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Chronic hypoxia *in vivo* reduces placental oxidative stress

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Abstract

Decreased placental oxygenation and increased oxidative stress are implicated in the development of preeclampsia. Oxidative stress arises from imbalance between pro-versus anti-oxidants and can lead to biological oxidation and apoptosis. Because pregnant women living at high altitude (3100 m, HA) have lowered arterial PO₂ and an increased incidence of preeclampsia, we hypothesized that HA placentas would have decreased anti-oxidant enzyme activity, increased oxidative stress (lipid peroxidation, protein oxidation and nitration) and greater trophoblast apoptosis than low-altitude (LA) placentas. We measured enzymatic activities, lipid and protein oxidation and co-factor concentrations by spectrophotometric techniques and ELISA in 12 LA and 18 HA placentas. Immunohistochemistry (IHC) was used to evaluate nitrated proteins and specific markers of apoptosis (activated caspase 3 and M30). Superoxide dismutase activity was marginally lower ($p=0.05$), while glutathione peroxidase activity ($p<0.05$), thioredoxin concentrations ($p<0.005$) and thioredoxin reductase activity $p<0.01$ were all reduced in HA placentas. Decreased anti-oxidant activity was not associated with increased oxidative stress: lipid peroxide content and protein carbonyl formation were lower at HA ($p<0.01$). We found greater nitrotyrosine residues in the syncytiotrophoblast at 3100 m ($p<0.05$), but apoptosis did not differ between altitudes. Our data suggest that hypoxia does not increase placental oxidative stress *in vivo*. Nitrate stress may be a consequence of hypoxia but does not appear to contribute to increased apoptosis. Lowered placental concentrations of anti-oxidants may contribute to the susceptibility of women living at HA to the development of preeclampsia, but are unlikely to be etiological.

Introduction

Preeclampsia is a leading cause of maternal and fetal mortality, pre-term birth and neonatal intensive care unit admissions. Epidemiological, clinical and molecular studies support that placental hypoxia is involved in preeclampsia. The incidence of the syndrome is doubled at high altitude (>2700 m) [1-3]. Human and experimental animal studies at >3000 m support that the blood entering the intervillous space has a PO₂ approximately 20% lower than at sea level [4]. Uterine blood flow and oxygen delivery are reduced by ~33%, even in normal human pregnancy at high altitude [5]. Finally, molecular studies show that global patterns of gene expression are similar in high altitude versus preeclamptic placentas and in placental explants subjected to 3% oxygen in organ culture [6]. The high altitude placenta is thus a valuable model

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to distinguish hypoxia-related biochemical pathways uniquely associated with preeclampsia versus benign or adaptive responses to hypoxia.

Oxidative stress, an imbalance between the cellular generation of reactive oxygen species (ROS) and the capacity of anti-oxidants to prevent oxidative damage, may play a pivotal role in preeclampsia (reviewed in [7]). Oxidative stress can result in lipid peroxidation (which compromises mitochondrial ATP production and can stimulate pro-apoptotic events), protein carbonylation or nitration/nitrosylation (which alters protein conformation and function), and accumulation of DNA damage. ROS are primarily a byproduct of aerobic respiration, and are involved in normal extracellular and intracellular signaling processes [8]. Excess ROS can be produced by enzymatic processes involving oxidases, while excessive mitochondrial ROS production can cause damage which escalates ROS production by the mitochondrial electron transport chain, leading to further dysfunction. ROS can also increase where there are decreased concentrations of non-protein ROS-scavenging antioxidants (Vitamins A, C, E, and metabolic co-factors such as glutathione and thioredoxin) or decreased production of antioxidant enzymes. Antioxidant enzymes defend against free radical damage; their regulation depends mainly on the oxidant status of the cell [9]. Antioxidant enzymes are reduced in preeclamptic placentas [10] and oxidative stress is increased in placentas of mothers with preeclampsia [10,11].

