

Resveratrol partially prevents oxidative stress and metabolic dysfunction in pregnant rats fed a low protein diet and their offspring

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Key points

- Maternal protein restriction during pregnancy increases both maternal and offspring oxidative stress and leads to metabolic dysfunction.
- Maternal low protein diet during pregnancy increases maternal and offspring corticosterone.
- Resveratrol administration partially prevents both maternal and offspring adverse outcomes induced by maternal protein restriction during pregnancy.

Abstract Protein restriction in pregnancy produces maternal and offspring metabolic dysfunction potentially as a result of oxidative stress. Data are lacking on the effects of inhibition of oxidative stress. We hypothesized that maternal resveratrol administration decreases oxidative stress, preventing, at least partially, maternal low protein-induced maternal and offspring metabolic dysfunction. In the present study, pregnant wistar rats ate control (C) (20% casein) or a protein-restricted (R) (10% casein) isocaloric diet. Half of each group received resveratrol orally, 20 mg kg⁻¹ day⁻¹, throughout pregnancy. Post-delivery, mothers and offspring ate C. Oxidative stress biomarkers and anti-oxidant enzymes were measured in placenta, maternal and fetal liver, and maternal serum corticosterone at 19 days of gestation (dG). Maternal (19 dG) and offspring (postnatal day 110) glucose, insulin, triglycerides, cholesterol, fat and leptin were determined. R mothers showed metabolic dysfunction, increased corticosterone and oxidative stress and reduced anti-oxidant enzyme activity vs. C. R placental and fetal liver oxidative stress biomarkers and anti-oxidant enzyme activity increased. R offspring showed higher male and female leptin, insulin and corticosterone, male triglycerides and female fat than C. Resveratrol decreased maternal leptin and improved maternal, fetal and placental oxidative stress markers. R induced offspring insulin and leptin increases were prevented and other R changes were offspring sex-dependent. Resveratrol partially prevents low protein diet-induced maternal, placental and sex-specific offspring oxidative stress and metabolic dysfunction. Oxidative stress is one mechanism programming offspring metabolic outcomes. These studies provide mechanistic evidence to guide human pregnancy interventions when fetal nutrition is impaired by poor maternal nutrition or placental function.

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Abbreviations C, control diet; Cres, control diet + resveratrol; dG, days of gestation; GPx, glutathione peroxidase; HOMA, homeostatic model assessment; MDA, malondialdehyde; PND, postnatal day; R, restricted diet; Rres, restricted diet + resveratrol; ROS, reactive oxygen species; SOD, superoxide dismutase.

Introduction

Poor fetal and neonatal nutrition result in developmental programming of multiple organ systems in humans and animals (Raghunath *et al.* 2009; Allen, 2012; Connor *et al.* 2012; Martin-Gronert & Ozanne, 2013; Nathanielsz *et al.* 2013; Hanson & Gluckman, 2014; Chan *et al.* 2015). These alterations in life course phenotype are the result of developmental programming, which can be defined as 'the response to a specific challenge to the mammalian organism during a critical developmental time window that alters the trajectory of development, modifying the organism's phenotype with effects on health that can persist throughout the life course' (Wadhwa *et al.* 2009; Gallo *et al.* 2013; Nathanielsz *et al.* 2013). One of the commonest and earliest experimental challenges studied is maternal protein restriction in pregnant rats. Maternal low protein diets result in male and female offspring experiencing metabolic dysfunction (Holemans *et al.* 2003; Armitage *et al.* 2004; Zambrano *et al.* 2005a; Langley-Evans & Sculley, 2006; Martin-Gronert & Ozanne, 2013), hypertension (Woods *et al.* 2001), as well as cardiovascular (Barros *et al.* 2015; Nascimento *et al.* 2014), behavioural (Almeida *et al.* 1996; Reyes-Castro *et al.* 2012) and reproductive (Engelbregt *et al.* 2000; Zambrano *et al.* 2014a) dysfunction.

Adequate perinatal nutrition is important because macro- and/or micronutrient deficiency during development increases oxidative stress by generation of reactive oxygen species (ROS) and decreases overall anti-oxidant activity (Mistry & Williams, 2011) associated with offspring insulin resistance (Zambrano *et al.* 2005a; Zambrano *et al.* 2006; Padmavathi *et al.* 2009). Under normal developmental conditions, ROS production is balanced by the removal of free radicals by anti-oxidant mechanisms (Halliwell, 2006) and oxidative stress only occurs when ROS generation exceeds the scavenging capacity of cellular anti-oxidant mechanisms as a result of excessive ROS production and/or inadequate anti-oxidant intake or synthesis.

Oxidative stress has been proposed as a causative agent of maternal pregnancy problems (e.g. recurrent pregnancy loss, pre-eclampsia and gestational diabetes) (Agarwal *et al.* 2012). There is also evidence to support the view that inadequate maternal nutrition and other challenges increase maternal and fetal oxidative stress, as well as ROS damage, and are also responsible for developmental programming of offspring disease (Giussani *et al.* 2012; Richter *et al.* 2012; Tarry-Adkins *et al.* 2013; Vega *et al.* 2015). In adults, chronic metabolic disorders may result from ROS generation and can be improved with dietary anti-oxidants (Willcox *et al.* 2004). Flavonoids and other polyphenols have powerful anti-oxidant activities *in vitro* and *in vivo*, including the ability to scavenge a range

of ROS (Heijnen *et al.* 2001; Santos & Mira, 2004). Resveratrol isolated from the oriental medicinal plant *Polygonum capsidatum* (Nonomura *et al.* 1963) exerts a variety of pharmacological effects, such as anticancer (Bhat & Pezzuto, 2002; Carter *et al.* 2014) and anti-inflammation actions (Jang *et al.* 1999). Resveratrol exerts a strong inhibitory effect on production of ROS and free radical scavenging properties in many experimental systems (Fremont, 2000; Martinez & Moreno, 2000; Bhat *et al.* 2001; Delmas *et al.* 2013; Cottart *et al.* 2014).

The effects of resveratrol on programming challenges include maternal hypoxia (Dolinsky *et al.* 2011; Bourque *et al.* 2012; Rueda-Clausen *et al.* 2012), maternal alcohol exposure (Kumar *et al.* 2011), maternal lipopolysaccharide treatment (Rose *et al.* 2014), maternal valproic acid inducing abnormal neural development (Bambini-Junior *et al.* 2014) and phthalates challenging male reproductive development (Botelho *et al.* 2009). Maternal resveratrol treatment has never been used to determine the effects of anti-oxidant therapy in relation to programming by maternal low protein diets. In one study, lazaroid, a 21-aminosteroid anti-oxidant inhibitor of lipid peroxidation (Cambonie *et al.* 2007), was shown to prevent adult hypertension, vascular dysfunction and microvascular rarefaction in male offspring following fetal exposure to a low-protein diet. No metabolic end-points were determined.

In a recent review, two leading researchers of oxidative stress as a mechanism underlying hypoxia and developmental origins of cardiovascular dysfunction state that mechanisms 'remain elusive, precluding the identification of potential clinical therapy' (Giussani & Davidge, 2013; Giussani *et al.* 2014). The same limitation to understanding the responsible mechanism is present in relation to programming by maternal undernutrition. One early study showed that in rat offspring, developmental programming by maternal low protein diets results from increased adrenocortical activity. Offspring adrenalectomy prevented programming of hypertension while administration of glucocorticoids to raise offspring corticosterone levels to those observed following fetal exposure to a low protein diet restored the hypertension (Gardner *et al.* 1997). We therefore determined changes in corticosterone in both mothers and offspring. Therefore, in the absence of any prior data evaluating resveratrol treatment on metabolic outcomes resulting from a low protein diet, the present study was based on the hypothesis that resveratrol administration during pregnancy decreases oxidative stress and prevents, at least partially, maternal, fetal and offspring metabolic outcomes resulting from low protein during pregnancy and improves offspring metabolism. We also hypothesized that there would be differences with respect to the sex of the offspring.

