigested and digested genomic samples of DNA from a 4-month control animal were also included on each gel to verify digestion efficiency. Telomere signals were analyzed using Adobe Photoshop (Adobe Systems Inc. San Jose, CA, USA) and Alpha Ease Software (Alpha Innotech, San Leandro, CA, USA). Telomere length was measured as described previously (Tarry-Adkins *et al.*, 2006).

Gene expression analysis

An initial panel of 38 candidate genes was developed to test which molecular pathways might be altered in the somatic oviduct following exposure to chronic gestational hypoxia. These genes were chosen based on (i) previous work on the effects of developmental programming on ovarian, para-ovarian adipose tissue, and oviductal gene expression (Aiken *et al.*, 2015; Aiken *et al.*, 2016; Tarry-Adkins *et al.*, 2018) (ii) knowledge of programming mechanisms in other organ systems in the same gestational hypoxia rat model (Camm *et al.*, 2010; Giussani *et al.*, 2012; Herrera *et al.*, 2012) and (iii) relevant literature review. RNA was extracted from snap-frozen oviducts using a miRNeasy mini kit (Qiagen, Hilden, Germany) following manufacturers' instructions, with the addition of a DNaseI digestion step to ensure no genomic DNA contamination. RNA quantification was performed using a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA (1 μg) was used to synthesize cDNA using oligo-dT primers and M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA). Gene expression was determined using custom designed primers (Sigma, Poole, Dorset, UK) and SYBR Green reagents (Applied Biosystems, Warrington, UK) as previously described (Tarry-Adkins *et al.*, 2009). Primer sequences are in supplementary table 1. Quantification of gene expression was performed using a Step One Plus RT-PCR machine (Applied Biosystems, Warrington, UK). Equal efficiency of the reverse transcription of RNA from all groups was confirmed through quantification of expression of the house-keeping gene *ppia*, the expression of which did not differ between groups.

Protein quantification

Due to the extremely small amount of tissue available, limited protein quantification was performed. Genes were selected for protein expression analysis on the basis of (i) RNA quantification results and (ii) rationale from previous studies in the same model. Protein was extracted from whole tissue lysates of snap-frozen oviducts, as described previously (Tarry-Adkins *et al.*, 2015; Tarry-Adkins *et al.*, 2018). Protein (20µg) was loaded onto 10%, 12% or 15% polyacrylamide gels, dependent upon the molecular weight of the protein to be measured. The samples were electrophoresed and transferred to polyvinylidene fluoride membranes. Detection steps used the following primary antibodies; P53 (R & D Systems; cat no: MAB1355, 1:1000, RRID:AB_357649), P16^{INK} (Abcam, Cambridge, UK; cat no: Ab189034, 1:1000, RRID:AB_2737282), OGG1 (Novus Biologicals; cat no: NB100-106,1:1000, RRID:AB_10104097), MRE11 (ProteinTech, Cambridge, UK, cat no: 10744-1-AP, 1:1000, RRID:AB2145118), KU70 (ProteinTech, Cambridge, UK, cat no: 10723-1-AP, 1:1000, RRID:AB_), KU80 (Novus, cat no: NB100-508, 1:1000, RRID:AB_2218756), Total Ox Phos rodent antibody cocktail (Abcam, Cambridge, UK, cat no: Ab110413, 1:5000, RRID:AB_2629281), HIF1α (Abcam: cat no: Ab51608, 1:1000, RRID:AB_880418), GP91^{phox} (ProteinTech, Cambridge, UK; cat no: 19013-1-AP, RRID:AB_1342287), P47^{phox} (ProteinTech, Cambridge, UK; cat. no: 15551-1-AP, 1:1000, RRID:AB_11182937), XO (Santa-Cruz, Wimbledon, Middlesex, UK; cat. no: SC-20991, 1:200, RRID:AB_2214858), HMOX1 (ProteinTech, Cambridge, UK, cat no: 20960-1-AP, 1:1000, RRID:AB_10732601), Catalase (Abcam, Cambridge, UK, cat. no.: Ab1877-10, 1:10000, RRID:AB_187710), MnSOD (Upstate, Watford, UK; cat. no.: 06-984, RRID:AB_310325), CuZnSOD (ProteinTech, Cambridge, UK; cat. no.: 10269-1-AP, 1:1000, RRID:AB_2193750). Anti-rabbit secondary antibodies (Cell Signaling Technology, Danvers, MA, USA, 1:2000) were utilised for all primary antibodies except P53, which required an anti-mouse secondary antibody (Cell Signaling Technology (Danvers, MA, USA), 1:2000). Equal protein loading was confirmed by staining electrophoresed gels with Coomassie Blue (Bio-Rad, Hemel Hempstead, Herts, UK) to visualize total protein. To ensure that the chemiluminescent signal changed in a linear manner, the ratio between loading controls (100% and 50% pooled sample) was confirmed for each detected protein.

