Selenium deficiency as a model of experimental pre-eclampsia in rats

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Abstract

Epidemiological studies and *in vitro* analysis demonstrate correlations between selenium status and human pre-eclampsia (PET). Selenium is an essential component in the anti-oxidant proteins glutathione peroxidase and thioredoxin reductase, which are produced in lower amounts in pre-eclamptic placenta. This study examined the effect of modulating dietary selenium content in pregnant rats. Rats were fed diets containing no selenium, 239 µg/kg selenium or 1000 µg/kg selenium, four weeks prior to and following conception. Significant pregnancy-specific increases in systolic blood pressure (116.4 ± 5.2 mmHg vs 108 ± 6.8 mmHg vs 111.4 ± 4.7 mmHg) and proteinuria (9.68 ± 2.12 µg/ml vs 5.93 ± 1.59 µg/ml vs 4.43 ± 0.96 µg/ml) were demonstrated in animals fed a selenium free-diet when compared with normal or high selenium diets. Placental weight and pup number were not affected by selenium deprivation, however a significant decrease in the pup weight was evident. Selenium deprivation caused dose-dependent decreases in liver glutathione peroxidase (28.55 ± 3.82 mmoles/min/mg vs 34.68 ± 8.64 mmoles/min/mg) and thioredoxin reductase (2.37 ± 1.25 U/mg vs 6.68 ± 1.82 U/mg) activity, whereas superoxide dismutase activity remained constant. Placental activity of these enzymes also decreased leading to oxidative stress as measured by increased lipid peroxides (17.92 ± 1.78 µmoles/mg vs 8.30 ± 5.52 µmoles/mg) and protein carbonyls in tissue extracts from selenium-free animals. These results suggest that selenium deficiency in pregnant rats leads to symptoms similar to those seen in human PET and may provide an experimental model for studying this complex disease.

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Introduction

Affecting 6–8% of all pregnancies, pre-eclampsia (PET) is the leading cause of maternal morbidity in the western world and is characterised by hypertension, proteinuria, edema and platelet aggregation. Despite its prevalence and severity the pathophysiology of this multi-system disorder is still poorly understood. Placental oxidative stress has been shown to be a key feature in the pathogenesis of PET (for reviews see Walsh 1998, Hubel 1999, Redman & Sargent 2000). Oxidative stress is defined as an imbalance between the cellular generation of reactive oxygen species (ROS) and the capacity of anti-oxidants to prevent oxidative damage. The expression and activity of important anti-oxidant proteins are decreased in placental tissues from pre-eclamptic mothers, resulting in an imbalance between pro-oxidants and anti-oxidants leading to oxidative stress. Two of these anti-oxidant proteins are glutathione peroxidase and thioredoxin reductase, enzymes that have selenocysteine within their active site and are selenium-dependent for activity.

Whole blood and plasma levels of selenium are lower in pregnant women when compared with non-pregnant

women (Mihailovic *et al.* 2000) and decrease as gestation proceeds (Zachara *et al.* 1993). In 1990, Lu reported an increased incidence of pregnancy-induced hypertension in selenium-deficient regions of China and it was later found that selenium supplementation could be used to lower this high incidence of disease. Furthermore, decreased levels of selenium have been observed in patients with PET (Rayman *et al.* 2003). In humans, as in other mammals, selenium depletion leads to loss of glutathione peroxidase and thioredoxin reductase activity, although to date no one has examined the placental expression of these proteins during PET and related this to selenium status.

A major limitation of the investigations into the pathophysiology of PET is the fact that the disorder does not occur naturally in animals other than humans and two species of higher order primates, the patas monkey and the lowland gorilla (Zuspan 1991, Faas & Schuiling 2001). Numerous research groups have developed a variety of rodent models that in one way or another induce similar symptoms to those seen during human PET. These include nitric oxide inhibition (Baylis & Engels 1992), low dose endotoxin administration (Faas *et al.* 1994), sympathetic

nervous system stimulation (Kanayama *et al.* 1997, Khatun *et al.* 1999), uteroplacental ischemia (Losonczy *et al.* 1992), hyperinsulinemia (Podjarny *et al.* 1998) and spontaneously hypertensive models (Sharkey *et al.* 2001). In this report we have studied the pregnancies of rats fed a selenium-deficient diet and carried out preliminary biochemical analysis on placental tissues from these animals to demonstrate that changes seen in selenium-deficient rat pregnancies are similar to those seen in human placental tissues during PET.

