

Effect of high oxygen on placental function in short-term explant cultures

Nicole G. Reti · Martha Lappas · Berthold Huppertz ·
Clyde Riley · Mary E. Wlodek · Phil Henschke ·
Michael Permezel · Gregory E. Rice

Received: 27 August 2006 / Accepted: 22 December 2006 / Published online: 21 February 2007
© Springer-Verlag 2007

Abstract *Ex situ* culture of human gestational tissues has been routinely used as a model to investigate tissue function. The objective of this study was to determine the effect of varying oxygen concentrations on human term placental explants over a 24-h time period. Specifically, the effect of incubating placental explants in oxygen concentrations of 8%, 21% or 95% on tissue viability, metabolism and cell death was measured by assessing glucose consumption, lactate production, release of lactate dehydroge-

nase, parathyroid hormone-related protein (PTHrP), tumour necrosis factor- α (TNF- α) and 8-isoprostane, immunoreactivity for cleaved-caspase-9 and immunohistochemistry for the caspase-3-cleaved cytokeratin-18 neopeptide, M30. Exposure to higher oxygen concentrations significantly increased the rates of glucose consumption and lactate production. Apoptosis was significantly increased under conditions of higher oxygen as evidenced by increased M30 in placental explant sections. Similarly, hyperoxia significantly increased the releases of PTHrP, TNF- α and 8-isoprostane. Thus, incubation of placental explants with oxygen concentrations of 95% and, to a lesser extent, 21% oxygen was associated with the modulation of multiple cellular response pathways including those associated with tissue viability and cell death. These data are consistent with the hypothesis that hyperoxia activates pathways and mechanisms involved in cellular metabolism, necrosis and apoptosis, thereby shifting the balance from a steady state towards cell death.

N. G. Reti · M. Lappas (✉) · M. Permezel · G. E. Rice
Department of Obstetrics and Gynaecology,
Mercy Perinatal Research Centre, Mercy Hospital for Women,
University of Melbourne,
163 Studley Road,
Heidelberg, Victoria 3084, Australia
e-mail: mlappas@unimelb.edu.au

B. Huppertz
Institute of Cell Biology, Histology and Embryology,
Medical University Graz,
Graz, Austria

C. Riley · G. E. Rice
Gynaecological Cancer Research Centre,
Royal Women's Hospital,
Melbourne, Victoria 3053, Australia

M. E. Wlodek
Department of Physiology, University of Melbourne,
Melbourne, Victoria, Australia

P. Henschke
Mercy Hospital for Women,
Heidelberg, Victoria 3084, Australia

G. E. Rice
Translational Proteomics, Baker Medical Research Institute,
Prahran, Victoria, Australia

Keywords Term placental explants · Short-term cultures · Oxidant stress · Metabolite activity · Apoptosis · Human

Introduction

The placenta has a defined life span and hosts specific adaptive mechanisms that allow for its survival over a restricted period of time together with the maintenance of its vital functions (Schneider 2000). Throughout gestation, placental tissue is exposed to changes in ambient oxygen concentrations. Starting with a partial pressure of oxygen (pO₂) of <20 mm Hg (~2% O₂) during the early first trimester of pregnancy, there is a progressive rise in oxygen concentration to a pO₂ within the placenta of

>50 mm Hg (~7% O₂) at the end of the first trimester. This rise in placental pO₂ is associated with the onset of the maternal intervillous circulation (Jauniaux et al. 2000). The haemochorial interface is established only at this time and represents the major route of oxygen and nutrient supply to the growing fetus, with the syncytiotrophoblast acting as the placental barrier between maternal blood and fetal placental tissues (Aplin 1991). The role of oxygen in the regulation of placental function is crucial and has major implications for a successful outcome in human pregnancy.

In the past, we and other investigators have performed incubations of gestational tissue explants in the presence of carbogen gas (95% O₂ and 5% CO₂; Lappas et al. 2004; Nguyen et al. 1994; Farrugia et al. 2000) on the basis of the concept of the partial compensation for the lack of haemoglobin by an increase in the amount of physically dissolved oxygen. Recently, increasing concern has been voiced about the potentially toxic effects of unphysiologically high partial pressures of oxygen (Schneider 2000; for reviews, see Miller et al. 2005; Newby et al. 2005). Furthermore, recent studies have also shown that ambient oxygen concentrations have marked effects on the behaviour of both term and early placental explants, with disturbances in oxygen delivery being implicated in certain complications of pregnancy (Huppertz et al. 2003; Caniggia et al. 2000a,b).

The objective of this study has been to determine the effects of varying oxygen concentrations on placental tissue viability, metabolism and cell death during short-term cultures (up to 24 h). The hypothesis tested is that high oxygen concentrations during incubation periods of up to 24 h might result in reduced placental tissue function. Explant cultures from term placentae have been incubated under three different oxygen concentrations (8%, 21% and 95% oxygen). In particular, our study has focused on certain markers of viability, metabolism and apoptosis in order to demonstrate the differences observed in placental tissue in response to varied oxygen concentrations.

Materials and methods

Participants and patient samples

Collection and processing of placentae was approved by the research ethics committee of Mercy Health and Aged Care. Written informed consent was obtained from participating women. Placentae ($n=12$) were collected from women with uncomplicated pregnancies at term (37–42 weeks of gestation) undergoing elective Caesarean section. Indications for Caesarean section included repeat Caesarean section and breech presentation.

Tissue explants

The placenta was collected within 10 min of delivery and transferred to our laboratory within the hospital. Tissue processing commenced immediately and involved the removal of a placental lobule from the central region of the placenta. The placental lobule was rinsed in cold RPMI (RPMI-1640 medium developed at the Roswell Park Memorial Institute), pH 7.4. RPMI medium was supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin and 2 g/l sodium bicarbonate and had a starting glucose concentration of 11.1 mmol/l. Placental villous tissue explants were obtained by blunt dissection of parts of the villous trees and removal of visible connective tissue, vessels and calcium deposits. All tissue fragments (approximately four from each placenta; 50 mg each) were placed in RPMI at 37°C in a humidified atmosphere of either one of three incubators for a pre-incubation period of 1 h. The incubators (for details, see below and Table 1) included; (1) a humidified chamber with an atmosphere of carbogen (95% O₂/~722 mmHg and 5% CO₂), (2) a standard 5% CO₂ in air incubator (21% O₂/~160 mmHg and 5% CO₂) and (3) a controlled oxygen incubator with regulated 8% O₂/~60.8 mmHg and 5% CO₂. Following this equilibration period, tissues were transferred to 24-well tissue culture plates (200 mg wet weight/well) in 2 ml fresh pre-equilibrated RPMI 1640. Plates were then incubated in their respective incubation chambers for 3, 6 and 24 h. After each time point, tissue and incubation medium were collected separately and stored at -80°C until further analysis or were fixed in formalin.