Acute (hours to days), chronic (weeks) and lifelong high-altitude hypoxic exposure increases circulating markers of oxidative stress in humans [12,13]. The elevated incidence of preeclampsia at high altitude, and the broad similarity in gene expression between preeclamptic and high-altitude placentas [6] led us to the hypothesis that high-altitude placentas would have altered pro- versus anti-oxidant defenses, resulting in increased oxidative damage. In this comparison of high versus low altitude placentas, we focused first on the levels of key endogenous anti-oxidants including superoxide dismutase (total SOD activity), glutathione peroxidase, thioredoxin and thioredoxin reductase. The accumulation of lipid peroxides and protein carbonyls was measured as an indicator of oxidative damage. We also examined the degree of protein nitration, a by-product of nitric oxide (NO) and superoxide interaction leading to conversion to peroxynitrite, as increased nitration is commonly noted in preeclampsia [14]. As oxidative stress is often considered as the event precipitating the increased placental apoptosis observed in preeclampsia [15] we further tested whether differences in oxidative stress would translate into increased apoptosis.

Methods

Subjects

Participants were pregnant women residing at 1600 m (n=12, 625 mmHg barometric pressure) and 3100 m (n=18, 530 mmHg barometric pressure). Participants resided at their respective altitudes of residence for a minimum of three months prior to conception, throughout pregnancy and delivery. All 1600 m subjects were born and raised at 1600 m (n=3) or lower. One high-altitude mother was native to 3100 m, 4 were native to towns ≥ 2400 m and the remainder were born and raised at low altitude. All gave informed consent to procedures approved by the ethics committees of the participating institutions. We included only women who were primiparous, healthy, and without conditions predisposing to preeclampsia such as renal disease, diabetes or obesity. Women were excluded if they had an abnormal oral glucose tolerance test or developed other complications of pregnancy.

Sample collection and preparation

Placentas were collected immediately following delivery and washed in cold PBS until the majority of blood was cleared. Fresh tissue was sampled from multiple sites within the villous core and flash frozen in liquid nitrogen. Randomly chosen blocks of full depth tissue were

collected for paraffin embedding. Placental extracts were prepared and protein measured as previously described [10,16].

Assays

Total superoxide dismutase (SOD) activity was evaluated via a spectrophotometric assay based upon the inhibition of pyrogallol oxidation; one unit of SOD activity is the amount that causes 50% inhibition pyrogallol oxidation. SOD activity was measured every 5 min over 1 hr at 405 nm, as previously detailed [10,16], and expressed as units per milligram of protein (U/mg). The inter- and intra-assay variation were 1.40% and 0.35%, respectively.

Glutathione peroxidase activity was determined spectrophotometrically by coupling the oxidation of glutathione and NADPH using glutathione reductase, as we have previously reported [10]. Glutathione peroxidase activity was standardized against protein concentrations and expressed as moles/min/mg protein. The inter – and intra-assay variation were 0.43% and 1.25%, respectively.

Thioredoxin concentrations were determined using a previously described double-antibody capture-linked immunoabsorbent assay (ELISA) [10,16] and expressed as ng/mg of extracted protein. The inter- and intra-assay variation were 1.14% and 2.96% respectively.

The activity of thioredoxin reductase was measured using the thioredoxin reductase and NADPH dependent reduction of insulin with and without thioredoxin [10,16]. The inter- and intra-assay variation were 3.66% and 2.36% respectively.

The degree of lipid peroxidation in placental extracts was determined using a Lipid Peroxidation Assay Kit (Calbiochem). The assay evaluates lipid peroxidation levels through the reaction of malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE) with the chromogenic reagent 1-methyl-2-phenylindole at 45°C, which results in the production of a stable chromophore with maximal absorbance at 586 nm. A standard curve was created using 4-HNE, samples were measured in duplicate and lipid peroxide concentrations are expressed as μM MDA + 4-HNE/mg protein. Inter- and intra-assay variation were 3.71% and 3.8% respectively.