Materials and Methods

Eight week old virgin female Wistar rats were housed at a constant temperature (22 °C) in a 12 h light dark cycle environment with free access to food and drinking water. Animals were randomised into four groups ($n = 6$) and fed one of three diets that differed in their levels of selenium for 4 weeks. The basal diet was selenium-free, containing 30% torula yeast, 59% sucrose, 5% coconut oil (vitamin E free), 5% premixed minerals (Hubbel, Mendel & Wake-man salt mix, ICN, Seven Hills, Australia) and 1% premixed vitamins (vitamin diet fortification mixture, ICN). One experimental group received the selenium-free diet whilst another received the same diet supplemented with 1000 µg/kg sodium selenite (Sigma). One group of animals were fed standard rat chow, which contained 239 µg/kg of total selenium. The animals from each diet group were then cycled and mated with fertile males at proestrous, with a positive vaginal smear for sperm the day after proestrous indicating day 0 of pregnancy. The remaining animals continued on their diets as non-pregnant controls.

Urine samples were collected by applying pressure to the bladder and collecting excreted urine with a capillary tube on days 7 and 21 and stored at -80 °C before analysis for urinary protein concentrations. Blood pressures were taken three times within 5 min on days 0, 7, 14 and 21 by tail cuff method (ABI Instruments, Sydney, Australia, using a 15 mm occlusion cuff and pulse transducer.

On day 21 pregnant rats were anaesthetised using sodium pentobarbital (60 mg/kg IP) and the pups delivered by caesarean section. Placental weight, fetal number and fetal weight were recorded. Placentas and the mother's liver and kidneys were collected, weighed and stored at -80 °C before biochemical analysis. The project was approved by the Griffith University Animal Ethics committee.

Processing of tissue samples and protein estimations

All samples were weighed and homogenized in four volumes of phosphate buffered saline (PBS) containing proteolytic enzyme inhibitors (Complete-Mini, Roche, Mannheim, Germany) using an Ultra-Turex homogeniser (IKA-Werke GMBH, KG Staufen, Germany). Samples were then centrifuged for 30 min at 4000 r.p.m. and the supernatant collected for biochemical analysis. The protein

concentrations of tissue homogenates was determined by the BCA Protein Assay kit (Pierce, Rockford, IL, USA) following the manufacturer's recommended protocols. Urea is an interfering substance for the BCA Protein Assay kit so rat urine samples were analysed using the Coomassie Plus Protein Assay kit (Pierce) following the manufacturer's recommended protocols. Bovine serum albumin (BSA) was used as the protein standard and assayed together with dilutions of placental extracts. Protein concentrations were determined from the standard curve and expressed as µg protein per ml of tissue extract (µg/ml).

Glutathione Peroxidase Assay

Glutathione peroxidase activity was determined by a modified method of Paglia and Valentine (1967). Activity was determined spectrophotometrically by coupling the oxidation of glutathione and NADPH using glutathione reductase. Briefly, 900 µl of assay mix containing 0.1 M KPi (pH 7.0), 2 mM EDTA, 0.5 U/ml glutathione reductase, 10 mM glutathione and 0.3 mM reduced NADPH was placed into a Varian CARY 50 spectrophotometer (Varian Palo Alto, CA, USA) set at 340 nm. Diluted tissue extract (50 µl of 1/10 dilution) was added to the cuvette along with 50 µl of 20 mM tert-butyl hydroperoxide, a suitable substrate for glutathione peroxidase. The decrease in A340 was determined over a 3 min period and rate calculations were performed using Varian Cary software. Glutathione peroxidase activity was standardised against protein concentrations and expressed as mmoles per minute per mg of protein (mmoles/min/mg).

