Article

Does altered oxygenation or reactive oxygen species alter cell turnover of BeWo choriocarcinoma cells?

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Abstract

This study assessed the effect of 20 and 6% ambient oxygen (O_2) or 5–50 µmol/l hydrogen peroxide (H_2O_2) on apoptosis, necrosis, proliferation and fusion of BeWo cells. The expression of p53, Mdm2 and Bax was assessed by western blotting. Apoptosis was increased in cells cultured in 6% O_2 tension and 50 µmol/l H_2O_2 ($P < 0.05$, $P < 0.01$ by ADP:ATP ratio). In the same conditions, cell viability as estimated by the MTT assay was decreased (6% O_2 $P < 0.01$, 50 µmol/l H_2O_2 $P < 0.05$). Human chorionic gonadotrophin secretion was decreased by culture in 6%O_2 and 50 µmol/l H_2O_2 ($P < 0.05$). Cell fusion was also decreased by treatment with 50 µmol/l H_2O_2 ($P < 0.05$). Treatment with 50 µmol/l H_2O_2 was associated with increased expression of p53 and decreased expression of Mdm2 ($P < 0.05$). This study provides evidence that BeWo cell turnover is altered following exposure to hypoxia or ROS. It is concluded that BeWo cell culture is an appropriate model for investigating the regulation of trophoblast cell turnover. In addition, these data support a role for p53 in mediating altered trophoblast cell turnover in response to oxidative stress.

Keywords: *apoptosis, BeWo choriocarcinoma cell line, hypoxia, reactive oxygen species, trophoblast*

Introduction

Cell turnover is a tightly regulated event; in a tissue in a state of equilibrium a balance must be maintained between cell proliferation and cells lost by damage or death. In villous trophoblast, nuclei in the syncytiotrophoblast undergo morphological changes similar to apoptosis and some degenerate nuclei are gathered together in syncytial knots, which are then lost into the maternal circulation (Huppertz and Kingdom, 2004). The syncytiotrophoblast is maintained by proliferation and fusion of underlying cytotrophoblast cells (Huppertz and Kingdom, 2004). The equilibrium between maintenance and loss of syncytiotrophoblast is disturbed in severe early-onset pre-eclampsia and intrauterine growth restriction (IUGR), both of which are associated with increased apoptosis (Smith *et al.*, 1997; Leung *et al.*, 2001), increased formation of syncytial knots (Heazell *et al.*, 2006) and disordered proliferation (Macara *et al.*, 1996). Similar disruption in cell turnover has also been observed in cases of missed miscarriage (Hempstock *et al.*, 2003). Investigation of the regulation of apoptosis and proliferation in trophoblast may provide insights into the pathophysiology of these conditions.

The aberrant turnover of villous trophoblast in pre-eclampsia and IUGR is thought to result from exposure to hypoxia and/or oxidative stress, as severe pre-eclampsia and IUGR are associated with a failure of conversion of spiral uterine arteries from convoluted vessels to wide flaccid conduits required for the delivery of maternal blood to the placenta (Meekins *et al.*, 1994; Naicker *et al.*, 2003). It is postulated that this reduction in blood flow results in placental hypoxia or hypoxia–reperfusion injury. This is supported by evidence that placentas in pre-eclampsia show similar gene expression to placental explants cultured in hypoxic conditions (Soleymanlou *et al.*, 2005).

Cell turnover is a tightly regulated process controlled by many proteins. In many cell types, apoptosis is induced by cell damage such as insults from hypoxia or oxidative stress. p53 is increased in response to different noxious stimuli including these conditions and has been termed the 'guardian of the genome', as damaged cells are destroyed (Prives and Hall, 1999). p53 is negatively regulated by Mdm2, which targets the p53 protein for destruction via the proteosome (Iwakuma and Lozano, 2003). p53 promotes the transcription of Mdm2 providing a negative feedback loop preventing unwanted apoptosis in healthy cells. The balance of p53 and Mdm2 is essential in determining cell survival (de Rozieres *et al*., 2000). p53 promotes transcription of downstream pro-apoptotic factors such as Bax (Miyashita and Reed, 1995). These factors are present in villous trophoblast at the mRNA and protein level, indicating that they may have a role in regulating cell turnover (Fulop *et al*., 1998; Qiao *et al*., 1998; Allaire *et al*., 2000). Preliminary data suggest that expression of p53 is increased in villous trophoblast of pregnancies complicated by IUGR (Levy *et al*., 2002) and following exposure to hypoxia (Levy *et al*., 2000; Heazell *et al*., 2008).