Statistical Analysis

All data were initially analyzed using a 2-way ANOVA with gestational hypoxia/normoxia as the independent variable. Raw p values were transformed to take account of the false discovery rates. Maternal environmental effects were compared between groups using 2-tailed Student's T tests. Data are represented as means ± SEM. Where p values are reported, an alpha level <0.05

was considered statistically significant. All data analysis was conducted using the R statistical software package version 2.14.1 (R Foundation for Statistical Computing, Vienna, Austria). In all cases, n refers to the number of litters, and n=7-8 for all groups. The adequacy of the sample size was determined via a power calculation based on the effect sizes for somatic ovarian expression for ageing-related genes a previous rodent developmental programming model (Aiken *et al.*, 2016) using an alpha level of 0.05 to give power of 0.8. Sample analysis was performed using project codes to blind the investigators to the experimental groups.

Results

There was no impact of chronic gestational hypoxia on either maternal food intake during gestation (normoxia 79±2g/kg/day^{-1} v. hypoxia 70±3g/kg/day^{-1}) or length of gestation (normoxia 20±1 days v. hypoxia 20±1 days).

Maintenance of oviductal telomere length

At 4 months of age, there were significantly more very short (1.3-4.2kB, $p<0.001$) telomeres in the oviducts of gestational hypoxia-exposed adult females compared to the normoxic group (Figure 1A). There were no significant differences between the hypoxia and normoxia-exposed groups in the proportion of telomeres that were short (4.2-8.6kB), long (8.6-45.5kB) or very long (45.5-145kB).

Cell-cycle markers of ageing

Alongside the increase in very short telomeres observed in hypoxia-exposed tissues, there was an increase in cell-cycle markers that increase with cellular ageing. Gene expression of *p21* was significantly increased in the hypoxia-exposed group compared to the controls ($p<0.04$). There was also a trend towards increased *p53* expression ($p=0.09$), but this did not reach statistical significance (Table 1). At the protein expression level, there was no significant difference in P16ink levels between groups, but there was a significant increase in P53 ($p<0.05$; Table 2)

DNA damage repair mechanisms

Gene expression of *Ogg1* was elevated in the hypoxia-exposed group compared to the normoxic group (1294±135 v. 1710±132 units; $p<0.05$) (Table 1). At the protein level, the elevation of OGG1 in the hypoxia-exposed group was of borderline significance ($p=0.08$; Table 2). By contrast *Mre11* expression was decreased by more than 50% in the hypoxia-exposed group compared to the controls (723±119 v. 307±79, $p<0.05$) (Table 1), however there was no difference in MRE11 protein expression between the experimental groups (Table 2). There was a trend towards an overall reduction in the catalytic subunit of the DNA protein kinase (*DNA*

pkcs) that is required for double-stranded break repair and telomere maintenance ($p<0.1$; Table 1), but no differences in the expression of either of the components of the binding subunit, *Ku70 or Ku80* (Figure 1B). However, at the protein level, there was a significant deficit of KU70 in the oviducts of animals exposed to gestational hypoxia ($p<0.05$), with no difference in KU80 levels (Figure 1C).

There was no significant difference between hypoxia-exposed and normoxic groups in expression of any other DNA damage sensing or early repair mechanisms that were included in the candidate genetic screen; *Neil1*, *Nthl1* or *Xrcc1* (Table 1).

Mitochondrial Biogenesis

Mitochondrial DNA (mtDNA) copy number was reduced in hypoxia-exposed animals compared to controls ($p<0.05$, Figure 2A). The expression of *Tfam* was significantly reduced in oviducts of animals exposed to gestational hypoxia compared to normoxic controls ($p<0.05$; Figure 2B). *Pgc1α* also showed reduced expression in the hypoxia-exposed group ($p<0.05$; Figure 2C). There was no difference between groups in expression of *Nrf2* or *Lonp1* (Table 1). Hence, there is evidence that mtDNA biogenesis may be impaired in the oviduct after exposure to chronic gestational hypoxia.

We further investigated the gene expression of components of the mitochondrial respiratory complex. There was significant reduction in gene expression of complex I ($p<0.01$) and complex IV ($p<0.05$) in the hypoxia-exposed group compared to the normoxia group. There was also a significant reduction in the gene expression of citrate synthase (*Cs*) ($p<0.05$; Table 1). There was no difference in the expression levels of complex II, complex III or cytochrome C (*Cycs*). However, there was no significant difference in protein expression between the hypoxia-exposed and normoxia-exposed groups in any of the tested mitochondrial respiratory components (Table 2).

Oxidative stress and anti-oxidant defense capacity

There was no direct evidence of increased oxidative stress markers in any of the pathways tested in the oviducts at either the gene expression or protein levels (*Hif1α, Gp91phox, P22phox, P47phox, Xo, Gpx1, Hmox1)* (Table 1 and Table 2).

In terms of antioxidant defense capacity, there was no significant difference in gene expression of *Catalase*, *Cuzusod* or *Ecsod* in the hypoxia-exposed compared to the normoxia group. However there was an increase in *MnSOD* expression at both the RNA and protein level (Figure 2 D&E), which is in keeping with the suggestion that mitochondrial biogenesis may be

suboptimal in the gestational oviduct. MnSOD is the specific mitochondrial isoform of the powerful superoxide dismutase group of anti-oxidants. Increased expression of MnSOD may thus indicate a successful attempt to buffer the impact of excess free radical generation resulting from impaired mitochondrial biogenesis.

