Effects of Taurine Depletion on Human Placental Syncytiotrophoblast Renewal and Susceptibility to Oxidative Stress

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Abbreviations

CTB	Cytotrophoblast
ETC	Electron transport chain
FGR	Fetal growth restriction
hCG	Human chorionic gonadotrophin
PE	Pre-eclampsia
ROS	Reactive oxygen species
STB	Syncytiotrophoblast
TauT	Taurine transporter

1 Introduction

Taurine (2-aminoethanesulfonic acid) is the most abundant free amino acid in the human placenta (Philipps et al. 1978). In syncytiotrophoblast (STB), the solute transporting epithelium of the placenta, the activity of the taurine transporter (TauT) in the maternal-facing microvillous membrane of the human placental syncytiotrophoblast (STB) is important to achieve a high intracellular taurine concentration and maintain a gradient that favours taurine efflux towards the fetus, where it is necessary for organogenesis (Sturman 1988; Han et al. 2000).

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We have previously reported reduced STB TauT activity in the pregnancy complication pre-eclampsia (PE) (Desforges et al. 2013a), a severe high blood pressure condition which is often accompanied by fetal growth restriction (FGR). In non-placental cells, taurine protects against damage caused by factors which are elevated in PE such as inflammatory cytokines and reactive oxygen species (ROS) (Wojtecka-Lukasik et al. 2006; Oriyanhan et al. 2005). Emerging evidence suggests that the cytoprotective role of taurine is related to its ability to promote mitochondrial function (Jong et al. 2010, 2012). Interestingly, mitochondrial dysfunction has been demonstrated in placentas from pregnancies complicated by PE and FGR (Muralimanoharan et al. 2012; Mando et al. 2014). Therefore, the reduction in STB taurine uptake in PE, in addition to restricting taurine efflux to the fetus, could compromise STB function and cytoprotection.

In the human placenta, renewal of STB is essential to preserve its function as a solute transporting epithelium and endocrine/paracrine organ, maintaining nutrient delivery to the fetus and producing hormones that sustain pregnancy. STB is renewed during pregnancy by a process of cellular turnover involving proliferation of the underlying cytotrophoblast cells (CTB) followed by differentiation, fusion, and incorporation of their nuclei into the STB (Huppertz et al. 2006; Heazell and Crocker 2008). In addition to supplying cellular energy, mitochondria are involved in a range of processes important for maintenance and function of STB, such as intracellular signalling, cellular differentiation, and cell death.

In PE and FGR there is reduced CTB fusion (Langbein et al. 2008; Vargas et al. 2011), and increased CTB apoptosis (Longtine et al. 2012) which leads to placental insufficiency. Using CTB *in vitro*, we showed that siRNA-mediated TauT knockdown reduced intracellular taurine, inhibited the differentiation and fusion of cells to form multinucleate syncytia, and increased susceptibility to TNF α-mediated apoptosis (Desforges et al. 2013b).

We hypothesise that the reduction in TauT activity and intracellular taurine in PE impairs STB renewal and lowers cytoprotection to damaging factors present in the maternal environment by compromising mitochondrial function. To test this hypothesis, STB renewal and susceptibility to oxidative stress was determined in the placental villous explant model following β-alanine-mediated intracellular taurine depletion. In separate studies using the BeWo choriocarcinoma cell line as a model of CTB, markers of mitochondrial function and oxidative stress were examined following β-alanine-mediated intracellular taurine depletion.

2 Methods

2.1 Intracellular Taurine Depletion in Placental Explants and Effect of Oxidative Stress

Human term placentas (38–40 weeks gestation) were obtained within 30 min of caesarean section from uncomplicated singleton pregnancies following written informed consent as approved by the Central Manchester Research Ethics Committee.

Placental villous fragments were dissected and maintained in explant culture for 7 days as described previously (Siman et al. 2001). Intracellular taurine depletion was achieved by incubating explants in the presence of 10 mM β alanine for the duration of culture. HPLC of homogenized explant tissue confirmed that 10 mM β alanine reduced intracellular taurine by ~95 % (data not shown). To study the effect of oxidative stress following intracellular taurine depletion, placental explants were treated with 1 mM H_2O_2 from day 5 of culture. Explant culture medium was collected daily to measure hCG, secreted by differentiated STB, using a commercially available ELISA (DRG Diagnostics, Germany) according to the manufactures' instructions. Routine IHC was used to detect cytokeratin 7 (an epithelial marker allowing assessment of STB regeneration) and 8-hydroxyguanosine (marker of oxidative DNA damage) in formalin-fixed wax-embedded explant tissue samples.