Placental extracts were analyzed for protein carbonyl concentrations by ELISA. This assay measures protein carbonyls in biological samples after reaction with 2,4-dinitrophenyl hydrazine (DNPH) [10,16]. Each sample was analyzed in triplicate and samples were quantified by comparison with oxidized BSA standards. Inter- and intra-assay variation were 2.6% and 6.7% respectively.

Immunohistochemistry

IHC was performed on a subset of 12 placentas from each altitude. Samples representing 3 different locations/placenta were randomly chosen for IHC analyses. We examined activated caspase-3 (rabbit monoclonal, Cell Signaling, Danvers MA), M30, the cleaved epitope of cytokeratin 18 that results from caspase-3 induced apoptosis (mouse monoclonal, M30 CytoDEATH, Roche, NJ), and nitrotyrosine (clone 1A6 mouse monoclonal, Upstate Biochemical, Charlottesville VA). Endogenous peroxidase was blocked (3% H_2O_2 in methanol) and antigen retrieval was performed by incubating slides in DAKO Target Retrieval Solution for 30 minutes at 95°C (DAKO, Carpinteria, CA). All primary and secondary antibody incubations were conducted at 37°C in a humidified chamber (activated caspase 3, 1:200; M30, 1:50 for 2.5 hours; nitrotyrosine 1:100 for 2 hours). All slides were incubated with specific biotinylated secondary antibodies for 1 hour (Sigma, St. Louis MO), followed by detection using the Vectastain ABC Kit for 45 min. Color was developed for 10 minutes with

NovaRed substrate solution (Vector Laboratories, Burlingame, CA), and counterstaining and mounting was according to standard protocols.

For an additional positive control we treated cultured BeWo choriocarcinoma cells (seeded in 2-well Lab-Tek slides at 10^5 cells per well) with 2.5 μ M camptothecin (Sigma, St Louis, MO) to induce apoptosis. Cells were fixed on the slide with cold ethanol/acetone mixture (1:1) and IHC performed as described above.

Image analysis

IHC data were analyzed using a digital analysis program (Image Pro, Media Cybernetics Silver Springs, MD) which permitted quantification of the percentage of cells positively stained for activated caspase 3 and M30. Positive controls (tonsil for Caspase-3, colon carcinoma for M30 and kidney and ischemic brain for nitrotyrosine) and negative controls (omission of primary antibody) were run for each batch of slides. The number of fields to be examined per slide was set at 12 after preliminary analyses showed that variability in the readings stabilized at 12 fields, and did not differ from observations of 16, 24, or 32 fields.

Statistical Analysis

Results are shown as mean $\pm$ SD. Maternal and infant characteristics and placental measures of oxidative stress were compared between low and high altitude using Student's *t* test. Correlation analysis was performed by linear or non-linear regression. The caspase 3 and M30 IHC results were analyzed using the Mann-Whitney U test. Evaluation of nitrotyrosine staining was based on a qualitative scale of -, +, ++, +++ representing no, weak, moderate or strong staining. Conversion of the qualitative data to ordinal data (0-3) was used for the nitrotyrosine IHC analyses in order to permit statistical analysis using the Mann-Whitney U [17]. Consistent with the requirements of the Mann-Whitney U, the variances were similar between the low versus high altitude samples. These data are reported as medians and inter-quartile ranges. Data are reported as significant where $p < 0.05$.

Results

Subject characteristics

Participating women were similar in demographic and obstetric characteristics at low versus high altitude (Table 1). Birth weight was lower at high altitude, despite similarity in placental weight, birth length and gestational age.

