REPRODUCTION
RESEARCH

Maternal omega-3 fatty acid intake increases placental labyrinthine antioxidant capacity but does not protect against fetal growth restriction induced by placental ischaemia–reperfusion injury

Megan L Jones, Peter J Mark and Brendan J Waddell

School of Anatomy, Physiology and Human Biology, The University of Western Australia, 35 Stirling Highway, Crawley, Western Australia 6009, Australia

Correspondence should be addressed to B J Waddell; Email: brendan.waddell@uwa.edu.au

Abstract

Placental oxidative stress plays a key role in the pathophysiology of several placenta-related disorders. Oxidative stress occurs when excess reactive oxygen species (ROS) damages cellular components, an outcome limited by antioxidant enzymes; mitochondrial uncoupling protein 2 (UCP2) also limits ROS production. We recently reported that maternal dietary omega-3 polyunsaturated fatty acid (n-3 PUFA) supplementation reduced placental oxidative damage and enhanced fetal and placental growth in the rats. Here, we examined the effect of n-3 PUFAs on placental antioxidant defences and whether n-3 PUFA supplementation could prevent growth restriction induced by placental ischaemia–reperfusion (IR), a known inducer of oxidative stress. Rats were fed either standard or high-n-3 PUFA diets from day 1 of pregnancy. Placentas were collected on days 17 and 22 in untreated pregnancies (term = day 23) and at day 22 following IR treatment on day 17. Expression of several antioxidant enzyme genes (*Sod1*, *Sod2*, *Sod3*, *Cat*, *Txn1* and *Gpx3*) and *Ucp2* was measured by quantitative RT-PCR in the placental labyrinth zone (LZ) and junctional zone (JZ). Cytosolic superoxide dismutase (SOD), mitochondrial SOD and catalase (CAT) activities were also analyzed. Maternal n-3 PUFA supplementation increased LZ mRNA expression of *Cat* at both gestational days (2- and 1.5-fold respectively; $P<0.01$) and female *Sod2* at day 22 (1.4-fold, $P<0.01$). Cytosolic SOD activity increased with n-3 PUFA supplementation at day 22 (1.3-fold, $P<0.05$). *Sod1* and *Txn1* expression decreased marginally (30 and 22%, $P<0.05$). JZ antioxidant defences were largely unaffected by diet. Despite increased LZ antioxidant defences, maternal n-3 PUFA supplementation did not protect against placental IR-induced growth restriction of the fetus and placental LZ.
Reproduction (2013) **146** 539–547

Introduction

The developing fetus requires substantial amounts of fatty acids to support rapid cellular growth and activity, and among these, the omega-3 polyunsaturated fatty acids (n-3 PUFAs) are particularly important (Haggarty 2010). Maternal dietary supplementation with n-3 PUFAs has beneficial effects on pregnancy outcomes, including increased gestation length (Szajewska *et al*. 2006), reduced risk of pregnancy complications (Olsen *et al*. 2000, Oken *et al*. 2007, Zhou *et al*. 2012), and increased fetal growth (Olsen *et al*. 1990, Jones *et al*. 2013*b*). Potentially, n-3 PUFAs may exert beneficial effects via their involvement in several physiological pathways, including anti-oxidative pathways. Indeed, we recently reported that maternal dietary n-3 PUFA supplementation reduced placental oxidative damage and increased fetal and placental growth in rats (Jones *et al*. 2013*b*).

Oxidative stress occurs when cellular production of reactive oxygen species (ROS), by-products of cellular respiration, exceeds the protective capacity of local antioxidant defences and thus damages cellular components (Burton & Jauniaux 2011). ROS levels are limited by a range of antioxidant enzymes including the superoxide dismutases (SODs), which catalyze conversion of superoxide to H_2O_2 and oxygen. Three isoforms of the SOD enzyme exist: cytosolic SOD1 and extracellular SOD3 are Cu/Zn dependent, whereas mitochondrial SOD2 is Mn dependent. H_2O_2, itself a strong oxidizer, may then be inactivated by catalase (CAT), glutathione peroxidase (GPX) and/or the thioredoxin (TXN) system (Burton & Jauniaux 2011). Alternatively, oxidative damage may be limited by inhibition of ROS production. For example, it has been proposed that uncoupling proteins (UCPs) may limit ROS generation by uncoupling oxidative phosphorylation (Mailloux & Harper 2011), a primary metabolic source of ROS. Oxidative stress is commonly associated with inflammation and *vice versa*, as pro-inflammatory cytokines are produced in response to ROS and

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-13-0282
Online version via www.reproduction-online.org

subsequently stimulate further ROS production by target cells (Burton & Jauniaux 2011).

Placental ROS generation is high due to the high metabolic activity of placental cells (Myatt & Cui 2004), and so all major antioxidant systems are present within the placenta (Myatt & Cui 2004, Perkins 2006, Jones *et al*. 2010). Several placenta-related disorders such as pre-eclampsia (PE), intrauterine growth restriction (IUGR), miscarriage, and gestational diabetes mellitus are characterized by increased placental oxidative stress (Coughlan *et al*. 2004, Biri *et al*. 2006, 2007, Jauniaux *et al*. 2006, Myatt 2010). In the case of PE, shallow implantation leads to intermittent placental blood flow through retention of vascular reactivity (Burton & Jauniaux 2011). This predisposes the placenta to ischaemia–reperfusion (IR) injury and is associated with heightened oxidative stress and inflammation (Burton & Jauniaux 2011). An experimental rat model of placental IR injury, termed 'uterine artery occlusion' (UAO), increases placental oxidative damage (Ishimoto *et al*. 1997, Nagai *et al*. 2008) and inhibits fetal growth (Ishimoto *et al*. 1997, Nakai *et al*. 2002, Thaete & Neerhof 2006, Yamazaki *et al*. 2006, Nagai *et al*. 2008).

In this study, we tested the hypothesis that maternal dietary n-3 PUFA supplementation increases placental antioxidant defences in an otherwise normal pregnancy. Placental mRNA expression and activity of the major antioxidant enzymes and *Ucp2* were measured at days 17 and 22 of rat gestation (term = day 23), covering the major period of fetal growth. We also examined whether maternal n-3 PUFA supplementation could prevent placental IR-induced fetal growth restriction, potentially via anti-oxidative and anti-inflammatory mechanisms.

Materials and methods

Animals and diets

Nulliparous albino Wistar rats, 8–12 weeks old, were obtained from the Animal Resources Centre (Murdoch, WA, Australia) and maintained under controlled conditions as described previously (Hewitt *et al*. 2006). Rats were mated overnight, with day 1 of pregnancy designated as the day on which spermatozoa were present in a vaginal smear. On day 1 of pregnancy, mothers were placed on either a standard (Std) or high n-3 PUFA (Hn3) iso-caloric semipure diet (Specialty Feeds, Glenn Forrest, WA, Australia). Both diets consisted of 5% total fat; detailed fatty acid composition of the diets is described by Jones *et al*. (2013*b*). Mothers were either left untreated or placental IR was induced on day 17 of gestation (see below). All procedures involving animals were conducted under approval by the Animal Ethics Committee of The University of Western Australia.