Lipid peroxidation

There was a significant increase in the gene expression of *Alox12* (a key component of the lipoxygenase pathway) in the hypoxia-exposed group compared to the controls ($p<0.05$; Table 2). There was no difference in the gene expression levels of *Alox15* between the hypoxia-exposed and control groups.

Discussion

We show evidence of accelerated ageing in the oviducts of female offspring in early-mid adulthood, following exposure to chronic gestational hypoxia. Accelerated ageing is demonstrated at a cellular level by decreased telomere length and increased expression of markers of cellular ageing, in particular *p21* and *p53*. The observed decrease in oviductal telomere length was accompanied by a specific post-transcriptional reduction in KU70, which is a key functional sub-unit of the DNA-activated protein kinase required for telomere length maintenance (Jette & Lees-Miller, 2015). The observed up-regulation of *Ogg1* in the oviducts of the hypoxia-exposed animals is in keeping with an increase in oxidative DNA damage. *Ogg1* excises 7,8-dihydro-8-oxoguanine (8-oxoG) from damaged DNA, which limits the impact of ubiquitous oxidative damage accumulated during normal ageing (Radicella *et al.*, 1997). Hence the observed increase in *Ogg1* suggests a greater exposure to oxidative DNA damage in the oviducts following gestational hypoxia.

There was also clear evidence that mitochondrial biogenesis is reduced in the oviduct following exposure to chronic gestational hypoxia. In particular, the key regulatory genes controlling mitochondrial biogenesis (*Tfam* and *Pgc1α*) were both down-regulated in the hypoxia-exposed group compared to the controls. *Tfam* is the master regulator of mitochondrial biogenesis via gene expression from the mitochondrial genome (Picca & Lezza, 2015) and *Pgc1α* regulates mitochondrial biogenesis via nuclear gene expression (Picca & Lezza, 2015). Alongside the observed reduction in mtDNA copy number, there is thus evidence that both key mechanisms regulating mitochondrial biogenesis are impaired following exposure to gestational hypoxia. Evidence of a mitochondrial deficit is particularly interesting as oviductal function depends on ciliary motility and coordinated smooth muscle contraction (Halbert *et al.*, 1976; Bylander *et al.*, 2013; Zhao *et al.*, 2015). Both of these processes are dependent on normal mitochondrial

function and ATP production (Dirksen & Zeira, 1981; Lydrup & Hellstrand, 1986), in particular in the ciliated cells of the oviduct epithelium. Oviductal ultra-structure, including mitochondria in the ciliated epithelial cells, appears to be established mainly during late fetal life (Kenngott *et al.*, 2008; Zhao *et al.*, 2015), which correlates with the timing of exposure to a chronic hypoxic environment in our study.

There is remarkably little published evidence regarding oviductal phenotype in other developmental programming models, despite the plethora of studies that have examined ovarian reserve (Bernal *et al.*, 2010; Aiken *et al.*, 2013; Chan *et al.*, 2015a; Aiken *et al.*, 2016). However, at least one previous study has examined the impact of a maternal low protein diet on mtDNA copy number and telomere length in the oviduct (Aiken *et al.*, 2013). In keeping with our findings here, oviductal telomere length was shown to be particularly sensitive to the early life environment, more so than the somatic ovarian tissue (Aiken *et al.*, 2013), an effect that was magnified with increasing age (Aiken *et al.*, 2013). In the current study, we observe the same highly significant reduction in telomere length in young animals near the start of reproductive life. An important point for future development of this work is to test directly whether oviductal shortening in response to gestational hypoxia is magnified later in reproductive life. Interestingly, in response to a maternal low protein diet, oviductal mtDNA copy number was increased compared to the controls, which contrasts with our finding here. This suggests that reduced mitochondrial biogenesis is a specific effect of gestational hypoxia rather than a generic impact of early life stress on the oviduct. The relatively small number of proteins in the developing oviduct affected by exposure to gestational hypoxia also points towards a highly specific impact on cellular ageing within the oviduct, rather than ubiquitous tissue damage caused by the adverse early life environment. We also did not observe ubiquitous up-regulation of markers of oxidative stress in the oviducts (*Hif1α, Gp91phox, P22phox, P47phox, Xo, Gpx1, Hmox1)*, which are normally highly sensitive to generic tissue damage adding further evidence that the effect reported is highly specific.

In keeping with the strong evidence of reduced mitochondrial biogenesis in the hypoxia-exposed oviducts, we also observed an increase in mitochondrial-specific antioxidant defense. MnSOD was up-regulated in the hypoxia-exposed group compared to the controls, indicating that there may be an increase in reactive oxygen species produced. Mitochondria are the major intracellular source of reactive oxygen species, but there was no direct evidence of an increase in any of the oxidative stress markers that were assayed in this study. However this may become apparent as the animals age.