Thioredoxin reductase assay

Thioredoxin reductase activity was measured spectrophotometrically in a 96-well plate using the method of Holmgren and Bjornstedt (1995). This assay measures the thioredoxin reductase- and NADPH-dependent reduction of insulin with and without thioredoxin. Briefly, 20 µl of the 1/10 diluted tissue extracts were added to 120 µl of assay mix containing 50 mM KPi, 5 mM EDTA and 0.324 mg/ml insulin in a 96 well plate. Recombinant thioredoxin (10 µl of 1.4 mg/ml) was added to one well of each sample, while a further 10 µl of assay mix was added to the second well to measure background absorbance. Each sample was assayed in at least duplicate. After incubation at 37 °C for 20 min the reaction was stopped by the addition of 50 µl of 0.4 mg/ml 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB) in 6 M guanidine-HCl. The plate was incubated for 10 min at room temperature in the dark and plates were then read at 412 nm. Thioredoxin reductase activity was determined by subtracting the level of spontaneous insulin reduction from the thioredoxin-, thioredoxin reductase-, NADPH-dependent reduction of insulin. Thioredoxin reductase activity was expressed as units per gram of protein (U/mg).

Superoxide dismutase assay

Total superoxide dismutase (SOD) activity was measured in triplicate, spectrophotometrically in a 96-well plate using the method of Ahmed *et al.* (2003). This assay is based upon the inhibition of pyrogallol oxidation, with one unit of SOD activity the amount that causes 50% inhibition of the oxidation of pyrogallol. SOD activity was measured every 5 min over 1 h at 405 nm for 20 μ l of 1/100 dilution of placental tissue extract with 180 μ l of Tris–cacodylic acid buffer (0.05 M, pH 8.2) containing 0.001 M diethylenetriamine pentaacetic acid (DTPA) and 0.2 mM pyrogallol. Superoxide dismutase activity was determined from the time-dependent inhibition of pyrogallol and expressed as units per milligram of protein (U/mg).

Lipid peroxidation assay

Lipid peroxidation levels in tissue extracts were determined using a lipid peroxidation assay kit (Calbiochem, San Diego, CA, USA), following the manufacturer's recommended protocol. This assay determines lipid peroxidation levels through the reaction of malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE) with chromogenic reagent 1-methyl-2-phenylindole at 45°C, which results in the production of a stable chromophore with maximal absorbance at 586 nm. Tissue extracts were incubated for 40 min with 1-methyl-2-phenylindole and 10 M methanesulphonic acid at 45°C, samples were then cooled on ice and centrifuged at 15000 *g* for 10 min to obtain clear supernatant samples that were read at 586 nm. 4-HNE was used to establish a standard curve, the gradient of which indicated the molar extinction coefficient used in the equation $[\text{MDA} + 4 - \text{HNE}] = (\text{sample absorbance}) \times 5/\epsilon$ with sample lipid peroxidation concentrations expressed as μ moles/mg protein.

Protein carbonyl assay

Placental extracts were analysed for protein carbonyl concentrations by the previously described ELISA method of Buss *et al.* (1997). This assay measures protein carbonyls in biological samples after reaction with 2,4-dinitrophenyl hydrazine (DNP). Samples are then non-specifically adsorbed onto Nunc Immunosorb plates where protein bound DNP was detected with anti-DNP-biotin-antibody labelled with streptavidin-biotinylated horseradish peroxidase. Finally, reaction with *o*-phenylenediamine and hydrogen peroxide in 50 mM NaHPO₄ plus citric acid resulted in color development after 25 min, with absorbances read at 450 nm after stopping the reaction with sulphuric acid. Each sample was analysed in triplicate and samples were quantified by comparison with oxidized BSA standards.

Data analysis

All data was processed using the Graph Pad Prism version 3.0 (Graph Pad Software Inc, San Diego, CA, USA) statistical package with $P < 0.05$ considered significant. One-way analysis of variance with Newman–Keuls method for pair-wise multiple comparisons was used for analysis and Grubb's test for outliers was the basis for exclusion where appropriate.

Results

Physiological data

The elimination of selenium from the diet of rats over a 4 week period prior to conception resulted in a significant pregnancy-dependent increase in systolic blood pressure from 109.7 ± 6.80 mmHg in non-pregnant animals to 116.4 ± 5.21 mmHg at gestation week 3 ($P < 0.001$) (Fig. 1A). Pregnant rats fed a standard diet containing 239 μ g Se/kg or a high selenium diet (1000 μ g Se/kg) did

Figure 1 (A) Effect of pregnancy on systolic blood pressure in rats fed a selenium-free diet, demonstrating a significant pregnancy-dependent increase in systolic blood pressure as a result of a selenium deficiency (*** $P < 0.001$). (B) Effect of varying the dietary selenium intakes on systolic blood pressure in pregnant rats. A diet deficient in selenium significantly increased systolic blood pressure when compared with both 239 μ g/kg and 1000 μ g/kg selenium diets. (* $P < 0.05$).