Endogenous anti-oxidants

The activities of anti-oxidant enzymes in placental tissues from low vs. high altitude are illustrated in Figure 1. Comparison of endogenous SOD between altitudes revealed marginally greater SOD activity at low altitude (2.0 ± 0.7 U/mg protein) than at high altitude (1.4 ± 0.6 U/mg protein, $p = 0.05$, Figure 1A). Glutathione peroxidase (Figure 1B) activity was greater at low altitude (16.4 ± 3.5) than at high altitude (11.9 ± 4.1 mmoles/min/mg protein, $p < 0.05$). The placental concentration of thioredoxin was greater at low altitude (122.1 ± 34.6) than at high altitude (80.4 ± 24.4 ng/mg protein, $p < 0.005$, Figure 1C). Consistent with this, thioredoxin reductase activity was twice as high in low (16.5 ± 7.8) vs. high altitude placentas (8.9 ± 5.4 U/mg protein, $p < 0.01$, Figure 1D). However, the decreased anti-oxidant activity at high altitude did not appear to result in oxidative stress, as the placental content of lipid peroxides (μ M MDA+4HNE/mg protein) was 2-fold less in the high altitude (7.6 ± 4.2) than the low altitude placentas (15.7 ± 9.8 , $p < 0.01$, Figure 2A). We also observed significantly less protein carbonyl formation in placentas from high altitude (80.5 ± 24.4 U/mg, $p < 0.01$, Figure 2B) versus low altitude (122.1 ± 34.6 U/mg, $p < 0.01$, Figure 2B). In summary, anti-

oxidant substrates and activity were decreased at high altitude, but this did not translate into increased oxidative stress.

Nitrotyrosine residues

IHC for nitrotyrosine revealed no difference between low and high altitude in the qualitative staining for nitrated proteins in endothelium or villous stroma. However staining was greater in the syncytiotrophoblast and extravillous trophoblast at high altitude ($p < 0.05$, Table 2, Figure 3). Thus, despite less oxidative stress as measured by lipid peroxidation and protein carbonylation in high altitude placentas, there was evidence for increased nitrative stress in the trophoblast.

Markers of apoptosis

We next examined whether differences in oxidative stress might have translated into differences in placental tissue apoptosis. In the control tissues, activated caspase-3 was detected in $0.06 \pm 0.08\%$ of tonsil cells (Figure 4A) while the negative control (Figure 4B) showed no staining. In the camptothecin-treated BeWo cells, $0.13 \pm 0.12\%$ of cells were positive (data not shown). Levels in placenta were more than an order of magnitude lower; at low altitude $0.005 \pm 0.004\%$ of cells (Figure 4C) were positive, while at high altitude $0.006 \pm 0.003\%$ of cells were positively stained (Figure 4D, $p = \text{NS}$).

M30, a downstream marker of caspase-3-related apoptosis, was present in $0.3 \pm 0.2\%$ of the control tissue, colon adenocarcinoma (Figure 4E). The camptothecin-treated BeWo cells (Figure 4F) showed $6.3 \pm 2.6\%$ positive staining for M30. There was positive staining in $0.03 \pm 0.02\%$ of cells in low altitude placentas (Figure 4G) and $0.04 \pm 0.03\%$ of cells in high altitude placenta (Figure 4H, $p = \text{NS}$). In summary, there were no differences in levels of apoptosis between the high versus low altitude placentas.

Oxidative stress, infant sex and gestational age

There was no difference in the placentas of male versus female infants in the measures reported above. There was no relationship between gestational age or placental weight and the dependent variables.

Discussion

The hypothesis that placentas subjected to high-altitude hypoxia would have increased oxidative stress was not supported. Instead endogenous placental anti-oxidant activity was lower, lipid peroxidation and protein carbonylation were reduced and no differences in apoptosis were found. Nitrotyrosine residues were increased in the high altitude placental trophoblast. Thus increased nitrative stress, but not oxidative stress, is associated with chronic hypoxia in the human placenta. Our findings are summarized in Figure 5. We conclude that mild chronic hypoxia does not increase placental lipid and protein oxidation *in vivo*, and that syncytiotrophoblast nitration is not necessarily associated with increased apoptosis.