Oviducts are a relatively homogeneous tissue, with very low levels of telomerase expression (Lee *et al.*, 2001). This is a significant advantage for our study, which provides novel insight into this relatively under-studied yet crucial part of the female reproductive system. A limitation of the study is the inherently tiny amount of tissue available from each experimental animal (average oviductal weight ≤5mg (Sen & Talwar, 1973)). This meant that the assays performed on protein, RNA and DNA had to be strictly prioritised rather than testing all potential genes and proteins of interest. The extremely small mass of the tissue also meant that we were unable assign tissue for histological examination, or cell-type specific analysis. These are important aims for future work. In particular, future work should focus on whether the muscularis or the epithelium or both are affected by the phenotype described. Either could plausibly have a significant influence on oviductal function and future fertility. Accelerated ageing in the muscularis could affect efficient transport of gametes or conceptus, thus influencing the future risk of ectopic pregnancy. Accelerated ageing in the epithelium could influence the composition of the oviductal fluid, and hence the culture medium for the early embryo. Assessing oviductal function *in vivo*, including assessing fertility outcomes, would help to verify the implications of our results and refine our understanding of the phenotype. This should form the basis of future programmes of work.

Oviduct-related infertility is a key cause of female sub-fertility, accounting for ~30% of cases (Kawwass *et al.*, 2013), and increases with advancing maternal age (Maheshwari *et al.*, 2008). Our work suggests that there may be a developmentally programmed component to the acceleration in cellular ageing and hence oviductal dysfunction observed in women ≥ 35 years (Maheshwari *et al.*, 2008). The age of the animals studied here equates to early in reproductive life, and hence the observed evidence of cellular ageing in the oviducts is even more striking. Aside from infertility, ageing of the oviducts is a significant risk factor predisposing to tubal ectopic pregnancy (Nybo Andersen *et al.*, 2000), which can be a fatal complication of oviductal dysfunction (Farquhar, 2005). The risk of ectopic pregnancy increases sharply with maternal age from 1.4% of all pregnancies in women aged 21 years, to 6.9% of pregnancies in women above the age of 44 (Nybo Andersen *et al.*, 2000). The active role of the oviductal epithelium in the pathogenesis of ectopic pregnancy is becoming increasingly clear (reviewed in (Horne & Critchley, 2012)) as is the requirement for normal regulation of smooth muscle contractility (Shaw *et al.*, 2010). Hence, our finding that adult females who have been exposed to chronic gestational hypoxia show accelerated ageing and dysregulated mitochondrial biogenesis in the oviducts may have potential clinical significance not only for patients with difficulty conceiving but also in understanding risk factors for ectopic pregnancy.

Conclusion

Large numbers of pregnancies world-wide are exposed to chronic gestational hypoxia, either through pregnancy at high altitude or through utero-placental insufficiency (Ducsay, 1998; Kuzmina *et al.*, 2005; Postigo *et al.*, 2009; Giussani *et al.*, 2016). The recognition of the adverse impact of lower than normal oxygenation during pregnancy on ageing of the oviducts, with attendant consequences for gamete and embryo transport in potential next generation mothers, is an important area for further research and exploration.

Additional information

Competing interests

The authors have no competing interests to declare.

Author contributions

CEA conceptualised the study, analysed and interpreted the data, and drafted the manuscript. JTA, AMS, AMN, TJA, AR, MJS and EJC acquired and analysed the data, and critically revised the manuscript. DAG and SEO conceptualised and designed the study, and drafted the manuscript. All of the authors approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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References

Aiken CE, Tarry-Adkins JL & Ozanne SE. (2013). Suboptimal nutrition in utero causes DNA damage and accelerated aging of the female reproductive tract. *FASEB J* **27,** 3959-3965.

Aiken CE, Tarry-Adkins JL & Ozanne SE. (2015). Transgenerational Developmental Programming of Ovarian Reserve. *Sci Rep* **5,** 16175.

Aiken CE, Tarry-Adkins JL, Penfold NC, Dearden L & Ozanne SE. (2016). Decreased ovarian reserve, dysregulation of mitochondrial biogenesis, and increased lipid peroxidation in female mouse offspring exposed to an obesogenic maternal diet. *FASEB J* **30,** 1548-1556.

Bernal AB, Vickers MH, Hampton MB, Poynton RA & Sloboda DM. (2010). Maternal undernutrition significantly impacts ovarian follicle number and increases ovarian oxidative stress in adult rat offspring. *PLoS One* **5,** e15558.

Bylander A, Lind K, Goksor M, Billig H & Larsson DG. (2013). The classical progesterone receptor mediates the rapid reduction of fallopian tube ciliary beat frequency by progesterone. *Reprod Biol Endocrinol* **11,** 33.

Camm EJ, Hansell JA, Kane AD, Herrera EA, Lewis C, Wong S, Morrell NW & Giussani DA. (2010). Partial contributions of developmental hypoxia and undernutrition to prenatal alterations in somatic growth and cardiovascular structure and function. *Am J Obstet Gynecol* **203,** 495 e424-434.

Camm EJ, Martin-Gronert MS, Wright NL, Hansell JA, Ozanne SE & Giussani DA. (2011). Prenatal hypoxia independent of undernutrition promotes molecular markers of insulin resistance in adult offspring. *FASEB J* **25,** 420-427.

Chan KA, Bernal AB, Vickers MH, Gohir W, Petrik JJ & Sloboda DM. (2015a). Early life exposure to undernutrition induces ER stress, apoptosis, and reduced vascularization in ovaries of adult rat offspring. *Biol Reprod* **92,** 110.