Placental IR induction by UAO

On day 17 of gestation, mothers were anaesthetized by i.p. injection of 40 mg/kg body weight (BW) ketamine (Parnell Laboratories, Sydney, NSW, Australia) and 0.05 mg/kg BW medetomidine hydrochloride (Pfizer). UAO was conducted according to the technique of Tanaka *et al*. (1994). Briefly, midline abdominal incisions (~3 cm) were made sequentially in the skin and muscle layers, and the uterine horns exposed. Microvascular clamps (Fine Science Tools, North Vancouver, BC, Canada) were placed on the lower and upper ends of uterine vessels of the right uterine horn for 30 min, during which time the vasculature visually darkened as oxygen was depleted. This effect was notably isolated to the occluded area. Body temperature was closely monitored and regulated to 37 °C via a warming pad and infra-red warming lamp. Further heat loss was minimized by placement of temporary sutures in the skin layer to close the incision. Following removal of the microvascular clamps, the muscle and skin layers were sutured separately. Animals were then administered the analgesic, buprenorphine hydrochloride (0.01 mg/kg BW, s.c.; Reckitt Benckiser, West Ryde, NSW, Australia) and 10 ml 0.18% sterile saline with 4% glucose for rehydration. Atipamezole hydrochloride (0.5 mg/kg BW; medetomidine hydrochloride reversal agent; Pfizer) was administered by i.m. injection to assist in recovery from anaesthesia. Control animals underwent a 'Sham' procedure, which was identical to the UAO procedure, except that microvascular clamps were not placed on uterine vessels.

Tissue collection

Rats were anaesthetized with isoflurane/nitrous oxide at either day 17 (untreated pregnancies only) or day 22 (untreated pregnancies and placental IR study) of gestation. For untreated pregnancies, three fetus–placenta pairs were obtained from the mid-region of each uterine horn (total of six per mother) and weighed. For IR-treated pregnancies (Sham and UAO), six fetus–placenta pairs were obtained from the right uterine horn in Sham-treated mothers, six fetus–placenta pairs were obtained from the 'occluded' (right) uterine horn for UAO-treated mothers, and six from the 'contralateral' (left) uterine horn. Each placenta was then dissected into labyrinth zone (LZ) and junctional zone (JZ), which were weighed individually and snap frozen in liquid nitrogen. Measurement of genes that are differentially expressed between placental zones confirms adequate zonal separation by this method (Mark *et al*. 2009). A blood sample obtained from the maternal dorsal aorta was mixed with 10:1 (vol:vol) 0.6 M EDTA and centrifuged at 13 000 ***g*** for 6 min to obtain plasma. All tissues and plasma samples were snap frozen in liquid nitrogen and stored at −80 °C until further analysis. Fetal sex was determined by PCR amplification of the *Sry* gene in day 17 fetuses as described previously (Jones *et al*. 2013*b*), and at day 22 by measuring anogenital distance (Imperato-McGinley *et al*. 1986).

RNA and cDNA sample preparation

Total RNA was isolated from placental zones using Tri-Reagent (Molecular Resources Centre, Cincinnati, OH, USA) as per the manufacturer's instructions. Total RNA (1 μg) was used as a template for cDNA synthesis by murine Moloney leukemia virus Reverse Transcriptase RNase H Point Mutant and random

hexamer primers (Promega) as per the manufacturer's instructions. The resultant cDNAs were purified using the Ultraclean PCR Cleanup kit (MoBio Industries, Solana Beach, CA, USA).

Real-time quantitative RT-PCR

Analyses of mRNA expression levels for the antioxidant genes *Sod1*, *Sod2*, *Sod3*, *Cat*, *Txn1*, *Gpx3*, and *Ucp2* for the pro-inflammatory genes tumor necrosis factor α (*Tnf*) and prostaglandin-endoperoxide synthase-2 (*Ptgs2*) and for the reference genes *Ppia*, *Sdha*, and *Ywhaz* were performed by real-time RT-PCR on the Rotorgene 6000 (Corbett Industries, Sydney, NSW, Australia) using 0.2 μM of primers, SYBR Green (Molecular Probes, Eugene, OR, USA) at 1/40 000 of stock, and 0.25 U of Immolase DNA polymerase (Bioline, Alexandria, NSW, Australia) per reaction. Primer pairs for all genes of interest (Table 1) were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast (Rozen & Skaletsky 2000)). Each of the selected primer pairs were positioned to span introns to ensure no product was amplified from genomic DNA, and the resulting amplicons were sequenced to confirm specificity. Standard curves for each amplicon were generated with tenfold serial dilutions of gel-extracted (QIAEX II, Qiagen) PCR products using the Rotorgene 6000 Software. All samples were normalized against *Ppia*, *Sdha* and *Ywhaz* using the GeNorm algorithm (Vandesompele *et al.* 2002).

Placental antioxidant enzyme activity

Activities of SOD and CAT were measured in placental zone homogenates of untreated pregnancies using commercial assay kits (Cat #706002 and 707002 respectively; Cayman Chemical Company, Ann Arbor, MI, USA). LZ and JZ tissues (~40 mg of each) were homogenized using the POLYTRON-Aggregate (Kinematica, Lucerne, Switzerland). For the SOD activity assay, tissues were homogenized in 10 vol of 20 mM HEPES buffer (pH 7.2, containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose). Samples were initially centrifuged at 1500 ***g*** for 5 min at 4 °C, and the cell lysate supernatant was again centrifuged at 10 000 ***g*** for 15 min to obtain aqueous and pelleted portions; the aqueous phase contained cytosolic SOD and the pelleted portion contained mitochondrial SOD. The pelleted portion was resuspended in 30 μl 20 mM HEPES buffer (pH 7.2, containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose). For the CAT activity assay, tissues were homogenized in 10 vol 50 mM potassium phosphate buffer (pH 7.0, containing 1 mM EDTA) and then centrifuged at 10 000 ***g*** for 15 min at 4 °C and the cell lysate supernatant was obtained. All samples were stored at −80 °C until they were assayed for protein concentration by Bradford assay. Activity assays were performed as per the manufacturer's instructions and read at either 440 or 540 nm (SOD and CAT activity assays respectively) on the Labsystems Multiskan plate reader (Labsystems, Vantaa, Finland). Values are expressed as units per gram protein.

Measurement of F_2-isoprostanes

Levels of F_2-isoprostanes were measured in female placental LZ samples of IR-treated animals. Tissues (~50 mg) were ground to a fine powder with a porcelain mortar and pestle chilled with liquid nitrogen. F_2-isoprostanes were extracted and then measured in duplicate by gas chromatography–mass spectrometry using electron capture chemical ionization as described previously (Barden *et al.* 2012).

Table 1 Primers and PCR conditions used to measure rat placental expression of antioxidant genes, *Ucp2*, pro-inflammatory genes, and reference genes by real-time RT-PCR.

