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N-acetylcysteine, xCT and suppression of Maxi-chloride channel activity in human placenta

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ABSTRACT

Introduction: Placental oxidative stress features in pregnancy pathologies but in clinical trials antioxidant supplementation has not improved outcomes. N-acetylcysteine (NAC) stimulates glutathione production and is proposed as a therapeutic agent in pregnancy. However, key elements of N-acetylcysteine biology, including its cellular uptake mechanism, remains unclear. This study explores how the cystine/glutamate transporter xCT may mediate N-acetylcysteine uptake and how N-acetylcysteine alters placental redox status.
Methods: The involvement of xCT in NAC uptake by the human placenta was studied in perfused placenta and *Xenopus* oocytes. The effect of short-term N-acetylcysteine exposure on the placental villous proteome was determined using LC-MS. The effect of N-acetylcysteine on Maxi-chloride channel activity was investigated in perfused placenta, villous fragments and cell culture.
Results: Maternoplacental N-acetylcysteine administration stimulated intracellular glutamate efflux suggesting a role of the exchange transporter xCT, which was localised to the microvillous membrane of the placental syncytiotrophoblast. Placental exposure to a bolus of N-acetylcysteine inhibited subsequent activation of the redox sensitive Maxi-chloride channel independently of glutathione synthesis. Stable isotope quantitative proteomics of placental villi treated with N-acetylcysteine demonstrated changes in pathways associated with oxidative stress, apoptosis and the acute phase response.
Discussion: This study suggests that xCT mediates N-acetylcysteine uptake into the placenta and that N-acetylcysteine treatment of placental tissue alters the placental proteome while regulating the redox sensitive Maxi-chloride channel. Interestingly N-acetylcysteine had antioxidant effects independent of the glutathione pathway. Effective placental antioxidant therapy in pregnancy may require maintaining the balance between normalising redox status without inhibiting physiological redox signalling.

1. Introduction

Placental oxidative stress is a feature of pregnancy pathologies, including pre-eclampsia, fetal growth restriction and preterm birth [1]. However, in clinical trials supplementation with vitamin C and E has been ineffective, if not counterproductive [1]. There is thus a need for better treatments for managing unregulated oxidative stress in pregnancy. N-acetylcysteine (NAC) is an antioxidant which is widely used as a nutritional supplement [2]. The reported safety profile of NAC makes it a candidate for use in pregnancy conditions where placental oxidative stress is a concern, such as pre-eclampsia. However, the biology of NAC is poorly understood, it is not clear how NAC is taken up into cells or the extent to which it can affect placental redox status.

Reactive oxygen species (ROS) and reactive nitrogen species are generated when electrons leak out from the electron transport chain or by enzymes such as xanthine oxidase and NADPH oxidase [3]. In the placenta, low levels of free radicals are known to be involved in physiological processes such as trophoblast proliferation, invasion, and

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angiogenesis [4]. Excess free radicals are mopped up by antioxidants but if the delicate balance between ROS/reactive nitrogen species production and removal by antioxidants is disrupted, oxidative stress occurs which can result in damage to cellular DNA, proteins and lipids [5]. Oxidative stress due to premature initiation of maternal blood flow to the placenta in the first trimester of pregnancy has deleterious effects on fetal growth [6]. However, randomised controlled trials of antioxidant treatment in pregnancy using vitamin C and E indicated adverse maternal and fetal outcomes including reduced birth weight and generational hypertension [1]. While oxidative stress is pathophysiological, the generation of free radicals has a crucial physiological role in terms of redox signalling [7]. For instance, oxidative stress can alter the activity of redox-sensitive transcription factors which in turn regulate apoptosis, cell differentiation and pro-inflammatory markers [8]. Attempts to treat placental oxidative stress may be detrimental if treatments block physiological redox signalling.

NAC has clear clinical benefits in some circumstances, for instance following paracetamol overdose where NAC provides cysteine for glutathione synthesis [9]. NAC may be beneficial in a wide range of other disorders, including cancers and cystic fibrosis [10,11]. Additionally, NAC has been administered in pregnancy in small trials in humans and animals [12–14], and has been found to restore nitric oxide mediated vasodilation in pre-eclampsia [15]. Compared to vitamin A and C, NACs antioxidant effect is primarily mediated indirectly via glutathione synthesis which may lead to a more physiological antioxidant effect that avoids supressing normal redox signalling.

There are no known NAC transport mechanisms but NAC has been demonstrated to cross the human placenta [16]. We hypothesise that NAC is transported by the glutamate-cystine antiporter xCT (*SLC7A11*) that combines with 4F2 cell-surface antigen heavy chain (4F2hc) to form the functional transporter. Physiologically, xCT provides cystine for glutathione synthesis and its activity may therefore be a rate-limiting determinant of glutathione synthesis [17,18]. NAC has been proposed to be an activator of xCT but it is not clear whether xCT transports NAC itself, is a *cis*-activator of xCT, or is broken down to cysteine which *trans*-stimulates xCT. The *SLC7A11* gene encoding the xCT protein is expressed in human placenta but xCT activity has not previously been demonstrated [19–21].

NAC has been shown to inhibit the volume regulatory decrease mediated by the Maxi-chloride channel in response to hydrogen peroxide, therefore implying that NAC has antioxidant effects [18, 19]. Maxi-chloride channels have ubiquitous expression in all cells of the body and are thought to be involved in the process of cellular regulatory volume decrease allowing the efflux of a range of anions including ATP, glutamate and taurine in order to restore normal cell volume [22].

This study aims to determine how NAC is transported into the human placenta and what effects it may have on placental redox status and function. We hypothesised that xCT is expressed on the MVM of the placental syncytiotrophoblast and helps to regulate placental oxidative stress.

2. Methods

Placentas were collected with written informed consent from women at term, who had uncomplicated pregnancies, delivering at the Princess Anne Hospital in Southampton with approval from the Southampton and Southwest Hampshire Local Ethics Committee (11/SC/0529).

2.1. Placental perfusions

Placentas were perfused using the isolated perfused placental cotyledon methodology of Schneider et al. [23,24], as adapted in our laboratory (15, 20). Placentas were perfused with Earle's Bicarbonate Buffer (EBB: 5 mmol L^{-1} glucose, 1.8 mmol L^{-1} $CaCl_2$, 0.4 mmol L^{-1} $MgSO_4$, 116.4 mmol L^{-1} NaCl, 5.4 mmol L^{-1} KCl, 26.2 mmol L^{-1} $NaHCO_3$, 0.9 mmol L^{-1} NaH_2PO_4, 1 g/l bovine serum albumin), gassed with 5% CO_2 and 95% O_2, via the fetal catheter going into the chorionic plate fetal artery at 6 ml/min. Where fetal venous outflow was ≥95% of fetal arterial inflow, the maternal-side arterial perfusion with EBB was established 15 min later at 14 ml/min using roller pumps. Maternal perfusion buffer contained 58.5 nmol L^{-1} of ^{14}C-glutamate (PerkinElmer, Massachusetts, USA) and 1.8 mmol L^{-1} creatinine which was used as a marker of paracellular diffusion. To stimulate glutamate exchange, 16 μmol boli of a range of solute carrier (SLC) transporter substrates; glutamate (n = 14 placentas), NAC (n = 7 placentas), glutamine (n = 5 placentas), taurine (n = 5 placentas) and serine (n = 5 placentas) in EBB were perfused into the maternal and fetal circulations. Samples were collected at regular time points from maternal and fetal venous outflow (every 30 s for the first 5 min, then at 8, 13, 21 and 25 min).