The relevance of a control sample obtained at 1600 m as opposed to sea level is of concern in interpreting our results. The 9% difference in arterial oxygen tension between sea level and 1600 m has no effect on pregnancy outcome [18,2], nor on placental structure at altitudes < 2200 m [19]. The 24% difference in arterial PO_2 at 3100 m measurably impacts placental morphology and function (reviewed in [4]). As the PO_2 in the intervillous space near term is only ~ 40 mmHg, or 6% O_2 [20], at 3100 m intervillous PO_2 could be reduced by an additional 2-3%. This yields a placental oxygen level that would be similar to the 3% oxygen used in some *in vitro* placental studies to mimic hypoxia and preeclampsia [21,6]. That the high altitude placenta is hypoxic is consistent with our prior reports showing elevated HIF-1 α , VEGF and

sFlt-1 in the high altitude placenta but not at the lower altitude of 1600 m [22,6,23]. We show in Table 3 our previously published data from preeclampsia and controls at sea level. It is apparent that 1600 m is similar to sea level, and that preeclampsia and 3100 m parallel one another except in the measures of protein carbonylation and lipid peroxide.

Oxidative stress has been invoked as the link between placental insults (ischemiareperfusion, hypoxia, inflammation) and the increased apoptosis observed in preeclampsia (reviewed in [14,15]. The rarity of cells positive for apoptosis in our study is similar to what has been observed in normal pregnancy using similar markers and techniques [24], and lower than in preeclampsia [25]. Thus, at least when measured with IHC, high altitude placentas do not have increased apoptosis, consistent with reduced oxidative stress.

There is controversy over what are the best markers for measurement of oxidative stress. We chose to measure 4-HNE+MDA, protein carbonylation and nitration as endpoints because they reflect a broad range of cellular responses to excess ROS, and can be compared with a wide spectrum of tissue studies examining oxidative damage. There are data showing both increase [26,27], and decrease in ROS with hypoxia [28], depending on tissue type and experimental design. While more direct markers such as ROS and NO production would be useful, they cannot be measured in these ex-vivo samples. The simplest explanation for our data is that ROS production is diminished and anti-oxidant enzymes are down-regulated, or, more importantly, not up-regulated in high-altitude placentas as a result of lower oxygen tension. This explanation is supported by *in vivo* experimental animal studies in which hypoxia did not change multiple parameters of oxidative status in the placenta, whereas they were increased in hyperoxia [29]. In contrast, *in vitro* studies show that hypoxia produces increased lipid peroxidation in human trophoblast [30] and increased trophoblast apoptosis that is reversible by caspase inhibition [31]. Differences between *in vivo* and *in vitro* data may well be due to the experimental conditions. *In vitro* studies obligatorily shift the placenta from a ~6% oxygen environment to 21% room air for digestion and isolation of trophoblast, followed by re-entry into an extremely low (<1-2%) oxygen environment. This produces hypoxia-re-oxygenation, which induces greater oxidative stress and apoptosis than hypoxia alone [32,33].

Systemic oxidative stress is associated with an altered balance between reactive oxygen species (ROS) and nitric oxide; several possible explanations for our findings derive from this. Excess superoxide anion, largely mitochondrial in origin, is most commonly implicated as the source of increased ROS in preeclampsia [34,35,14]. Of relevance to our findings in light of the increased nitration of trophoblast is that NO also serves as an effective scavenger of superoxide (forming peroxynitrite) and of reactive lipid radicals (e.g. peroxyl). Thus an alternative explanation is that NO may be acting as a superoxide sink in the high-altitude placenta. This explanation is attractive as increased NO scavenging combined with low SOD activity would contribute to lower hydrogen peroxide and hence the reduced lipid peroxidation seen in the high altitude placenta. Alternatively, or in addition, it may be that superoxide production is similar at low versus high altitude, but the relative decrease we report in SOD, combined with a faster reaction rate of NO and superoxide relative to SOD [36] channels more of the superoxide towards the formation of peroxynitrite instead of hydrogen peroxide (Figure 5). If there were increased peroxynitrite production in the high altitude placenta, it could inhibit SOD (Mn) though not SOD (Cu/Zn) activity [37,38] and inactivate glutathione peroxidase [39]. This mechanism would be consistent with our data and would further escalate NO scavenging of superoxides (Figure 5). Uterine vascular eNOS and NO are increased by hypoxia [40], as are NO metabolites in the circulation of experimental animals [41] and humans with preeclampsia [42]. There is increased eNOS in the syncytiotrophoblast and endothelium in preeclamptic placentas [43,44] and in *in vitro* placental hypoxia [45]. Thus stimulated NO production may be elevated in both preeclampsia and in chronic hypoxia, and account for the parallel between our data on placental hypoxia and the increased nitrotyrosine staining seen in preeclampsia.