Various in-vitro models have been used to investigate the effects of altered oxygenation and ROS on trophoblast cell turnover including: culture of whole placental tissue, isolated primary trophoblast and choriocarcinoma cell lines. Apoptosis may be induced *in vitro* in placental explants following exposure to reactive oxygen species (ROS) (Moll *et al*., 2007), hypoxia–reoxygenation injury (Hung *et al*., 2002) or hypoxia (Heazell *et al*., 2008). Hypoxia also induces apoptosis in isolated cytotrophoblasts (Levy *et al*., 2000). In accordance with other in-vitro models, BeWo choriocarcinoma cells undergo proliferation, followed by fusion to form multinucleate syncytia, thereby mirroring the formation of syncytiotrophoblast *in vivo*. As a result, BeWo cells have been used to investigate some aspects of trophoblast cell turnover, including fusion and formation of syncytia, apoptosis and proliferation (Kudo *et al*., 2003b; Al-Nasiry *et al*., 2006; Bae *et al*., 2007; Hu *et al*., 2007). Despite several investigations of the effects of oxygen (O_2) on fusion and nutrient transport by BeWo cells, there has been no investigation of the effects of O_2 or ROS on apoptosis, which may add to understanding of common pregnancy complications. It was postulated here that exposing BeWo cells to reduced O_2 tension or ROS in the form of hydrogen peroxide (H_2O_2) would alter cell turnover including: apoptosis, proliferation and cell fusion. In addition, it was postulated that changes in cell turnover would be associated with altered expression of p53, Mdm2 and Bax.

Materials and methods

Unless otherwise stated, reagents were obtained from Sigma-Aldrich Chemical Company (Poole, Dorset, UK).

Cell culture

BeWo cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK) and had been stored in liquid nitrogen. For experimentation, cells were rapidly thawed using Dulbecco's modified Eagle's medium-F12 supplemented with 10% fetal bovine serum, 30 mg/l penicillin, 50 mg/l streptomycin and 146 µg/l glutamine warmed to 37°C and transferred to a 75 cm^3 flask and grown to confluence. When confluent, cells were washed with warm phosphate-buffered saline (PBS), and treated with 5 ml 0.05% trypsin–EDTA solution for 2 min. Cells were collected in 10 ml of warmed culture medium and counted using a haemocytometer (Marienfeld, Germany). Cells were then seeded into either 6-well plates (1.5×10^6/well) or 96-well plates ($10–50 \times 10^5$/well) for subsequent culture (Corning Inc., NY, USA). Cells were cultured for 48 h in 20% ambient O_2 (control). To assess the effects of reduced oxygenation cells were cultured for 48 h in 6% atmospheric O_2 for 48 h or in 20% O_2 for 24 h followed by 6% O_2 for 24 h. The effect of ROS was investigated by culture in 20% O_2 for 24 h, then 5, 10 or 50 µmol/l H_2O_2 was added and cells were cultured for a further 24 h (**Figure 1**).

Assessment of apoptosis

Apoptosis was assessed using two methods. Firstly, by the Apopercentage kit according to the manufacturers' instructions (Biocolor, Northern Ireland, UK). The details of reagents used for this method are under patent and cannot be disclosed. Briefly, cells were cultured on 96-well plates at a density of 5×10^5/well. Eight wells were cultured per experimental condition for five separate passages ($n = 5$). After the treatment period, the culture medium was replaced with medium containing dye, which is taken up by apoptotic cells. The cells were returned to their culture environment for a further 30 min. Cells were then gently washed with warmed PBS and photographs taken using an inverted microscope (Leica, Germany). Cells were then treated with dye release reagent for 10 min at room temperature and absorbance read at 550 nm using a spectrophotometer (Molecular Devices, Wokingham, UK). Apoptosis was also assessed using a commercially available ADP:ATP ratio kit (Apoglow kit; Cambrex, Verviers, Belgium) with modifications to the manufacturers' instructions. This method has been previously used on primary trophoblast culture (Crocker *et al*. 2003). Briefly, BeWo cells were cultured in a white walled 96-well plate at a density of 2×10^5/well; eight wells were cultured per experimental condition for five separate passages ($n = 5$). Following culture, cells were treated to release ADP and ATP, then combined with a luciferin reagent, which releases light in the presence of ATP. A reading was taken immediately using a luminometer (Molecular Devices) (reading A) and the light emission was allowed to decay for 20 min. A further reading was then taken (reading B) and ADP-converting reagent added, and a final reading taken after 2 min (reading C). The ADP:ATP ratio was calculated by (reading C–reading B)/reading A.

Assessment of necrosis

Necrotic cell death was quantified using a commercially available lactate dehydrogenase (LDH) kit, which measures the reduction of a tetrazolium to formazan salt in the presence of NADH, which is generated by the conversion of lactate to pyruvate by LDH (Roche Applied Science, Lewes, UK). Conditioned culture media was defrosted on ice and combined with the reagents as per manufacturer's instructions. Unconditioned culture medium was used as a negative control. Absorbance was measured at 490 nm (Molecular Devices). The coefficient of variation for this assay was 5.97%.

Assessment of mitochondrial viability

The reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a formazan salt was used as a measure of cell viability as previously described (Al-Nasiry

et al., 2006). Cells were seeded onto 96-well plates at densities of 1×10^5/well, eight wells were cultured per experimental condition for between six to eight separate passages (n = 6–8). Following culture, the culture medium was replaced with that containing 1 mmol/l MTT. The cells were returned to their original culture environment for 3 h; cells were then washed gently in warmed PBS and lysed using dimethyl sulphoxide. The absorbance of the resulting solution at 550 nm was measured using a spectrophotometer (Molecular Devices).