In the above experiments, volume regulated glutamate efflux by the Maxi-chloride channel from the isolated perfused human placenta was determined following the cessation of tracer perfusion at 145 min (time zero). The maternal buffer was switched to normal EBB and perfused for 30 min to wash ^{14}C-glutamate out of the maternal and fetal circulations, with samples collected every 10 min. Perfusate samples were collected every 5 min from maternal and fetal venous outflow. At 31 min, maternal EBB was switched to EBB +50 mmol L^{-1} urea (n = 12) in EBB for 10 min. Uptake of urea by the placental syncytiotrophoblast will lead to osmotically driven water uptake and cellular swelling. When sampling, approximately 1.5 ml of venous exudate was collected from maternal and fetal venous outflows. At the end of the experiments, the perfused tissue was blotted dry and weighed.

2.2. Placental villous fragments

xCT ^{14}C-glutamate efflux experiments: Villous fragments (10 mg) were dissected from human term placentas and cultured for 2.5 h in Tyrode's buffer (135 mmol L^{-1} NaCl, 5 mmol L^{-1} KCl, 1.8 mmol L^{-1} $CaCl_2$, 1 mmol L^{-1} $MgCl_2.H_2O$, 10 mmol L^{-1} HEPES, 5.6 mmol L^{-1} glucose, pH 7.4) containing ^{14}C-glutamate (4.5 nM). Fragments were washed three times in Tyrode's buffer before an efflux buffer (containing Tyrode's alone, 1 mmol L^{-1} glycine, 1 mmol L^{-1} cystine, 1 mmol L^{-1} NAC or 1 mmol L^{-1} glutamate) was added for 5 min ^{14}C-glutamate efflux into the media was analysed by liquid scintillation counting.

Maxi-chloride channel experiments: Villous fragments (approx. 10 mg) were dissected from human term placentas and cultured for 2.5 h in Tyrode's buffer containing ^{14}C-glutamate (4.5 nM) with and without 500 μmol L^{-1} NAC or cystine or 100 μmol L^{-1} buthionine sulfoximine (BSO, glutathione synthesis inhibitor). Fragments were washed three times in Tyrode's buffer before an efflux buffer (containing Tyrode's alone or urea Tyrode's (55 mmol L^{-1} NaCl, 5 mmol L^{-1} KCl, 1.8 mmol L^{-1} $CaCl_2$, 1 mmol L^{-1} $MgCl_2.6H_2O$, 10 mmol L^{-1} HEPES, 5.6 mmol L^{-1} glucose, 160 mmol L^{-1} urea, pH 7.4)) was added for 5 min.

^{14}C-glutamate efflux from fragments was measured by liquid scintillation counting. Each experimental condition was performed in duplicate, with three fragments per duplicate (n = 5 placentas). Results were adjusted for protein using the BCA protein assay kit (Life technologies, UK).

2.3. Reverse transcriptase -PCR

RNA was extracted from 30 mg term placental tissue using the RNEasy Mini Kit (Qiagen, UK) according to the manufacturer's instructions and primary term human cytotrophoblast cells (isolated as described previously Simner et al., 2020) using RNAzol (Sigma Aldrich, UK) [25]. The integrity of total RNA was confirmed by gel electrophoresis. RNA (0.5 μg) was reverse transcribed with 0.5 μg random hexamer primer, 200 units MMLV (Moloney Murine Leukemia Virus) reverse transcriptase, 25 units recombinant RNasin ribonuclease inhibitor and 0.5 mm each of dATP, dCTP, dGTP and dTTP in a final reaction volume of 25 μl in 1 × MMLV reaction buffer (Promega, UK). Oligonucliotide

primers were designed for *SLC7A11* (xCT NM_014331.3) forward 5′-GGCAGTGACCTTTTCTGAGC-3′ and reverse 5′- TGTTCTGGTTATTTTCT CTGACATT -3′ using Primer 3 and synthesised by Eurogentec (Seraing, Belgium). The mRNA expression of *SLC7A11* was measured by PCR with 0.2 μg cDNA added to primers (1 μmol l^{-1}) with polymerase (2 X Mastermix, (Promega, UK)). Thermal cycling was performed under the following conditions: 94 °C for 3 min; 40 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; and then 72 °C for 4 min. PCR products were run on an agarose gel and visualized under ultra violet light. Sequencing (GATC-Biotech, Germany) confirmed PCR product identity.

2.4. cRNA synthesis

Xenopus laevis oocyte experiments were used to further characterise the xCT transporter system. Oocytes allow large quantities of the protein of interest to be synthesised where it can subsequently be targeted to the plasma membrane. The relative absence of endogenous channels and transporters mean *Xenopus* transporter studies may be more reliable compared to non-stable transfection cell culture systems where other channels and transporters may complicate analyses. For the transporter to be functional, both *SLC7A11* (xCT) and *SLC3A2* (4F2hc) have to be expressed. Plasmids containing the cDNA of *SLC7A11* (xCT) tagged to green fluorescent protein (GFP; C terminus) and its heavy chain *SLC3A2* (4F2hc) were obtained from Cambridge Bioscience and were linearized using restriction enzyme specific to *Age*I sites (Promega, Southampton, UK). cRNA was synthesised from the T7 promoter of the plasmid using the Ambion mMachine mMessage kit (Life Technologies, UK) according to the manufacturer's instructions.

2.5. Xenopus oocytes

Xenopus oocytes were obtained from the European *Xenopus* Resource Centre (Portsmouth, UK). Oocytes were treated with collagenase (2 mg/ml) in OR2 buffer (2.5 mmol L^{-1} KCl, 82.5 mmol L^{-1} NaCl, 1 mmol L^{-1} $CaCl_2$, 1 mmol L^{-1} Na_2HPO_4, 1 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} HEPES) for 1 h at room temperature to release individual oocytes from the ovary. Oocytes were then incubated in ND91 buffer (2 mmol L^{-1} KCl, 91 mmol L^{-1} NaCl, 1.8 mmol L^{-1} $CaCl_2$, 1 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} HEPES, 1% Penicillin/Streptomycin and 0.1% Gentamycin Sulphate) at 18 °C, the optimum temperature for oocyte maintenance. Oocytes were injected with 10 ng of cRNA for both xCT and 4F2hc in 56 nl of water, using a microinjector. Control oocytes were injected with an equivalent volume of water.