Figure 2 Effect of dietary selenium concentration on urinary protein concentration in pregnant and non-pregnant rats: a pregnancy-dependent increase in urinary protein concentration was observed in selenium-deficient rats (** $P < 0.01$).

not display this increase (Fig. 1B). Systolic blood pressures for rats fed the selenium-free diet (116.4 ± 5.21 mmHg) increased significantly when compared with week three blood pressures of rats fed both the normal diet (108 ± 6.80 , $P = 0.014$) and the high selenium diet (111.4 ± 4.78 , $P < 0.01$) (Fig. 1B). Significantly increased urinary protein concentrations were measured in pregnant rats with the selenium-free diet (Fig. 2). Non-pregnant animals on the selenium free diet demonstrated mean urinary protein concentrations of 6.46 ± 1.13 µg/ml that, when compared with pregnant animals on the same diet, demonstrated significantly increased urinary protein concentrations of 9.68 ± 2.12 µg/ml at term ($P < 0.01$), an increase not seen in other diet groups. The comparison of term urinary protein concentrations for the selenium-free diet (9.68 ± 2.12 µg/ml) with both the standard (5.94 ± 1.60 µg/ml, $P < 0.01$) and high selenium (4.43 ± 0.96 µg/ml, $P < 0.001$) treatment groups demonstrated significant differences (Fig. 2). There was no significant difference in placental weight or pup number between the groups (data not shown). However, pup

Figure 3 Effect of dietary selenium concentration on pup weights, illustrating that a selenium deficient diet resulted in significantly decreased pup weights (*** $P < 0.001$).

weight was significantly reduced in rats from the selenium-free group (4.57 ± 0.34 grams) when compared with both the standard diet group (5.726 ± 0.452 grams, $P < 0.001$) and the high selenium group (5.49 ± 0.26 grams, $P < 0.001$) (Fig. 3).

Liver concentrations of anti-oxidant proteins

Selenium deficiency resulted in a significant reduction in the liver concentrations of selenium-dependent anti-oxidant proteins. Thioredoxin reductase activity (Table 1) was significantly reduced for the selenium-free pregnant group (2.37 ± 1.25 U/mg protein, $P < 0.001$) when compared with the pregnant animals on the standard diet (6.68 ± 1.82 U/mg protein). Increasing dietary selenium intake to 1000 µg/Kg of food during pregnancy was shown to significantly increase liver thioredoxin reductase activity (12.13 ± 1.94 U/mg protein, $P < 0.05$). There was a significant difference ($P < 0.05$) between pregnant (6.681 ± 1.82 U/mg protein) and non-pregnant animals (12.45 ± 6.16 U/mg protein) on the standard rat chow diet, indicating that pregnancy may be associated with a decrease in systemic anti-oxidant activity (Table 1).

Glutathione peroxidase activity in liver homogenates (Table 1) was significantly reduced in pregnant animals on selenium-free diets (28.55 ± 3.82 mmoles/min/mg) when compared with pregnant animals on normal diets (34.68 ± 8.64 mmoles/min/mg, $P < 0.001$). Increasing the selenium content of the diet of pregnant rats increased the liver activity of glutathione peroxidase to 62.26 ± 8.10 mmoles/min/mg ($P < 0.001$). When compared with the non-pregnant control group (49.10 ± 9.83 mmoles/min/mg) there was a significant decrease in glutathione peroxidase activity in those animals on a standard rat chow diet when they became pregnant (34.68 ± 8.64 mmoles/min/mg, $P < 0.01$).

There were no significant changes in superoxide dismutase levels in animals receiving various selenium diets, whether pregnant or not (Table 1).