Methods

Care and maintenance of animals

All procedures described in the present study were approved by the Animal Experimentation Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición 'Salvador Zubirán', Mexico, and were conducted in accordance with the guidelines of Mexican law on animal protection (NOM-062-ZOO-1999).

Details of maternal diet, breeding and management have been reported previously in detail (Zambrano *et al.* 2005b). Female albino adult Wistar rats, aged 15–17 weeks, weighing 280 ± 3 g (mean \pm SD), were fed a Purina 5001 rodent diet (LabDiet, St. Louis, MO, USA) and maintained under a 12:12 h light/dark cycle (lights on 07.00 h) at 22–23°C. Founder female rats were mated overnight with proven male breeders. Females recruited to the study underwent a daily vaginal swab and the day that spermatozoa were present in the vaginal smear was designated as the day of conception (day 0). Only rats that became pregnant within 5 days of introduction of a fertile male were studied. Pregnant rats were placed in individual cages and randomly allocated to eat either control (C: 20% casein; $n = 32$) or restricted isocaloric diet (R: 10% casein; $n = 33$) (Zambrano *et al.* 2005b). Food and water were provided *ad libitum*. Pregnant rats were weighed daily during pregnancy and lactation. Food was provided as flat biscuits and food intake was measured daily. Half of the control rats were randomly assigned to receive resveratrol (Cres), as were half of the R mothers (Rres).

Maternal resveratrol intervention

Pharmacokinetic properties of resveratrol indicate that it cannot be dissolved in water, it has a short half-life (Baur & Sinclair, 2006) and, once administered, it is rapidly metabolized in the body (Delmas *et al.* 2006). Because of these characteristics, resveratrol was dissolved in an aqueous solution of carboxymethylcellulose 0.5% that was also used as the vehicle (Das *et al.* 2008). The dose of resveratrol (Sigma-Aldrich, St Louis, MO, USA) fed per rat was 20 mg $(\text{kg body weight})^{-1} \text{ day}^{-1}$. The resveratrol or vehicle was administered orally by gavage from 0 days of gestation (dG) until delivery. At 19 dG, half of the females from each group were killed to obtain serum, maternal and fetal tissue. Six rats per group were allowed to deliver to provide offspring for studies at postnatal day (PND) 110. Post-delivery mothers were fed with the C diet. Offspring were weaned at PND 21 and fed the Purina 5001 diet throughout the study.

Maternal phenotype, blood parameters and fetal characteristics at 19 dG

Serum and tissue collection. At 19 dG (chosen to avoid effects of parturition) at 6.00 h, food was removed from

pregnant rats. Between 10.00 and 11.00 h, pregnant rats were rapidly killed by decapitation by experienced personnel who were trained to use the rodent guillotine (Thomas Scientific, Swedesboro, NJ, USA). To ensure homogeneity of study subjects, litters >14 or <10 pups were excluded. Trunk blood was collected, allowed to clot at 4°C for 1 h, centrifuged at 1500 g for 15 min at 4°C and serum stored at -75°C .

Through a midline abdominal incision, the maternal liver was removed, cleaned, weighed and frozen at -75°C . The uterine horns were exposed and fetuses were rapidly killed by decapitation, counted and weighed. Each placenta was cleaned, weighed and male and females from each litter pooled together as one sample for each sex. Fetal livers were isolated and weighed. All male livers from each litter were pooled to produce a single sample for the litter. Females were treated similarly. Livers were immediately frozen in liquid nitrogen.

Adult offspring phenotype

Offspring were studied at PND 110. One male and one female per litter ($n = 6$) were randomly chosen, fasted for 4 h and killed by decapitation, and the trunk blood and liver were obtained and stored at -75°C for later protein quantification, as well as for determination of biomarkers of oxidative stress (ROS and anti-oxidant enzymes). Visceral fat depots located inside the thorax (mediastinal) and abdomen (omental, perirenal, retroperitoneal, epididymal, periovarian, perivescical and parametrial) were excised and weighed. The adiposity index was calculated as total adipose tissue (g) body weight (g) $^{-1}$.

Biochemical analysis

Serum measurements. Glucose, triglycerides and cholesterol were determined enzymatically with a Synchron CX auto analyser (Beckman Coulter, Fullerton, CA, USA) and corticosterone, insulin and leptin were determined by radioimmunoassay (Diagnostic Products Corp, Los Angeles, CA, USA; Linco Research, St Charles, MO, USA) (Guzman *et al.* 2006; Zambrano *et al.* 2006). Homeostatic model assessment (HOMA) was calculated from $\text{HOMA} = \text{glucose} (\text{mmol}^{-1}) \times \text{insulin} (\mu\text{U ml}^{-1}) / 22.5$ (Zambrano *et al.* 2006; Rasmussen *et al.* 2009).

Tissue homogenates and protein determination. Next, 100 mg of frozen placenta and liver were homogenized in 1 ml of 0.9% saline solution at 4°C, and protein was quantified by the Bradford method (Bradford, 1976). Lipid peroxidation was determined at the time of tissue homogenization. All determinations were performed in duplicate and averaged for statistical analysis.

Lipid peroxidation assay. Lipid peroxidation was assessed by malondialdehyde (MDA) content (Buege & Aust, 1978), measured by a thiobarbituric acid spectrometry assay, as described previously (Vega *et al.* 2015) in serum and liver. Tissue MDA was normalized to protein concentration. Intra- and inter-assay CVs were $\leq 3\%$ and $\leq 5\%$, respectively.

ROS assay. ROS formation was estimated as reported previously (Vega *et al.* 2015), adapted for tissue homogenates and expressed as dichlorofluorescein formed [$\text{nmol} (\text{mg protein})^{-1} \text{min}^{-1}$].

Superoxide dismutase activity. Superoxide dismutase (SOD) activity was measured using a commercial kit (RANSOD by Randox Laboratories, Crumlin, UK; catalogue numbers SD125 and SD126) and normalized to protein concentration. Intra- and interassay CVs were $\leq 7\%$ and $\leq 8\%$, respectively.

Glutathione peroxidase activity. Glutathione peroxidase (GPx) activity was measured using a commercial kit (RANSEL by Randox Laboratories; catalogue number RSD504). Data were normalized to protein concentration. Intra- and inter-assay CVs were $\leq 3\%$ and $\leq 5\%$, respectively.

Statistical analysis. For 19 dG studies, 10 C mothers, 10 Cres, 14 R and seven Rres mothers were studied. One animal of each sex from six different litters was studied per postnatal group. All data are reported as the mean \pm SEM. Analysis of maternal diet effects and resveratrol intervention was by ANOVA with Tukey's *post hoc* test where appropriate. $P < 0.05$ was considered statistically significant.

Results

Maternal characteristics

Maternal body weight and food intake during gestation. Maternal body and liver weight at 19 dG and the end of pregnancy were similar among groups. At the end of gestation, maternal weight gain was lower in R than C. At the beginning and end of lactation, Cres mothers weighed more than R. Resveratrol administration did not modify food intake (Table 1).

Placenta and fetal measures. Litter size and sex ratio were similar among groups. Placental and fetal body and liver weight data are reported in Table 1. Male and female R fetal weights were lower than C fetuses and resveratrol administration partially prevented this decrease in males. For both sexes, the fetal:placental ratio was lower in the

R group. This decrease was prevented in males by maternal resveratrol treatment. In resveratrol exposed females, the ratio did not differ from either controls or R offspring (Table 1). Female but not male liver fetal weight was lower in R and higher in Cres compared to C (Table 1).

Oxidative stress markers in mothers at 19dG

At 19 dG, maternal serum and liver MDA were higher in R compared to the C group (Fig. 1A and B). At 19 dG, the low protein diet increased maternal liver ROS (Fig. 1C). Resveratrol treatment in pregnancy decreased serum MDA and hepatic ROS in both Cres and Rres compared to the corresponding untreated groups, C and R (Fig. 1A and C). Liver SOD activity was lower in Cres, R and Rres relative to C (Fig. 1D). GPx activity was lower in R compared to C (Fig. 1E).