2.6. Trans-stimulation studies

Two to three days after injection, oocytes injected with the cRNA of interest were incubated for 30 min in 20 μmol L^{-1} (50 μCi/l) ^{14}C-glutamate. After washing three times in 1 ml of ND91 buffer, oocytes were incubated for 5 min in ND91 buffer alone, ND91 buffer containing xCT substrates (10 mmol L^{-1} NAC or glutamate) or control buffer (10 mmol L^{-1} glycine) to stimulate glutamate efflux. Each experiment was performed >5 times, within each experiment each condition was studies in triplicate and within each replicate there were 10 oocytes. ^{14}C-glutamate efflux was measured by liquid scintillation counting.

2.7. Western blotting methodology

Western blotting was carried out using human placental syncytiotrophoblast microvillous membrane (MVM) (n = 4) and basal membrane (BM) (n = 4) vesicle preparations [26,27] with protein extracted in RIPA buffer and 1 x DTT reducing agent. Alkaline phosphatase enrichments for the MVM preparations were 24.9 ± 1.7 fold, mean ± SEM, $n = 17$. BM marker enrichment of dihydroalprenolol binding in the BM preparations compared to initial homogenates was 28.8 (7.9)-fold (mean and SEM.; $n = 10$). 45 μg protein was loaded onto 4–12% SDS-PAGE gels and transferred onto PVDF membranes. Blots were incubated with xCT goat anti-human, (ab60171, AbCAM) primary antibody and left to incubate overnight at 4 °C before being washed for 10 min (x 3) in PBS-T. Blots were then incubated with a 1/20,000 dilution of rabbit anti-goat secondary antibody (ab60171) for 60 min. Immunoreactive signals were visualized using enhanced chemiluminescence (SuperSignal West Femto, ThermoScientific, UK) at a ratio of 1:1. The membrane was incubated with β-actin antibody (Sigma-Aldrich, UK; 1:10,000), left for 60 min and washed for 10 min (x 3) in PBS-T to assess protein loading.

2.8. Cell culture methodology

Human embryonic kidney 293 cells (HEK 293, American Type Culture Collection) were plated on 35 mm culture dishes (Sigma-Aldrich, UK) at a density of 1.5 × 10^5 cells/well and maintained at 37°C in an incubator with 95% O_2–5% CO_2. Cells were cultured in DMEM media supplemented with 10% fetal bovine serum, 1 mmol L-1 glutamine and 10% penicillin-streptomycin (Sigma-Aldrich, UK). To determine whether NAC inhibits glutamate uptake via xCT, cells at 70% confluency were transfected with purified 4 μg xCT-GFP plasmid DNA and 4F2hc plasmid DNA (Cambridge Bioscience, UK) using Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. Cells were left to express xCT and 4F2hc protein for 24 h before ^{14}C-glutamate uptake assays were performed. The media was removed from non-transfected (control), 4F2hc transfected and xCT-GFP plus 4F2hc transfected cells. The cells were washed with 37°C sodium free Hank's balanced salt solution (HBSS; 140 mmol L^{-1} N-methyl-D-glucamine, 5.4 mmol L^{-1} KCl, 2.5 mmol L^{-1} $CaCl_2$, 1 mmol L^{-1} $MgCl_2$, 0.4 mmol L^{-1} KH_2PO_4, 10 mmol L^{-1} HEPES and 5 mmol L^{-1} glucose (pH 7.4)). Once washed, the cells were incubated at 37°C for 20 min with sodium free HBSS containing 1.08 μmol L^{-1} of ^{14}C-glutamate alone or with 1 mmol L^{-1} unlabelled glutamate, cystine, NAC, aspartate or 10 μmol L^{-1} of the xCT inhibitor Quisqualate (Sigma Aldrich, UK) to stimulate uptake. All NAC containing solutions were made fresh on the day of the experiment to minimise any risk of decomposition. Following media removal, the cells were washed with 2 ml ice-cold sodium free HBSS to stop uptake. The cells were then lysed with 0.1 mol/l potassium phosphate containing 0.5% Triton x-100. In order to determine ^{14}C-glutamate uptake into cells, 500 μl cell lysate were added to 2 ml scintillation fluid for liquid scintillation counting whilst the remaining 500 μl was stored at −20 and retained for protein determination via the BCA protein assay kit (Life technologies, UK).

For kinetic experiments to determine how xCT substrates altered ^{14}C-glutamate uptake, HEK293 cells were transfected with xCT-4F2hc as described above. At 24 h post transfection, vehicle transfected (control), 4F2hc transfected and xCT-GFP plus 4F2hc transfected cells were washed with 37°C sodium free HBSS. The cells were incubated at 37°C for 20 min with sodium free HBSS containing ^{14}C-glutamate alone or with 30 μmol L^{-1}, 100 μmol L^{-1}, 300 μmol L^{-1}, or 3000 μmol L-1 of unlabelled cystine or NAC.

For Maxi-chloride experiments, BeWo cells were cultured in DMEM supplemented with Ham's F12 nutrient mixture and 10% FBS, 2 mmol/l L-glutamine, 100 units/ml of penicillin G sodium and 100 μg/ml of streptomycin. Cells were plated out in triplicate at a density of 2.5 × 10^5 cells/well. After 48 h, the media was removed, the cells were washed in warm control Tyrode's buffer (135 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 1.8 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgCl_2.6H_2O$, 10 mmol l^{-1} HEPES, 5.6 mmol l^{-1} Glucose, pH 7.4) before being replaced with control Tyrode's buffer containing 0.22 μmol/l ^{14}C-glutamate and incubated for 2 h at 37 °C to preload the cells with ^{14}C-glutamate.

At 90 min, the treatment group of cells had NAC added to a final concentration of 500 μmol/l. At the end of the 2 h, the radioactive control Tyrode's buffer was aspirated and kept for liquid scintillation counting. The cells were then washed for four times in ice cold control Tyrode's buffer to remove remaining tracer and prevent further uptake. Warm control Tyrode's buffer was added for 1 min before being

collected. This was repeated for a total of 5 min. Either 1 ml control Tyrode's, urea Tyrode's (55 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 1.8 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgCl_2.6H_2O$, 10 mmol l^{-1} HEPES, 5.6 mmol l^{-1} glucose, 160 mmol l^{-1} urea, pH 7.4) or urea Tyrode's containing 5 mmol l^{-1} NAC was then added to the cells for 1 min and collected. This buffer was iso-osmotic unlike the buffer used in the perfusions. This was repeated for a total of 10 min. The cells were then lysed in 0.3 mol/l NaOH for 1 h before the lysate was collected. Samples were analysed by liquid scintillation counting to determine ^{14}C-glutamate efflux and uptake.

2.9. Quantitative proteomics sample processing and analysis

Incubation of villous tissue with NAC: Villous fragments (approx. 10 mg) were dissected from human term placentas and cultured for 4 h in Tyrode's buffer or Tyrode's buffer containing 500 μmol L^{-1} NAC. Following incubation tissue was briefly washed in Tyrode's buffer and snap frozen on dry ice and stored at −80C for quantitative proteomics.