The oxygen concentration of the culture medium was obtained on a Radiometer ABL 700 series blood gas analyser. Culture medium alone was sampled by aspirating medium into a blood gas syringe by utilising a direct gas-tight IV connection tube primed with medium. The syringe was purged of any gas, sealed and placed in ice until analysis. The concentration of dissolved oxygen in the culture medium was assessed following simultaneous 3-h, 6-h and 24-h cultures in each of the three incubation chambers: a Sanyo MCO-17AI, a Sanyo MCO-18M and a Ratek shaking water bath.

Determination of total protein

Total protein content was measured by extracting proteins from placental explants. Approximately 200 mg tissue was placed in 600 µl RIPA buffer (50 mM TRIS, 1% IGEPAL, 0.1% deoxycholate, 0.1% SDS, 150 mM NaCl, 10 µg/ml aprotinin, 1 mM EDTA). Samples were homogenised (2×20-s bursts) by using a metal blade tissue homogeniser (Ultra-turrax, S25N 8G dispersing tool; Jenke and Kunkel, Staufen, Germany) and incubated on ice for 1 h. Tissue homogenates were then centrifuged at 8,765g

Table 1 Theoretical and measured oxygen concentrations

Incubation chamber	Length of incubation (hours)	Theoretical pO ₂		Measured pO ₂ (n=3)		Oxygen concentration in culture medium (mg O ₂ /l) ^a
		mm Hg	% O ₂	mm Hg	% O ₂	
		Ratek shaking water bath	3	722.0	95.0	
	6	722.0	95.0	206.0±1.2	27.1±0.2	11.2
	24	722.0	95.0	561.0±13.5	73.8±1.8	30.5
Sanyo MCO-17AI	3	160.0	21.0	177.0±1.3	23.3±0.2	9.6
	6	160.0	21.0	167.0±1.7	22.0±0.2	9.1
	24	160.0	21.0	160.0±3.1	21.1±0.4	8.7
Sanyo MCO-18M	3	60.8	8.0	137.0±3.5	18.0±0.5	7.4
	6	60.8	8.0	119.0±2.3	15.7±0.3	6.5
	24	60.8	8.0	104.0±8.3	13.7±1.1	5.7

^a Calculated according to Henry's law: $e_p = e_{kc}$ where p is the partial pressure of the solute above the solution, c is the concentration of the solute in the solution and k is the Henry's Law constant (the value for k is 769.2 l atm/mol)

for 15 min. Supernatant was collected as the protein extract; the pellet was discarded. The protein extract (50 µl) was solubilised by 50 µl 2 M NaOH and 100 µl 1 M HCl with 10 min boiling in between. Total protein was measured by the bicinchoninic acid (BCA) procedure (BCA Pierce Protein Assay Kit, Rockford, Ill.) by using a 96-well plate (NUNC). A microplate reader (BioRad, Hercules, Calif., USA) read absorbance at 595 nm. Bovine serum albumin (BSA) was used as a reference standard. Total protein for placental explant tissues ranged between 2.8 and 6.2 mg/ml.

Determination of lactate dehydrogenase, glucose and lactate

To assess cell membrane permeability during culture, the release of lactate dehydrogenase (LDH) into the incubation medium and total tissue LDH activity were determined as described previously (Farrugia et al. 2000). The absorbance was quantified by using a 96-well microplate reader at a wavelength of 340 nm (BioRad). LDH release was calculated as LDH activity in the medium divided by total protein in the tissue. An EML 105 electrolyte and metabolite analyser determined the glucose and lactate concentration in the incubation medium of the placental explants. The rates of glucose consumption and lactate production were measured in units of micromoles per milligram per hour.

Determination of tumour necrosis factor-alpha, 8-isoprostane and parathyroid hormone-related protein

The release of tumour necrosis factor-alpha (TNF-α) into the explant incubation medium was quantified by sandwich enzyme-linked immunosorbent assay according to the manufacturer's instructions (BioSource International, Camarillo, Calif., USA). The limit of detection of the TNF-α assay was 7.2 pg/ml. The release of 8-isoprostane into the incubation medium was assayed by using a

commercially available competitive enzyme immunoassay kit according to the manufacturer's specifications (Cayman Chemical, Ann Arbor, Mich., USA). The limit of detection of the assay was 5 pg/ml. The release of parathyroid hormone-related protein (PTHrP) into the incubation medium was quantified by means of a sensitive and specific amino-terminal PTHrP radioimmunoassay as described previously (Grill et al. 1991). All data were corrected for total protein and expressed as picograms per milligram protein (TNF-α and 8-isoprostane) and millimoles per milligram protein (PTHrP).

Western blotting

The relative abundance of pro- and cleaved caspase-9 in protein extracts was analysed by Western blotting with a rabbit polyclonal anti-caspase-9 antibody (H-170, diluted 1:200; Santa Cruz, Calif., USA). Proteins (50 µg) were separated on an 18% polyacrylamide gel (BioRad) and transferred to a polyvinylidene fluoride membrane (BioRad) as previously described (Lappas et al. 2003). Protein expression was identified by co-migration with a positive control (Jurkat cell extracts: cytochrome-C-treated and control) and by comparison with the mobility of a protein standard. Goat anti-rabbit IgG horseradish peroxidase conjugate (Santa Cruz), diluted 1:10,000, was used as the secondary antibody. Cleaved-caspase-9 protein levels were normalised against its inactive form, pro-caspase-9. Protein bands were semi-quantified by densitometric analyses by Quantity One Quantitation Software Version 4.3.1 (BioRad).