Maternal metabolic variables at 19dG

Maternal serum glucose was similar in C and R. Resveratrol reduced blood glucose in C mothers (Fig. 2A). Serum insulin concentration and HOMA were higher in R than C. Although resveratrol significantly improved both of these key metabolic variables, the reductions were only by 17% and 22% (Fig. 2B and C). Serum leptin levels were higher in R compared to C, Cres and Rres (Fig. 2D). Serum triglyceride and cholesterol levels were similar in all four groups (Fig. 2E and F).

At 19 dG, maternal serum corticosterone was higher in R mothers than C. This increase was reduced by 20% after the administration of resveratrol (Fig. 3A).

Placental oxidative stress markers

At 19 dG, male and female placental MDA was higher in R and lower in male Cres and Rres compared to C (Fig. 4A and B). ROS levels were higher in both male and female R placentas compared to C placentas (Fig. 4C and D). Placental SOD activity was higher in female R than C without any change in males (Fig. 4E and F). Placental GPx activity was higher in male R compared to the C, Cres and Rres, and lower in female Cres and Rres compared to R (Fig. 4G and H).

Fetal liver oxidative stress markers

At 19 dG, liver MDA and ROS levels were higher in male and female R fetuses compared to C (Fig. 5A–D). ROS was lower in female Rres than the other three groups (Fig. 5D). SOD activity was higher in male R compared to C, Cres and Rres. By contrast, female SOD activity was lower in R compared to C, whereas Rres showed the lowest value (Fig. 5E and F). Male GPx liver activity was

Table 1. Maternal, placenta and fetal characteristics

	C	Cres	R	Rres	P
Maternal					
Body weight (g) at onset pregnancy	280 ± 4	283 ± 5	278 ± 4	278 ± 2	0.62
Maternal body weight (g) at 19 dG	407 ± 10	394 ± 10	375 ± 8	391 ± 9	0.17
Maternal liver weight (g) at 19 dG	12.8 ± 0.6	12.6 ± 0.5	11.7 ± 0.3	11.2 ± 0.2	0.07
Maternal body weight (g) at the end of pregnancy	423 ± 11	409 ± 9	389 ± 9	407 ± 8	0.13
Maternal weight gain (g) through the whole of gestation	143 ± 7 ^a	127 ± 7 ^{ab}	113 ± 6 ^b	131 ± 8 ^{ab}	0.05
Food intake (g) during gestation	579 ± 10	581 ± 11	579 ± 7	580 ± 6	0.753
Maternal body weight (g) at the beginning of lactation	314 ± 7 ^{ab}	336 ± 7 ^a	295 ± 7 ^b	311 ± 9 ^{ab}	0.01
Maternal body weight (g) at the end of lactation	278 ± 6 ^{ab}	292 ± 5 ^a	268 ± 3 ^b	284 ± 6 ^{ab}	0.02
Litter size	11.7 ± 0.33	11.4 ± 0.27	11.3 ± 0.19	11.4 ± 0.3	0.707
Male:female ratio	1.1 ± 0.13	0.9 ± 0.08	1.2 ± 0.07	1.1 ± 0.08	0.318
Placenta					
Male placenta weight (g) at 19 dG	0.51 ± 0.01	0.52 ± 0.02	0.48 ± 0.02	0.46 ± 0.01	0.07
Female placenta weight (g) at 19 dG	0.51 ± 0.01	0.52 ± 0.02	0.49 ± 0.02	0.48 ± 0.02	0.38
Fetuses					
Male body weight (g) at 19 dG	3.1 ± 0.07 ^a	3.2 ± 0.05 ^a	2.4 ± 0.04 ^b	2.7 ± 0.08 ^c	0.001
Male fetus:placenta ratio at 19 dG	6.1 ± 0.15 ^a	6.1 ± 0.21 ^a	4.9 ± 0.19 ^b	5.8 ± 0.16 ^a	0.001
Male liver weight (g) at 19 dG	0.18 ± 0.006	0.19 ± 0.008	0.18 ± 0.008	0.18 ± 0.005	0.91
Female body weight (g) at 19 dG	3.1 ± 0.05 ^a	3.2 ± 0.03 ^a	2.5 ± 0.1 ^b	2.7 ± 0.05 ^{ab}	0.001
Female fetus:placenta ratio at 19 dG	5.9 ± 0.14 ^{ab}	6.2 ± 0.21 ^a	5.1 ± 0.33 ^b	5.6 ± 0.14 ^{ab}	0.023
Female liver weight (g) at 19 dG	0.17 ± 0.004 ^a	0.19 ± 0.01 ^b	0.14 ± 0.004 ^c	0.18 ± 0.004 ^{ab}	0.001

Data are the mean ± SEM. One-way ANOVA, $P < 0.05$ for data not sharing at least one letter. Number per group: C = 10, Cres = 10, R = 14 and Rres = 7 at 19 dG. $n = 6$ per group during lactation.

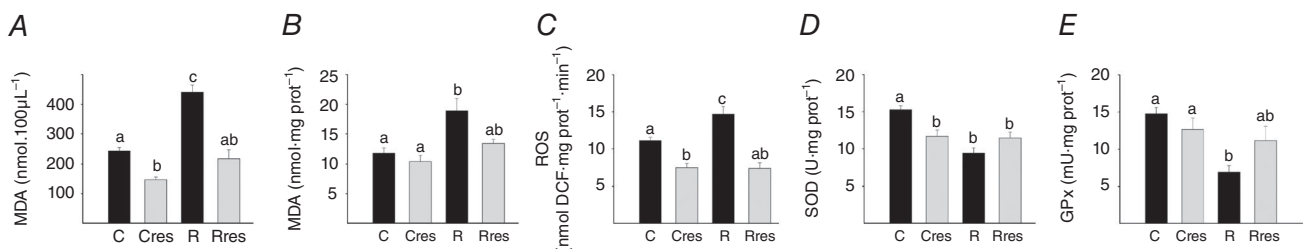
lower in Cres compared to C and Rres compared to R. Female GPx was higher in R compared to C (Fig. 5G and H).

Adult offspring phenotype

At PND 110, Cres males weighed less than C (Fig. 6A). Total fat and adiposity index were lower in male Cres compared to C (Fig. 6B and C). Female body weight was higher in Rres compared to C and Cres, and total fat and adiposity index were higher in female R and Rres compared to C and Cres (Fig. 6G–I). Fat depot distribution is shown in Table 2. Male offspring retroperitoneal and perirenal fat was decreased in Cres compared to C and R. Retroperitoneal and perirenal and parametrial fat were greater in R and Rres females compared to C and Cres.

Male epididymal fat was decreased in Cres compared to C and R and lower in Rres compared to R. Female periovarian and perivescical, male and female mediastinal and omental fat were similar among groups (Table 2). Male and female serum corticosterone levels were higher in the R group than C. In male offspring, maternal resveratrol administration prevented the R induced increase in corticosterone. In female offspring, corticosterone in Rres did not differ from either controls or R offspring (Fig. 3B and C).

Serum leptin was higher in R males and females compared to all other groups (Fig. 6D and J). Male serum triglycerides in R were higher than C, Cres and Rres, with no differences among female offspring groups (Fig. 6E and K). Male and female serum cholesterol were similar among groups (Fig. 6F and L).

**Figure 1. Maternal oxidative stress biomarkers at 19 dG**

Serum MDA (A), liver MDA (B), liver ROS (C), liver SOD (D) and GPx (E) activities. Data are the mean ± SEM; $P < 0.05$ when not sharing at least one letter. Number per group: C = 10, Cres = 10, R = 14 and Rres = 7.

For both sexes, serum glucose levels were similar among groups. Insulin and the HOMA index were higher in R in both sexes compared to all other groups (Fig. 7).

Offspring oxidative stress parameters at PND 110

Serum MDA was higher in R compared to C, Cres and Rres in both male and female offspring (Fig. 8A and F). Liver MDA concentration in male offspring was lower in Cres compared to R and lower in Rres compared to C and R (Fig. 8B), whereas, in female offspring, liver MDA was higher in R compared to C, Cres and Rres (Fig. 8G). Liver ROS concentration was higher in R compared to the rest

of the groups for male offspring, whereas female offspring groups were similar (Fig. 8C and H). Liver SOD and GPx activities were lower in R male offspring compared to the other male groups (Fig. 8D and E). In female offspring, SOD Cres was higher than R and Rres (Fig. 8I), whereas GPx was higher than the other three groups and R was lower than C (Fig. 8J).