Fresh frozen villous fragment tissue specimens were homogenized in 0.5 M triethylammonium bicarbonate (TEAB), 0.05% sodium dodecyl sulphate (SDS) with pulsed probe sonication (Misonix, Farmingdale, NY, USA) and syringe titration with 22-gauge syringe needle under ice-cold conditions. Cell lysates were then centrifuged (16,000 g, 10 min, 4 °C) and supernatants were measured for protein content using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, US). A total of 100 μg of protein was used per sample, adjusted to the highest volume. Proteins were reduced [tris 2-carboxyethyl phosphine hydrochloride (TCEP), 4 μl of 50 mmol L^{-1} solution, incubation at 60 °C for 1 h], alkylated [methyl-methanethiosulfonate (MMTS), 2 μl of 200 mmol L^{-1} solution, incubation at room temperature for 15 min) and enzymatically proteolyzed using trypsin (1:25). Peptides from each sample were labelled using the isobaric tag for relative and absolute quantitation (iTRAQ) reagents (AB Sciex, Framinghamn, MA, USA). Labelled peptides were mixed and analysed using two-dimensional liquid chromatography and tandem mass spectrometry as reported previously [28]. The labelling scheme was as follows: 113 = control 1, 114 = NAC 1, 115 = control 2, 116 = NAC 2, 117 = control 3, 118 = NAC 3, 119 = control 4, 121 = NAC 4. Reporter ion intensities were median-normalized. We considered the mean $\log_2$ transformed ratio of each NAC treated cells versus each respective control. A heteroschedastic, one-sample *t*-test with the Benjamini, Krieger and Yekutieli step-up method for multiple testing correction was performed. Proteins identified with $q < 0.05$, were considered differentially expressed in NAC treated cells versus control. – 'Proteins that were differentially expressed in NAC treated cells versus control were identified ($q < 0.05$)'. MetaCore (Clarivate Analytics, Philadelphia, PA, USA) was used to identify pathway maps significantly enriched with the differentially expressed proteins. The threshold of significance for the pathway analysis was set at false discovery rate (FDR) ≤ 0.05. The FDR adjusts the p value to take into account the number of observations made and minimise false positive results. All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011425 (Reviewer account details: Username: reviewer25729@ebi.ac.uk Password: VIH27nwJ).

2.10. Statistics

For the perfusion experiments area under the curve (AUC) was generated using the trapezium rule with the two points before the bolus (t = −1 and −2 min) and the points following (t = +15 and + 17 min) defined as baseline. To determine whether the AUC was greater than 0 (i.e. indicating efflux), the data were analysed using a Wilcoxon signed rank test. For Maxi-chloride channel perfusions, the data were not normally distributed so analysis was performed using a non-parametric two tailed *t*-test with a Mann-Whitney *U* test post hoc. A Wilcoxon signed rank test was used to determine whether pre-treatment with NAC inhibited glutamate efflux in response to urea.

For the oocyte experiments, ^{14}C-glutamate efflux responses from non-injected oocytes were subtracted from the injected oocyte responses before ^{14}C-glutamate efflux results for each substrate were compared to that of buffer alone using a two-tailed Dunnett's *t*-test. To compare the efflux of ^{14}C-glutamate in response to unlabelled glutamate from xCT-4F2hc injected, 4f2hc alone injected and non-injected oocytes, a one-way ANOVA was used.

In placental villous fragment experiments, ^{14}C-glutamate efflux results for each substrate were compared to that of Tyrode's buffer alone using a two-tailed Dunnett's *t*-test.

For cell culture experiments, data were analysed using a one-way ANOVA with a Dunnett's multiple comparisons posthoc test whereby the efflux of ^{14}C-glutamate from treated cells was compared to the efflux of ^{14}C-glutamate in response to control buffer. Data are presented as mean and SEM or median and interquartile range. Results were deemed significant when $p < 0.05$.

3. Results

3.1. Gene and protein expression and localisation of xCT in human placenta

Expression of *SLC7A11* (xCT) mRNA was demonstrated by rtPCR in term human placenta and primary term cytotrophoblast (Fig. 1a). DNA sequencing of the PCR product confirmed its identity. Using Western blotting, an antibody for xCT identified a 72 kDa protein in microvillous membrane (MVM) but not basal membrane (BM) preparations (Fig. 1b). The expected molecular weight of xCT is 57 kDa and it is unclear why it was observed as a 70 kDa band on the Western blot, but may reflect post-translational modifications such as glycosylation. This MVM localisation protein expression is consistent with the data from the placental perfusion experiments below.

3.2. N-acetylcysteine (NAC) stimulates glutamate efflux from perfused placenta, xenopus oocytes and villous fragments

The addition of a NAC bolus to the maternal but not the fetal circulation stimulated release of ^{14}C-glutamate from the perfused placenta ($p < 0.05$, n = 6, Fig. 1c and d). Glutamine (n = 5) and taurine (n = 5) which are not xCT substrates did not stimulate exchange for ^{14}C-glutamate.

^{14}C-glutamate efflux from xCT/4F2hc expressing oocytes was *trans*-stimulated by 10 mmol L^{-1} glutamate ($p < 0.001$, n = 5 independent experiments) or 10 mmol L^{-1} NAC ($p < 0.001$, n = 6 independent experiments) but not by 10 mmol L^{-1} glycine which is not an xCT substrate (n = 4 independent experiments) or ND91 buffer alone (n = 6 independent experiments, Fig. 2a). Time course experiments demonstrated that maximal efflux had occurred by 2.5 min. No ^{14}C-glutamate efflux could be *trans*-stimulated from oocytes injected with 4F2hc alone (n = 3 independent experiments each with 3 × 10 oocytes per group).

^{14}C-glutamate efflux from placental villous fragments was *trans*-stimulated by 1 mmol L^{-1} glutamate ($p < 0.001$, n = 4), 500 μmol L^{-1} and 1 mmol L^{-1} cystine ($p < 0.007$, n = 4), 500 μmol L^{-1} and 1 mmol L^{-1} NAC ($p < 0.005$, n = 4) but not by 1 mmol L^{-1} glycine which is not an xCT substrate or Tyrode's alone (n = 4 experiments, Fig. 2b).