Chan KA, Tsoulis MW & Sloboda DM. (2015b). Early-life nutritional effects on the female reproductive system. *J Endocrinol* **224,** R45-62.

Depmann M, Faddy MJ, van der Schouw YT, Peeters PH, Broer SL, Kelsey TW, Nelson SM & Broekmans FJ. (2015). The Relationship Between Variation in Size of the Primordial Follicle Pool and Age at Natural Menopause. *J Clin Endocrinol Metab* **100,** E845-851.

Dirksen ER & Zeira M. (1981). Microtubule sliding in cilia of the rabbit trachea and oviduct. *Cell Motil* **1,** 247-260.

Ducsay CA. (1998). Fetal and maternal adaptations to chronic hypoxia: prevention of premature labor in response to chronic stress. *Comp Biochem Physiol A Mol Integr Physiol* **119,** 675-681.

Farquhar CM. (2005). Ectopic pregnancy. *Lancet* **366,** 583-591.

Giussani DA, Bennet L, Sferruzzi-Perri AN, Vaughan OR & Fowden AL. (2016). Hypoxia, fetal and neonatal physiology: 100 years on from Sir Joseph Barcroft. *J Physiol* **594,** 1105-1111.

Giussani DA, Camm EJ, Niu Y, Richter HG, Blanco CE, Gottschalk R, Blake EZ, Horder KA, Thakor AS, Hansell JA, Kane AD, Wooding FB, Cross CM & Herrera EA. (2012). Developmental programming of cardiovascular dysfunction by prenatal hypoxia and oxidative stress. *PLoS One* **7,** e31017.

Giussani DA & Davidge ST. (2013). Developmental programming of cardiovascular disease by prenatal hypoxia. *J Dev Orig Health Dis* **4,** 328-337.

Giussani DA, Phillips PS, Anstee S & Barker DJ. (2001). Effects of altitude versus economic status on birth weight and body shape at birth. *Pediatr Res* **49,** 490-494.

Gonzalez-Candia A, Veliz M, Araya C, Quezada S, Ebensperger G, Seron-Ferre M, Reyes RV, Llanos AJ & Herrera EA. (2016). Potential adverse effects of antenatal melatonin as a treatment for intrauterine growth restriction: findings in pregnant sheep. *Am J Obstet Gynecol* **215,** 245 e241-247.

Gonzalez-Rodriguez P, Jr., Tong W, Xue Q, Li Y, Hu S & Zhang L. (2013). Fetal hypoxia results in programming of aberrant angiotensin ii receptor expression patterns and kidney development. *Int J Med Sci* **10,** 532-538.

Halbert SA, Tam PY & Blandau RJ. (1976). Egg transport in the rabbit oviduct: the roles of cilia and muscle. *Science* **191,** 1052-1053.

Herrera EA, Camm EJ, Cross CM, Mullender JL, Wooding FB & Giussani DA. (2012). Morphological and functional alterations in the aorta of the chronically hypoxic fetal rat. *J Vasc Res* **49,** 50-58.

Ho SM, Cheong A, Adgent MA, Veevers J, Suen AA, Tam NNC, Leung YK, Jefferson WN & Williams CJ. (2017). Environmental factors, epigenetics, and developmental origin of reproductive disorders. *Reprod Toxicol* **68,** 85-104.

Horne AW, Brown JK, Nio-Kobayashi J, Abidin HB, Adin ZE, Boswell L, Burgess S, Lee KF & Duncan WC. (2014). The association between smoking and ectopic pregnancy: why nicotine is BAD for your fallopian tube. *PLoS One* **9,** e89400.

Horne AW & Critchley HO. (2012). Mechanisms of disease: the endocrinology of ectopic pregnancy. *Expert Rev Mol Med* **14,** e7.

Jette N & Lees-Miller SP. (2015). The DNA-dependent protein kinase: A multifunctional protein kinase with roles in DNA double strand break repair and mitosis. *Prog Biophys Mol Biol* **117,** 194-205.

Kawwass JF, Crawford S, Kissin DM, Session DR, Boulet S & Jamieson DJ. (2013). Tubal factor infertility and perinatal risk after assisted reproductive technology. *Obstet Gynecol* **121,** 1263-1271.

Kenngott RA, Neumuller C & Sinowatz F. (2008). Prenatal differentiation of bovine oviductal epithelium: an electron microscopic study. *Anat Histol Embryol* **37,** 418-426.

Keyes LE, Armaza JF, Niermeyer S, Vargas E, Young DA & Moore LG. (2003). Intrauterine growth restriction, preeclampsia, and intrauterine mortality at high altitude in Bolivia. *Pediatr Res* **54,** 20-25.

Kuzmina IY, Hubina-Vakulik GI & Burton GJ. (2005). Placental morphometry and Doppler flow velocimetry in cases of chronic human fetal hypoxia. *Eur J Obstet Gynecol Reprod Biol* **120,** 139-145.

Lee YL, Lee KF, Xu JS, Wang YL, Tsao SW & Yeung WS. (2001). Establishment and characterization of an immortalized human oviductal cell line. *Mol Reprod Dev* **59,** 400-409.

Lydrup ML & Hellstrand P. (1986). Rate of oxidative and glycolytic metabolism in the guinea-pig oviduct in relation to contractility and hormonal cycle. *Acta Physiol Scand* **128,** 525-533.