Histology and immunohistochemistry

Placental explants were fixed in neutral phosphate-buffered 4% formaldehyde solution and then embedded in paraffin. Serial sections (5 µm) were cut, mounted on poly-L-lysine-coated glass slides, incubated overnight at 37°C and

subsequently deparaffinised by using xylene and a graded series of ethanol. Haematoxylin and eosin staining was performed on a series of slides assigned for histology to analyse qualitatively the morphological characteristics of placental explants. For immunohistochemical analysis, slides were immersed in citrate buffer (pH 6.0, 10 mM) and heated in a microwave oven for antigen retrieval. Endogenous peroxidases were inactivated by incubation in H_2O_2 . Non-specific binding sites were blocked (1% BSA in phosphate-buffered saline/Tween-20) for 10 min in a humidified chamber followed by incubation in primary antibody solution (1 h at room temperature) containing mouse monoclonal M30 CytoDEATH, (Roche Molecular Biochemicals, Mannheim, Germany), diluted 1:50. M30 antibody recognises a neo-epitope, which is exposed after cleavage of cytokeratin-18 by caspases (Kadyrov et al. 2001). Normal mouse control IgG (Santa Cruz; diluted 1:400) was used as a negative control; placental explants treated with cycloheximide (10 $\mu\text{g}/\text{ml}$) and 95% O_2 for 24 h were employed as positive controls. Slides were incubated in sheep anti-mouse IgG biotin-conjugated secondary antibody (Chemicon International, Temecula, Calif., USA) for 30 min. Reactions were performed by using a standardised sequence based on the streptavidin-biotin technique for the detection of a biotinylated link antibody. Detection of the primary antibody was achieved by addition of 3,3-diaminobenzidine substrate (Roche). Slides were counterstained with haematoxylin and mounted. Control reactions carried out with the substitution of mouse IgG for the primary antibody were always negative. Leica Qwin Version 3 Image Analysis Software (Leica Microsystems Imaging Solutions, Cambridge, UK) was used for the quantitative analysis of positive (M30) brown staining in the placenta sections. Five random fields of view (FOV) per section were selected for quantitative analysis. The ratio of M30 staining was expressed as a percentage ($\pm\text{SEM}$). Thus, percentages of staining per FOV per section per sample were collated and categorised according to oxygen concentration and length of incubation. Two slides were consecutively prepared for each sample, one as a positive and the other as a negative control slide. Each slide was analysed at $\times 100$ magnification.

Statistical analysis

Statistical analyses were performed by using a commercially available statistical software package (Statgraphic Plus version 3.1; statistical graphics, Rockville, Md., USA). The homogeneity of the data was assessed by Bartlett's test and, when significant, data were logarithmically transformed before further analysis. Sample comparisons were analysed by an analysis of variance (ANOVA) in which the oxygen concentration and length of incubation were factors.

The Tukey HSD (honestly significantly different) test was used for post hoc testing. Statistical differences were indicated by a *P*-value of less than 0.05. Data are expressed as mean ($\pm\text{SEM}$).

Results

Theoretical and measured oxygen concentrations

The concentration of dissolved oxygen in the culture medium was assessed following simultaneous 3-h, 6-h and 24-h cultures in each of the three incubation chambers. The oxygen concentration of the incubation medium from each incubator at each time point was measured in triplicate and the mean oxygen concentration ($\pm\text{SEM}$) was calculated (see Table 1).

Effect of oxygen concentration on cell membrane integrity and metabolite activity

The effect of oxygen on placental cell membrane integrity was assessed by the release of the intracellular enzyme LDH; the effect of oxygen on metabolite activity was measured by glucose consumption and lactate production. LDH release was significantly greater at 95% oxygen ($0.13\text{E}-2\pm 0.7\text{E}-4$) compared with 21% and 8% oxygen ($0.09\text{E}-2\pm 1.1\text{E}-4$ and $0.08\text{E}-2\pm 1.2\text{E}-4$, respectively) at 3 h incubation ($P<0.05$; Fig. 1a). There was a significant progressive decline in glucose consumption between the 3-h and 24-h time points in both the 21% and 8% groups ($P<0.05$; one-way ANOVA, Tukey HSD post-hoc test). In contrast, the rate of glucose consumption significantly increased at 6 h in 95% O_2 (0.77 ± 0.13) compared with 21% and 8% O_2 (0.37 ± 0.07 and 0.26 ± 0.07 , respectively; $P<0.05$). Subsequently, glucose consumption declined in the 95% O_2 group to reach levels comparable with those of the 21% and 8% O_2 groups (Fig. 1b). In conjunction, the rate of lactate production in placenta was significantly greater at 3 h in 95% O_2 (1.17 ± 0.12) compared with 21% and 8% O_2 (0.77 ± 0.07 and 0.74 ± 0.15 , respectively; $P<0.05$; one-way ANOVA, Tukey HSD post-hoc test; Fig. 1c). Of note, the pH values of the incubation medium remained unchanged (pH 7.4) throughout the incubation period.

Effect of oxygen concentration on morphological characteristics of placental explants

Qualitative analysis was performed to determine the effect of oxygen concentration on the general morphology of placental explants. With increasing oxygen, signs of detachment and thinning of the syncytiotrophoblast became