3.3. NAC inhibits glutamate uptake in xCT-4F2hc transfected HEK293 cells

Under sodium free conditions, HEK293 cells transfected with both xCT and 4F2hc mediated significantly more ^{14}C-glutamate uptake compared to control non-transfected and xCT only transfected cells ($p < 0.05$, n = 3 independent experiments in triplicate, Fig. 3a). The addition of 1 mmol L^{-1} glutamate ($p < 0.001$), cystine ($p < 0.001$) and NAC ($p < 0.001$) all significantly inhibited ^{14}C-glutamate uptake in xCT-4F2hc

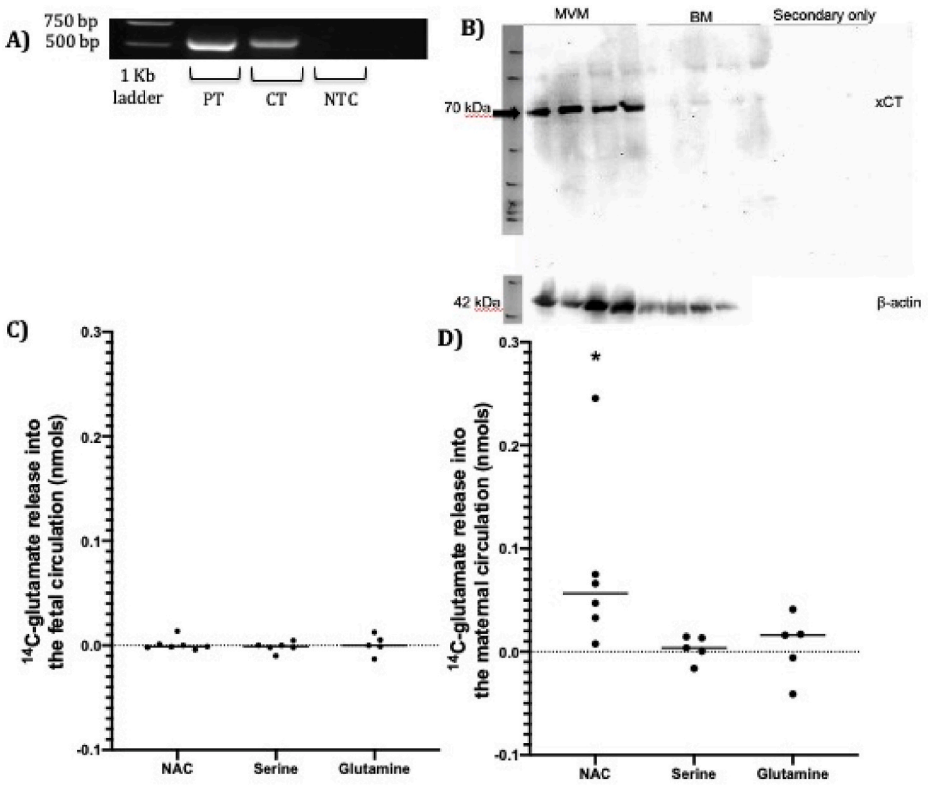

Fig. 1. xCT activity and immunoreactivity was observed on the maternal but not fetal side of the placenta. A) *SLC7A11* (xCT) mRNA expression in placental tissue (PT), isolated cytotrophoblast (CT) and in the no template control sample (NTC). **B**) xCT protein was localised to MVM but not BM preparations of the placental syncytiotrophoblast by Western blotting. Perfusion of the fetal **C)** and maternal **D)** circulation with NAC stimulated glutamate release into the maternal circulation of the isolated perfused human placenta (*$p < 0.05$, $n = 6$ perfusions). Serine ($n = 6$ perfusions) and glutamine ($n = 5$ perfusions) had no significant effect. The data are presented as median and interquartile range and analysed via a Wilcoxon signed rank test.

transfected cells as did 10 μmol L^{-1} of the xCT inhibitor, quisqualate ($p < 0.001$). Aspartate was added as a negative control (non xCT substrate) and did not alter ^{14}C-glutamate uptake in xCT-4F2hc transfected cells ($n = 3$).

3.4. Inhibition of xCT mediated glutamate uptake by cystine and NAC

In xCT-4F2hc transfected cells, cystine was shown to completely inhibit xCT-4F2hc mediated ^{14}C-glutamate uptake at all concentrations tested while NAC showed dose dependant inhibition of xCT mediated ^{14}C-glutamate uptake. This suggests that if NAC is an xCT substrate, then it is a lower affinity substrate than cystine (Fig. 3b).

3.5. Effects of NAC on the villous proteome

In total 8934 proteins were quantitatively profiled across all placental villous fragment samples (peptide level $q < 0.05$). Of these, 171 were differentially expressed in NAC treated fragments ($n = 4$) versus control ($n = 4$) with 80 proteins being downregulated and 91 being upregulated. Pathway Map analysis using MetaCore showed that pathways associated with oxidative stress, apoptosis, immune response and signal transduction were significantly over-represented among differentially expressed proteins (DEPs, Table 1 and Supplementary Figures 1–2 and Supplementary Table 1). Specific oxidative stress pathways altered by NAC treatment included role of ASK1 under oxidative stress ($q = 0.04$) and ROS induced cellular signalling ($q = 0.04$) Ingenuity Pathway Analysis (IPA) suggested that pathways associated with the acute phase response in the placenta were significantly inhibited following NAC treatment.

3.6. NAC inhibits osmotically activated glutamate release from placental tissues

Following placental perfusion of the maternal circulation with 50 mmol L^{-1} urea, an increase in ^{14}C-glutamate was observed in the maternoplacental venous outflow ($n = 6$, $p < 0.05$) but not in the fetoplacental circulation (Fig. 4a). In placentas pre-treated with 10 mmol L^{-1} NAC, no efflux of ^{14}C-glutamate was observed following a urea bolus (Fig. 4b, $n = 6$, $p < 0.05$).

Placental villous fragments treated with iso-osmotic urea Tyrode's stimulated release of ^{14}C-glutamate compared to Tyrode's alone ($p < 0.05$, $n = 6$, Fig. 4c). ^{14}C-glutamate release could be prevented by pre-treatment with 500 μmol L^{-1} NAC ($p < 0.01$, $n = 5$), 500 μmol L^{-1} cystine ($p < 0.01$, $n = 3$) and 500 μmol L^{-1} NAC with 100 μmol L^{-1} BSO ($p < 0.05$, $n = 3$) but not with 100 μmol L^{-1} BSO alone. ^{14}C-glutamate uptake did not differ between groups.

BeWo cells challenged with iso-osmotic urea containing solutions stimulated release of ^{14}C-glutamate ($p < 0.01$, $n = 3$) and this could be prevented by pre-treatment with 100 μmol/L NAC ($p < 0.05$, $n = 3$). Following urea treatment, cells pre-treated with dithiothreitol (DTT) released significantly less ^{14}C-glutamate compared to urea alone cells ($p < 0.05$, $n = 3$) and this was at a similar level of inhibition achieved by NAC treatment suggesting both DTT and NAC inhibit Maxi-chloride mediated glutamate efflux via the same mechanism (Fig. 4d).

4. Discussion

This study provides a mechanism by which N-acetylcysteine (NAC) could be taken up into the human placenta and demonstrates effects of NAC on placental tissue. NAC uptake into the placenta is likely to be mediated by the cystine/glutamate exchanger xCT which we localised to the microvillous membrane of the placental syncytiotrophoblast. We demonstrate that NAC causes rapid changes in function and the expression of redox sensitive protein pathways such as inhibition of apoptosis and the acute phase response, suggesting that NAC may have beneficial effects on the placenta.