Gene	Forward/reverse primer	Annealing temperature (°C)	Amplicon size (bp)	$MgCl_2$ (mM)
Sod1	F: 5′-CGTCATTCACTTCGAGCAGA-3′ R: 5′-AAAATGAGGTCCTGCAGTGG-3′	60	145	3
Sod2	F: 5′-GGCCAAGGGAGATGTTACAA-3′ R: 5′-GCTTGATAGCCTCCAGCAAC-3′	60	149	3
Sod3	F: 5′-TCAGAGGCTCTTTCTCAGGC-3′ F: 5′-CTGCTAAGTCGACACCGGAC-3′	60	195	2
Cat	F: 5′-ACATGGTCTGGGACTTCTGG-3′ R: 5′-CAAGTTTTTGATGCCCCTGGT-3′	61	197	3
Txn1	F: 5′-AGCTGATCGAGAGCAAGGAA-3′ R: 5′-TCAAGGAACACCACATTGGA-3′	60	160	3
Gpx3	F: 5′-GGCTTTGTGCCTAATTTCCA-3′ R: 5′-CCCACCAGGAACTTCTCAAA-3′	60	188	3
Ucp2	F: 5′-TCATCACTTTCCCTCTAGACACC-3′ R: 5′-AAGCTCATCTGGCGCTGTAG-3′	57	190	2
Tnf	F: 5′-TACTGAACTTCGGGGTGATTGGTCC-3′ R: 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′	60	295	3
Ptgs2	F: 5′ GAAGGGACACCCTTTCACAT 3′ R: 5′-TGGGGAGACCATGGTAGAAC-3′	59	178	4
Ppia	F: 5′-AGCATACAGGTCCTGGCATC-3′ R: 5′-TTCACCTTCCCAAAGACCAC-3′	62	127	3
Sdha	F: 5′-TGGGGCGACTCGTGGCTTTC-3′ R: 5′-CCCCGCCTGCACCTACAACC-3′	60	134	2
Ywhaz	F: 5′-GACGGAGCTGAGGGACATCTGC-3′ R: 5′-GGCTGCGAAGCATTGGGGATCA-3′	60	75	2

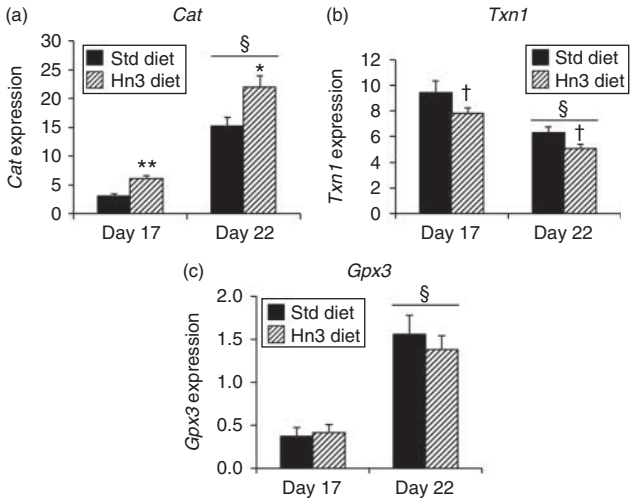

Figure 1 Labyrinth zone expression of (a) *Cat*, (b) *Txn1*, and (c) *Gpx3* mRNAs at days 17 and 22 of pregnancy (fetal sex pooled). Mothers were fed either a Std or Hn3 diet from day 1 of pregnancy. Values are mean ± S.E.M. (n=7–8 per group). *P<0.01 and **P<0.001 compared with corresponding Std diet group (two-way ANOVA and *post hoc* LSD tests or unpaired *t*-test); †P<0.05 overall compared with Std diet (three-way ANOVA) and §P<0.001 compared with day 17 (three-way ANOVA).

Pro-inflammatory cytokine quantitation

Levels of the pro-inflammatory cytokines, tumor necrosis factor α (TNFa), interleukin 6 (IL6) and IL1b, were measured in maternal plasma of IR-treated animals using MILLIPLEX MAP Kit; Rat Cytokine/Chemokine (Cat #RCYTO-80K; Merck Millipore, Billerica, MA, USA). Samples were centrifuged at 13 000 ***g*** for 5 min prior to analysis. The assay was performed as per the manufacturer's instructions and read on CS1000 Autoplex Analyzer (PerkinElmer, Waltham, MA, USA), using Luminex xPONENT program (Luminex, TX, USA).

Statistical analysis

All analyses were conducted using Genstat version 14 (VSN International Ltd., Hemel Hempstead, UK). Where data were not normally distributed (based on residual plot analyses), values were log transformed prior to statistical analysis. In all instances, '*n*' refers to the number of litters analyzed. Variation in placental SOD activity was assessed by five-way ANOVA, with variation attributed to subcellular location (cytosolic or mitochondrial), maternal diet, gestational age, placental zone, and fetal sex. Variation in CAT activity and placental gene expression was assessed by four-way ANOVA, with variation attributed to maternal diet, gestational age, placental zone, and fetal sex. Variation in plasma levels of pro-inflammatory cytokines was assessed by two-way ANOVA, with variation attributed to maternal diet and gestational age. For all ANOVAs, when a significant interaction was seen between sources of variation, separate comparisons were conducted by four-, three-, two-way ANOVA, and/or unpaired *t*-tests as appropriate. Where the *F* test reached statistical significance (P<0.05), subsequent *post hoc* analyses were performed using least significant difference (LSD) tests (Snedecor & Cochran 1989).

Results

We have previously reported that maternal n-3 PUFA supplementation in this same cohort of animals increased fetal (6%) and placental (12%) weights at day 22, the latter attributable primarily to enhanced growth of the LZ (Jones *et al*. 2013*b*). Furthermore, levels of the oxidative stress marker, F_2-isoprostanes, were reduced in both the placental LZ (31 and 11% at days 17 and 22 respectively) and JZ (29% lower at day 22) with the Hn3 diet (Jones *et al*. 2013*b*).

Effects of n-3 PUFAs on placental antioxidant gene expression

Labyrinth zone

The maternal Hn3 diet increased LZ expression of *Cat* at both day 17 (twofold, P<0.001) and day 22 (1.5-fold, P<0.01; Fig. 1a) and *Sod2* expression in female LZ at day 22 (1.4-fold; P<0.01; Fig. 2b); male *Sod2* expression was unaffected by Hn3 diet (data not shown). By contrast, *Sod1* and *Txn1* expression decreased overall in response to maternal Hn3 diet (30%, P<0.05 and 22%, P<0.05; Fig. 1a and 2b), and LZ expression of *Sod3*, *Gpx3*, and *Ucp2* was unaffected.