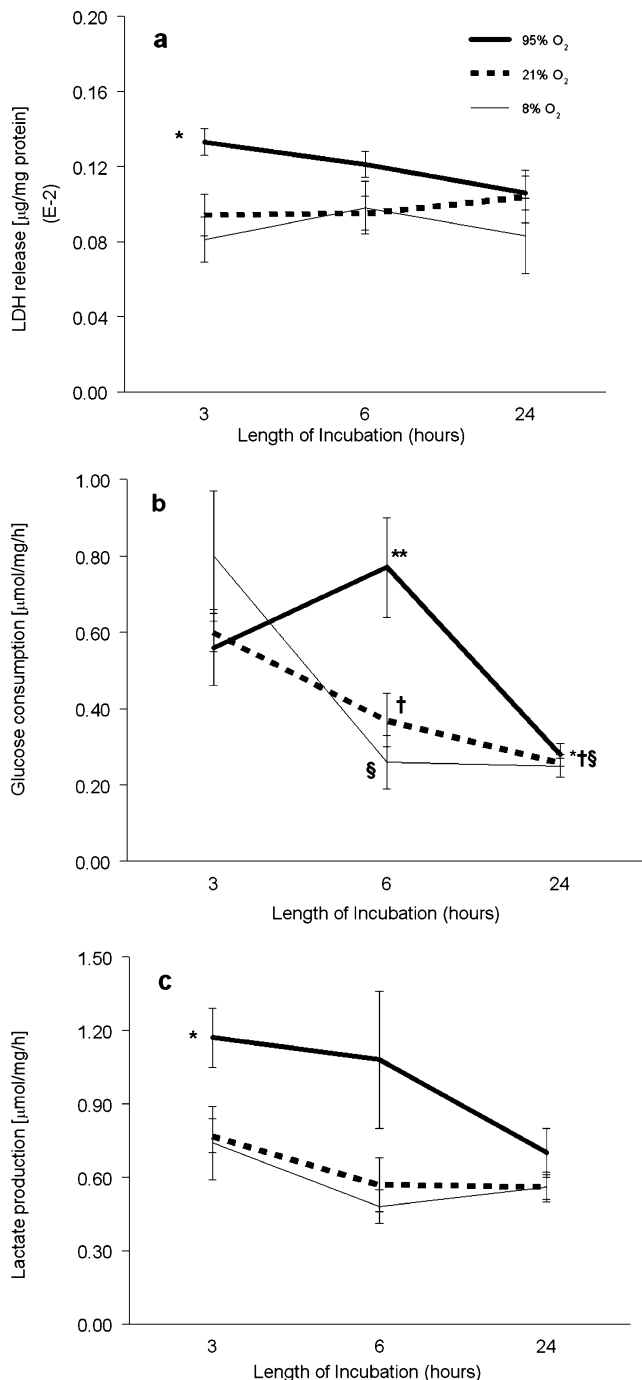


Fig. 1 **a** Release of LDH from placental explants incubated at 95%, 21% and 8% oxygen. * $P < 0.05$ vs. 21% and 8% O₂ at 3 h. **b** Effect of oxygen concentration on the rate of glucose consumption in placental explants. Note the significant effect of oxygen and length of incubation on the rate of glucose consumption. ** $P < 0.05$ vs. 21% and 8% O₂, * $P < 0.05$ vs. 6 h at 95% O₂, † $P < 0.05$ vs. 3 h at 21% O₂, § $P < 0.05$ vs. 3 h at 8% O₂. **c** Effect of oxygen concentration on the rate of lactate production in placental explants. Note the significant effect of oxygen concentration on lactate production. * $P < 0.05$ vs. 21% and 8% O₂

apparent. Detachment of the syncytiotrophoblast was more often observed at 95% compared with 21% oxygen. Necrotic sites within the syncytiotrophoblast also became visible as oxygen increased (Fig. 2).

Effect of oxygen concentration on apoptosis in placental explants

To determine whether oxygen had an effect on apoptosis, we examined the levels of staining of the caspase-3-cleaved cytokeratin 18 neo-epitope, M30 (Fig. 3). In general, M30 staining increased with increasing incubation time and oxygen concentration. Compared with time zero, M30 staining at 24 h was significantly increased in both 95% ($6.13\% \pm 1.15$ vs. $0.52\% \pm 0.19$) and 21% ($2.75\% \pm 0.37$ vs. $0.80\% \pm 0.36$) oxygen ($P < 0.05$). Similarly, M30 staining increased between 6 h and 24 h in both the 95% ($6.13\% \pm 1.15$ vs. $2.14\% \pm 0.77$) and 21% ($2.75\% \pm 0.37$ vs. $1.33\% \pm 0.32$) oxygen groups ($P < 0.05$). At 24 h, M30 staining was significantly stronger at 95% oxygen ($6.13\% \pm 1.15$) than at 21% ($2.75\% \pm 0.37$) and 8% ($2.85\% \pm 0.73$) oxygen ($P < 0.05$; Fig. 3a–g).

The relative abundance of pro- and cleaved caspase-9 was determined in all placental explant samples. No significant effect of oxygen concentration was seen on either pro- or cleaved-caspase-9 expression (Fig. 4).

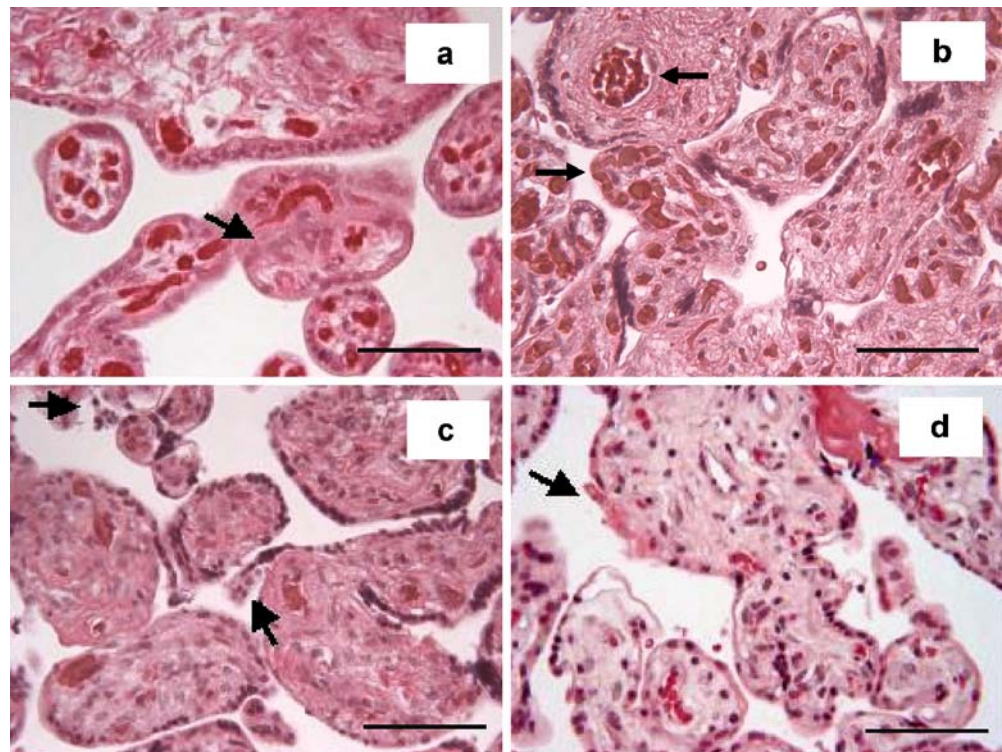
Effect of oxygen concentration on the release of TNF- α , PTHrP and 8-isoprostane

We and others have previously demonstrated that TNF- α and PTHrP are responsive to oxidant stress challenge (Coughlan et al. 2004; Hastings et al. 2002). In this study, we aimed to determine the effect of variable oxygen concentration on the release of TNF- α and PTHrP from placental tissue explants. TNF- α release was significantly increased as a result of exposure to 95% oxygen concentration at all time points as compared with 21% and 8% O₂ ($P < 0.05$) with the magnitude of the difference being greatest at 6 h (Fig. 5a). Additionally, TNF- α release in the 21% and 8% oxygen groups increased to a lesser extent but this was significant at 6 h compared with 3 h ($P < 0.05$; Fig. 5a). TNF- α release also significantly decreased at 24 h in 21% O₂ but not 8% O₂ (Fig. 5a).