Previously, we have shown that the glutamate gradient is important on both the basal membrane (BM) and microvillous membrane (MVM) of the syncytiotrophoblast for the uptake of xenobiotics from the fetal and maternal circulation respectively [29,30]. This current study

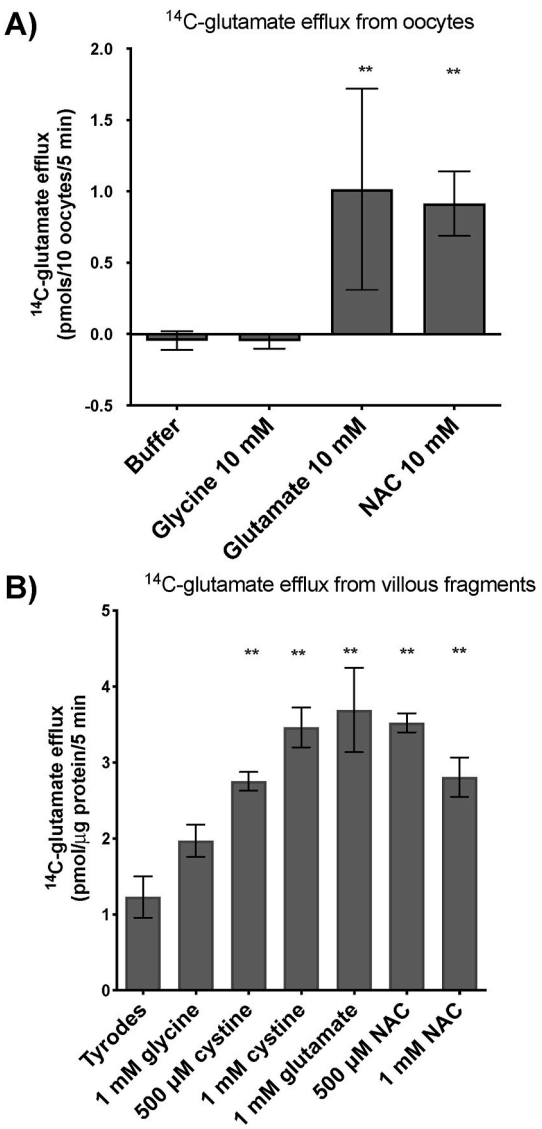

Fig. 2. NAC stimulates glutamate efflux from *Xenopus* oocytes expressing xCT-4F2hc and placental villous fragments. A) NAC mediates xCT mediated ^{14}C-glutamate efflux from *Xenopus* oocytes. Compared to non-injected controls, efflux of ^{14}C-glutamate was stimulated by glutamate (n = 5 experiments, **p < 0.01 vs buffer alone) and NAC (n = 6 experiments, **p < 0.01 vs buffer alone) but not by glycine. **B)** NAC and cystine stimulate ^{14}C-glutamate efflux from placental villous fragments. Compared to Tyrodes alone, ^{14}C-glutamate efflux was stimulated by 500 µmol L^{-1} cystine (**p < 0.01), 1 mmol L^{-1} cystine (**p < 0.01), glutamate (**p < 0.01), 500 µmol L^{-1} NAC (**p < 0.01) and 1 mmol L^{-1} NAC (**p < 0.01) (n = 4 experiments, duplicate conditions, 3 fragments per replicate). All data are presented as mean ± SEM.

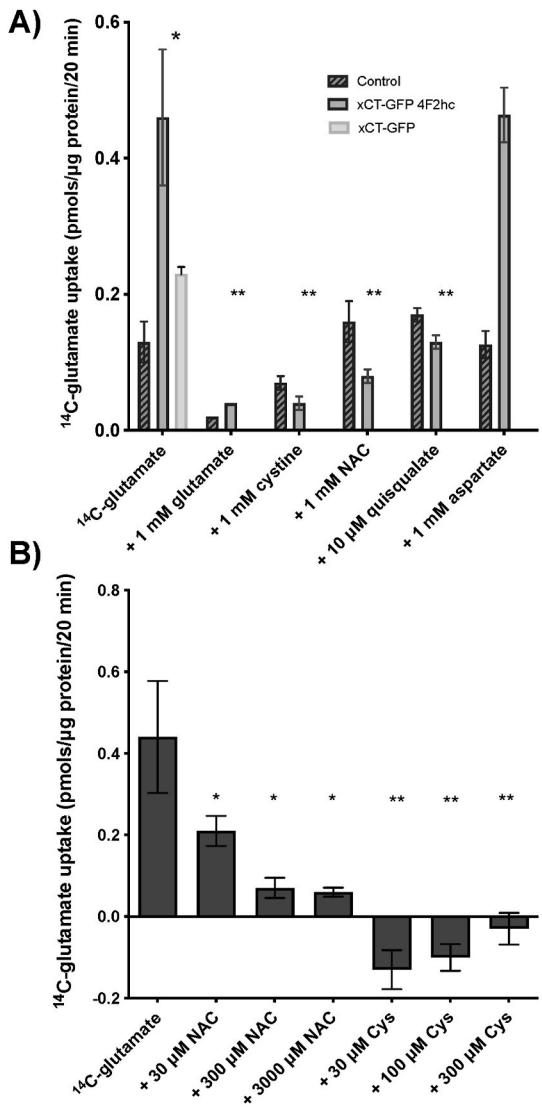

Fig. 3. NAC and cystine inhibit ^{14}C-glutamate uptake in xCT-4F2hc transfected HEK293 cells. A) ^{14}C-glutamate uptake in sodium free conditions was higher in xCT-4F2hc transfected cells compared to vehicle transfected cells (*p < 0.05). In xCT-4F2hc transfected cells the xCT inhibitor quisqualate, glutamate, cystine and NAC all significantly inhibited ^{14}C-glutamate uptake compared to ^{14}C-glutamate uptake alone (**p < 0.001) but aspartate did not (n = 3). **B)** ^{14}C-glutamate uptake in sodium free conditions was significantly decreased in xCT-4F2hc cells with increasing NAC (*p < 0.05) and cystine concentrations (**p < 0.001) compared to ^{14}C-glutamate uptake alone. 30 µmol L^{-1} cystine decreased ^{14}C-glutamate significantly more than 30–300 µmol L^{-1} NAC (*p < 0.05), suggesting that NAC is a weaker xCT substrate compared to cystine. Uptake is adjusted for uptake of ^{14}C-glutamate in vehicle-transfected cells (n = 3 experiments). All data are presented as mean ± SD.

suggests that the glutamate gradient may also be important for providing the placenta with antioxidant precursors from the maternal circulation via the activity of xCT. Glutathione synthesis relies upon the availability of glycine, glutamate and cysteine within the cell [31]. Within the placenta and many other tissues, glutamate and glycine levels are high [19], so cysteine availability becomes rate limiting for glutathione synthesis. Inside the cell, cysteine is used for glutathione production but outside the cell it is quickly oxidised to form cystine. As the majority of cysteine is derived from the diet, cystine uptake into the cell via xCT is important [32]. The expression of xCT on the MVM may facilitate glutathione biosynthesis by mediating the influx of glutathione's precursors cystine. The glutamate gradient will therefore drive the uptake of cystine via xCT.