LZ antioxidant enzyme expression varied with gestational age (Figs 1, 2 and 3). Most notably, mRNA expression of *Cat*, *Gpx3* and *Ucp2* increased from days 17 to 22 (pooled diet groups; all P<0.001). Conversely, *Sod1* and *Txn1* decreased over the same period

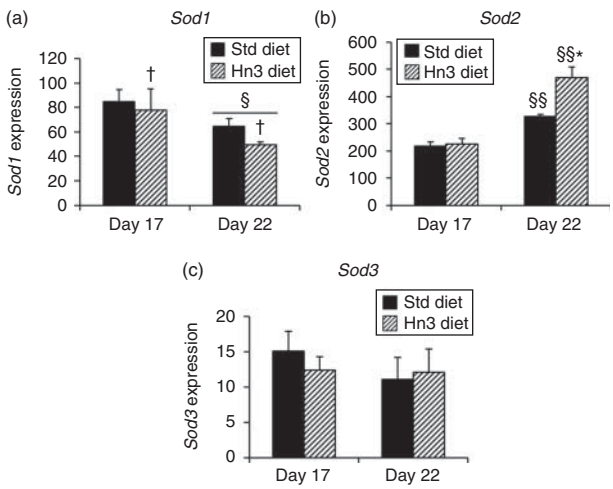

Figure 2 Labyrinth zone expression of (a) *Sod1*, (b) *Sod2* (female only), and (c) *Sod3* mRNAs at days 17 and 22 of pregnancy (fetal sex pooled except for *Sod2*). Mothers were fed either a Std or Hn3 diet from day 1 of pregnancy. Male *Sod2* data is not presented because expression was unaffected by maternal diet. Values are mean ± S.E.M. (n=7–8 per group). *P<0.01 compared with corresponding Std diet group (two-way ANOVA and *post hoc* LSD tests or unpaired *t*-test); †P<0.05 overall compared with Std diet (three-way ANOVA); §P<0.01 overall compared with day 17 (pooled diet groups, three-way ANOVA) and §§P<0.001 compared with day 17 value of same diet group (unpaired *t*-test).

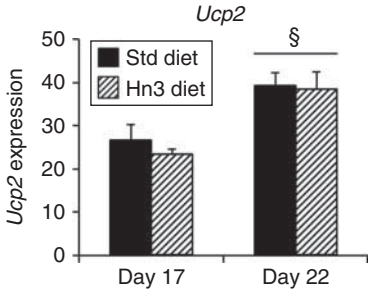

Figure 3 Labyrinth zone expression of *Ucp2* mRNAs at days 17 and 22 of pregnancy (fetal sex pooled). Mothers were fed either a Std or Hn3 diet from day 1 of pregnancy. Values are mean ± S.E.M. (n=7–8 per group). §P<0.001 compared with day 17 (three-way ANOVA).

(overall, P<0.01 and P<0.001), whereas that of *Sod3* did not vary with gestational age. These gestational age effects were unaffected by Hn3 diet, with the exception of *Sod2* in females, where a significant interaction term between gestational age and diet was observed (P<0.01). Specifically, the *Sod2* gestational increase from days 17 to 22 was greater in the Hn3 group (2.1-fold increase, P<0.001) than the Std group (1.5-fold increase, P<0.001; Fig. 2b).

Junctional zone

JZ mRNA expression of antioxidant enzymes and *Ucp2* was largely unaffected by the Hn3 diet, with the exception of *Txn1*, which increased in female JZ at day 17 (2.5-fold, P<0.01), and *Sod3*, which decreased overall (both gestational days, 42–45%, P<0.001) (Supplementary Figures 1, 2 and 3, see section on supplementary data given at the end of this article). JZ expression of antioxidant genes was generally higher in male compared with female samples, most notably for *Sod2*, *Cat*, and *Txn1* (see Supplementary Figures 1 and 2). Despite this, diet and gestational age effects were similar in the JZ of males and females.

JZ expression of several antioxidant enzymes changed with gestational age and these shifts were variably affected by the maternal Hn3 diet (Supplementary Figures 1, 2 and 3). For example, while the Hn3 diet dampened gestational increases in both *Sod1* and *Txn1* expression in males, it increased *Cat* expression over this period in both sexes (each P<0.001). Moreover, the gestational decline in JZ *Txn1* expression in females was enhanced by the Hn3 diet (P<0.05).

Effects of n-3 PUFAs on placental SOD and CAT enzyme activities

Placental levels of SOD and CAT activity were similar in males and females, and so data were pooled for all further analyses. In the LZ, the Hn3 diet increased cytosolic SOD activity at day 22 (29%; P<0.05; Fig. 4a) but had no effect on mitochondrial SOD and CAT activities on either day (Fig. 4b and c). In the JZ, the Hn3 diet increased cytosolic SOD activity only at day 17 (32%; P<0.01) and had no effect on JZ mitochondrial SOD and CAT activities (Supplementary Figure 4, see section on supplementary data given at the end of this article).

The maternal Hn3 diet also variably affected gestational changes (i.e. between days 17 and 22) in placental SOD and CAT activities (Fig. 4, Supplementary Figure 4). Most notably, the Hn3 diet prevented the gestational decline in LZ activity of cytosolic SOD (Fig. 4a), whereas JZ activity of cytosolic SOD decreased only in Hn3 group over the same period (15% decrease; P<0.05; Supplementary Figure 4a).

Placental IR

Fetal and placental weights

UAO treatment affected growth outcomes of males and females similarly, and so data were pooled for further analysis (Fig. 5). Maternal Hn3 diet increased fetal weight overall (4.5% increase, pooled treatment groups; P<0.05), consistent with observed growth outcomes in untreated pregnancies (Jones *et al.* 2013*b*). In UAO-treated mothers, fetal weight of the occluded horn was reduced overall compared with Sham (pooled diet groups, P<0.01), although by LSD test this was significant only in the Hn3 group (7.7% reduction, P<0.05), and was a strong trend in the Std diet group (8.3% reduction, P=0.053). Fetal weight of the contralateral horn was unaffected by UAO irrespective of maternal diet. In Sham-treated animals, placental weight increased with maternal Hn3 diet (9%, P<0.05), again

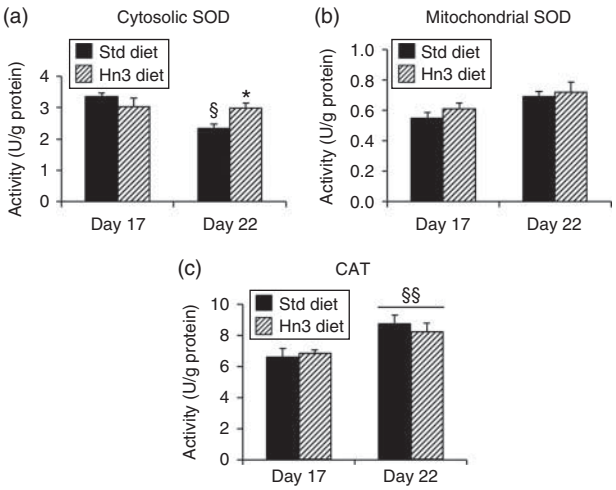

Figure 4 Labyrinth zone activity of (a) cytosolic SOD, (b) mitochondrial SOD, and (c) CAT at days 17 and 22 of pregnancy (fetal sex pooled). Mothers were fed either a Std or Hn3 diet from day 1 of pregnancy. Values are mean ± S.E.M. (n=6–8 per group). *P<0.05 compared with corresponding Std diet group (two-way ANOVA); §P<0.01 and §§P<0.001 compared with day 17 (three- or two-way ANOVA).