Incubation of placental explants under conditions of high oxygen concentration caused a significant increase in the release of PTHrP. In 95% O₂, there was a dramatic increase in the release of PTHrP at 24 h (5.99 ± 0.858) compared with 21% and 8% O₂ (2.12 ± 0.209 and 2.03 ± 0.229 , respectively; $P < 0.05$). A similar but much diminished rise in PTHrP release was noted between 6 and 24 h in both the 21% and 8% O₂ groups at 24 h ($P < 0.05$; Fig. 5b).

At the 24-h time point, a significant increase was recorded in the release of 8-isoprostane at 95% oxygen (837.40 ± 263.69) compared with 21% and 8% oxygen (361.24 ± 70.34 and 136.71 ± 22.74 , respectively; $P < 0.05$; Fig. 5c). In addition, an overall increase occurred in the release of 8-isoprostane between 6 h and 24 h for all oxygen concentrations ($P < 0.05$; Fig. 5c).

Fig. 2 Effect of oxygen concentration on morphological characteristics of placental explants. Preparations stained with haematoxylin and eosin to show the general morphology and structural integrity of placental explants prior to incubation (**a**) and at 6 h at 21% O₂ (**b**), at 24 h at 21% O₂ (**c**) and at 24 h at 95% O₂ (**d**). Note the thick trophoblast present prior to incubation (*solid arrow* in **a**), pooling of blood vessels and evidence of necrotic cells (*solid arrows* in **b**), detachment of syncytiotrophoblast cells (*solid arrows* in **c**) and absence of trophoblast cover (*solid arrow* in **d**). Bars 55 μm



Discussion

Tissue explants derived from human placental tissue delivered at term provide information on the way that the placenta behaves late in gestation and suggest a mechanism for studying the impact of *in vitro* conditions on placental tissue survival and function. In this study, our data have demonstrated that increasing oxygen concentrations significantly affect the viability, morphology, metabolism and cell death of placental explant cultures. Collectively, the data indicate that oxygen concentrations of 95% and, to a lesser extent, 21% induce significant adverse changes in placental tissue explants. In particular, cell membrane integrity (as assessed by the release of LDH), metabolic functions (such as glucose consumption) and lactate production are all significantly affected by a 95% oxygen concentration. Further, exposure to this level of oxygen results in an increased release of autocoids (TNF- α and PTHrP) and of the oxidative stress marker, 8-isoprostane. Finally, placental explants demonstrate a higher incidence of apoptosis when exposed to both 95% and 21% oxygen over a 24-h period, compared to 8% oxygen, as evidenced by the increase in M30 staining. These data provide information important to the planning of future experiments, particularly those in which placental explants are to be incubated with supplemental oxygen.

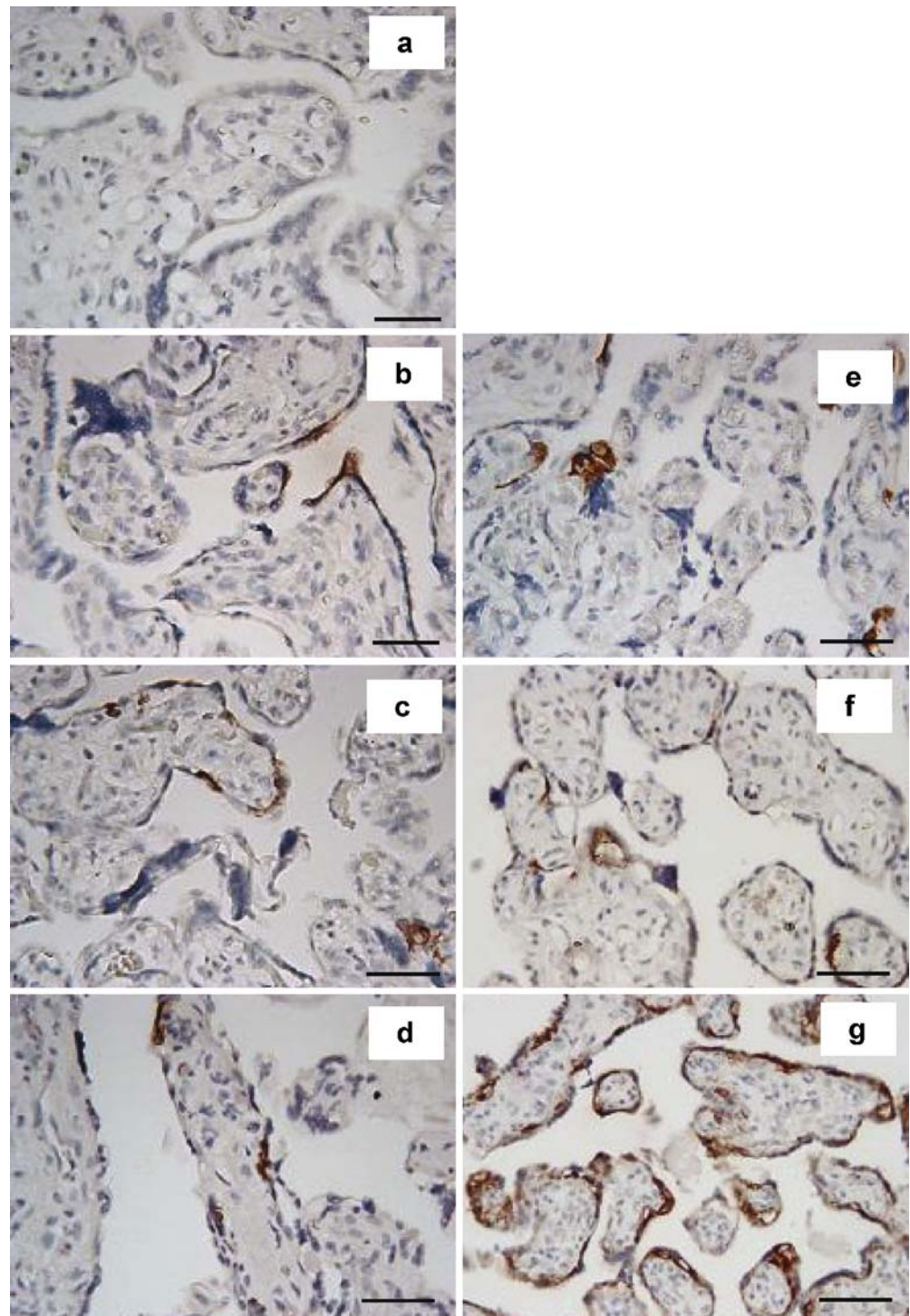
The release of LDH has been commonly used as a necrosis marker in explant cultures (Crocker et al. 2004; Long et al. 2004). In this study, we have used this assay as