Maheshwari A, Hamilton M & Bhattacharya S. (2008). Effect of female age on the diagnostic categories of infertility. *Hum Reprod* **23,** 538-542.

Nio-Kobayashi J, Abidin HB, Brown JK, Iwanaga T, Horne AW & Duncan WC. (2016). Cigarette smoking alters sialylation in the Fallopian tube of women, with implications for the pathogenesis of ectopic pregnancy. *Mol Reprod Dev* **83,** 1083-1091.

Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J & Melbye M. (2000). Maternal age and fetal loss: population based register linkage study. *BMJ* **320,** 1708-1712.

Parada-Bustamante A, Orostica ML, Reuquen P, Zuniga LM, Cardenas H & Orihuela PA. (2016). The role of mating in oviduct biology. *Mol Reprod Dev* **83,** 875-883.

Pelosi E, Simonsick E, Forabosco A, Garcia-Ortiz JE & Schlessinger D. (2015). Dynamics of the ovarian reserve and impact of genetic and epidemiological factors on age of menopause. *Biol Reprod* **92,** 130.

Phillips TJ, Scott H, Menassa DA, Bignell AL, Sood A, Morton JS, Akagi T, Azuma K, Rogers MF, Gilmore CE, Inman GJ, Grant S, Chung Y, Aljunaidy MM, Cooke CL, Steinkraus BR, Pocklington A, Logan A, Collett GP, Kemp H, Holmans PA, Murphy MP, Fulga TA, Coney

AM, Akashi M, Davidge ST & Case CP. (2017). Treating the placenta to prevent adverse effects of gestational hypoxia on fetal brain development. *Sci Rep* **7,** 9079.

Picca A & Lezza AM. (2015). Regulation of mitochondrial biogenesis through TFAM-mitochondrial DNA interactions: Useful insights from aging and calorie restriction studies. *Mitochondrion* **25,** 67-75.

Postigo L, Heredia G, Illsley NP, Torricos T, Dolan C, Echalar L, Tellez W, Maldonado I, Brimacombe M, Balanza E, Vargas E & Zamudio S. (2009). Where the O2 goes to: preservation of human fetal oxygen delivery and consumption at high altitude. *J Physiol* **587,** 693-708.

Practice Committee of the American Society for Reproductive M. (2015). Role of tubal surgery in the era of assisted reproductive technology: a committee opinion. *Fertil Steril* **103,** e37-43.

Radicella JP, Dherin C, Desmaze C, Fox MS & Boiteux S. (1997). Cloning and characterization of hOGG1, a human homolog of the OGG1 gene of Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **94,** 8010-8015.

Richter HG, Camm EJ, Modi BN, Naeem F, Cross CM, Cindrova-Davies T, Spasic-Boskovic O, Dunster C, Mudway IS, Kelly FJ, Burton GJ, Poston L & Giussani DA. (2012). Ascorbate prevents placental oxidative stress and enhances birth weight in hypoxic pregnancy in rats. *J Physiol* **590,** 1377-1387.

Robertson SA, Chin PY, Schjenken JE & Thompson JG. (2015). Female tract cytokines and developmental programming in embryos. *Adv Exp Med Biol* **843,** 173-213.

Roost M, Altamirano VC, Liljestrand J & Essen B. (2009). Priorities in emergency obstetric care in Bolivia--maternal mortality and near-miss morbidity in metropolitan La Paz. *BJOG* **116,** 1210-1217.

Sen KK & Talwar GP. (1973). Similarities and differences in the binding of oestradiol-17beta to rat oviduct and uterus. *J Reprod Fertil* **35,** 369-372.

Shaw JL, Denison FC, Evans J, Durno K, Williams AR, Entrican G, Critchley HO, Jabbour HN & Horne AW. (2010). Evidence of prokineticin dysregulation in fallopian tube from women with ectopic pregnancy. *Fertil Steril* **94,** 1601-1608 e1601.

Tarry-Adkins JL, Aiken CE, Ashmore TJ & Ozanne SE. (2018). Insulin-signalling dysregulation and inflammation is programmed trans-generationally in a female rat model of poor maternal nutrition. *Sci Rep* **8,** 4014.

Tarry-Adkins JL, Chen JH, Smith NS, Jones RH, Cherif H & Ozanne SE. (2009). Poor maternal nutrition followed by accelerated postnatal growth leads to telomere shortening and increased markers of cell senescence in rat islets. *FASEB J* **23,** 1521-1528.

Tarry-Adkins JL, Fernandez-Twinn DS, Madsen R, Chen JH, Carpenter A, Hargreaves IP, McConnell JM & Ozanne SE. (2015). Coenzyme Q10 Prevents Insulin Signaling Dysregulation and Inflammation Prior to Development of Insulin Resistance in Male Offspring of a Rat Model of Poor Maternal Nutrition and Accelerated Postnatal Growth. *Endocrinology* **156,** 3528-3537.

Tarry-Adkins JL, Ozanne SE, Norden A, Cherif H & Hales CN. (2006). Lower antioxidant capacity and elevated p53 and p21 may be a link between gender disparity in renal telomere shortening, albuminuria, and longevity. *Am J Physiol Renal Physiol* **290,** F509-516.