Like cystine, NAC is broken down within the cell to produce cysteine and so it can also provide substrates for glutathione synthesis. However, it is not clear how NAC uptake is mediated. NAC is reported to be an 'activator' of the transporter xCT, suggesting a relationship between the two but this has not been well described in the literature [33]. There are three possibilities; first that NAC is a substrate of xCT, second that NAC binds the extracellular face and *trans*-activates efflux of substrates without itself being transported and finally that two NAC molecules are converted to cystine externally and then converted to cysteine inside the cell. The data presented here are consistent with NAC being a substrate for xCT. NAC stimulated glutamate efflux in *Xenopus* oocytes expressing xCT, cells transfected with xCT and in placental villous fragments. As

Table 1
Using a 5% false discovery rate (FDR - a global estimate of the false positives present in the results) we observed 171 proteins to be differentially expressed between NAC treated fragments compared to control placental fragments, 80 of which were upregulated and 91 downregulated. Metacore pathway analysis showed pathways associated with oxidative stress, immune response, apoptosis and signal transduction were significantly over-represented among differentially expressed proteins.

Network	Total	P value	FDR	In data
Cytoskeleton Actin filaments	176	1.488E-08	2.009E-06	16
Development Skeletal muscle development	144	2.723E-06	1.838E-04	12
Muscle contraction	173	9.132E-05	4.109E-03	11
Translation Regulation of initiation	127	1.809E-04	6.106E-03	9
Protein folding ER and cytoplasm	43	5.648E-04	1.463E-02	5
Cytoskeleton Regulation of cytoskeleton rearrangement	183	6.500E-04	1.463E-02	10
Protein folding Response to unfolded proteins	69	7.696E-04	1.484E-02	6
Immune response Phagosome in antigen presentation	243	1.654E-03	2.595E-02	11
Cytoskeleton Intermediate filaments	81	1.785E-03	2.595E-02	6
Cell adhesion Cell-matrix interactions	211	1.923E-03	2.595E-02	10
Immune response Phagocytosis	222	2.791E-03	3.425E-02	10
Cell adhesion Integrin-mediated cell-matrix adhesion	214	7.046E-03	7.839E-02	9
Signal transductionESR2 pathway	77	7.548E-03	7.839E-02	5
Inflammation Kallikrein-kinin system	185	9.303E-03	8.327E-02	8
Development Blood vessel morphogenesis	228	1.047E-02	8.327E-02	9
Proteolysis ECM remodeling	85	1.134E-02	8.327E-02	5
Inflammation IL-6 signaling	119	1.172E-02	8.327E-02	6
Proteolysis Connective tissue degradation	119	1.172E-02	8.327E-02	6
Protein folding Folding in normal condition	119	1.172E-02	8.327E-02	6
Development Keratinocyte differentiation	58	1.351E-02	9.120E-02	4
Cell adhesion Cell junctions	162	1.476E-02	9.489E-02	7
Blood coagulation	94	1.695E-02	1.040E-01	5
Translation Translation initiation	171	1.932E-02	1.134E-01	7
Cell adhesion Platelet aggregation	158	4.040E-02	2.273E-01	6
Response to hypoxia and oxidative stress	162	4.474E-02	2.416E-01	6
Cytoskeleton Macropinocytosis and its regulation	85	4.660E-02	2.420E-01	4
Reproduction Feeding and Neurohormone signaling	210	5.048E-02	2.524E-01	7

xCT is an exchanger it is unlikely that NAC could transactivate glutamate efflux in the absence of an extracellular substrate. However, while we provide evidence consistent with NAC uptake by xCT, we cannot conclusively exclude other possibilities. Further kinetic and HPLC studies which demonstrate direct uptake of NAC into the cell are required to definitively determine whether NAC is an xCT substrate. Assuming NAC is a xCT substrate, our cell culture data indicate that it is a lower affinity substrate than cystine.

This study found that NAC induced rapid changes in function of the redox sensitive Maxi-chloride channel and the expression of redox sensitive protein pathways, specifically inhibiting the acute phase response and altering the role of ASK1 under oxidative stress and ROS induced cellular signalling pathways. This suggests that NAC may downregulate apoptotic and inflammatory pathways and could therefore have a positive effect on the placenta. However, as NAC treatment also altered pathways associated with the immune response and signal transduction, the use of NAC in pregnancy needs to be carefully considered.

It is well established that the Maxi-chloride channel is activated in response to osmotic stress, mediating glutamate, taurine, aspartate and ATP efflux when opened [22]. However, this study is the first to demonstrate volume regulated glutamate release from intact human placenta. Furthermore, we showed that this volume regulated release was inhibited by the antioxidant NAC. We propose that this release is mediated by the Maxi-chloride channel, which in turn mediates glutamate efflux. Indeed the volume regulated release mediated by the Maxi-chloride channel has been shown to be sensitive to hydrogen peroxide and this effect was inhibited by NAC in patch clamping experiments with placental membranes [22,34,35]. It is unclear however whether NAC inhibits Maxi-chloride channel opening directly by scavenging free radicals or indirectly by contributing to glutathione synthesis. In this study, we demonstrate that NAC but not the glutathione inhibitor buthionine sulfoximine (BSO), inhibited volume regulated glutamate release suggesting that NAC directly scavenges the free radicals that are required for Maxi-chloride channel activation (Fig. 5). This is interesting as NAC is known to work through increasing glutathione production, for instance in treating paracetamol overdose, but the absence of an effect of BSO suggests NAC is having biologically relevant antioxidant effects independent of glutathione.

The volume regulated release of glutamate observed in placental perfusion experiments and BeWo cell culture was abolished following pre-treatment with NAC and DTT which are both antioxidants. Consistent with previous patch clamping studies this suggests that redox signals regulate the Maxi chloride channel [34]. This could be mediated directly via chemical modification of the extracellular regions of the channel protein or indirectly via the stimulation of glutathione production and intracellular redox signalling. If the direct extracellular route is the case this may be a way by which the mother's redox status affects placental function.

Maxi-chloride channel activation is initiated by osmotically induced cellular swelling [36]. However, *in vivo,* events such an osmotic shock to the placenta are unlikely and the role of the channel is unclear. One possibility is that Maxi-chloride channel activation occurs when there is a mismatch between the uptake and efflux of nutrients in the syncytiotrophoblast, leading to cellular swelling. Another possibility is that this channel is involved in volume regulation following cytotrophoblast fusion. Opening of the channel releases solutes leading to regulatory volume decrease but this may also have an autocrine role. The Maxi-chloride substrates glutamate and ATP have receptors on the placental surface so may act in an autocrine manner [37]. Glutamate has been suggested to have autocrine signalling roles in other tissues such as the pancreas [38], and ATP has itself been found to act as an autocrine regulator of the Maxi-chloride channel.