2.2 Effect of Intracellular Taurine Depletion on Markers of Mitochondrial Function in Trophoblast Cells

BeWo choriocarcinoma cells were incubated in the presence or absence of 5 mM β-alanine for 72 h before isolation of mitochondria using a mitochondria/cytosol fractionation kit (Abcam). Western blot analysis of subunits in mitochondrial electron transport chain (ETC) complex proteins I–V was then performed using a Total OXPHOS antibody cocktail (Abcam, 1:200 dilution). Nitrocellulose membranes were stripped and re-probed for VDAC (Cell signalling, 1:1,000 dilution). Developed film was scanned and the mean signal intensity of the immunoreactive species determined using Image J software. To account for any variability in sample loading, signal intensity of complex proteins I-V in each sample was normalised to the corresponding VDAC signal intensity.

Mitochondrial morphology and ROS in BeWo cells were assessed using MitoTracker® and CellRox® fluorescent probes respectively. To study the effect of oxidative stress following β-alanine-mediated intracellular taurine depletion, BeWo cells were treated for 1 h with 1 mM H_2O_2 prior to immunofluorescence.

2.3 Statistical Analysis

Paired data from placental villous explants were analysed by Wilcoxon matched pairs signed rank or Friedman test as appropriate. Western blotting data were analysed by Mann Whitney. A calculated p value of <0.05 was considered statistically significant using GraphPad Prism version 5.

3 Results

3.1 *Intracellular Taurine Depletion Compromises STB Renewal*

During placental villous explant culture, STB sheds over the first 2–3 days and thereafter is regenerated and maintained by processes of cellular turnover resembling those *in vivo*. STB regeneration *in vitro* is accompanied by increased hCG secretion into the explant medium (Siman et al. 2001; Audette et al. 2010). Cytokeratin 7 IHC allowed visualization of both the shed STB and newly regenerated STB (Fig. 1a). STB regeneration on day 7 of culture was assessed by