Discussion

Compelling epidemiological evidence indicates that reduced maternal nutrition and poor fetal nutrient delivery have long-term effects on offspring health. We

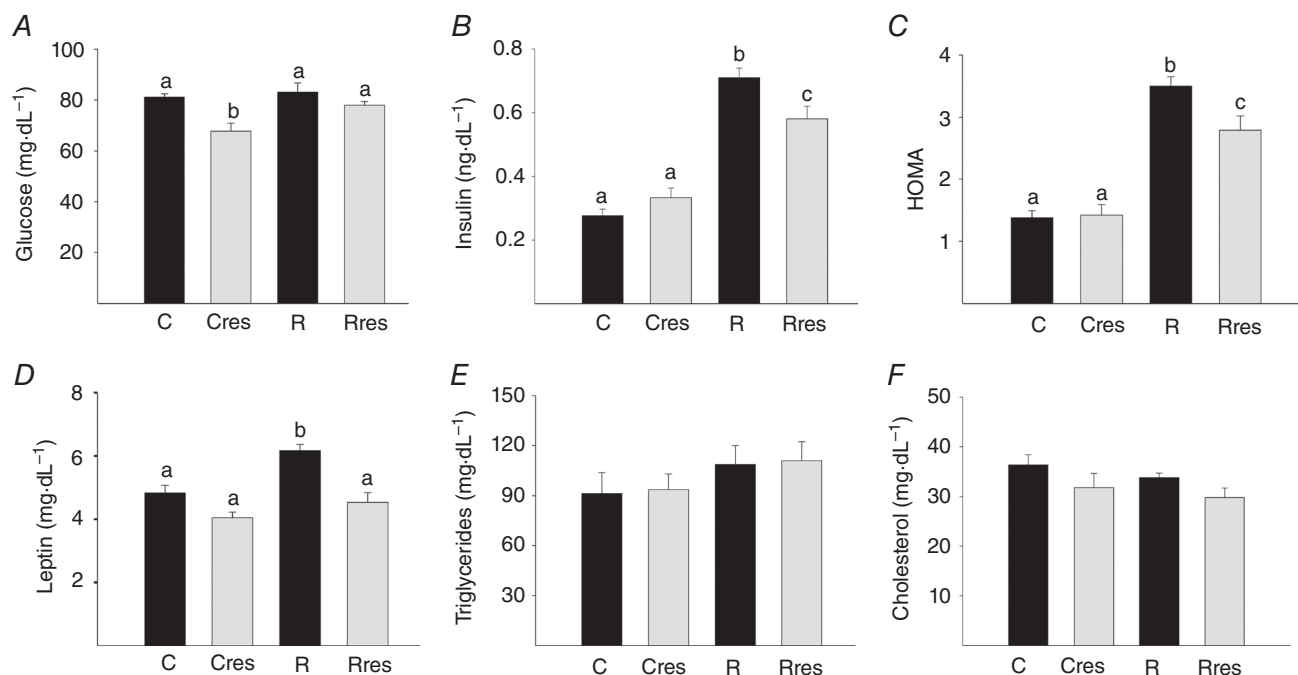


Figure 2. Maternal metabolism parameters at 19 dG

Glucose (A), insulin (B), HOMA (C), leptin (D), triglycerides (E) and cholesterol (F). Data are the mean \pm SEM; $P < 0.05$ when not sharing at least one letter. Number per group: C = 10, Cres = 10, R = 14 and Rres = 7.

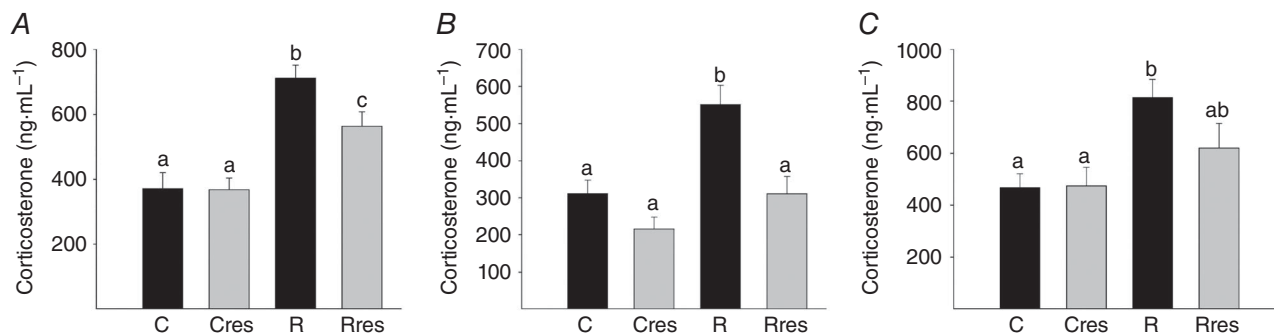


Figure 3. Serum corticosterone levels

Maternal at 19 dG (A), male (B) and female (C) offspring at PND 110. Data are the mean \pm SEM; $P < 0.05$ when not sharing at least one letter. Number per group: C = 10, Cres = 10, R = 14 and Rres = 7.

studied a common anti-oxidant intervention to determine the role of oxidative stress in programming the metabolic dysfunction seen in both sexes resulting from a maternal low protein diet in pregnancy. Pregnancy is accompanied by a high maternal energy and oxygen requirement to satisfy increasing maternal and fetal demands and

therefore probably increases oxidative stress (Gitto *et al.* 2002). We observed that the low protein diet increased oxidative stress in pregnant mothers compared to mothers fed the control diet. The placenta is very sensitive to oxidative stress (Gupta *et al.* 2005). Placental changes in oxygen tension at the beginning of pregnancy help to

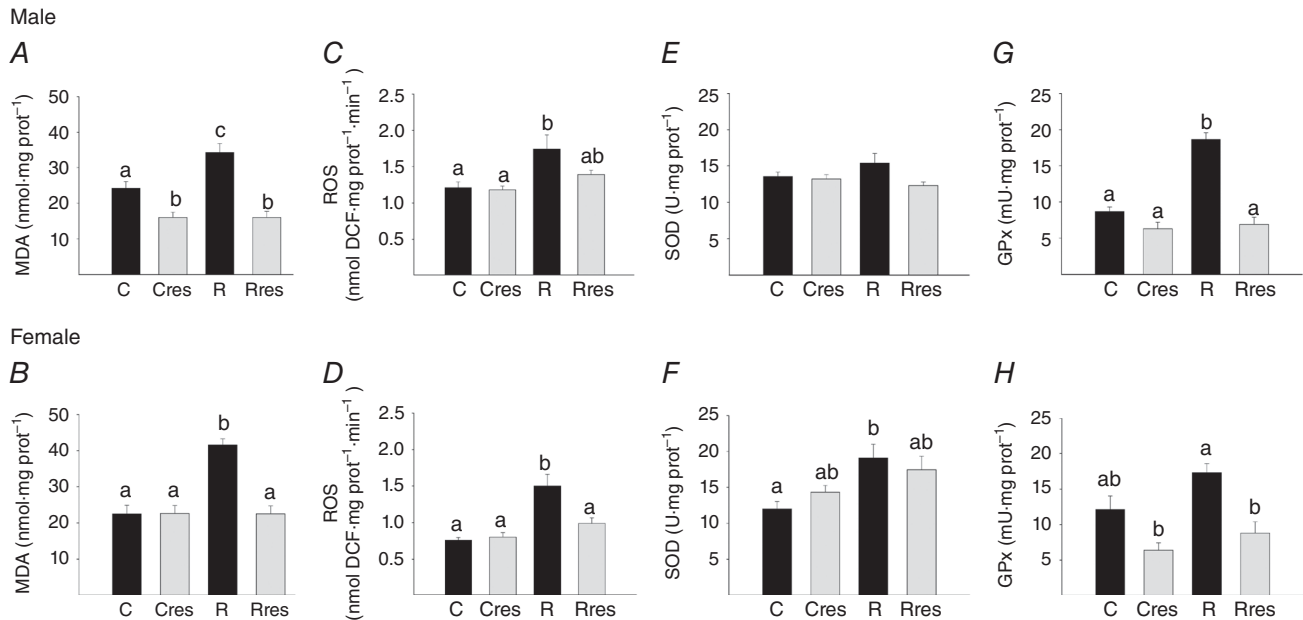


Figure 4. Placental oxidative stress biomarkers at 19 dG
 Male MDA (A), ROS (C), SOD (E), GPx (G) and female MDA (B), ROS (D), SOD (F) and GPx (H). Data are the mean ± SEM; *P* < 0.05 when not sharing at least one letter. Number per group: C = 10, Cres = 10, R = 14 and Rres = 7.