a viability marker of cell membrane integrity. As demonstrated in studies previously performed in our laboratory, the length of incubation itself has no significant effect on the release of LDH over a 24-h period (Laham et al. 1997). In contrast, the release of LDH at 3 h in 95% oxygen was significantly greater than that in 21% and 8% oxygen, returning to baseline at 24 h. Although novel in placental tissue, this finding is consistent with the outcomes previously demonstrated in a murine lung model, whereby severe hyperoxia (>95% O₂) induces a significant increase in LDH activity compared with 21% O₂ (Hesse et al. 2004).

In this study, the rates of glucose consumption and lactate production significantly increase under high oxygen concentration. Similarly, hyperoxia has been demonstrated to induce a significant increase in cellular glucose consumption in human lung microvascular endothelial cells incubated in 95% oxygen (Ahmad et al. 2006). Elevation of glucose uptake and lactate production in response to hyperoxia, in conjunction with the activation of signalling pathways associated with tissue injury, may represent a compensatory mechanism to facilitate survival (Ahmad et al. 2006). These results also demonstrate the decline in glucose consumption from the 3-h to 24-h incubation time points. The reduced glucose consumption at 24 h may reflect reduced substrate availability at this time point.

Previous studies have indicated that exposure to elevated concentrations of oxygen (hyperoxia) can cause the activation of apoptotic pathways in some tissues. In the present study, we have demonstrated that following the incubation of placental

Fig. 3 M30 antibody expression in placental explants. **a** Prior to incubation. **b** At 6 h at 8% O₂. **c** At 24 h at 8% O₂. **d** At 6 h at 21% O₂. **e** At 24 h at 21% O₂. **f** At 6 h at 95% O₂. **g** At 24 h at 95% O₂. M30 antibody staining increased with O₂ concentration and time. At 24 h, 95%>21% O₂ ($P<0.05$). At 95%, 24 h>0 h, 3 h and 6 h ($P<0.05$). Bars 130 μm



explants in 95% oxygen for 24 h, the intensity and extent of staining for the caspase-3-cleaved cytokeratin 18 neo-epitope, M30, was significantly greater than at 21% and 8% oxygen. Similarly at both 95% and 21% oxygen, but not at 8% oxygen, M30 staining increased between the incubation time points of 6 h and 24 h. M30 antibody, as an apoptotic detection marker, has proven to be superior to the TUNEL method for the detection of apoptosis in villous and extravillous trophoblast

(Kadyrov et al. 2001). These findings are supported by those previously described in placentae recovered from pregnancies complicated by preeclampsia, which is associated with a significantly higher M30 index than that measured in control placentas (Aban et al. 2004; Lee et al. 2005). Thus, in summary, hyperoxic conditions have been shown significantly to increase apoptosis in human term placental explants in vitro.

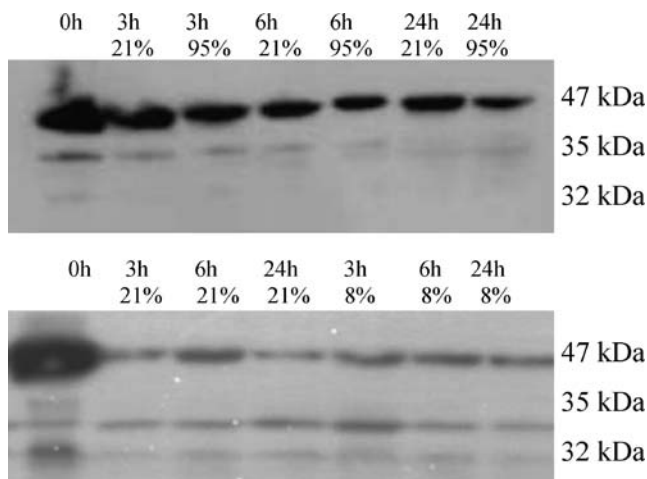


Fig. 4 Effect of oxygen concentration on pro- and cleaved-caspase-9 in placental explants. Results representative of Western blotting performed on placental explants to determine the relative abundance of pro- and cleaved-caspase-9 at 95%, 21% and 8% oxygen up to 24 h. In placental explants, both pro-caspase-9 (47 kDa) and cleaved-caspase-9 (32 and 35 kDa) were evident but there was no significant effect of oxygen or time

Oxidative stress has also been shown to induce apoptotic cell death by targeting mitochondria directly. Mitochondrial-dependent apoptosis has been shown to require the release of cytochrome c from mitochondria and the subsequent activation of a specific class of cytoplasmic proteases known as caspases (Takahashi et al. 2004). In the present study, cleaved-caspase-9 immunoreactivity is not significantly affected by oxygen concentration. In support of this finding, a previous study has demonstrated no significant change in caspase-9 activity in lung cells exposed to hyperoxia (analysed colorimetrically; Guthmann et al. 2005). Whether an increase in oxygen alters the activity of caspase-9, rather than its relative abundance, has yet to be determined. Further, whether an alternate apoptotic pathway, such as the death receptor pathway, is induced by hyperoxia in placental explants remains uncertain.