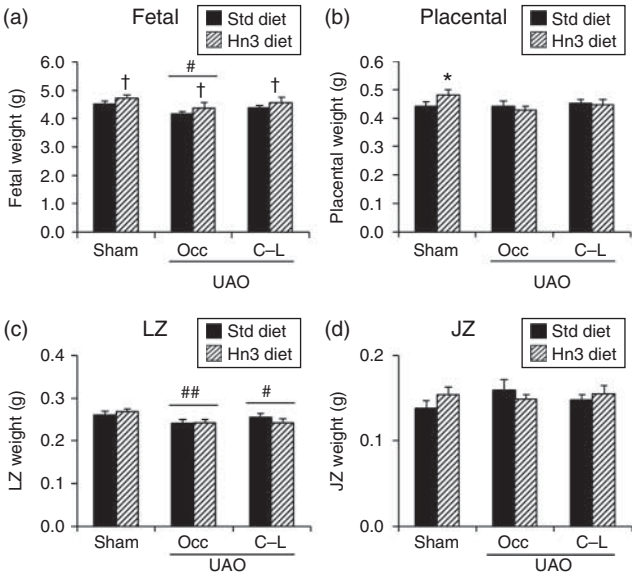

Figure 5 Fetal and placental weights in placental IR. (a) Fetal, (b) placental, (c) LZ, and (d) JZ weights at day 22 following Sham or UAO treatment (fetal sex pooled). In UAO-treated animals, weights of the occluded horn (Occ) and contralateral horn (C–L) are considered separately. Mothers were fed either a Std or a Hn3 diet from day 1 of pregnancy. Values are mean±S.E.M. (n=7–8 per group). $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with Sham (three-way ANOVA, *post hoc* LSD tests); $^{*}P<0.05$ compared with corresponding Std diet group (two-way ANOVA, *post hoc* LSD tests) and $^{\dagger}P<0.05$ overall compared with Std diet (three-way ANOVA).

consistent with untreated pregnancy (Jones *et al.* 2010). In UAO-treated mothers, total placental weight was unaffected in both diet groups, although LZ weight was reduced in both occluded (7.9–11.3%, $P<0.01$) and contralateral horns (2.5–10.9%; overall $P<0.05$, pooled diet groups); JZ weight was unaffected by UAO. Ratios between fetal, whole placental, and LZ weights did not differ with maternal diet or UAO treatment (data not shown).

Placental LZ F_2-isoprostanes

Levels of F_2-isoprostanes were measured in female LZ samples of IR-treated mothers (Sham and UAO). Levels decreased in response to maternal Hn3 dietary intake (19.4–33.6% decrease; pooled treatment groups, $P<0.05$; data not shown), consistent with untreated pregnancies (Jones *et al.* 2013*b*). UAO treatment did not affect LZ levels of F_2-isoprostanes (data not shown).

Maternal circulating levels and placental gene expression of pro-inflammatory mediators

Maternal TNFa levels were not affected by either maternal Hn3 or UAO treatment (data not shown). Maternal circulating levels of IL1b were increased with Hn3 diet (53%; $P<0.05$), consistent with our previous observations (Jones *et al.* 2013*a*). IL6 was undetectable in maternal plasma, and placental LZ *Tnf* expression was not affected by either the maternal Hn3 diet or the UAO treatment (Fig. 6a). *Ptgs2* expression was lower overall in female compared with male LZ (overall sex effect, $P<0.01$), but diet and UAO treatment effects were similar for each sex. The maternal Hn3 diet reduced *Ptgs2* expression in the Occ group of UAO-treated mothers ($P<0.05$ for each sex; Fig. 6b). Surprisingly, *Ptgs2* expression was higher in Sham- than in UAO-treated LZ (occluded or contralateral; 36–49% higher; overall treatment effect $P<0.001$; Fig. 6b). JZ expression of both *Tnf* and *Ptgs2* was unaffected by maternal diet or UAO treatment (data not shown).

Discussion

Mounting evidence suggests that oxidative stress in uteroplacental tissues plays a key role in the development of pregnancy complications, including PE and IUGR. Maternal dietary n-3 PUFA supplementation may be a possible therapeutic intervention for these disorders, given their ability to reduce placental oxidative damage (Jones *et al.* 2013*b*). The aim of this study was to investigate the impact of maternal n-3 PUFA supplementation on the antioxidant capacity of the late gestation placenta. The major findings were that maternal n-3 PUFA supplementation enhanced the expression, and to a lesser extent activity, of various antioxidant enzymes in the placental LZ. These observations extend our earlier report that maternal dietary n-3 PUFA intake reduced placental F_2-isoprostanes (a highly reliable marker of oxidative damage) and was associated with increased fetal and placental growth (Jones *et al.* 2013*b*). Despite enhanced basal LZ levels of antioxidant enzymes and reduced placental oxidative stress in otherwise normal pregnancies, dietary n-3 PUFA supplementation could not prevent IR-induced fetal and placental growth restriction.

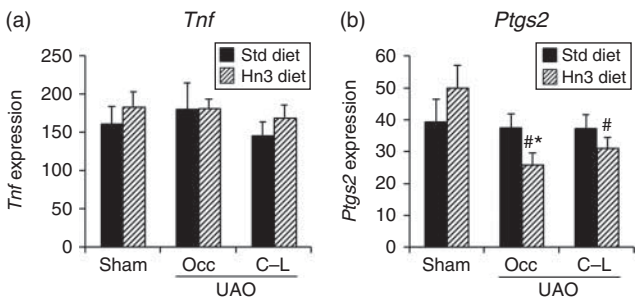

Figure 6 Labyrinth zone expression of (a) *Tnf* (pooled sexes) and (b) *Ptgs2* (female only) at day 22 following Sham or UAO treatment. In UAO-treated animals, the occluded horn (Occ) and contralateral horn (C–L) were considered separately. Mothers were fed either a Std or a Hn3 diet from day 1 of pregnancy. Values are mean±S.E.M. (n=7–8 per group). $^{\#}P<0.05$ compared with corresponding Sham value (two-way ANOVA, *post hoc* LSD tests) and $^{*}P<0.05$ compared with Std diet of same treatment group (two-way ANOVA, *post hoc* LSD tests).

The importance of limiting placental oxidative stress during normal gestation has recently been shown by two studies. First, Umekawa *et al*. (2008) demonstrated that global TXN1 overexpression reduced oxidative damage and enhanced fetal growth in a mouse model. Similarly, we recently reported reduced placental oxidative damage and increased fetal and placental growth with maternal dietary n-3 PUFA supplementation (Jones *et al*. 2013*b*). Together, these studies suggest that placental ROS tonically suppress fetal growth during normal pregnancy. Because the placental LZ is the site of maternal–fetal exchange, enhanced antioxidant capacity of this placental zone could significantly impact on fetal growth outcomes. In this study, LZ mRNA expression of *Cat* was increased at both gestational days in response to maternal n-3 PUFA supplementation, as was *Sod2* in female LZ at day 22. Concomitantly, expression of *Sod1* and *Txn1* was reduced, although these effects were marginal. These results point to a likely redundancy in the antioxidant defences, given that several antioxidant enzymes catalyze conversion of the same ROS molecule. Therefore, despite the marginal falls in *Txn1* and *Sod1*, corresponding increases in *Cat* and *Sod2* mRNA expression likely account for the previously reported reduction in F_2-isoprostanes (Jones *et al*. 2013*b*). Enhanced *Cat* expression in response to maternal Hn3 diet was not matched by higher CAT activity levels, but it is possible that this reflected lower sensitivity of the activity assay relative to quantitative PCR. Despite an overall slight reduction of *Sod1* mRNA expression in the placental LZ with maternal n-3 PUFA supplementation, corresponding cytosolic SOD activity was increased at day 22, presumably reflecting increased enzyme efficiency or activation, potentially via post-translational regulatory mechanisms. Overall, these results suggest that antioxidant capacity of the placental LZ was enhanced by maternal n-3 PUFA supplementation and may in part contribute to increased fetal and placental LZ growth (Jones *et al*. 2013*b*).