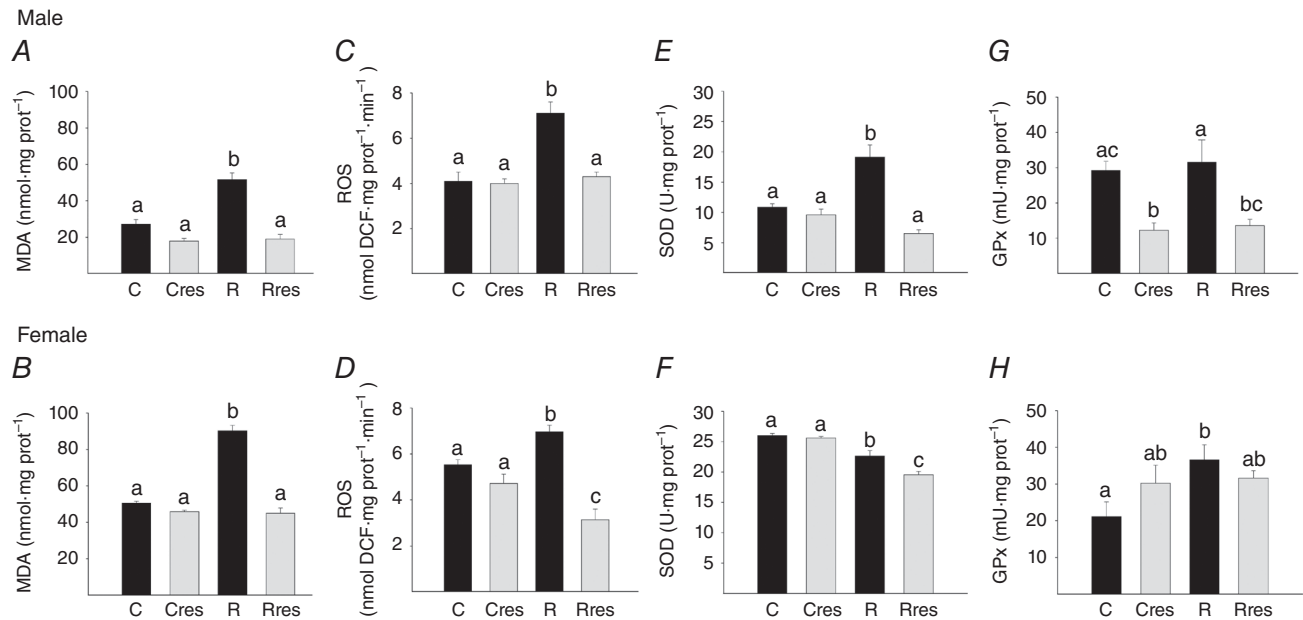


Figure 5. Liver oxidative stress in fetuses of 19 dG
 Male MDA (A), ROS (C), SOD (E), GPx (G) and female MDA (B), ROS (D), SOD (F) and GPx (H). Data are the mean ± SEM; *P* < 0.05 when not sharing at least one letter. Number per group: C = 10, Cres = 10, R = 14 and Rres = 7.

sustain the increased metabolic rate that occurs during the rapid fetal growth phase (Burton, 2009) and are associated with both increased circulating ROS, as well as increased activity of enzymes such as catalase, GPx and SOD (Jauniaux *et al.* 2000), which are responses that help to protect against increased oxidative stress. The results of the present study show that maternal protein restriction increases oxidative stress markers in placentas of both male and female fetuses, whereas there are some fetal sex-dependent differences in anti-oxidant responses. Maternal nutrient deficiency alters placental function and increased oxidative damage may play a role

in the observed reduction in the passage of nutrients from mother to fetus in the setting of reduced maternal nutrition and intra-uterine growth restriction (Jones *et al.* 2013; Rebelato *et al.* 2013). The results of the present study show that resveratrol has no effect on placental efficiency, measured as the fetal:placenta ratio in controls, but does increase placental efficiency in placentas of restricted mothers to levels similar to controls. This improvement in placental efficiency probably represents a beneficial outcome.

Although some studies show increased placental oxidative stress in the presence of maternal protein

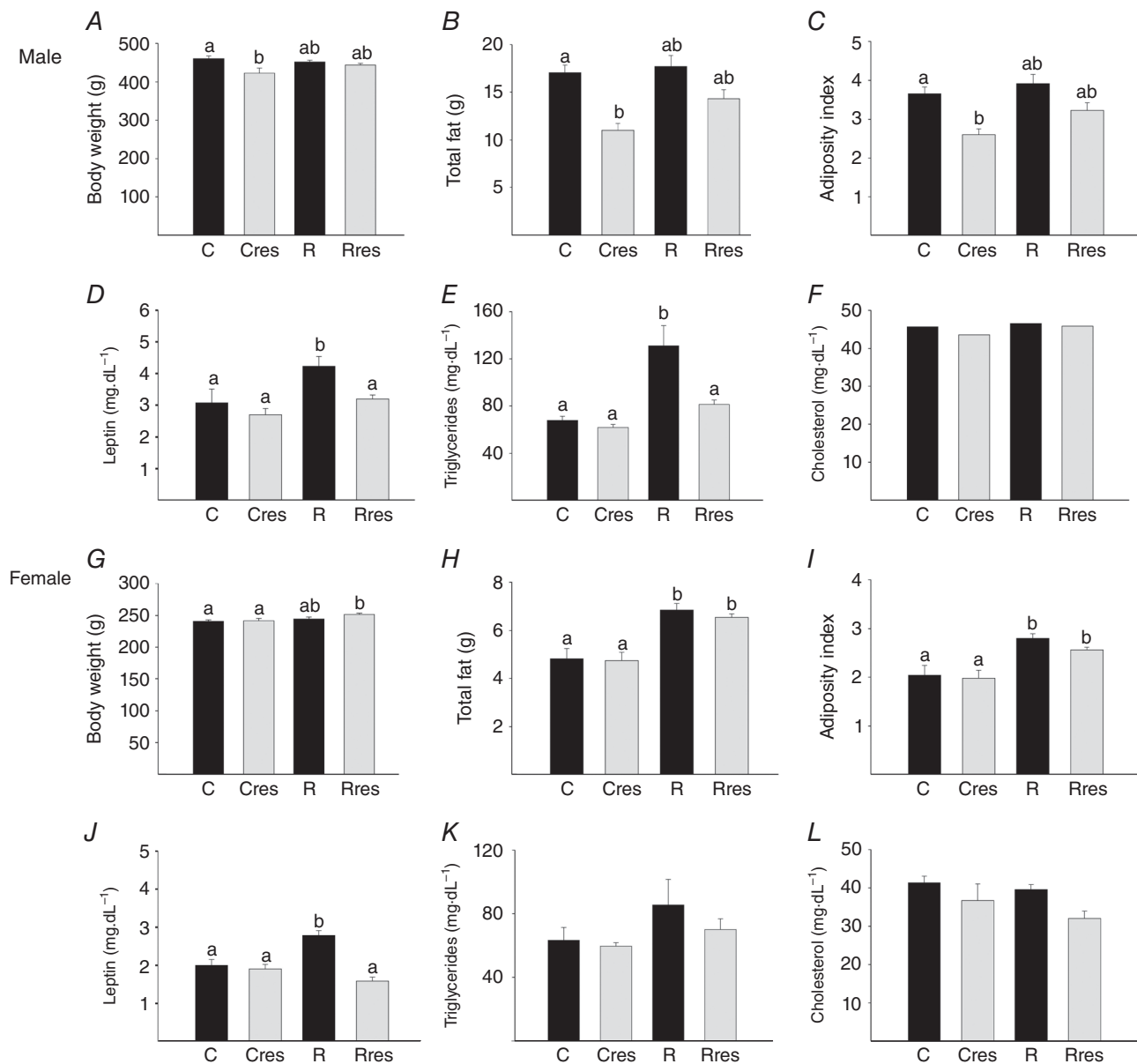


Figure 6. Offspring characteristics at PND 110

Male: body weight (A), total fat (B), adiposity index (C), leptin (D), triglycerides (E) and cholesterol (F). Female: body weight (G), total fat (H), adiposity index (I), leptin (J), triglycerides (K) and cholesterol (L). Data are the mean ± SEM; $P < 0.05$ when not sharing at least one letter. $n = 6$ per group.