Wang S & Larina IV. (2018). In vivo three-dimensional tracking of sperm behaviors in the mouse oviduct. *Development* **145**.

Zhao W, Zhu Q, Yan M, Li C, Yuan J, Qin G & Zhang J. (2015). Levonorgestrel decreases cilia beat frequency of human fallopian tubes and rat oviducts without changing morphological structure. *Clin Exp Pharmacol Physiol* **42,** 171-178.

Figure legends:

Figure 1 A) Oviductal telomere length in adult female rats exposed to gestational hypoxia compared to normoxia. **B)** Effect of gestational hypoxia compared to normoxia on gene expression of components (*Ku70* and *Ku80*) of the DNA-activated protein kinase (DNA-PK) in the oviducts. **C)** Effect of gestational hypoxia compared to normoxia on protein expression of KU70 and KU80. Data shown as mean ± SEM. Open bars: normoxia (21% oxygen) during gestation, grey bars: hypoxia (13% oxygen) during gestation. *p<0.05, ***p<0.001. n=7-8 for all groups (n refers to the number of litters)

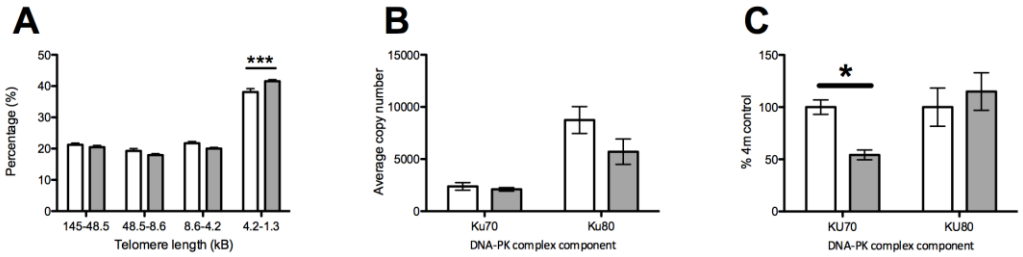
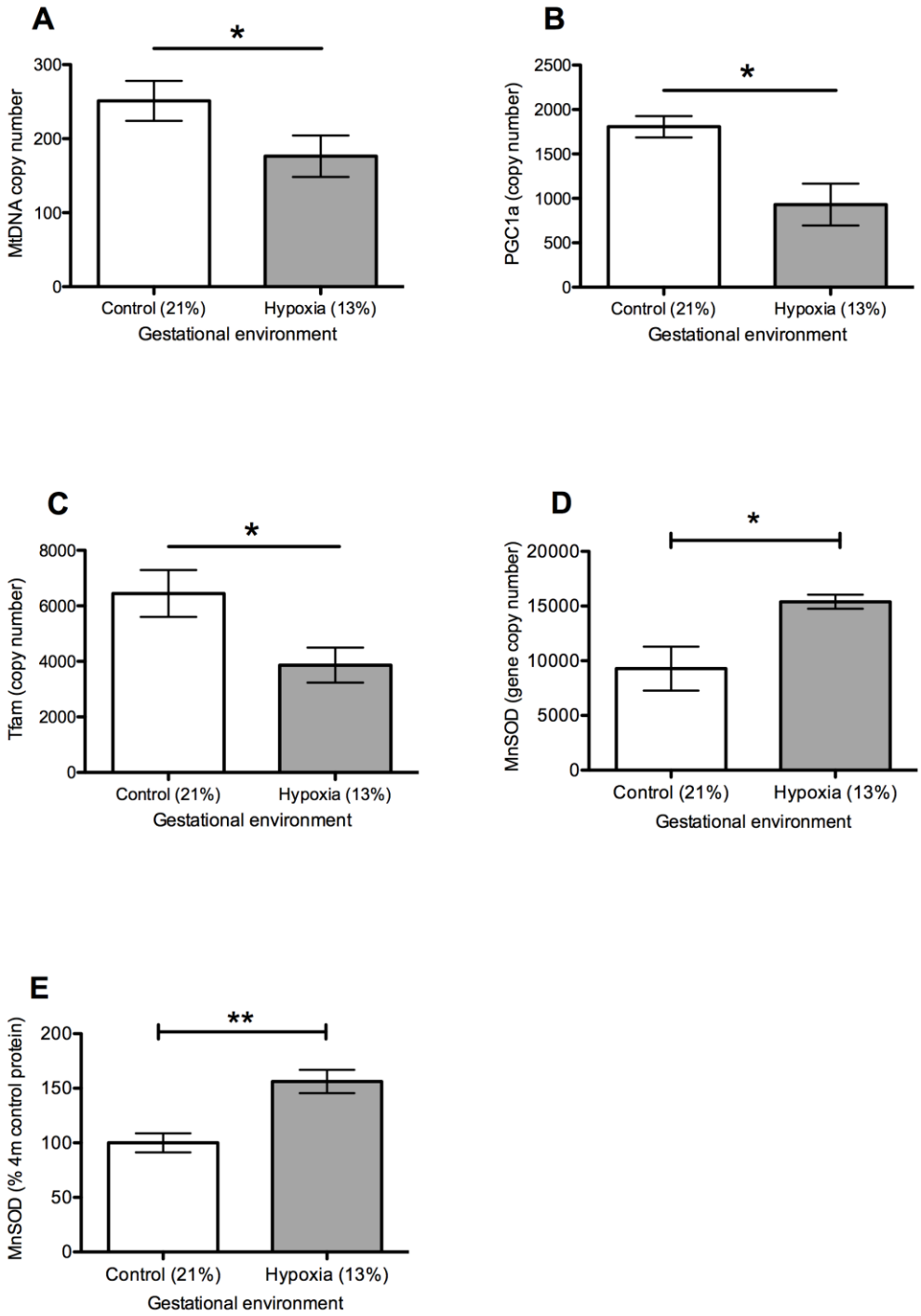