We have previously demonstrated that TNF- α release is increased by 20-fold when placental tissue is subjected to oxidative stress (Coughlan et al. 2004). In the current study, the release of TNF- α has been shown to be significantly greater at 95% O₂ compared with 21% and 8% oxygen. Evidence that hyperoxia represents an oxidative challenge is indicated by the response of the oxidative stress marker, 8-isoprostane, in placental tissue incubated under hyperoxic conditions. The release of 8-isoprostane at 95% oxygen is significantly greater than at 21% and 8% oxygen, thereby highlighting the oxidative challenge and limited capacity to induce tissue antioxidant defences. Furthermore, the ability of placental explants to synthesise 8-isoprostane under various oxygen conditions suggests that other family members of the F₂ isoprostanes might also be synthesised. The heightened response of TNF- α to hyperoxia and

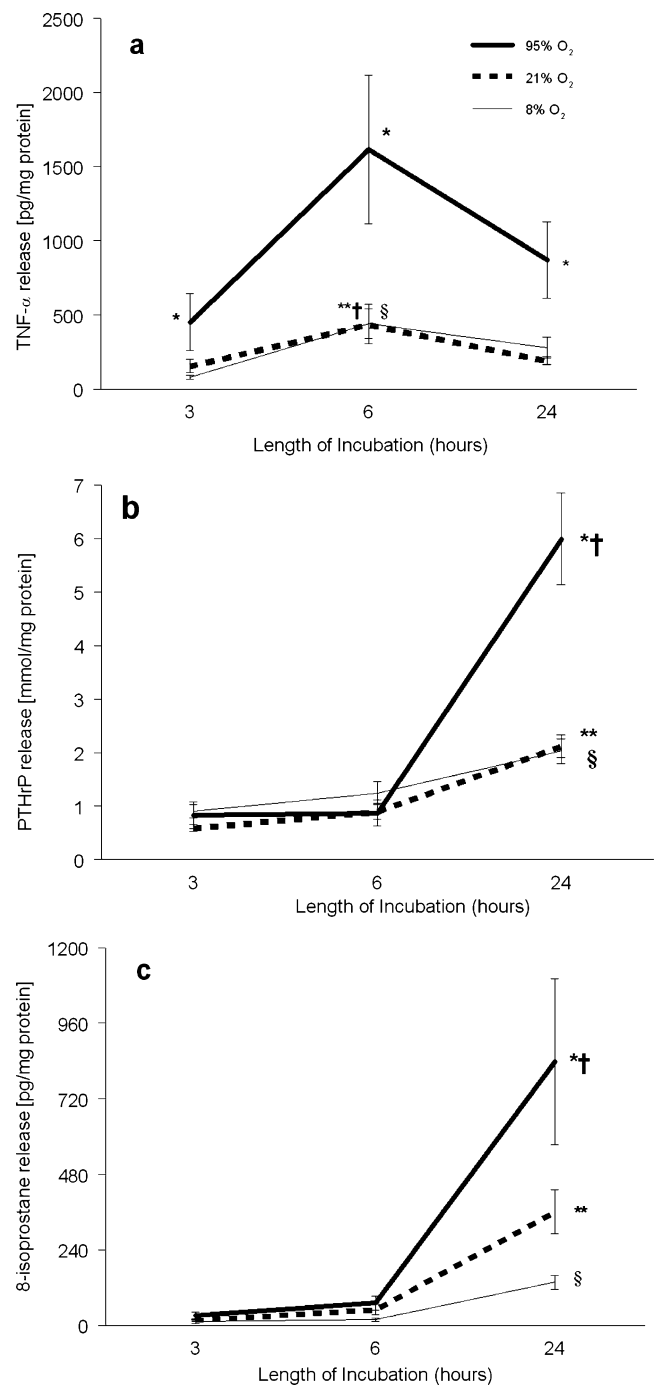


Fig. 5 **a** Effect of oxygen concentration on the release of TNF- α from placental explants. * P <0.05 vs. 21% and 8% O₂ at 3, 6 and 24 h, ** P <0.05 vs. 3 h at 21% O₂, § P <0.05 vs. 3 h at 8% O₂ and † P <0.05 vs. 24 h at 21% O₂. **b** Effect of oxygen concentration on the release of PTHrP from placental explants. * P <0.05 vs. 21% and 8% O₂ at 24 h, **†§ P <0.05 vs. 3 and 6 h at all oxygen concentrations. **c** Effect of oxygen concentration on the release of 8-isoprostane from placental explants. * P <0.05 vs. 21% and 8% O₂ at 24 h, **†§ P <0.05 vs. 3 and 6 h at all oxygen concentrations

oxidative stress in placental tissue may be a consequence of the inability of the cell to accommodate.

PTHrP is an autocrine/paracrine regulator of tissue development (Zerega et al. 1999), is involved in human

pregnancy (Farrugia et al. 2000; Wlodek et al. 1995) and has a role in mediating responses to extreme oxygen levels, such as those that occur in hyperoxic lung injury (Hastings et al. 2002). The data obtained in this study are consistent with increased PTHrP responses to extreme oxygen environments. In placental explants at 95% oxygen, PTHrP release is significantly increased. The response of PTHrP to oxidative stress imitates that of a pro-inflammatory cytokine and provides further evidence to highlight the vulnerability of placental villous tissue to oxidative challenges.

Although our oxygen measurements in culture medium differ somewhat from those previously measured in comparable experimental designs (Black et al. 2004), high, medium and low oxygen concentrations, resulting in significantly different experimental outcomes in placental explants, have been achieved. The discrepancy between the theoretical and actual values obtained may be, at least in part, attributable to the primary function of the blood gas analyser used to measure pO_2 in blood rather than in aqueous solution.

Concluding remarks

Newby et al. (2005) have recently proposed a set of guidelines for judging whether a culture model is suitable for its stated purpose; they state that different experimental conditions might provide the opportunity to examine reasons for investigators obtaining differing results in seemingly similar experiments. In the present study, we have demonstrated that, over a 24-h time period, incubation of placental tissue explants in 8% oxygen results in improved tissue viability when compared with explants incubated in 95% or even 21% oxygen. We suggest that investigation of additional incubation time points between 6 and 24 h may provide further insights into the impact of varying oxygen concentrations on other signalling pathways. This study demonstrates that increasing oxygen concentrations during placental villous explant cultures might impair, rather than improve, viability over extended incubation periods. Further, our data provide further evidence that oxidant stress, at least when induced by hyperoxia, plays a significant role in placental tissue dysfunction.

Acknowledgments The authors gratefully acknowledge the contribution made by the clinical research midwives (Val Bryant, Sarah Mitchell, Angie Denning, Melissa Ryan and Ellen Smith) at the Mercy Hospital for Women. They are also grateful to both Daphne Hards and Patricia Ho at the St Vincent's Institute for Medical Research for time spent on paraffin embedding and performing the PTHrP radioimmunoassay, respectively. Finally, they thank the Medical Research Foundation for Women and Babies for providing funding for the Leica Qwin Image Analysis System.