Placental concentrations of anti-oxidant proteins

There was no significant difference in placental thioredoxin reductase activity when comparing the selenium-free animals (11.06 ± 1.84 U/mg protein) with those on a standard diet containing 239 µg/Kg selenium (11.29 ± 3.14 U/mg). Placental thioredoxin reductase activity was significantly increased to 17.01 ± 4.09 U/mg protein ($P < 0.05$) with selenium supplementation of 1000 µg/kg food (Table 1). A similar trend was also seen when comparing glutathione peroxidase activity for the high selenium group (54.34 ± 6.73 mmoles/min/mg protein) with the selenium free (33.33 ± 6.11 mmoles/min/mg protein, $P < 0.001$) and standard diet (40.52 ± 4.87 mmoles/min/mg protein, $P < 0.01$) groups, with no significant difference between the latter two groups (Table 1). There was no significant difference in the level of placental superoxide dismutase activity in the placentae of animals from each dietary

Table 1 The effect of dietary selenium on anti-oxidant proteins in rat liver and placenta.

	Non-pregnant (mean $\pm$ S.D.) <i>n</i> = 6	Selenium-free (mean $\pm$ S.D.) <i>n</i> = 6	239 μ g Se/kg (mean $\pm$ S.D.) <i>n</i> = 6	1000 μ g Se/kg (mean $\pm$ S.D.) <i>n</i> = 6
Liver				
Glutathione peroxidase (mmoles/min/mg protein)	49.10 $\pm$ 9.83	28.55 $\pm$ 3.82 ^{a***}	34.68 $\pm$ 8.64 ^{b**}	62.26 $\pm$ 8.10 ^{a***}
Thioredoxin reductase (U/mg protein)	12.45 $\pm$ 6.16	2.37 $\pm$ 1.25 ^{a***}	6.68 $\pm$ 1.82 ^{b*}	12.13 $\pm$ 1.94 ^{a*}
Superoxide dismutase (U/mg protein)	2.37 $\pm$ 0.41	2.51 $\pm$ 0.28	2.25 $\pm$ 0.89	2.57 $\pm$ 0.38
Placenta				
Glutathione peroxidase (mmoles/min/mg protein)	–	33.33 $\pm$ 6.11	40.52 $\pm$ 4.87	54.34 $\pm$ 6.73 ^{a,c***}
Thioredoxin reductase (U/mg protein)	–	11.06 $\pm$ 1.84	11.29 $\pm$ 3.14	17.01 $\pm$ 4.09 ^{a*}
Superoxide dismutase (U/mg protein)	–	2.07 $\pm$ 0.14	2.23 $\pm$ 0.29	2.44 $\pm$ 0.24

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ^acompared to 239 μ g Se/kg group, ^bcompared to non-pregnant group, ^ccompared to the selenium-free group.

group although there was a trend towards an increase in activity correlating with higher selenium intakes (Table 1).

Placental oxidative stress

The oxidative state of placental tissues from all diet groups was measured via lipid peroxide and protein carbonyl concentrations. Placental lipid peroxidation was significantly increased with the elimination of selenium from the diet ($17.92 \pm 1.78 \mu\text{moles/mg}$) with a significant difference in MDA + 4-HNE concentrations when compared with both the standard ($8.30 \pm 5.52 \mu\text{moles/mg protein}$, $P < 0.05$) and high selenium ($7.81 \pm 3.79 \mu\text{moles/mg protein}$, $P < 0.05$) diet groups (Fig. 4A). Protein carbonyl concentrations were elevated in the placenta by the elimination of selenium from the diet, however this increase did not reach significance (Fig. 4B).

Discussion

In 1990 Lu observed that selenium levels in women with pregnancy-induced hypertension (PIH) were lower than women experiencing a normotensive pregnancy. It was later shown that selenium supplementation could be used to lower the incidence of PIH in a small group of pregnant women with high risk factors (Han & Zhou 1994). In 1996 Rayman *et al.* detected no difference in the serum levels of selenium in patients with PET when compared with non pre-eclampsic pregnancies, however in a later report the same authors measured the selenium concentrations in the toe-nails of pregnant women and found a significant correlation between pre-eclampsia and decreased selenium status prior to pregnancy (Rayman *et al.* 2003). Many of the studies examining selenium status during pregnancy have noted a decrease as gestation proceeds, presumably due to increased metabolic demand for selenium and plasma volume expansion (Zachara *et al.* 1993). Given the evidence suggesting that hypertensive disorders of

Figure 4 (A) Effect of dietary selenium concentration on placental lipid peroxidation in pregnant rats. A selenium-deficient diet resulted in a significant increase in placental levels of lipid peroxides (* $P < 0.05$). (B) Effect of dietary selenium concentration on placental protein carbonyl concentrations in rats. An elevation in protein carbonyl concentrations was observed after the elimination of selenium from the diet that did not reach significance.

pregnancy are associated with low selenium intake, we decided to examine the impact of varying selenium diets on pregnant rats.