In conclusion, our findings show no link between *in vivo* chronic placental hypoxia, oxidative stress and increased apoptosis. Chronic hypoxia does appear to contribute to increased nitrative stress. It is possible that there is preferential diversion of ROS to interact with NO to form peroxynitrites, and hence increased syncytiotrophoblast nitration, but this does not translate into increased apoptosis. These data suggest that oxidation as opposed to nitration may be the more important determinant of increased apoptosis in the preeclamptic placenta. Greater nitration is not inherently linked to increased apoptosis in our model, thus it is unlikely that increased nitrative stress in preeclampsia reflects a pathophysiological process unique to preeclampsia. It may instead be secondary to placental hypoxia, to generalized reduction in anti-oxidant defenses, or to metabolic derangement as in gestational diabetes [46,47]. Further investigation is necessary to determine the balance between NO and ROS under hypoxic conditions in the placenta and the role of reactive nitrative species in modulating ROS production. We also speculate that under conditions of chronic hypoxia, if anti-oxidant defenses are insufficiently induced, additional oxidative insults, e.g. short periods of ischemia, may render the system more vulnerable and this may contribute to the well-known increased susceptibility to preeclampsia at high altitude.

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Figure 1.

A: Superoxide dismutase activity was marginally ($p=0.05$) greater in the low altitude (blue bar, range 1.19-2.99) than high altitude placentas (red bar, range 0.56 – 0.91).

B: Glutathione peroxidase activity was greater ($p = 0.01$) in the low altitude (blue bar, range 11.63-21.89) than high altitude placentas (red bar, range 5.32 – 18.79).

C. Thioredoxin content was greater ($p = 0.002$) in low altitude (blue bar, range 75.58 – 176.8) than high altitude placentas (red bar, range = 41.20-122.0).

D. Thioredoxin reductase activity was also greater ($p = 0.009$) in low altitude (blue bar, range 6.22 – 27.01) than high altitude placentas (red bar, range 1.69 – 19.04).

Figure 2.

A: Oxidative stress, as indicated by lipid peroxidation, was greater ($p = 0.007$) in low altitude placentas (blue bar, range 4.89 – 29.81) when compared with high altitude placentas (red bar, range 3.42 – 21.22).

B: Oxidative stress, as reflected in protein carbonylation, was greater ($p=0.002$) in low altitude (blue bar, range 75.6 – 176.8) than high altitude placentas (red bar, range 41.2-122.0).

Figure 3.

- A: Kidney was used as positive control for nitrotyrosine staining (positive staining is a dark reddish brown). Note the intense staining in the renal tubules.
 B: Ischemic brain was used as a second positive control.
 C: Placenta with the primary antibody for nitrotyrosine omitted.
 D: A low altitude placenta showing staining of serum within blood vessels and the intervillous space, some stromal staining, but a relative absence of syncytiotrophoblast staining.
 E: A high altitude placenta showing intense syncytiotrophoblast staining.
 F: A high altitude placenta showing strong staining in the intermediate (extravillous) trophoblast.

Figure 4.

A: Tonsil was used as a positive control tissue for immunohistochemical detection of Activtaed Caspase-3. Positive staining is indicated by the reddish brown color in the cytoplasm of positively stained cells.