Release of human chorionic gonadotrophin (HCG)

The release of HCG by BeWo cells was measured in conditioned culture medium using a commercial quantitative radio-immunoassay in accordance with the manufacturer's instructions (ICN Pharmaceuticals; Basingstoke, UK). HCG expression was normalized to cell protein measured using the Lowry method (Biorad, Hercules, CA, USA). The coefficient of variation for this assay was 10.48%.

Assessment of cell fusion

BeWo cells were cultured on sterile glass cover slips (Fisher Scientific, Loughborough, UK) placed in 6-well plates seeded at a density of 1.5×10^6 cells/well. Following culture, cells were washed twice in warmed PBS and fixed in methanol for 30 min at –20°C. The cells were washed twice in PBS and non-specific antibody binding blocked by exposing cells to 1% bovine serum albumin (BSA) with 0.05% Tween for 20 min at room temperature. The coverslips were then incubated with primary antibody solution of mouse monoclonal anti-desmoplakin (Sigma-Aldrich, Clone ZK31, 19 µg/ml) in 1% BSA with 0.05% Tween for 1 h at room temperature. The coverslips were washed three times in PBS. A negative control was achieved by omission of primary antibody. The coverslips were then exposed to fluorescein-conjugated anti-mouse antibody (Dako-Cytomation, Ely, UK; 1:200) for 1 h at room temperature. The coverslips were washed three times in PBS then mounted in medium containing propidium iodide (Vector, Burlingame, CA, USA). The resulting slides were visualized using confocal laser scanning microscopy (Biorad) and 10 images per field were taken. BeWo cell fusion was quantified by two independent observers blinded to experimental conditions. Cell fusion was defined as the presence of three or more nuclei contained within a single cell membrane. To ensure that these represented fused cells, z-sections were taken of multinucleate cells (see **Figure 2**). A short time-lapse video recording, demonstrating a Z-section of BeWo cells cultured in 20% O_2 stained with anti-desmoplakin antibody (green) with nuclei stained with propidium iodide (red), is available online [http://www.rbmonline.com/Article/3394]. **Figure 2** shows a still image from the movie clip. Cells containing three or more nuclei can be seen in the bottom left and centre right of the image.

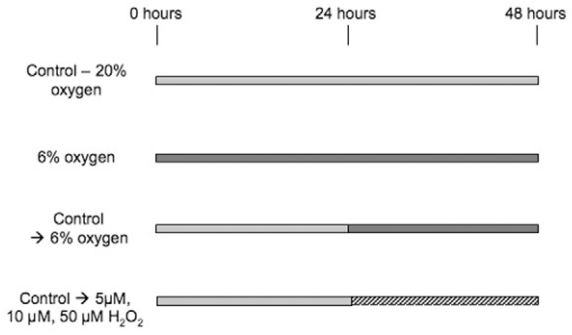

Figure 1. Schematic diagram to illustrate the experimental protocol, indicating duration of exposure of BeWo cells to either 20 (light grey) or 6% (dark grey) ambient O_2 and treatment with hydrogen peroxide (hatched). Culture continued for 48 h in total.

Western blotting

Cell lysate 20 µg was subjected to 10% discontinuous sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Separate blots were used for each protein of interest. Membranes were blocked for 1 h with 3% (w/v) non-fat dried milk in Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST) and then probed with mouse monoclonal antibodies to p53 (Clone D01, Merck Biosciences, Nottingham, UK, 1 µg/ml), Mdm2 (Clone 2A10, Merck Biosciences, 2 µg/ml), β-actin, (Clone AC15, Sigma, 1:10,000) or rabbit polyclonal antibodies to Bax (Abcam, Cambridge, UK; 1 µg/ml) overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) or goat anti-rabbit IgG (Dako-Cytomation, 1:1000) for 1 h at room temperature. Resulting bands were visualized on photo-sensitive film (Amersham Biosciences Ltd, Chalfont St Giles, UK) using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA). Densitometry was performed on a Bio-Rad 700 (BioRad) and bands of interest standardized against β-actin, which has been used previously to standardize protein expression in similar experiments (Bae *et al.* 2007).

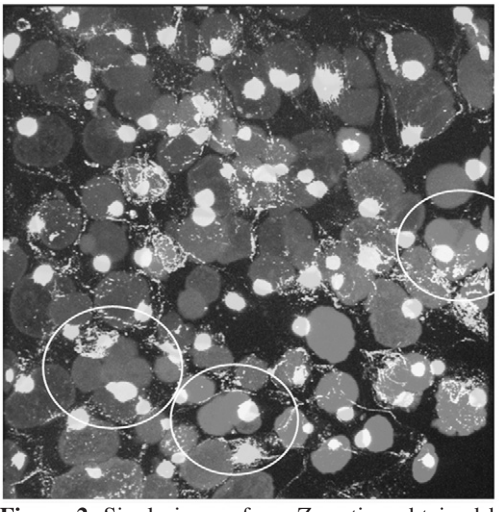

Figure 2. Single image from Z-section obtained by confocal laser microscopy demonstrating fusion of BeWo cells cultured at 20% O_2 by staining with anti-desmoplakin antibody with nuclei stained with propidium iodide. The anti-desmoplakin staining defines cell boundaries. Cells containing three or more nuclei are highlighted in white circles.