Table 2. Offspring (F₁) male and female fat depots weight at PND 110

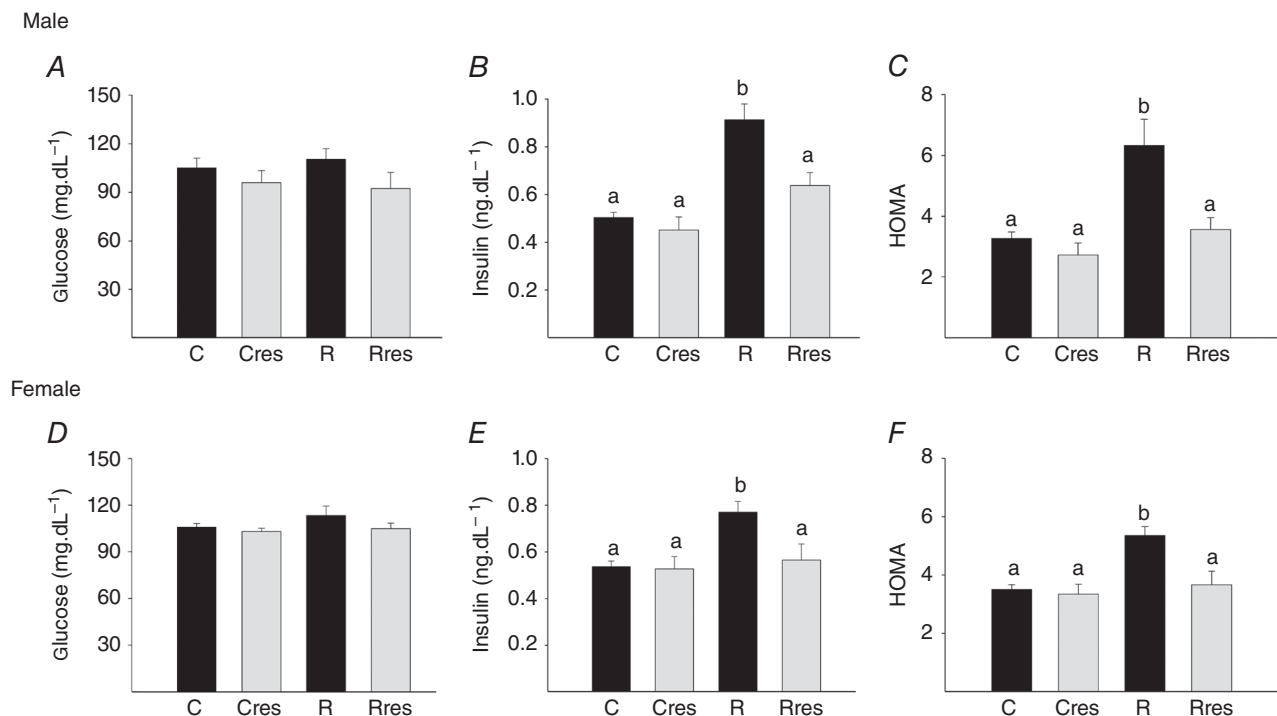
Fat (g)	C	Cres	R	Rres	P
Male					
Mediastinal	0.29 ± 0.02	0.23 ± 0.02	0.32 ± 0.04	0.36 ± 0.03	0.37
Retroperitoneal and perirenal	7.8 ± 0.4 ^a	4.8 ± 0.4 ^b	8.1 ± 0.7 ^a	6.6 ± 0.5 ^{ab}	0.001
Omental	0.8 ± 0.07	0.7 ± 0.1	0.7 ± 0.04	0.7 ± 0.04	0.12
Epididymal	8.1 ± 0.4 ^{ac}	5.2 ± 0.4 ^b	8.6 ± 0.5 ^a	6.6 ± 0.5 ^{bc}	0.001
Female					
Mediastinal	0.11 ± 0.02	0.11 ± 0.02	0.14 ± 0.01	0.13 ± 0.01	0.36
Retroperitoneal and perirenal	1.7 ± 0.2 ^a	1.7 ± 0.2 ^a	2.4 ± 0.1 ^b	2.4 ± 0.1 ^b	0.01
Omental	0.4 ± 0.06	0.4 ± 0.02	0.5 ± 0.08	0.4 ± 0.05	0.85
Parametrial	1.4 ± 0.1 ^a	1.4 ± 0.2 ^a	2.5 ± 0.1 ^b	2.1 ± 0.1 ^b	0.001
Periovarian and Perivescical	1.2 ± 0.08	1.1 ± 0.2	1.3 ± 0.1	1.5 ± 0.09	0.21

Data are the mean ± SEM. One-way ANOVA, $P < 0.05$ for data not sharing at least one letter. $n = 6$ per group.

restriction (Rebelato *et al.* 2013), we have been unable to find data on oxidative stress levels in fetal tissues in response to this challenge. However, other fetal programming models in the rat such as gestational diabetes have shown an increase in whole embryo lipid peroxidation and total thiols (Singh *et al.* 2011). In addition, hypoxic pregnancy promotes maternal and placental indices of oxidative stress, as well as enhanced aortic nitrotyrosine staining (Giussani *et al.* 2012; Richter *et al.* 2012). In the present study, hepatic lipid peroxidation

and ROS production were increased in male and female fetuses by the maternal low protein diet. The changes in anti-oxidant enzymes differed in male and female fetal liver tissues. Anti-oxidant sex differences are present in adult rats. Female rats have higher hepatic and cardiac vitamin E concentrations than males but lower hepatic GPx and muscle vitamin C (Tiidus *et al.* 1999).

Because few studies report tissue oxidative stress changes or status during resveratrol treatment (Cambonie *et al.* 2007), our second aim was to determine the

**Figure 7. Offspring metabolism at PND 110**

Male: glucose (A), insulin (B) and HOMA (C). Female: glucose (D), insulin (E) and HOMA (F). Data are the mean ± SEM; $P < 0.05$ when not sharing at least one letter. $n = 6$ per group.