B: Placenta with primary antibody for caspase 3 omitted. Placenta with primary antibody omitted for M30 had the same appearance (data not shown).

C: A low altitude placenta showing a rare positively stained cell in the syncytiotrophoblast.

D: A high altitude placenta showing an equally rare positively stained cell in the syncytiotrophoblast.

E: Adenocarcinoma of the colon, used as a positive control for M30, showed rare epithelial cells with strong staining (brownish red) with faint staining in the apical cytoplasm of other epithelial cells.

F: BeWo cells treated with camptothecin were used as an additional positive controls for M30 (note the much greater frequency of positive staining than in the normal placentas).

G: A low altitude placenta showing rare positive syncytiotrophoblast staining for M30. H. A high altitude placenta showing rare positive staining for M30 in the syncytiotrophoblast.

Figure 5.

The components of oxidative stress measured in the present study are shown in red. The hypothesis predicted that high altitude placentas would show decreased endogenous anti-oxidants such as total superoxide dismutase (SOD), a reduction in glutathione peroxidase and in thioredoxin reductase, and in thioredoxin, the substrate for thioredoxin reductase, predictions confirmed by our measurements. Due to reduced anti-oxidant protection we expected increased, but instead observed decreased lipid-peroxidation and protein carbonylation and no difference in apoptosis. Despite reduced lipid peroxidation and protein carbonylation, there was increased protein nitrosylation, specifically in the syncytiotrophoblast.

Table 1Maternal and Infant Characteristics (means $\pm$ SD)

	1600 m n=12	3100 m n=18
Age (years)	29 $\pm$ 3	28 $\pm$ 7
Height (cm)	169 $\pm$ 5	166 $\pm$ 7
Pre-pregnant weight (kg)	62 $\pm$ 6	63 $\pm$ 10
BMI (kg/m ²)	22 $\pm$ 2	23 $\pm$ 3
Weight gain with pregnancy (kg)	14 $\pm$ 5	15 $\pm$ 5
Birth weight (grams)	3365 $\pm$ 382	3132 $\pm$ 335 [*]
Gestational Age (weeks)	39.4 $\pm$ 1.7	39.7 $\pm$ 1.2
Baby Length (cm)	50.0 $\pm$ 3.0	49.6 $\pm$ 3.2
Placental weight (grams)	551 $\pm$ 166	625 $\pm$ 83
Ratio M/F	9M/3F	12M/6F

*
p < 0.05

Table 2

IHC analysis of nitrated proteins (medians and inter-quartile range)

	1600 m n=12	3100 m n=12
Syncytiotrophoblast	1.0 (1.5)	2.5 (1.0)*
Intermediate trophoblast	2.0 (1.0)	3 (1.0)*
Stroma	1.0 (0.5)	2.0 (2.0)
Endothelium	1.0 (1.0)	1.5 (1.0)

*
p < 0.05, Mann-Whitney U

Table 3

Sea level and preeclamptic data compared to the present study (mean±SD)

	<10 m control	<10 m preeclampsia	1600 m control	3100 m placental hypoxia
SOD (U/mg)	2.5±0.6	2.0±0.5 [-20]	2.0±0.7	1.4±0.6 [-30]
GPx (moles/min/mg)	17.3±6.1	11.5±3.1 [-34]	16.4±3.5	11.9±4.1 [-27]
THx(ng/mg)	107.0±18.4	91.1±21.2 [-15]	122.1±34.6	80.5±24.4 [-34]
THxRed (U/mg)	19.2±9.8	13.0±5.7 [-48]	16.5±7.8	8.9±5.4 [-46]
LPO (μmoles/mg)	5.4±4.0	20.7±7.8 [+283]	15.7±9.8	7.6±4.2 [-47]
PC (U/mg)	209.7±82.6	248.1±97.7 [+18]	122.1±34.6	80.5±24.4 [-34]

Data from reference 10