Statistical analysis

As the number of experiments undertaken numbered between five and nine passages, statistical significance was tested using either the Kruskal–Wallis test for unmatched non-parametric data or Friedman test for matched non-parametric data as normal distribution cannot be assumed. Friedman test was used for data obtained from the experiments using identical passages. Dunn's post-hoc test was performed as appropriate. Results are presented as median and interquartile range, and plotted as box and whisker plots (box = interquartile range, whiskers = total range).

Results

Effects of culture in 6% O_2 and H_2O_2 on apoptosis

BeWo cells cultured in 6% O_2 and in the presence of 50 µmol/l H_2O_2 demonstrated increased apoptosis, as measured by both the Apopercentage kit as evident by the increased uptake of pink dye solution (**Figure 3A**). In addition, cells staining with pink dye appeared rounded and condensed, which is suggestive of apoptosis. Furthermore, immunostaining with desmoplakin revealed some cells in 6% O_2 and 50 μmol/l H_2O_2 with increased nuclear:cytoplasmic ratio, a feature of apoptotic cells (**Figure 3B**).

The increase in apoptotic cells in 6% O_2 and 50 μmol/l H_2O_2 was evident by measurement of absorbance following lysis of BeWo cells (**Figure 4A**) and the ADP:ATP ratio measurement (**Figure 4B**). Exposure to 6% O_2 for 24 h or smaller doses of H_2O_2 did not significantly increase the rate of apoptosis. There was a wide range of LDH activity in conditioned culture medium from cells cultured in control conditions (**Figure 4C**). There was no significant increase in LDH activity following culture in 6% O_2 or in cells exposed to 5–50 µmol/l H_2O_2.

Effect of culture in 6% O_2 and H_2O_2 on cell viability, differentiation and fusion

The conversion of MTT was reduced significantly in cells exposed to 6% O_2 and 50 µmol/l H_2O_2 (**Figure 4D**). A similar pattern was noted in the amount of ATP extracted from BeWo cells (**Figure 4E**). The level of MTT or ATP was not affected by culture in 5 or 10 µmol/l H_2O_2. Transferring cells from 20 to 6% O_2 led to a value for MTT and ATP assays midway between those obtained for culture in 20 and 6% O_2.

Culture in 6% O_2 and 50 µmol/l H_2O_2 reduced the secretion of HCG into conditioned culture media (**Figure 5A**). However, only culture in 5 or 50 µmol/l H_2O_2 resulted in a significant decrease in formation of multinucleate BeWo cells ($P < 0.05$; **Figure 5B**). When all culture conditions were analysed, there was a weak correlation between HCG secretion and the percentage of nuclei contained within multinucleate BeWo cells, defined as more than three nuclei contained within a single cell membrane ($r^2 = 0.33$, $P < 0.0041$) (**Figure 5C**).

Effect of culture in 6% O_2 and H_2O_2 on expression of p53, Mdm2 and Bax

Western blotting for p53 demonstrated a single band at approximately 53 kD, treatment with 50 µmol/l H_2O_2 was associated with increased expression of p53 (**Figure 6A,B**). Mdm2 appeared as a doublet band at approximately 95 kD (**Figure 6A**). Densitometry revealed that treatment with 50 μmol/l H_2O_2 was associated with a reduction in Mdm2 expression (**Figure 6C**). The expression of p53 and Mdm2 was not altered by culture in 6% O_2. Bax appeared as a single band at approximately 24 kD. Densitometry showed no alteration in protein expression of Bax in any experimental condition (**Figure 6D**).

Discussion

These data demonstrate that culture in 6% O_2 and 50 µmol/l H_2O_2 alters BeWo cell turnover. The assessment of apoptosis here is strengthened by two different assessments of apoptotic cell death, the inclusion of a specific dye during apoptosis and the ADP:ATP ratio, which assesses the metabolic consequences of cell death. The findings of these assays were also supported by observations of cell morphology from phase contrast and confocal microscopy. Therefore, in common with native trophoblast, BeWo choriocarcinoma cells undergo apoptosis in response to decreased O_2 tension and ROS (Levy *et al.*, 2000; Moll *et al.*, 2007; Heazell *et al.*, 2008). Importantly for their use as a model of abnormal cell turnover, BeWo cells do not appear to be resistant to apoptosis. This finding is confirmed by studies demonstrating apoptosis of BeWo cells in response to stimuli capable of inducing apoptosis in other cell-types such as: zinc citrate compound (Bae *et al.*, 2007), tumour-necrosis factor-α (TNF-α) (Al-Nasiry *et al.*, 2006) and galectin-1 (Wiest *et al.*, 2005).