Rats were fed diets containing 0 µg/kg, 239 µg/kg and 1000 µg/kg selenium for 4 weeks prior to mating. There was no increase in either blood pressure or proteinuria during this period. Animals from each dietary group were mated and their blood pressures observed weekly from day 0 to day 21 of pregnancy. There was a significant increase in the systolic blood pressure of those animals on a selenium-free diet when compared with animals on a normal rat diet or a high selenium diet. We also observed an increase in proteinuria in these animals, suggesting that selenium deprivation leads to a pre-eclamptic like condition in rats deprived of selenium. We also examined the outcomes from these pregnancies and noted a decrease in pup weights in the selenium-free group but no change in pup number or placental weight.

In order to explore the biochemistry underlying these physiological changes we examined the liver activity of key anti-oxidant proteins thioredoxin reductase, glutathione peroxidase and superoxide dismutase. There was a dose-dependent relationship between selenium intake and liver activity of thioredoxin reductase and glutathione peroxidase but no change in superoxide dismutase activity. This is to be expected as thioredoxin reductase and glutathione peroxidase are selenium-dependent enzymes and we have shown in other studies that dietary selenium intake is a simple and applicable method of altering the endogenous activity of these proteins (Venardos *et al.* 2004). An interesting observation during these studies was the significant decrease in both thioredoxin reductase and glutathione peroxidase activity in pregnant animals fed a standard diet compared with non-pregnant animals on the same diet. This suggests that pregnancy is associated with decreased anti-oxidant protection, perhaps due to an increased demand for selenium. In humans, selenium levels decrease as gestation proceeds (Zachara *et al.* 1993).

It is now generally accepted that human PET is associated with an increase in placental oxidative stress (reviewed in Redman & Sargent 2001). The levels of reactive oxygen species, products of increased biological oxidation such as lipid peroxides, protein carbonyls and nitro-tyrosine residues are all elevated in pre-eclamptic placentae. Recent work from this laboratory and that of others (Walsh & Wang 1993, Wiktor *et al.* 2000) has shown that key anti-oxidants such as thioredoxin reductase and glutathione peroxidase are decreased in placental tissue from pre-eclamptics, adding to the oxidative stress in these tissues which may lead to increased apoptosis and even necrosis. In this study we found that animals on selenium-free diets and those on normal diets had decreased placental expression of thioredoxin reductase and glutathione peroxidase when compared with animals supplemented with 1000 µg/kg selenium. Perhaps selenium supplementation could be a simple applicable method of alleviating placental oxidative stress in humans

suffering from PET as has been shown to be the case in the Chinese study on PIH women (Han & Zhou 1994).

In rats on selenium-free diets there was a significant increase in placental oxidative stress as measured by the lipid peroxide and protein carbonyl content. The addition of selenium to the diets decreased this oxidative stress but this did not correspond to the increased expression of thioredoxin reductase and glutathione peroxidase. The expression of these proteins was similar in animals on selenium-free and normal diets yet the level of lipid peroxides was higher in selenium-free animals only. The level of protein carbonyls, a marker of protein oxidation, followed a similar trend but did not reach statistical significance. This suggests that another selenium dependent protein may be responsible for this decrease in selenium replete animals, perhaps selenoprotein P that has been shown to act as an extra-cellular glutathione peroxidase (Saito *et al.* 1999).

Research on PET has been hindered in the past by the lack of a suitable animal model of this complex disease. Various rodent models of PET have been explored, all of which display various symptoms analogous to human PET, but do not display the oxidative changes in the placental compartment that is a key feature of the disease. In this study we have shown that selenium deprivation is able to modulate the endogenous expression of key anti-oxidant proteins, leading to a state of placental oxidative stress resulting in physiological changes in pregnant rats similar to those seen during human PET.

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