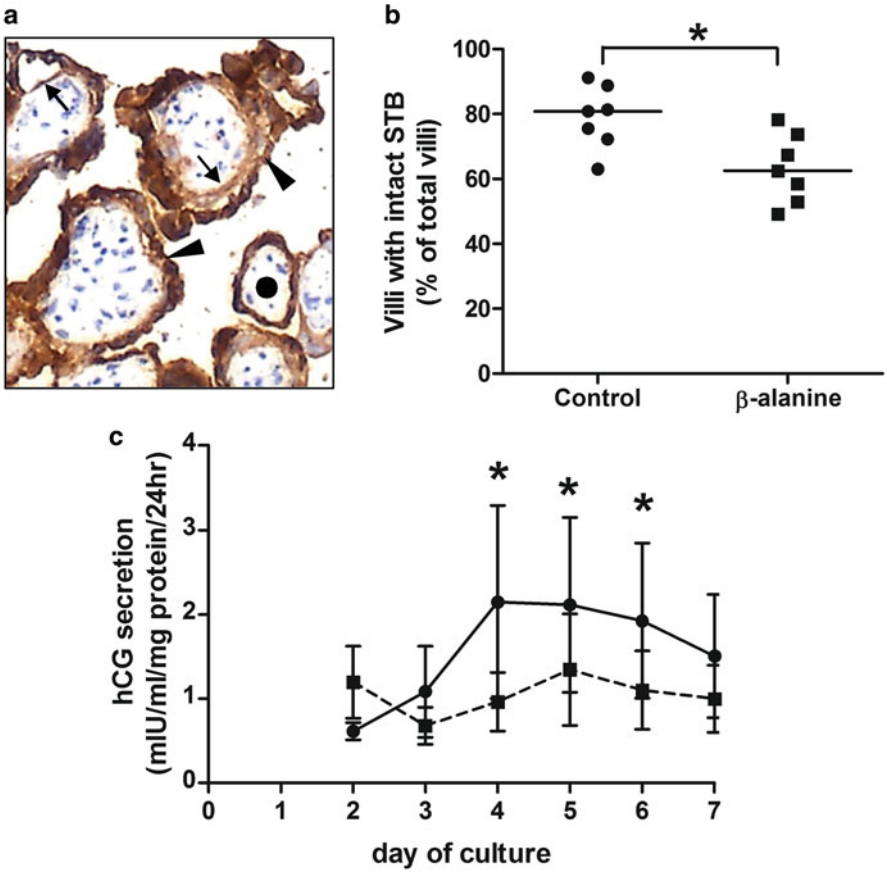


Fig 1 Analysis of STB renewal in placental villous explants. (**a**) Cytokeratin 7 IHC in explants fixed on day 7 of culture for analysis of STB shedding (*arrowheads*) and renewal (*arrows*). *Circle* indicates an example of a villus with intact STB. (**b**) Proportion of villi with intact STB (n = 7; *line* represents median). (**c**) Time course of hCG secretion (mean ± SE) by control (*circles*) and β-alanine-treated (*squares*) explants (n = 7). *$p < 0.05$, Wilcoxon matched pairs signed rank

expressing the number of villi with intact STB as a proportion of the total number of villi within a field of view. In control explants, complete STB regeneration was evident in 60–90 % of villi (Fig. 1b). In explants with intracellular taurine depletion, complete STB regeneration was significantly reduced compared to controls (Fig. 1b). As previously described (Siman et al. 2001; Audette et al. 2010), hCG secretion by control explants increased from day 2 to day 4 of culture (Fig. 1c). Intracellular taurine depletion with β-alanine significantly reduced hCG secretion compared to matched controls (Fig. 1c).

3.2 *Intracellular Taurine Depletion Increases STB Susceptibility to Oxidative Stress In Vitro*

IHC to detect 8-hydroxyguanosine (marker of oxidative DNA damage) in STB nuclei and cytoplasm (i.e. mitochondrial DNA) (Fig. 2a, b respectively) was performed in placental villous explants following chronic treatment (48 h) with 1 mM H_2O_2. Semi-quantitative analysis of staining revealed H_2O_2 treatment alone was insufficient to induce oxidative damage to either nuclear DNA or mitochondrial DNA in STB (Fig. 2c, d respectively). However, oxidative damage to both STB nuclear DNA (Fig. 2c) and mitochondrial DNA (Fig. 2d) was significantly higher in explants treated with H_2O_2 following intracellular taurine depletion with β-alanine when compared to control, H_2O_2 or β-alanine alone.

3.3 *Evidence of Mitochondrial Dysfunction in BeWo Cells Following Intracellular Taurine Depletion*

Western blot analysis of mitochondrial ETC complex proteins I–V in isolated mitochondria from BeWo cells (Fig. 3a) demonstrated significantly reduced expression of ETC complex V (ATP synthase) subunit in cells with β-alanine-mediated taurine depletion (range: 0.72–0.9) compared to controls (range: 0.75–2.5) (Fig. 3b).

Immunofluorescence to detect active mitochondria (Mitotracker®) and ROS (CellRox®) in BeWo cells demonstrated that under control conditions, mitochondria were diffusely spread throughout the cell and levels of ROS were minimal (Fig. 4; Control). Taurine depletion induced mitochondrial swelling and fragmentation of the diffuse mitochondrial network (Fig. 4; β-alanine). In addition, there was an increase in ROS which co-localised with the nuclei and mitochondria. A similar alteration to mitochondrial morphology was seen in positive control cells incubated with 30 μM antimycin A (an inhibitor of ETC complex III) for 1 h: mitochondria appeared swollen and clustered around cell nuclei (Fig. 4; Antimycin A). There was no apparent increase in ROS following H_2O_2 treatment of control cells (Fig. 4; H_2O_2). However, in cells with β-alanine-mediated taurine depletion, H_2O_2 treatment increased levels of ROS co-localised with nuclei and some mitochondria and also increased ROS in the cytoplasm.