The increase in apoptosis following culture in 6% O_2 and in the presence of 50 µmol/l H_2O_2 was associated with a decrease in the conversion of MTT. The MTT assay was developed as a measure of proliferation in homogenous cell populations, as the reduction of MTT by the mitochondria is thought to reflect cell number. In the present experiments, there were similar patterns in MTT assay and ATP production linking mitochondrial viability with MTT measurement. This finding questions whether MTT reduction may be directly related to mitochondrial activity, which may be reduced during periods of oxidative stress irrespective of cell number. In addition, the effect of syncytialization on the MTT assay is unclear. These concerns were addressed by Al-Nasiry *et al.*, who investigated proliferation and fusion of BeWo cells in the presence of forskolin, epidermal growth factor and tumour necrosis factor (Al-Nasiry *et al.*, 2006). BeWo cell fusion was associated with a fall in MTT conversion; at the same time a similar decrease in the Mib-1 marker of proliferation was noted (Al-Nasiry *et al.*, 2006), indicating that MTT was proportional to Mib-1 expression and therefore proliferation. In the light of these data, the fall in MTT conversion observed in these experiments may provide a reflection of cell number and indirectly proliferation. Alternatively, the decrease in MTT conversion may reflect a loss of mitochondrial viability, which might be expected in cells undergoing apoptosis. Further studies are required to determine the relationship between apoptosis, proliferation and

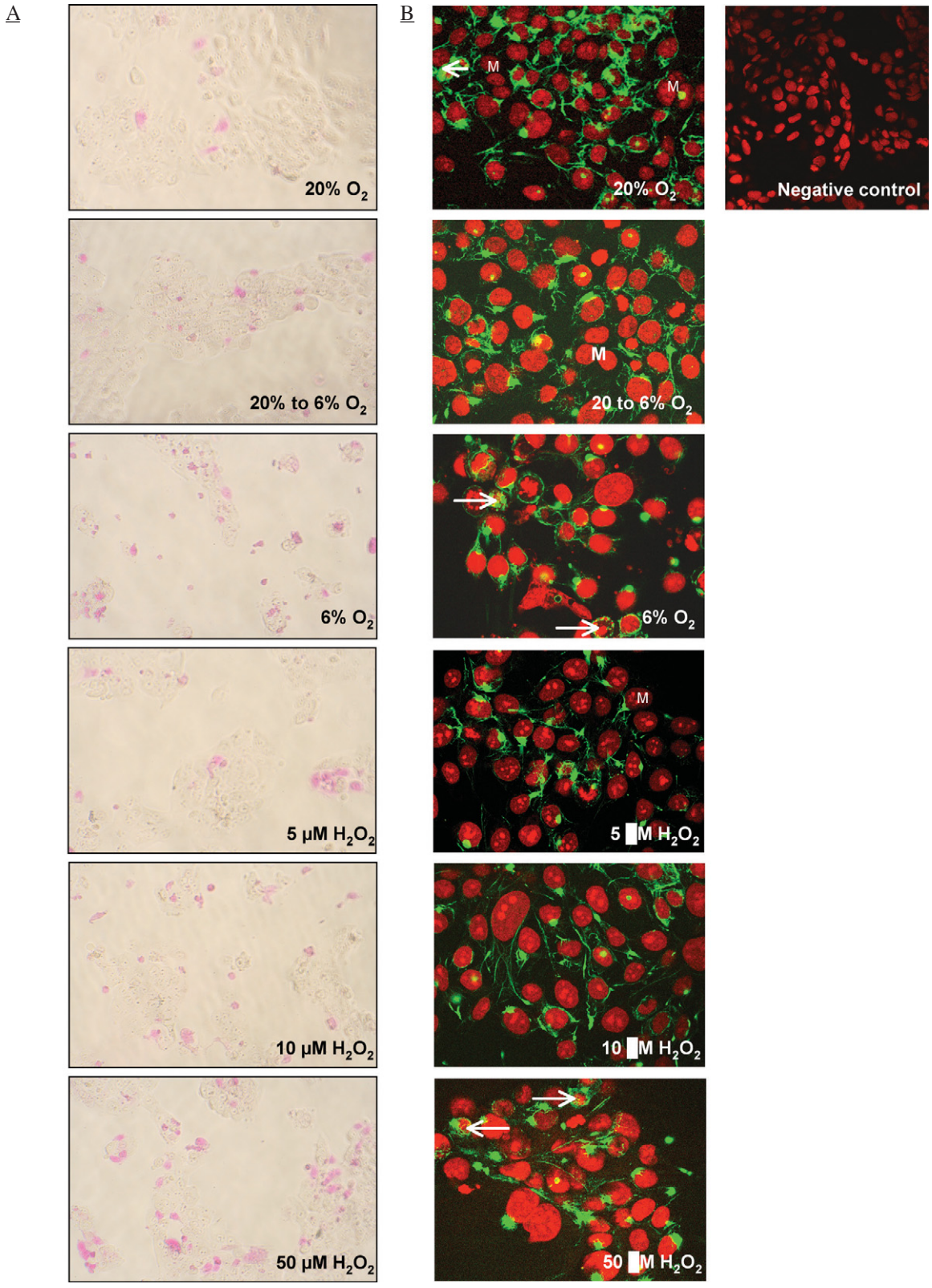

Figure 3. (**A**) Phase contrast micrographs demonstrating increased staining with pink dye following culture in 6% O_2 and 50 µmol/l H_2O_2 compared with control. Cells stained with dye in all conditions are dense and rounded, a morphological feature of apoptosis. (**B**) Cell fusion assessed by immunocytochemistry using anti-desmoplakin antibody (green) counterstained with propidium iodide (red). The anti-desmoplakin staining defines cell boundaries. BeWo cells cultured in control conditions show evidence of multinucleation (marked M). A small proportion of cells cultured in control conditions (20% O_2), and a greater number of cells cultured in 6% O_2 and 50 µmol/l H_2O_2 demonstrate some morphological features of apoptosis such as increased nuclear: cytoplasmic ratio (marked with open arrows). A negative control section shows no immunofluorescence.