References

- Aban M, Cinel L, Arslan M, Dilek U, Kaplanoglu M, Arpacı R, Dilek S (2004) Expression of nuclear factor-kappa B and placental apoptosis in pregnancies complicated with intrauterine growth restriction and preeclampsia: an immunohistochemical study. *J Exp Med* 204:195–202
- Ahmad A, Ahmad S, Chang L, Schaack J, White CW (2006) Endothelial Akt activation by hyperoxia: role in cell survival. *Free Radic Biol Med* 40:1108–1118
- Aplin J (1991) Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro. *J Cell Sci* 99:681–692
- Black S, Kadyrov M, Kaufmann P, Ugele B, Emans N, Huppertz B (2004) Syncytial fusion of human trophoblast depends on caspase 8. *Cell Death Differ* 11:90–98
- Caniggia I, Winter J, Lye S, Post M (2000a) Oxygen and placental development during the first trimester: implications for the pathophysiology of pre-eclampsia. *Placenta* 14(Suppl A):S25–S30
- Caniggia I, Mostachfi H, Winter J, Gassmann M, Lye S, Kuliszewski M, Post M (2000b) Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGF β 3. *J Clin Invest* 105:577–587
- Coughlan MT, Permezal M, Georgiou H, Rice G (2004) Repression of oxidant-induced nuclear factor-kappa B activity mediates placental cytokine responses in gestational diabetes. *J Clin Endocrinol Metab* 89:3585–3594
- Crocker IP, Tansinda D, Jones C, Baker P (2004) The influence of oxygen and tumour necrosis factor- α on the cellular kinetics of term placental villous explants in culture. *J Histochem Cytochem* 52:749–757
- Farrugia W, Ho PWM, Rice GE, Moseley J, Permezal M, Wlodek ME (2000) Parathyroid hormone-related protein(1–34) in gestational fluids and release from human gestational tissues. *J Endocrinol* 165:657–662
- Grill V, Ho PWM, Body JJ, Johanson N, Lee SC, Kukreja SC, Moseley JM, Martin TJ (1991) Parathyroid related-hormone: elevated levels in both humoral hypercalcemia of malignancy and hypercalcemia complicating metastatic breast cancer. *J Clin Endocrinol Metab* 73:1309–1315
- Guthmann F, Wissel F, Schachtrup C, Tolle A, Rudiger M, Spener F, Rustow B (2005) Inhibition of TNF alpha in vivo presents hyperoxia-mediated activation of caspase-3 in type II cells. *Respir Res* 6:1465–1475
- Hastings R, Ryan R, D'Angio C, Holm B, Patel A, Quintana R, Biederman E, Burton D, Defetos L (2002) Parathyroid hormone-related protein response to hyperoxic lung injury. *Am J Physiol Lung Cell Mol Physiol* 282:L1198–L1208
- Hesse A, Dorger M, Kupatt C, Krombach F (2004) Proinflammatory role of inducible nitric oxide synthase in acute hyperoxic lung injury. *Respir Res* 5:1–9
- Huppertz B, Kingdom J, Caniggia I, Desoye G, Black S, Korr H, Kaufmann P (2003) Hypoxia favours necrotic versus apoptotic shedding of placental syncytiotrophoblast into the maternal circulation. *Placenta* 24:181–190
- Jauniaux E, Watson AL, Hempstock J, Ba YP, Skepper JN, Burton GJ (2000) Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. *Am J Pathol* 157:2111–2122
- Kadyrov M, Kaufmann P, Huppertz B (2001) Expression of a cytokeratin 18 neo-epitope is a specific marker for trophoblast apoptosis in human placenta. *Placenta* 22:44–48
- Laham N, Brennecke SP, Rice G (1997) Interleukin-8 release from human gestational tissue explants: the effects of lipopolysaccharide and cytokines. *Biol Reprod* 57:616–620

- Lappas M, Permezel M, Rice GE (2003) N-acetyl-cysteine inhibits phospholipid metabolism, pro-inflammatory cytokine release, protease activity and NF- κ B DNA binding activity in human fetal membranes in vitro. *J Clin Endocrinol Metab* 88:1723–1729
- Lappas M, Permezel M, Georgiou HM, Rice GE (2004) Regulation of phospholipase isozymes by nuclear factor- κ B in human gestational tissues in vitro. *J Clin Endocrinol Metab* 89:2365–2372
- Lee H, Park H, Kim Y, Kim H, Ahn Y, Park B, Park J, Lee B (2005) Expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in human preeclamptic placenta: possible implications in the process of trophoblast apoptosis. *Placenta* 26:226–233
- Long A, Colitz CMH, Bomser J (2004) Apoptotic and necrotic mechanisms of stress-induced human lens epithelial cell death. *Exp Biol Med* 229:1072–1080
- Miller RK, Genbacev O, Turner MA, Aplin JD, Caniggia I, Huppertz B (2005) Human placental explants in culture: approaches and assessments. *Placenta* 26:439–448
- Newby D, Marks L, Lyall F (2005) Dissolved oxygen concentration in culture medium: assumptions and pitfalls. *Placenta* 26:353–357
- Nguyen H, Rice GE, Farrugia W, Wong M, Brennecke S (1994) Bacterial endotoxin increases type II phospholipase A2 immunoreactive content and phospholipase A2 enzymatic activity in human choriondecidua. *Biol Reprod* 50:526–534
- Schneider H (2000) Placental oxygen consumption. II. In vitro studies: a review. *Placenta* 21(Suppl A):S38–S44
- Takahashi A, Masuda A, Sun M, Centonze VE, Herman B (2004) Oxidative stress-induced apoptosis in association with alterations in mitochondrial caspase activity and Bcl-2-dependent alterations in mitochondrial pH (pHm). *Brain Res Bull* 62:497–504
- Wlodek ME, Ho PW, Rice GE, Moseley JM, Martin TJ, Brennecke SP (1995) Parathyroid hormone-related protein (PTHrP) concentrations in human amniotic fluid during gestation and at the time of labour. *Reprod Fertil Dev* 7:1509–1513
- Zerega B, Cermelli S, Bianco P, Cancedda R, Cancedda FD (1999) Parathyroid hormone [PTH(1-34)] and parathyroid hormone-related protein [PTHrP(1-34)] promote reversion of hypertrophic chondrocytes to a pre-hypertrophic proliferating phenotype and prevent terminal differentiation of osteoblast-like cells. *J Bone Mineral Res* 14:1281–1289