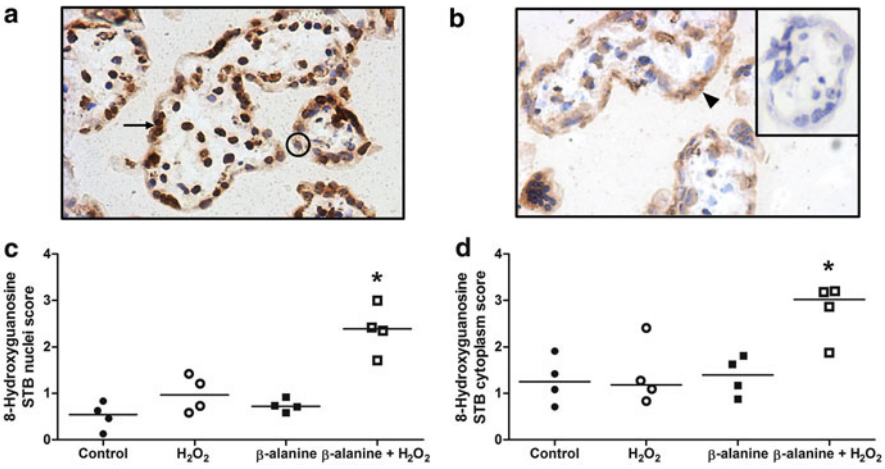
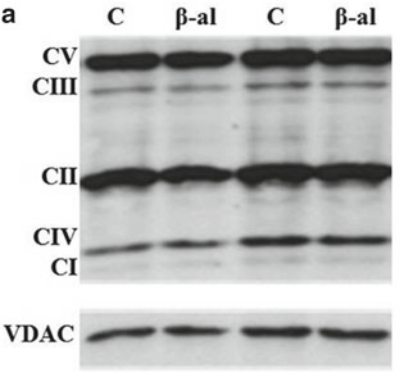


Fig. 2 IHC for oxidative DNA damage in STB following β-alanine-mediated taurine depletion and H_2O_2 treatment. Detection of oxidative damage to DNA in (**a**) the nucleus (arrow = +ve nuclei, circle = −ve nucleus) and (**b**) the cytoplasm (mitochondrial DNA; indicated by *arrowhead*) using positive staining for 8-hydroxyguanosine (*brown*). Counterstained with hematoxylin. *Inset*: negative control (non-immune IgG replaced primary antibody). (**c**, **d**) Semi-quantitative assessment of 8-hydroxyguanosine staining in STB using a scale of 0–4 (n = 4; *line* represents the median. *p < 0.05, Friedman with Dunn's post test)



b

ETC Complex	p value (control *vs* β-alanine)
I	0.83
II	0.09
III	1
IV	0.84
V	0.05*

Fig. 3 Western blot analysis of mitochondrial ETC proteins in BeWo cells following β-alanine-mediated taurine depletion. (**a**) Representative Western blot of complex I–V (CI–CV) subunit expression in control (C) and β-alanine-treated cells (β-ala). VDAC was used as a loading control. (**b**) Summary table of p values following comparison of subunit expression, normalized to VDAC, in control and β-alanine treated cells. n = 4/5; *p < 0.05, Mann Whitney