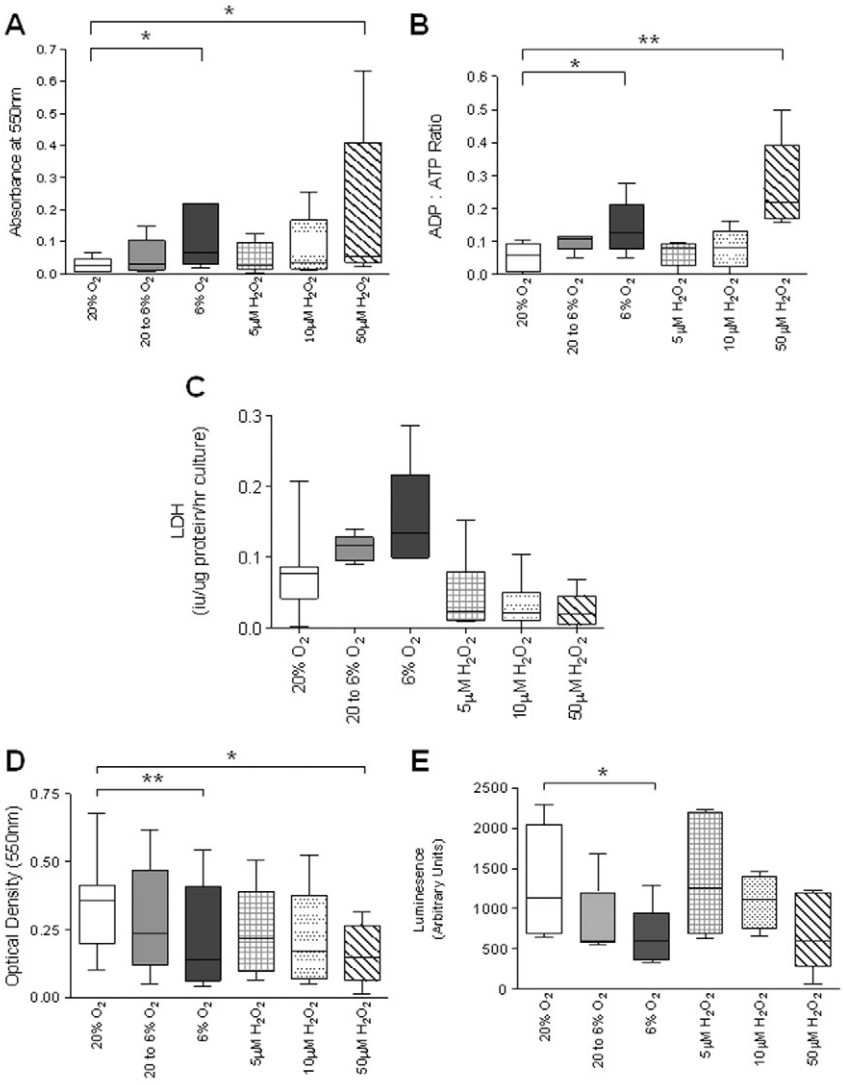

Figure 4. (**A**) Quantification of Apopercentage dye absorbed following lysis of cells demonstrating increased apoptosis in BeWo cells cultured in 6% O_2 and 50 μmol/l H_2O_2 (both *$P < 0.05$) ($n = 5$). (**B**) Apoptotic cell death as assessed by ADP:ATP ratio, demonstrating increased ADP:ATP ratio in BeWo cells cultured in 6% O_2 and 50 μmol/l H_2O_2 (*$P < 0.05$, **$P < 0.01$) ($n = 5$). (**C**) There was no significant difference in lactate dehydrogenase (LDH) secretion into culture media following culture in 6% O_2 or treatment with H_2O_2 ($n = 5$–9). (**D**) Absorbance at 550 nm indicating reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), demonstrating decreased viability of cells cultured in 6% O_2 and 50 μmol/l H_2O_2 compared with control (*$P < 0.05$, **$P < 0.01$) ($n = 6$). (**E**) ATP measured by luminescence is significantly reduced in cells cultured in 6% O_2 (*$P < 0.05$) ($n = 5$).

measurement of BeWo cell viability. However, it is notable that a decrease in cell number/proliferation is in keeping with an increase in apoptosis, as these events are inversely related in first and third trimester placental villous trophoblast *in vivo* (Chan *et al.*, 1999; Yamada *et al.*, 2001).