extent to which the anti-oxidant resveratrol can prevent the oxidative stress changes resulting from maternal ingestion of a low protein isocaloric diet. Resveratrol has high anti-oxidant activity (Martinez & Moreno, 2000; Bhat *et al.* 2001; Cottart *et al.* 2014), and modulates multiple signalling pathways, including phosphorylation of adenosine-monophosphate-activated protein kinase, adenylyl cyclase, sirtuin 1, cyclooxygenase, lipooxygenase and protein kinase C (Pirola & Frojdo, 2008). Bourque *et al.* (2012) showed that adverse fetal outcomes as a result of hypoxic pregnancies can be improved if pregnant rats consume resveratrol. They also showed that resveratrol crosses the placenta; however, it is uncertain whether the benefits of resveratrol are the result of its actions on the placenta or in the maternal–fetal system, or both (Bourque *et al.* 2012). Using transgenic mouse models, it was shown that AMPK and SIRT1 are key mediators of the metabolic health actions driven by resveratrol (Kulkarni & Canto, 2015). In the gestational diabetic rat, resveratrol improved maternal glucose and the lipid profile and prevented embryonic oxidative stress and apoptosis (Singh *et al.* 2013). However, no data exist on the ability of resveratrol to prevent unwanted effects on developmental programming mechanisms induced by maternal protein restriction in either the mother or her offspring. In addition, the resveratrol dose administered differs widely between studies (Table 3). In pregnant mice, a dose of 50 mg kg⁻¹ day⁻¹ resveratrol reduced the incidence of fetal malformations induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure *in utero* (Jang *et al.* 2008). In a prenatal pregnant rat restraint stress model, resveratrol administration of twice the mouse dose, 100 mg kg⁻¹ day⁻¹, inhibited mitochondrial loss in the offspring hippocampus (Cao *et al.* 2014). (Table 3). In

the present study, maternal resveratrol administration at less than half the mouse dose (20 mg kg⁻¹ day⁻¹) had an overall beneficial effect by decreasing maternal MDA and ROS. Resveratrol also prevented the increase in placental and fetal oxidative stress markers. One resveratrol study in female Japanese macaques (Roberts *et al.* 2014) addressed the potential for beneficial effects of resveratrol administration in the setting of maternal obesity and fetal overnutrition. Mothers were maintained on a Western-style diet and provided with resveratrol in the diet (0.37%) for 3 months before and during pregnancy. Because no data were provided on food intake, the dose of resveratrol consumed cannot be calculated. The findings of this interesting study indicated that resveratrol treatment improved uterine blood flow, reduced placental inflammation and improved fetal liver lipid deposition (Roberts *et al.* 2014). However, there was an unexpected enlargement of the fetal pancreas without a concomitant increase in islet mass, suggesting a potentially harmful direct action of resveratrol on the fetal pancreas (Roberts *et al.* 2014). These and other data raised some concerns regarding anti-oxidant treatment in general and resveratrol in particular in pregnancy. However, Table 3 shows the widely different doses and routes of administration in different studies. Much more data are required to establish the potential cost–benefit of using resveratrol to prevent programming. In the present study, no adverse effects were observed potentially as a result of the lower dose.

Hypertrophy of pancreatic β -cells and the heart is accompanied by the induction of anti-oxidant enzymes that may contribute to cell survival (Rathore *et al.* 1998; Laybutt *et al.* 2002). The production of anti-oxidant enzymes may increase or decrease in the setting of

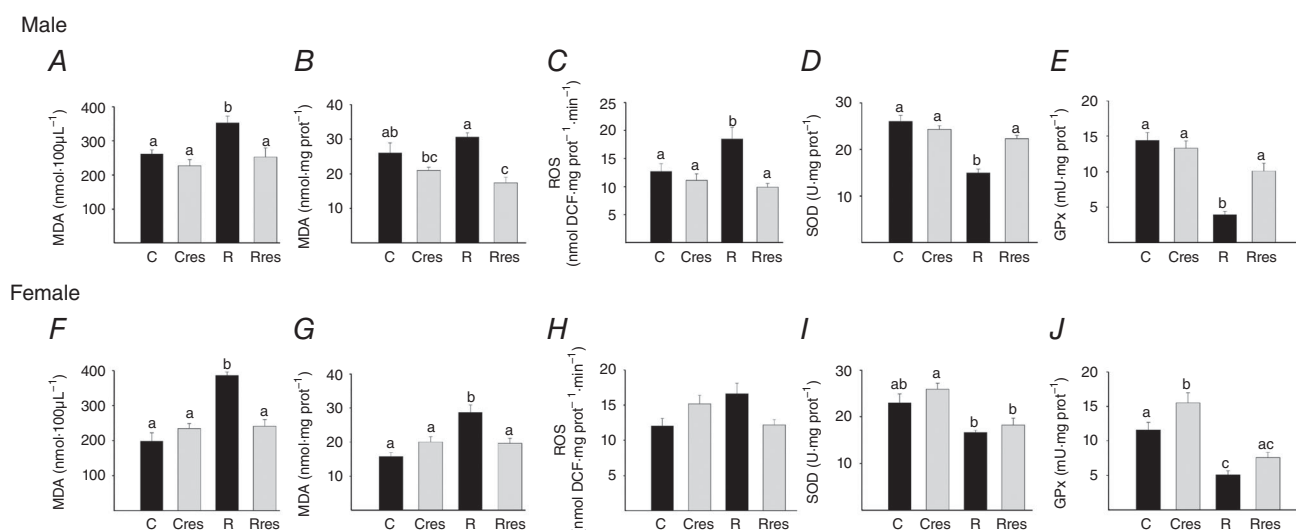


Figure 8. Offspring oxidative stress biomarkers at PND 110

Male: serum MDA (A), liver: MDA (B), ROS (C), SOD (D) and GPx (E). Female: serum MDA (F), liver: MDA (G), ROS (H), SOD (I) and GPx (J). Data are the mean \pm SEM; $P < 0.05$ when not sharing at least one letter. $n = 6$ per group.

Table 3. Review of publications using resveratrol to determine effects of oxidative stress in response to programming challenges

Species	Model	Dose	Period of intervention	Outcomes	Reference
Mouse C57BL/KsJ-Lep (db/+)	Genetic gestational diabetes mellitus	Oral gavage (10 mg kg ⁻¹ day ⁻¹)	Prior to and throughout pregnancy	Resveratrol intervention improved maternal glucose metabolism, insulin tolerance and reproductive outcomes. Hepatic AMPK activation was enhanced in both the mother and the offspring. Glucose 6-phosphatase production and activity were reduced	Yao <i>et al.</i> (2015)
Mouse C57BL/6J	Teratogenesis	Oral administration (50 mg kg ⁻¹ day ⁻¹)	Pregnancy 6 consecutive days from E8–E13	Resveratrol treatment reduced the incidence of fetal malformations induced by 2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin exposure <i>in utero</i>	Jang <i>et al.</i> (2008)
Wistar rats	Autism induced by valproic acid	Subcutaneous injections in DMSO (3.6 mg kg ⁻¹)	Pregnancy from E6.5 until E18.5	Resveratrol prevented valproic acid induced signs of autism	Bambini-Junior <i>et al.</i> (2014)
Wistar rat	Prenatal stress	Oral administration (10 mg kg ⁻¹ day ⁻¹)	Throughout pregnancy	Resveratrol treatment enhanced offspring number of newly born neurons and brain-derived neurotrophic factor expression in the hippocampus	Madhyastha <i>et al.</i> (2013)
Wistar rat	Prenatal stress	Oral administration (10 mg kg ⁻¹ day ⁻¹)	Throughout pregnancy	Resveratrol administration improved offspring spatial learning and memory and enhanced brain Na ⁺ , K ⁺ -ATPase activity	Sahu <i>et al.</i> (2013)
Wistar rats	Preeclampsia	Intragastric resveratrol administration 20 mg kg ⁻¹ day ⁻¹	Pregnancy twice daily during the entire pregnancy	Resveratrol administration reduced placental MDA levels, increased SOD activity and inhibited apoptosis	Zou <i>et al.</i> (2014)
Sprague-Dawley	Diabetic Embryopathy	Resveratrol administered by gavage (100 mg kg ⁻¹ day ⁻¹)	Pregnancy from day E3–E12	Embryos of diabetic rats treated with resveratrol showed normalized levels of retinoic acid receptors, and neuronal markers (GAP-43, total tau and neurofilament B)	Singh <i>et al.</i> (2012)
Sprague-Dawley	Prenatal stress	Oral gavage (100 mg kg ⁻¹ day ⁻¹)	Pregnancy	Resveratrol administration inhibited mitochondrial loss in the offspring hippocampus and activated Nrf2 and AMPK	Cao <i>et al.</i> (2014)

(Continued)