In contrast to the LZ, antioxidant responses of the JZ to maternal n-3 PUFA supplementation were minimal. While the LZ grows rapidly during late gestation to meet fetal demand, the JZ regresses during this time (Waddell *et al*. 2000). As such, it is perhaps unsurprising that antioxidant responses differ between the two placental zones in late gestation, and this highlights the need to consider these zones separately in rodent studies. The majority of antioxidant enzymes were more highly expressed in the placental LZ than in the JZ, the single exception being *Gpx3* expression, which was considerably higher in JZ, and this is consistent with our previous findings in chow-fed animals (Jones *et al*. 2010). Overall, the placental LZ appeared to exhibit enhanced antioxidant defences compared with the JZ. Accordingly, levels of F_2-isoprostanes were lower in the placental LZ than in the JZ (Jones *et al*. 2013*b*).

Placental ROS accumulation may also be controlled by limiting ROS generation. Previous studies have established an important role for *Ucp2* in limiting oxidative damage in other tissues, with *Ucp2*-null mice showing increased ROS production in several cell types (Arsenijevic *et al*. 2000, Chevillotte *et al*. 2007). Dietary n-3 PUFAs have been shown to increase *Ucp2* mRNA expression in mouse white adipose tissue *in vivo* (Hun *et al*. 1999) and in rat myocytes *in vitro* (Hatakeyama & Scarpace 2001). Despite these previous observations, maternal dietary supplementation with n-3 PUFAs did not affect placental *Ucp2* expression in this study. This may suggest that basal placental *Ucp2* expression is already relatively high in normal pregnancy and is thus unresponsive to further stimulation.

In addition to their anti-oxidative properties, the anti-inflammatory and pro-resolving actions of n-3 PUFAs are well documented (Calder 2012). Inflammation is closely associated with oxidative stress, given that pro-inflammatory cytokines produced in response to ROS accumulation subsequently stimulate further ROS production by target cells (Burton & Jauniaux 2011). Furthermore, we recently reported increased placental synthesis of pro-inflammatory cytokines towards term in the rat (Mark *et al*. 2013). Consequently, the beneficial effects of n-3 PUFA supplementation on oxidative status could be accounted for, in part, by their anti-inflammatory actions. Although levels of pro-inflammatory mediators (placental and maternal) were not suppressed by n-3 PUFA supplementation, placental levels of the n-3 PUFA-derived pro-resolving mediators, resolvins and protectins, were markedly increased (Jones *et al*. 2013*a*). This could be relevant to antioxidant capacity, as administration of resolvin D1 can limit oxidative stress-induced inflammation in the mouse (Spite *et al*. 2009), and protectin D1 confers protection from oxidative stress-induced apoptosis in human retinal pigment epithelial cells (Faghiri & Bazan 2010). Thus, the enhanced levels of placental resolvins and protectins induced by n-3 PUFA supplementation may protect against oxidative damage.

In this study, we show that expression and activity of antioxidant enzymes increase towards term, confirming our previous report in chow-fed animals (Jones *et al*. 2010). This is consistent with an up-regulation of placental antioxidant defences as placental oxygenation increases, which occurs on two occasions during normal gestation: following the rapid onset of maternal intra-placental circulation (10–12 weeks human gestation (Myatt & Cui 2004)) and during late gestation when fetal demand is maximal (Takehara *et al*. 1990, Perkins 2006).

Omega-3 PUFA administration has also been shown to confer protection against hepatic (Kim *et al*. 2013), renal (Ashtiyani *et al*. 2012), intestinal (Arisue *et al*. 2012, Brahmbhatt *et al*. 2013), cerebral (Pan *et al*. 2009), pulmonary (Lee *et al*. 2008), and myocardial (Zeghichi-Hamri *et al*. 2010, Gao *et al*. 2011) IR injury.

Furthermore, given our previous observation of reduced placental oxidative damage with maternal n-3 PUFA supplementation in normal pregnancy (Jones *et al.* 2013*b*) and increased LZ antioxidant defences as demonstrated here, we hypothesized that maternal n-3 PUFA supplementation would prevent placental IR-induced growth restriction. On the contrary, the adverse impact of IR on fetal and placental growth was similar in Std and Hn3 diet-fed mothers. This may suggest that while maternal n-3 PUFA supplementation is beneficial in a normal pregnancy setting, its capacity to protect against a major IR insult is limited.

UAO treatment did not affect markers of placental LZ oxidative damage (F_2-isoprostanes), maternal or placental inflammation. This may be because these were analyzed 5 days following UAO, thereby allowing time for tissue recovery and resolution of inflammation. As such it would be of interest to measure oxidative and inflammatory markers nearer to the time of UAO treatment. As fetal growth is maximal between days 17 and 22, collecting tissues at day 22 allowed us to best examine the effect of UAO on fetal and placental growth outcomes. Fetal growth was restricted by around 8% after UAO treatment, somewhat greater than that observed in other studies utilizing this model (e.g. as little as 4% fetal weight restriction 5 days after treatment (Yamazaki *et al.* 2006)).

In conclusion, this study demonstrates that maternal dietary supplementation with n-3 PUFAs increases the antioxidant capacity of the placental LZ, which may contribute to reduced placental oxidative status and increased fetal and placental growth outcomes (Jones *et al.* 2013*b*). Despite this increased antioxidant capacity, maternal n-3 PUFA supplementation did not protect against placental IR-induced fetal and placental LZ growth restriction.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0282.

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.

Funding

This work was supported by the National Health and Medical Research Council of Australia (grant number 572621).

Acknowledgements

The authors wish to acknowledge Prof. Trevor Mori (School of Medicine and Pharmacology, The University of Western Australia) for analysis of F_2-isoprostanes, and the technical assistance of Ms Tracey Lee-Pullen (Centre for Microscopy, Characterization and Analysis, The University of Western Australia, Australia) for the Milliplex cytokine assay.

References

Arisue A, Shimojima N, Tomiya M, Shimizu T, Harada D, Nakayama M, Tomita H, Shinoda M, Tanabe M, Maruyama I *et al.* 2012 Effect of an omega-3 lipid emulsion in reducing oxidative stress in a rat model of intestinal ischemia–reperfusion injury. *Pediatric Surgery International* **28** 913–918. (doi:10.1007/s00383-012-3144-0)

Arsenijevic D, Onuma H, Pecqueur C, Raimbault S, Manning BS, Miroux B, Couplan E, Alves-Guerra MC, Goubern M, Surwit R *et al.* 2000 Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. *Nature Genetics* **26** 435–439. (doi:10.1038/82565)

Ashtiyani SC, Najafi H, Kabirinia K, Vahedi E & Jamebozorky L 2012 Oral omega-3 fatty acid for reduction of kidney dysfunction induced by reperfusion injury in rats. *Iranian Journal of Kidney Diseases* **6** 275–283.