The altered cell turnover was reflected in the fusion and differentiation of BeWo cells. Previous studies have utilized BeWo cells as a model of syncytium formation, which is markedly increased by the addition of forskolin (Kudo *et al.*, 2003b; Hu *et al.*, 2007; Nampoothiri *et al.*, 2007). In agreement with previous data, an association was observed between HCG secretion and the percentage of nuclei contained within multinucleate cells (Kudo *et al.*, 2003a). In common with other studies of BeWo cells, HCG secretion was diminished by hypoxia (Kudo *et al.*, 2003b; Hu *et al.*, 2007). However, no concurrent fall in multinucleation was observed, despite the relationship between the two observations. This suggests that morphological and biochemical differentiation may be separated by culture in 6% O_2. Alternatively, the data may reflect the low percentage of multinucleate cells, approximately 10%, compared with studies using forskolin that report up to 80% of nuclei within multinucleate syncytia (Nampoothiri *et al.*, 2007). The fall in HCG secretion following treatment with H_2O_2 concurs with data from isolated primary cytotrophoblasts (Kharfi Aris *et al.*, 2007), and the reduction in HCG secretion in response to lowered O_2 here was similar to that reported in normal term placental villous explants (Crocker *et al.*, 2004). Therefore, alterations in HCG release from BeWo cells exposed to oxidative stress are similar to those seen in native trophoblast.

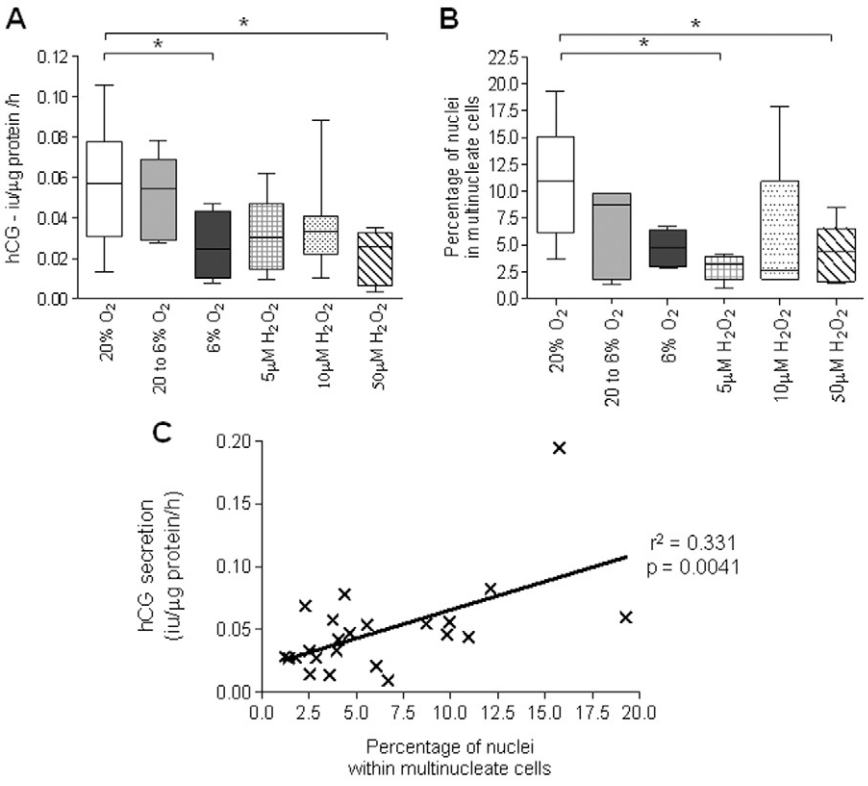

Figure 5. (**A**) Human chorionic gonadotrophin (HCG) secretion was reduced in BeWo cells cultured in 6% O_2 and exposed to 50 µmol/l H_2O_2 (*$P < 0.05$) ($n = 5–9$). (**B**) The percentage of nuclei contained in multinucleate cells, defined as three or more nuclei enveloped by a single membrane, was significantly reduced by culture in 5 and 50 µmol/l H_2O_2 (*$P < 0.05$) ($n = 5–9$). (**C**) There was a weak correlation between HCG secretion and the proportion of nuclei in multinucleate cells.