Table 3. Continued

Species	Model	Dose	Period of intervention	Outcomes	Reference
Mouse C57BL/6J, eNOS ^{-/-} and COMT ^{-/-}	Fetal growth restriction	Diet supplemented with resveratrol (4 g kg ⁻¹ diet)	Pregnancy between E0.5 and E18.5	Resveratrol supplementation significantly increased uterine artery blood flow velocity and fetal weight in COMT ^{-/-} but not in eNOS ^{-/-} mice. No effects on litter size and placental weight	Poudel et al. (2013)
Sprague-Dawley	Breast cancer	7ppm in diet	Pregnancy starting at E7	Resveratrol administration exerted protective effects against breast tumour development	Papoutsis et al. (2015)
Sprague-Dawley	In utero LPS exposure induced dopaminergic deficits	40 g of resveratrol-enriched diet (120 mg of resveratrol per kg of rodent diet)	Pregnancy from E3-E22.5	Dietary resveratrol intervention protected offspring against loss of striatal dopamine, dopamine metabolites and tyrosine hydroxylase expression	Rose et al. (2014)
Sprague-Dawley	Maternal hypoxia	4 g kg ⁻¹ in high fat diet	Postnatal (offspring 3 weeks of age) for 9 weeks	Resveratrol improved cardiac recovery from ischaemia-reperfusion injury and attenuated superoxide levels in male and female offspring	Shah et al. (2016)
Rat	Hypoxia	4 g kg ⁻¹ diet	Pregnancy from E7-E21	Resveratrol was detected in plasma of hypoxic dams and fetuses (performed by ultra-performance liquid chromatography). Resveratrol administration reversed fetal death in hypoxic pregnancies	Bourque et al. (2012)
Japanese macaques	Maternal obesity	High fat diet containing 0.37% of resveratrol	Prior to and throughout pregnancy	Resveratrol administration stimulated placental docosahexaenoic acid uptake capacity, AMPK activation and transporter expression	O'Tierney-Ginn et al. (2015)
Japanese macaques	Western style diet	Diet containing 0.37% of resveratrol	3 months before breeding season and pregnancy	Resveratrol administration caused a significant increase in islet capillary density	Pound et al. (2014)
Japanese macaques	Western style diet	Diet containing 0.37% of resveratrol	3 months before breeding season and pregnancy	Resveratrol administration reduced maternal weight by 30% and improved glucose tolerance, increased uterine artery volume blood flow and reduced placental inflammation and triglycerides deposition. Resveratrol administration also altered fetal pancreatic development (fetal pancreatic mass was enlarged by 42% with increased proliferation)	Roberts et al. (2014)

oxidative stress in ways that remain unexplained. One recent study showed that a low protein diet during gestation and lactation in rats increased the production of ROS and decreased anti-oxidant activity levels in heart tissue associated with increased susceptibility to developing metabolic and cardiac diseases in adulthood (Nascimento *et al.* 2014). Thus, when cells are exposed to oxidative stress, they may increase the activity and expression of anti-oxidant enzymes as a defence mechanism against free radicals. However, excessive free radical production may result in damage of the molecular machinery required to produce these enzymes (Rodriguez *et al.* 2004).

The final aim of the present study was to determine the extent to which resveratrol administration and the accompanying modifications in oxidative stress outcomes prevent the well-documented offspring metabolic programming changes produced by maternal low protein diets. Our overall hypothesis was that resveratrol administration during pregnancy would at least partially prevent maternal oxidative stress increases, thereby improving adult offspring adverse metabolic outcomes. The lowering of glucose in controls in the presence of unchanged insulin and the lowering of HOMA in both restricted mothers at the end of gestation and their offspring indicates a beneficial effect of resveratrol on both maternal and offspring insulin sensitivity. One interesting observation was the effect of resveratrol in reducing serum MDA and liver ROS and serum glucose in Cres mothers without altering insulin. In male Cres offspring, body weight and fat were also reduced. These findings indicate that Res may have maternal metabolic effects that are insulin independent and also potential direct effects on offspring adipose tissue. The beneficial effects of maternal resveratrol treatment on both C and R offspring were sex-dependent. For example, resveratrol decreased fat in C males and triglycerides in R males with no effects in females. Other studies have shown anti-oxidant sex differences, and conclude that changes in a sex- and tissue-specific manner are the result of changes in the post-transcriptional mechanisms in some enzymes such as GPx (Riese *et al.* 2006).

The present study shows sex differences in the offspring response to the low protein diet and the effect of resveratrol. For example, the R diet increases triglycerides in male but not female offspring. Also, liver ROS increases in male but not female offspring of R mothers. Mitochondria are the major source of free radicals in cells. Mitochondria from female rats generate half the amount of hydrogen produced by males and have higher levels of mitochondrial reduced glutathione (Borras *et al.* 2007). Cellular levels of anti-oxidant proteins, including catalase, glutathione peroxidase, peroxiredoxin, superoxide dismutase and thioredoxin, are regulated in sex-dependent manner in metabolic tissues in ways that

may result in females having a greater capacity to combat oxidative stress than males (Chaudhari *et al.* 2014). Further studies will need to be conducted to address these findings.

Carefully controlled interventions are essential for determining causative mechanisms in developmental programming with the aim of developing evidence based interventions (Nathanielsz *et al.* 2013). Recent studies have addressed potential benefits of pharmacological interventions in pregnancy on the short- and long-term health of both the mother and child (Duque-Guimaraes & Ozanne, 2013). For example, in rats, glycine (Jackson *et al.* 2002) and folic acid (Lillycrop *et al.* 2010) enrichment of maternal restricted diets prevent alterations in offspring liver metabolism and hypertension. In rodents, an improvement in offspring glucose homeostasis is observed when maternal low protein diets are supplemented with the amino acid taurine (Mortensen *et al.* 2010). Interventions with natural products, drugs or isolated compounds have shown beneficial results in offspring of mothers with poor nutrition. For example, maternal anti-oxidant supplementation (vitamin A, E, C and selenium) in rats fed a Western diet partially prevented offspring adiposity and normalized glucose tolerance (Mortensen *et al.* 2010). Genistein supplementation in mice during gestation protects offspring from susceptibility to obesity (Dolinoy *et al.* 2006). The administration of the potent inhibitor of lipid peroxidation lazaroid to protein-restricted mothers reduced the cardiovascular changes in adult offspring (Cambonie *et al.* 2007). In our model, the anti-oxidant compound resveratrol improved the maternal metabolism of protein-restricted diets and, as a result, prevented the adverse effects on offspring metabolism in adulthood. The differences between male and female offspring merit further investigation.

Maternal undernutrition in the rat from the beginning of pregnancy (Zambrano *et al.* 2005b; Guzman *et al.* 2006) or at late pregnancy increases maternal corticosterone levels (Lesage *et al.* 2001). In the present study, serum corticosterone was 91% higher in R compared to C mothers and resveratrol administration reduced this increase to 51%. Glucocorticoids play a major role in developmental programming, as shown by the beneficial effects of maternal adrenalectomy on the programming of hypertension by maternal low protein diets (Langley-Evans, 1997). Glucocorticoids have been proposed as fundamental orchestrators of developmental programming (Zambrano *et al.* 2014b). We propose that the 50% inhibition of the maternal corticosterone increase resulting from the low protein diet seen following resveratrol administration contributes to the prevention of offspring adverse outcomes independently of the direct effects of decreased ROS. Additional beneficial effects of resveratrol are probably independent of corticosterone-mediated effects.

In summary, pregnancy leads to a physiological state of maternal oxidative stress that is increased by a maternal low protein diet. The present study demonstrates increased oxidative stress in offspring of low protein mothers. Maternal intervention with resveratrol during pregnancy decreased maternal and offspring oxidative stress and the resultant damage in a sex-specific manner. The efficacy of this intervention supports a causative role for oxidative stress in the developmental programming of metabolic outcomes. The data reported in the present study indicate that oxidative stress is a major mechanism by which poor fetal nutrition leads to developmental programming of adult disease, indicating potential interventions when fetal nutrition is impaired either by a lack of maternal nutrition or impaired placental function.

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Additional information

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

CCV, LAR, GLRG, CJB and AMVM researched data. CCV, FL, GACC, PWN and EZ were responsible for the study design, preparation and review of the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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