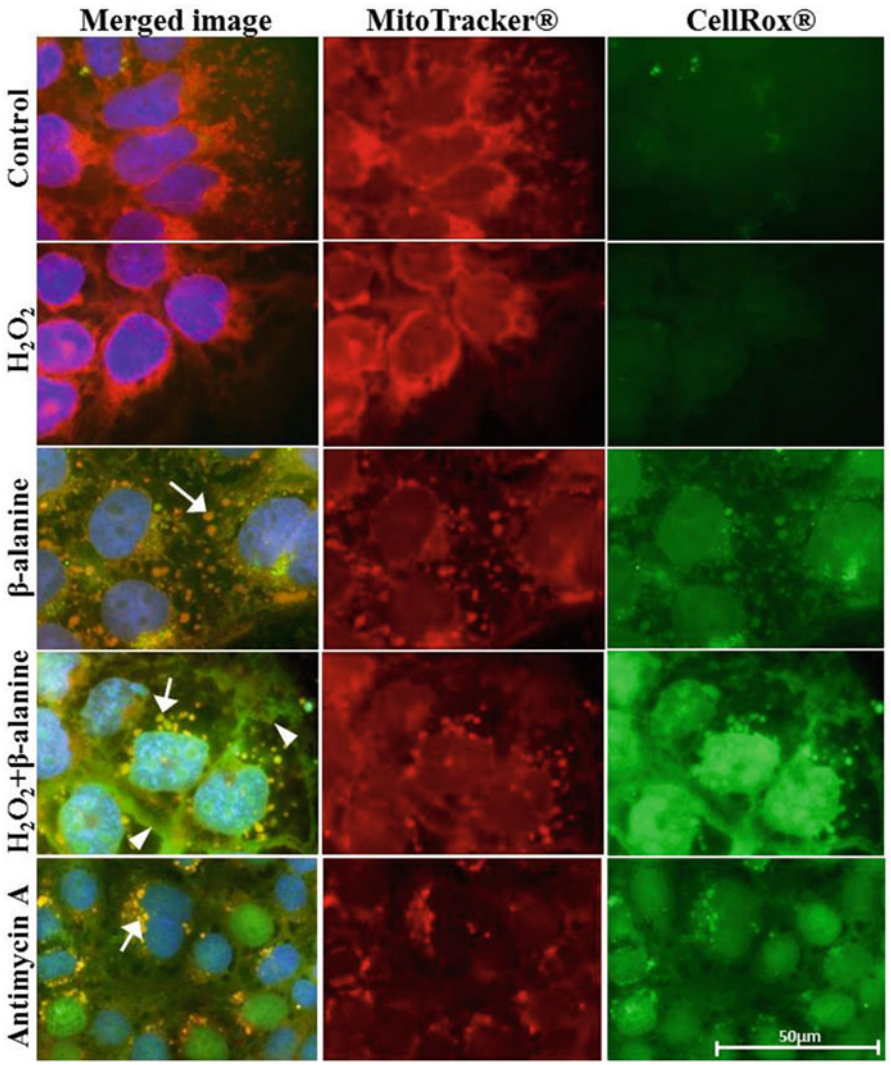


Fig. 4 Mitochondrial morphology and ROS in BeWo cells following β-alanine-mediated taurine depletion and H_2O_2 treatment. CellRox® (*green*) to detect ROS and MitoTracker® (*red*) to detect mitochondria. Merged image with DAPI nuclear counterstain (*blue*). ROS can be seen in the cytoplasm (*arrowheads*) and co-localised with swollen mitochondria (*arrows*) and nuclei. Antimycin A included as a positive control. All images were captured at the same exposure to allow comparison of fluorescence intensity. Representative images of n = 6

4 Discussion

In previous studies we have demonstrated compromised CTB differentiation *in vitro* following intracellular taurine depletion (Desforges et al. 2013b). Differentiation of CTB is a key event in the process of STB turnover *in situ*, preceding CTB fusion with STB and the subsequent incorporation of fresh cellular material. Here we have provided further evidence that intracellular taurine is necessary for maintenance of STB by demonstrating impaired renewal of STB in placental villous explants following intracellular taurine depletion.

Dysregulated STB turnover is evident in several pregnancy complications and in PE it arises from reduced CTB fusion (Langbein et al. 2008; Vargas et al. 2011) and increased CTB apoptosis (Longtine et al. 2012). The etiology of PE is poorly understood but the disease is thought to arise from partial failure of placentation, leading to compromised uteroplacental blood flow and ischemia-reperfusion injury of the placenta (Huppertz 2008). This can induce both local and systemic oxidative stress, defined as an imbalance between ROS and antioxidant capacity. Indeed, numerous studies have shown that women with PE have increased circulating markers of oxidative stress and reduced antioxidant capacity (Siddiqui et al. 2010; Hubel 1999) along with increased oxidative stress in the placenta (Myatt and Cui 2004). *In vitro* experiments show that ROS damage the placenta through effects on STB turnover (Moll et al. 2007). Elevated ROS and reduced placental TauT activity in PE are therefore likely contributors to disrupted STB turnover associated with this pregnancy complication.