Barden AE, Corcoran TB, Mas E, Durand T, Galano J-M, Roberts LJ, Paech M, Muchatuta NA, Philips M & Mori TA 2012 Is there a role for isofurans and neuroprostanes in pre-eclampsia and normal pregnancy? *Antioxidants & Redox Signaling* **16** 165–169. (doi:10.1089/ars.2011.4214)

Biri A, Onan A, Devrim E, Babacan F, Kavutcu M & Durak Ä 2006 Oxidant status in maternal and cord plasma and placental tissue in gestational diabetes. *Placenta* **27** 327–332. (doi:10.1016/j.placenta.2005.01.002)

Biri A, Bozkurt N, Turp A, Kavutcu M, Himmetoglu O & Durak I 2007 Role of oxidative stress in intrauterine growth restriction. *Gynecologic and Obstetric Investigation* **64** 187–192. (doi:10.1159/000106488)

Brahmbhatt V, Oliveira M, Briand M, Perrisseau G, Bastic Schmid V, Destaillats F, Pace-Asciak C, Benyacoub J & Bosco N 2013 Protective effects of dietary EPA and DHA on ischemia–reperfusion-induced intestinal stress. *Journal of Nutritional Biochemistry* **24** 104–111. (doi:10.1016/j.jnutbio.2012.02.014)

Burton GJ & Jauniaux E 2011 Oxidative stress. *Best Practice & Research. Clinical Obstetrics & Gynaecology* **25** 287–299. (doi:10.1016/j.bpobgyn.2010.10.016)

Calder PC 2012 Long-chain fatty acids and inflammation. *Proceedings of the Nutrition Society* **71** 284–289. (doi:10.1017/S0029665112000067)

Chevillotte E, Giralt M, Miroux B, Ricquier D & Villarroya F 2007 Uncoupling protein-2 controls adiponectin gene expression in adipose tissue through the modulation of reactive oxygen species production. *Diabetes* **56** 1042–1050. (doi:10.2337/db06-1300)

Coughlan MT, Vervaart PP, Permezel M, Georgiou HM & Rice GE 2004 Altered placental oxidative stress status in gestational diabetes mellitus. *Placenta* **25** 78–84. (doi:10.1016/S0143-4004(03)00183-8)

Faghiri Z & Bazan NG 2010 PI3K/Akt and mTOR/p70S6K pathways mediate neuroprotectin D1-induced retinal pigment epithelial cell survival during oxidative stress-induced apoptosis. *Experimental Eye Research* **90** 718–725. (doi:10.1016/j.exer.2010.03.002)

Gao J, Yasuda S, Tsuburaya R, Ito Y, Shiroto T, Hao K, Aizawa K, Kikuchi Y, Ito K & Shimokawa H 2011 Long-term treatment with eicosapentaenoic acid ameliorates myocardial ischemia–reperfusion injury in pigs *in vivo* – involvement of Rho-kinase pathway inhibition. *Circulation Journal* **75** 1843–1851. (doi:10.1253/circj.CJ-11-0209)

Haggarty P 2010 Fatty acid supply to the human fetus. *Annual Review of Nutrition* **30** 237–255. (doi:10.1146/annurev.nutr.012809.104742)

Hatakeyama Y & Scarpace PJ 2001 Transcriptional regulation of uncoupling protein-2 gene expression in L6 myotubes. *International Journal of Obesity* **25** 1619–1624. (doi:10.1038/sj.ijo.0801812)

Hewitt DP, Mark PJ & Waddell BJ 2006 Placental expression of peroxisome proliferator-activated receptors in rat pregnancy and the effect of increased glucocorticoid exposure. *Biology of Reproduction* **74** 23–28. (doi:10.1095/biolreprod.105.045914)

Hun CS, Hasegawa K, Kawabata T, Kato M, Shimokawa T & Kagawa Y 1999 Increased uncoupling protein2 mRNA in white adipose tissue, and decrease in leptin, visceral fat, blood glucose, and cholesterol in KK-Ay mice fed with eicosapentaenoic and docosahexaenoic acids in addition to linolenic acid. *Biochemical and Biophysical Research Communications* **259** 85–90. (doi:10.1006/bbrc.1999.0733)

Imperato-McGinley J, Binienda Z, Gedney J & Vaughan EDJ 1986 Nipple differentiation in fetal male rats treated with an inhibitor of the enzyme 5α-reductase: definition of a selective role for dihydrotestosterone. *Endocrinology* **118** 132–137. (doi:10.1210/endo-118-1-132)

Ishimoto H, Natori M, Tanaka M, Miyazaki T, Kobayashi T & Yoshimura Y 1997 Role of oxygen-derived free radicals in free growth retardation induced by ischemia–reperfusion in rats. *American Journal of Physiology* **272** H701–H705.

Jauniaux E, Poston L & Burton GJ 2006 Placental-related diseases of pregnancy: involvement of oxidative stress and implications in human evolution. *Human Reproduction Update* **12** 747–755. (doi:10.1093/humupd/dml016)

Jones ML, Mark PJ, Lewis JL, Mori TA, Keelan JA & Waddell BJ 2010 Antioxidant defenses in the rat placenta in late gestation: increased labyrinthine expression of superoxide dismutases, glutathione peroxidase 3, and uncoupling protein 2. *Biology of Reproduction* **83** 254–260. (doi:10.1095/biolreprod.110.083907)

Jones ML, Mark PJ, Keelan JA, Barden A, Mas E, Mori TA & Waddell BJ 2013*a* Maternal dietary omega-3 fatty acid intake increases resolvin and protectin levels in the rat placenta. *Journal of Lipid Research* **54** 2247–2254. (doi:10.1194/jlr.M039842)

Jones ML, Mark PJ, Mori TA, Keelan JA & Waddell BJ 2013*b* Maternal dietary omega-3 fatty acid supplementation reduces placental oxidative stress and increases fetal and placental growth in the rat. *Biology of Reproduction* **88** 37. (doi:10.1095/biolreprod.112.103754)

Kim K, Jung N, Lee K, Choi J, Kim S, Jun J, Kim E & Kim D 2013 Dietary omega-3 polyunsaturated fatty acids attenuate hepatic ischemia/reperfusion injury in rats by modulating toll-like receptor recruitment into lipid rafts. *Clinical Nutrition* **32** 855–862. (doi:10.1016/j.clnu.2012.11.026)

Lee JC, Bhora F, Sun J, Cheng G, Arguiri E, Solomides CC, Chatterjee S & Christofidou-Solomidou M 2008 Dietary flaxseed enhances antioxidant defenses and is protective in a mouse model of lung ischemia–reperfusion injury. *American Journal of Physiology. Lung Cellular and Molecular Physiology* **294** L255–L265. (doi:10.1152/ajplung.00138.2007)

Mailloux RJ & Harper M-E 2011 Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free Radical Biology & Medicine* **51** 1106–1115. (doi:10.1016/j.freeradbiomed.2011.06.022)