Figure 2 Effect of gestational hypoxia compared to normoxia on expression of mitochondrial biogenesis regulators and mitochondrial anti-oxidant defense in the oviducts. Data shown as mean ± SEM. Open bars: normoxia (21% oxygen) during gestation, grey bars: hypoxia (13% oxygen) during gestation. **A)** MtDNA copy number, **B)** *Tfam* gene expression, **C)** *Pgc1α* gene expression, **D)** *MnSOD* gene expression, **E)** MnSOD protein expression. *$p<0.05$, **$p<0.01$. n=7-8 for all groups (n refers to the number of litters)



Catherine Aiken is a University Lecturer and Honorary Consultant in Fetal and Maternal Medicine at the University of Cambridge. My work focuses on improving fetal and maternal health in the long-term, as well as the immediate outcomes of pregnancy and delivery. I study how the intrauterine environment impacts on the health and disease of offspring in later life using animal models of suboptimal nutrition during pregnancy, as well as how to make delivery safer for both mothers and babies using data from clinical cohorts. I am committed to improving maternal and child health in both developed and developing settings.

Gene	Normoxia	Hypoxia	
Ppia	32234 ±2363	28269±3394	NS
P53	10775±1237	13417±1332	0.09
P21	5188±1053	9292±1374	0.04
Alox12	3120±744	7714±2089	0.05
Alox15	925±225	854±147	NS
Ogg1	1294±135	1710±132	0.03
Neil1	769± 63	730±117	NS
Nth1	1505±27	1329±151	NS
Xrrc1	2675±375	2175±372	NS
Nrf2	11560±1704	7555±893	NS
Dna pkcs	2134±323	1421±192	0.1
Mre11	723±119	307±79	0.04
Ku70	2380±397	1533±389	NS
Ku80	8743±1410	5709±1219	NS
Bax	2093±199	1750±329	NS
Bcl2	4036±530	2599±293	0.05
BaxBcl2	0.41±0.02	0.5±0.08	NS
Tfam	6447±844	3866±632	0.04
Pgc1a	1806±121	903±236	0.01
Cs	18621±2551	9627±156	0.02
Lonp1	7518±874	7262±1035	NS
Cycs	27321±4613	15812±4446	0.08
Complex I	26745±721	22123±2086	0.01
Complex II	19112±3730	14311±1389	NS

Complex III	27555±4854	18414±1721	NS
Complex IV	46402±4883	33668±1533	0.05
Hif	8172±791	8276±628	NS
Gp91phox	6191±1727	6904±1023	NS
P22phox	5128±1081	7298±1030	NS
P47phox	1887±136	2620±631	NS
Xo	19493±2381	15989±1793	NS
Gpx1	67342±11501	34576±8409	NS
Hmox1	3492±202	3720±255	NS
Catalase	12593±1716	13651±280	NS
Nfkβ	6419±476	6073±307	NS
Mnsod	9286±2005	15399±577	0.04
Cuznsod	171954±8398	160528±13018	NS
Ecsod	35354±3730	23778±3163	NS

Table 1 Effect of gestational hypoxia compared to normoxia on gene expression in the oviducts of adult female rats. All reported p values have been adjusted to take account of multiple hypothesis testing. n=7-8 for all groups (n refers to the number of litters)

Protein	Normoxia	Hypoxia	
P53	100±17	158±19	0.05*
P16^{INK}	100±30	100±24	NS
OGG1	100±22	137±13	0.08
MRE11	100±30	77±22	NS
KU70	100±10	58±12	0.03*
KU80	100±18	115±18	NS
Complex I	100±36	142±56	NS
Complex II	100±29	150±38	NS
Complex III	100±15	96±18	NS
Complex IV	100±22	137±31	NS
Complex V	100±2	108±6	NS
CS	100±13	110±16	NS
HIF1α	100±12	124±15	NS
GP91^{phox}	100±27	97±15	NS
P47^{phox}	100±24	119±4	NS
XO	100±10	92±11	NS
HMOX1	100±44	37±11	NS
CATALASE	100±10	125±23	NS
MnSOD	100±9	156±10	<0.01**
CuZnSOD	100±30	94±23	NS

Table 2 Effect of gestational hypoxia compared to normoxia on protein expression in the oviducts of adult female rats. All reported p values have been adjusted to take account of multiple hypothesis testing. *p<0.05, **p<0.01. n=7-8 for all groups (n refers to the number of litters)