Our data also demonstrate the importance of intracellular taurine in STB/CTB for protection against ROS. ROS can react rapidly with DNA, proteins and lipids, thereby leading to oxidative damage, which impairs cellular function (Burton and Jauniaux 2011). Here we have shown that intracellular taurine depletion in placental villous explants rendered STB more vulnerable to oxidative DNA damage following treatment with H_2O_2. In experiments with taurine deplete BeWo cells, H_2O_2 treatment was associated with increased ROS in the cytoplasm. Interestingly, in control (i.e. taurine replete) cells and explant tissue, H_2O_2 treatment did not increase intracellular levels of ROS or induce oxidative DNA damage. There is some evidence that taurine functions as a free radical scavenger (Cheong et al. 2013), but there is little evidence that it upregulates the antioxidant defences of the cell. When cells are subjected to oxidative stress, mitochondria are capable of drawing upon their reserve capacity to serve the increasing energy demands for maintenance of organ function, cellular repair, or ROS detoxification. The final outcome is determined by a balance that may lean toward either apoptosis or cellular recovery. We did not explore apoptosis in the current study following intracellular taurine depletion and H_2O_2 treatment. However, in a previous study we found no effect of intracellular taurine depletion on basal levels of apoptosis in STB, but there was increased susceptibility to apoptotic cell death following treatment with the inflammatory cytokine TNFα (Desforges et al. 2013b). Collectively, these data indicate a cytoprotective role for taurine in STB similar to that reported for other non-placental cells (Wojtecka-Lukasik et al. 2006; Oriyanhan et al. 2005).

In non-placental cells, taurine is required for efficient translation of proteins in the ETC (Jong et al. 2010, 2012). Intracellular taurine is therefore important for maintaining ETC activity and enhancing mitochondrial reserve capacity. Biochemical modelling has also demonstrated an important role for taurine as a mitochondrial matrix buffer, stabilising oxidative metabolism (Hansen et al. 2006). These observations suggest the mechanism underlying the antioxidant activity of taurine is related to its role in promoting mitochondrial function. In the current study there were signs of mitochondrial dysfunction in taurine deplete BeWo cells evidenced by reduced expression of ETC complex V subunit (responsible for generation of ATP via oxidative phosphorylation), swelling of mitochondria, and fragmentation of the normally diffuse mitochondrial network. There was also increased ROS co-localising with the nuclei and mitochondria indicating a higher level of oxidative stress. This is the first evidence to suggest taurine has a role in the regulation of normal mitochondrial function in placenta. We speculate that the increased susceptibility of taurine deplete STB/CTB to damage following H_2O_2 treatment that we observed is related to mitochondrial dysfunction and insufficient capacity to detoxify accumulating ROS in these cells.

Our *in vitro* data suggest reduced placental taurine transport in PE could compromise placental development and increase CTB/STB susceptibility to death and damage through an effect on mitochondrial function. Structural, functional, and genetic changes in mitochondria have been reported in STB from patients with PE. For example, the mitochondria show swelling with a loss of cristae (Muralimanoharan et al. 2012), there is a reduction in the expression and activity of ETC complexes I and III and the ETC enzyme, cytochrome c oxidase (Muralimanoharan et al. 2012; Furui et al. 1994; Matsubara et al. 1997), and mutations in mitochondrial tRNA genes have been shown in two families with a high occurrence of PE (Folgero et al. 1996). It has yet to be determined whether these observations are contributing factors to the disease, or a consequence of ongoing oxidative stress which leads to mtDNA and/or protein damage.

5 Conclusion

In summary, these *in vitro* studies showed that intracellular taurine depletion in the placenta compromised STB regeneration and resulted in an increased susceptibility to oxidative stress, evidenced by nuclear and mitochondrial DNA damage. Intracellular taurine depletion was also associated with markers of mitochondrial dysfunction and increased ROS in trophoblast cells. The effects of taurine deficiency *in vitro* are consistent with dysregulated STB renewal, elevated oxidative stress and mitochondrial dysfunction that are features of placental pathology in PE. Reduced placental taurine uptake in PE could therefore be a contributing factor to the placental insufficiency associated with this pregnancy complication.

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