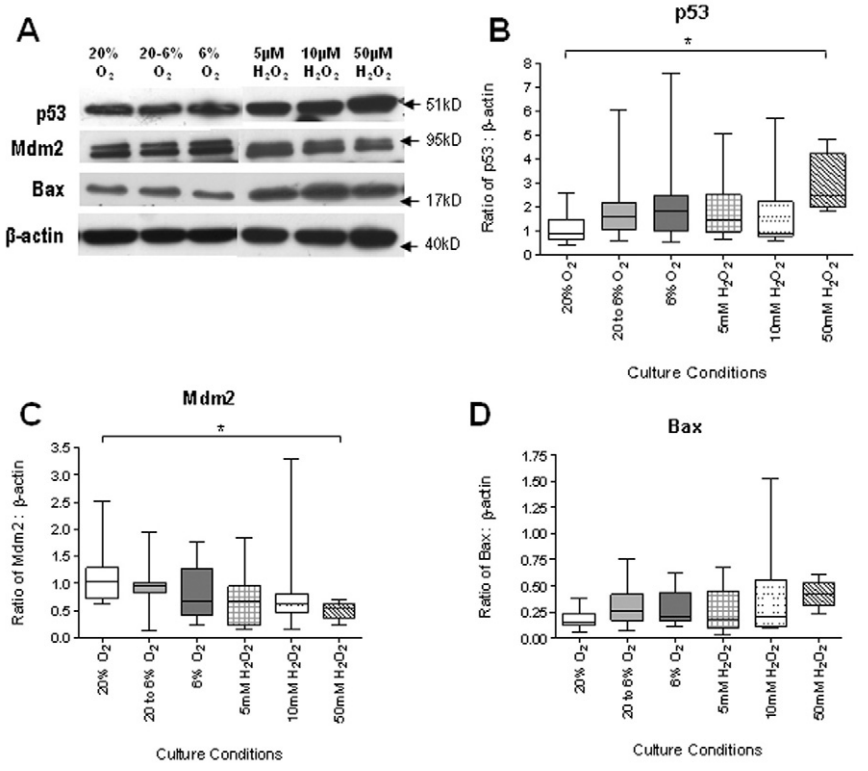

Figure 6. (**A**) Representative western blots for p53, Mdm2, Bax and β-actin. (**B**) Graph of densitometry of western blotting for p53 standardized to β-actin expression demonstrating an increase in p53 following exposure to 50 µmol/l H_2O_2 (*$P < 0.05$) ($n = 5–9$). (**C**) Densitometry of western blotting for Mdm2 standardized to β-actin, demonstrating a decrease in Mdm2 expression following exposure to 50 µmol/l H_2O_2 (*$P < 0.05$) ($n = 5–9$). (**D**) Densitometry of Bax standardized to β-actin demonstrating no change in response to altered oxygenation and reactive oxygen species ($n = 5–9$).

Apoptosis induced by oxidative stress in other methods of trophoblast culture is associated with altered p53 signalling (Levy *et al.*, 2000; Heazell *et al.*, 2008). In other cell types, the balance between p53 and Mdm2 is essential in determining cell survival (de Rozieres *et al.*, 2000), as Mdm2 is responsible for nuclear export and ubiquitination of p53, removing it from its site of action and breakdown by the proteosome (Haupt *et al.*, 1997). In agreement with previous data, it was found that p53 was expressed in BeWo cells (Haidacher *et al.*, 1995) and the presence of Mdm2 has been described in this cell type. In addition, p53 expression increased following exposure to 50 µmol/l H_2O_2 and Mdm2 expression decreased. Therefore, the findings of altered cell turnover in response to 50 µmol/l H_2O_2 were associated with an increase in pro-apoptotic p53 expression, which may result from reduced degradation in the absence of Mdm2 (Haupt *et al.*, 1997). In response to 6% O_2, p53 expression was not significantly increased, suggesting that p53-independent mechanisms may be present and capable of inducing apoptosis in BeWo cells. As BeWo cells are derived from choriocarcinoma, a malignant tumour of trophoblast origin, the ability of p53 to regulate apoptosis may be impaired, as both p53 and Mdm2 expression is increased in choriocarcinoma compared with normal first trimester villous tissue (Fulop *et al.*, 1998; Qiao *et al.*, 1998). However, p53 has been sequenced in molar pregnancies and choriocarcinoma and found to be wild type (Cheung *et al.*, 1994; Shi *et al.*, 1996; Fulop *et al.*, 1998).

The data presented in this and previous studies suggest that BeWo cells are a useful cellular model in which hypotheses regarding the regulation of placental cell turnover might be tested. However, interpretation of these findings presents some challenges. Firstly, the level of oxygenation used for control conditions in this and other studies is 20–21% O_2, which does not reflect appropriate in-vivo or in-vitro O_2 conditions for third trimester villous trophoblast, which are estimated to be 6–8% O_2 (Miller *et al.*, 2005; Schaaps *et al.*, 2005). However, following isolation from a metastatic choriocarcinoma, BeWo cells were cultured in 20% O_2 environment (Pattillo and Gey, 1968), which may have resulted in this level of oxygenation being adopted as normoxic conditions for this cell-line, suggesting that culture in 6%O_2 would be representative of hypoxia. Secondly, interpretation of data regarding the effects of mitogenic or apoptotic stimuli may be complicated by the addition of forskolin to stimulate fusion of BeWo cells. This is particularly important, as mononucleate trophoblast appears to be more susceptible to apoptosis than syncytiotrophoblast (Hu *et al.*, 2006). It was demonstrated that there is a low level of spontaneous cell fusion and that this appeared to be proportional to HCG secretion at 48 h. Therefore, where the effects of an agent or condition on apoptosis are being studied, it may aid clarity to omit treatment with forskolin.

Several studies have concluded that BeWo cells are a useful model to study trophoblast cell turnover (Rao *et al.*, 2003) and the expression of components of the intrinsic apoptotic pathway (White *et al.*, 2007). Using this model, it was demonstrated that ROS and hypoxia, stimuli implicated in the development of pre-eclampsia and IUGR, can induce apoptosis at concentrations (50 µmol/l) present in maternal serum (Kharfi *et al.*, 2005). Furthermore, these changes are associated with an increase in p53 expression, which is also observed in pregnancies complicated by IUGR (Levy *et al.*, 2002). Although findings of studies in choriocarcinoma cell lines must be extrapolated with caution, use of this model may facilitate investigation of the control of trophoblast cell turnover, for example whether upstream signalling proteins interact with p53 and whether stabilized p53 can induce changes in cell turnover. Ultimately BeWo cells may provide a useful technique to define the proteomic profile of an apoptotic phenotype in trophoblast, determining molecular targets to attenuate apoptosis, which may be useful in antagonising the increased apoptosis in pre-eclampsia and IUGR.

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