Mark PJ, Augustus S, Lewis JL, Hewitt DP & Waddell BJ 2009 Changes in the placental glucocorticoid barrier during rat pregnancy: impact on placental corticosterone levels and regulation by progesterone. *Biology of Reproduction* **80** 1209–1215. (doi:10.1095/biolreprod.108.073650)

Mark PJ, Lewis JL, Jones ML, Keelan JA & Waddell BJ 2013 The inflammatory state of the rat placenta increases in late gestation and is further enhanced by glucocorticoids in the labyrinth zone. *Placenta* **34** 559–566. (doi:10.1016/j.placenta.2013.04.006)

Myatt L 2010 Review: Reactive oxygen and nitrogen species and functional adaptation of the placenta. *Placenta* **31** S66–S69. (doi:10.1016/j.placenta.2009.12.021)

Myatt L & Cui X 2004 Oxidative stress in the placenta. *Histochemistry and Cell Biology* **122** 369–382. (doi:10.1007/s00418-004-0677-x)

Nagai R, Watanabe K, Wakatsuki A, Hamada F, Shinohara K, Hayashi Y, Imamura R & Fukaya T 2008 Melatonin preserves fetal growth in rats by protecting against ischemia/reperfusion-induced oxidative/nitrosative mitochondrial damage in the placenta. *Journal of Pineal Research* **45** 271–276. (doi:10.1111/j.1600-079X.2008.00586.x)

Nakai A, Taniuchi Y, Oya A, Asakura H, Koshino T & Araki T 2002 Windows of therapeutic opportunity on fetal growth retardation induced by transient intrauterine ischemia in rats. *Journal of Nippon Medical School* **69** 534–541. (doi:10.1272/jnms.69.534)

Oken E, Ning Y, Rifas-Shiman SL, Rich-Edwards JW, Olsen SF & Gillman MW 2007 Diet during pregnancy and risk of preeclampsia or gestational hypertension. *Annals of Epidemiology* **17** 663–668. (doi:10.1016/j.annepidem.2007.03.003)

Olsen SF, Olsen JR & Frische G 1990 Does fish consumption during pregnancy increase fetal growth? A study of the size of the newborn, placental weight and gestational age in relation to fish consumption during pregnancy *International Journal of Epidemiology* **19** 971–977. (doi:10.1093/ije/19.4.971)

Olsen SF, Secher NJ, Tabor A, Weber T, Walker JJ & Gluud C 2000 Randomised clinical trials of fish oil supplementation in high risk pregnancies. Fish oil trials in pregnancy (FOTIP) team. *BJOG: an International Journal of Obstetrics and Gynaecology* **107** 382–395. (doi:10.1111/j.1471-0528.2000.tb13235.x)

Pan H-C, Kao T-K, Ou Y-C, Yang D-Y, Yen Y-J, Wang C-C, Chuang Y-H, Liao S-L, Raung S-L, Wu C-W *et al.* 2009 Protective effect of docosahexaenoic acid against brain injury in ischemic rats. *Journal of Nutritional Biochemistry* **20** 715–725. (doi:10.1016/j.jnutbio.2008.06.014)

Perkins AV 2006 Endogenous anti-oxidants in pregnancy and preeclampsia. *Australian & New Zealand Journal of Obstetrics & Gynaecology* **46** 77–83. (doi:10.1111/j.1479-828X.2006.00532.x)

Rozen S & Skaletsky H 2000 Primer3 on the WWW for general users and for biologist programmers. *Methods in Molecular Biology* **132** 365–386.

Snedecor G & Cochran W 1989 *Statistical Methods*. Ames, IA: Iowa State University Press.

Spite M, Summers L, Porter TF, Srivastava S, Bhatnagar A & Serhan CN 2009 Resolvin D1 controls inflammation initiated by glutathione-lipid conjugates formed during oxidative stress. *British Journal of Pharmacology* **158** 1062–1073. (doi:10.1111/j.1476-5381.2009.00234.x)

Szajewska H, Horvath A & Koletzko B 2006 Effect of n-3 long-chain polyunsaturated fatty acid supplementation of women with low-risk pregnancies on pregnancy outcomes and growth measures at birth: a meta-analysis of randomized controlled trials. *American Journal of Clinical Nutrition* **83** 1337–1344.

Takehara Y, Yoshioka T & Sasaki J 1990 Changes in levels of lipoperoxide and antioxidant factors in human placenta during gestation. *Acta Medica Okayama* **44** 103–111.

Tanaka M, Natori M, Ishimoto H, Miyazaki T, Kobayashi T & Nozawa S 1994 Fetus–placenta–newborn: experimental growth retardation produced by transient period of uteroplacental ischemia in pregnant Sprague–Dawley rats. *American Journal of Obstetrics and Gynecology* **171** 1231–1234. (doi:10.1016/0002-9378(94)90138-4)

Thaete LG & Neerhof MG 2006 Endothelin and platelet-activating factor: significance in the pathophysiology of ischemia/reperfusion-induced fetal growth restriction in the rat. *American Journal of Obstetrics and Gynecology* **194** 1377–1383. (doi:10.1016/j.ajog.2005.11.019)

Umekawa T, Sugiyama T, Kihira T, Murabayashi N, Zhang L, Nagao K, Kamimoto Y, Ma N, Yodoi J & Sagawa N 2008 Overexpression of thioredoxin-1 reduces oxidative stress in the placenta of transgenic mice and promotes fetal growth via glucose metabolism. *Endocrinology* **149** 3980–3988. (doi:10.1210/en.2007-1682)

Vandesompele J, Preter KD, Pattyn F, Poppe B, Roy NV, Paepe AD & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** RESEARCH0034. (doi:10.1186/gb-2002-3-7-research0034)

Waddell BJ, Hisheh S, Dharmarajan AM & Burton PJ 2000 Apoptosis in rat placenta Is zone-dependent and stimulated by glucocorticoids. *Biology of Reproduction* **63** 1913–1917. (doi:10.1095/biolreprod63.6.1913)

Yamazaki K, Endo T, Kitajima Y, Manase K, Nagasawa K, Honnma H, Hayashi T, Kudo R & Saito T 2006 Elevation of both cyclooxygenase-2 and prostaglandin E2 receptor EP3 expressions in rat placenta after uterine artery ischemia–reperfusion. *Placenta* **27** 395–401. (doi:10.1016/j.placenta.2005.04.007)

Zeghichi-Hamri S, de Lorgeril M, Salen P, Chibane M, de Leiris J, Boucher F & Laporte F 2010 Protective effect of dietary n-3 polyunsaturated fatty acids on myocardial resistance to ischemia–reperfusion injury in rats. *Nutrition Research* **30** 849–857. (doi:10.1016/j.nutres.2010.10.010)

Zhou SJ, Yelland L, McPhee AJ, Quinlivan J, Gibson RA & Makrides M 2012 Fish-oil supplementation in pregnancy does not reduce the risk of gestational diabetes or preeclampsia. *American Journal of Clinical Nutrition* **95** 1378–1384. (doi:10.3945/ajcn.111.033217)

Received 2 July 2013
First decision 29 July 2013
Revised manuscript received 5 September 2013
Accepted 10 September 2013