This work provides new insights into the role of glutamate within the placenta and why the placenta maintains such a large glutamate concentration gradient between tissue and plasma. Previously, we have shown that intracellular glutamate is an important counter-ion that drives transport of xenobiotics and hormone precursors across both the MVM and BM of the placental syncytiotrophoblast [29,30]. Maintaining this gradient is energetically expensive but in addition to driving xenobiotic and hormone transport, high intracellular glutamate levels also allows the placenta to drive the uptake of cysteine for glutathione synthesis and to mediate rapid cellular volume decrease. Within the placenta, a high intercellular glutamate concentration is important in mediating a range of cellular processes including nutrient uptake, maintaining oxidative status as well as volume regulation and/or

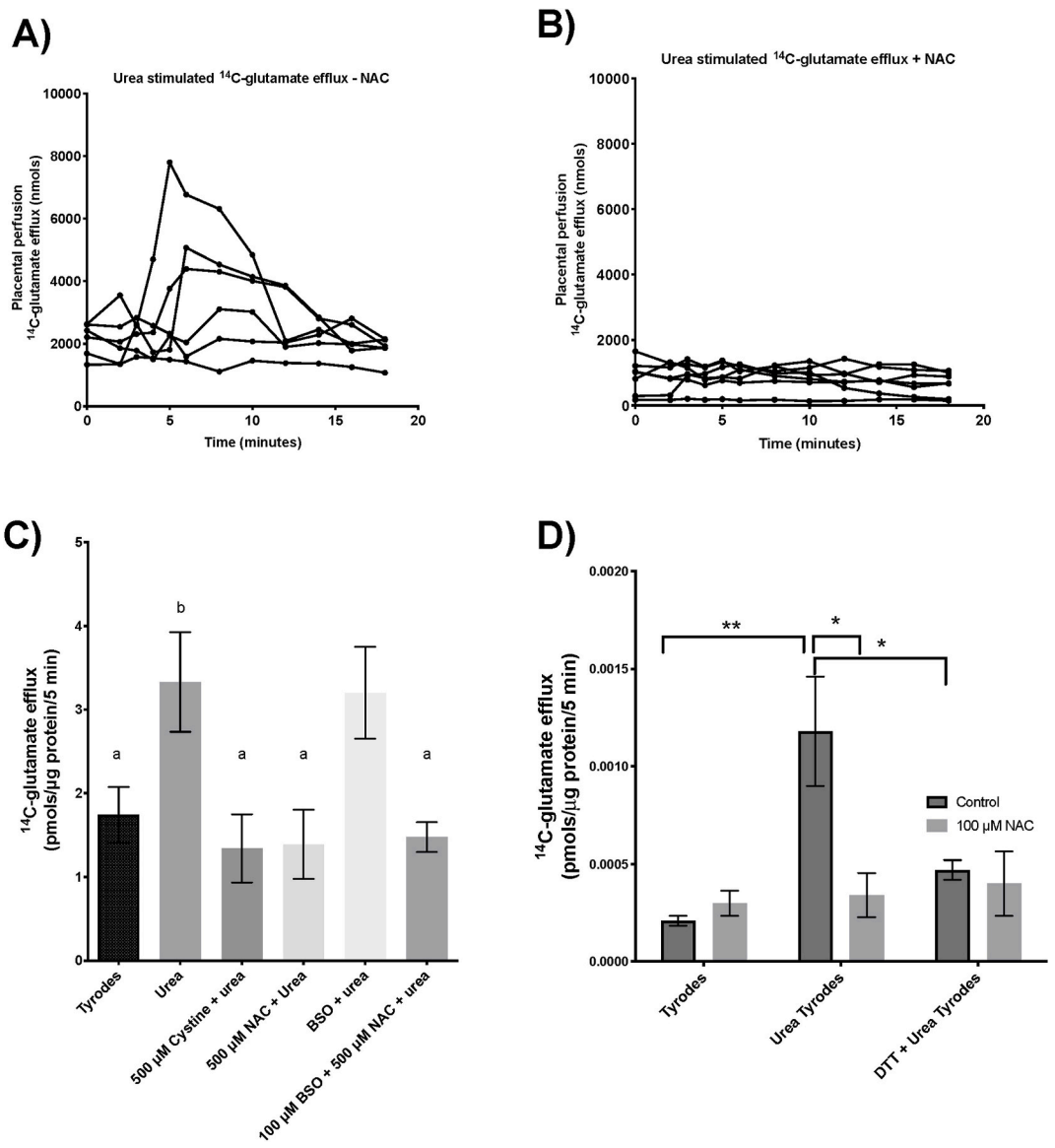

Fig. 4. Pre-treatment with NAC inhibits osmotically induced glutamate release from the MVM of the isolated perfused placenta, villous fragments and BeWo cells. A-B) In perfused placenta urea stimulates ^{14}C-glutamate efflux (A) but this is inhibited by pre-treatment with NAC (B) (n = 6 placentas for each condition AUC, *$p < 0.05$). **C)** In villous fragments urea stimulates ^{14}C-glutamate efflux ($p < 0.05$, n = 6 placentas, 3 fragments in duplicate per experimental group) and this is prevented by pre-treatment with NAC or cysteine. The glutathione synthesis inhibitor buthionine sulfoximine (BSO) with NAC pre-treatment inhibits ^{14}C-glutamate efflux ($p < 0.01$) but this was not seen with BSO alone. Data are presented as mean ± SEM. **D)** In BeWo cells urea stimulates ^{14}C-glutamate release (**$p < 0.01$, n = 4 experiments) and this is prevented by pre-treatment with NAC. DTT treatment also prevented ^{14}C-glutamate release compared to urea alone (*$p < 0.05$, n = 4 experiments). Data are presented as mean ± SEM.

autocrine signalling via the Maxi-chloride channel (Fig. 5).

High levels of oxidative stress are known to have deleterious effects in the first trimester of pregnancy and oxidative stress has been implicated in miscarriage and pre-eclampsia [39]. Before placental blood flow is established, extravillous trophoblast invade the maternal decidua and the spiral arteries are remodelled in order to create a low resistance circulation into the intervillous space. If this does not happen effectively, blood flow is impaired and hypoxia results which may last throughout the pregnancy and contribute to impaired placental function and subsequently poor fetal growth [8,40]. NAC could therefore be a potential beneficial supplement during pregnancy. Previous studies using antioxidants have had mixed results. One trial with vitamin C and vitamin E claimed to decrease the incidence of pre-eclampsia [41]. However, in a later trial in which both vitamin C (1000 mg) and vitamin E (400 IU) were given to women at risk of pre-eclampsia, there was no observed benefit but the incidence of women giving birth to low birth weight babies was higher in the experimental group compared to control [42].

There are few studies investigating NAC in pregnancy. One small randomised trial in women at risk of pre-eclampsia found that oral NAC was safe but did not prevent pre-eclampsia or affect gestation length [43]. There is therefore a lot of work to be done with regards to finding ways to treat oxidative stress pathologies in pregnancy. Although NAC has so far not been found to be beneficial to women with pre-eclampsia, this study suggests that NAC treatment can downregulate apoptotic and inflammatory pathways in the placenta. In addition, the administration of NAC administered to women potentially at risk of pre-eclampsia before they conceive may have beneficial effects on the endometrium and maternal vascular system facilitating effective implantation and conversion of the spiral arteries [43].

In conclusion, this study provides evidence that xCT mediates NAC uptake into the placenta and that NAC subsequently downregulates pathways associated with oxidative stress, inflammation and apoptosis.

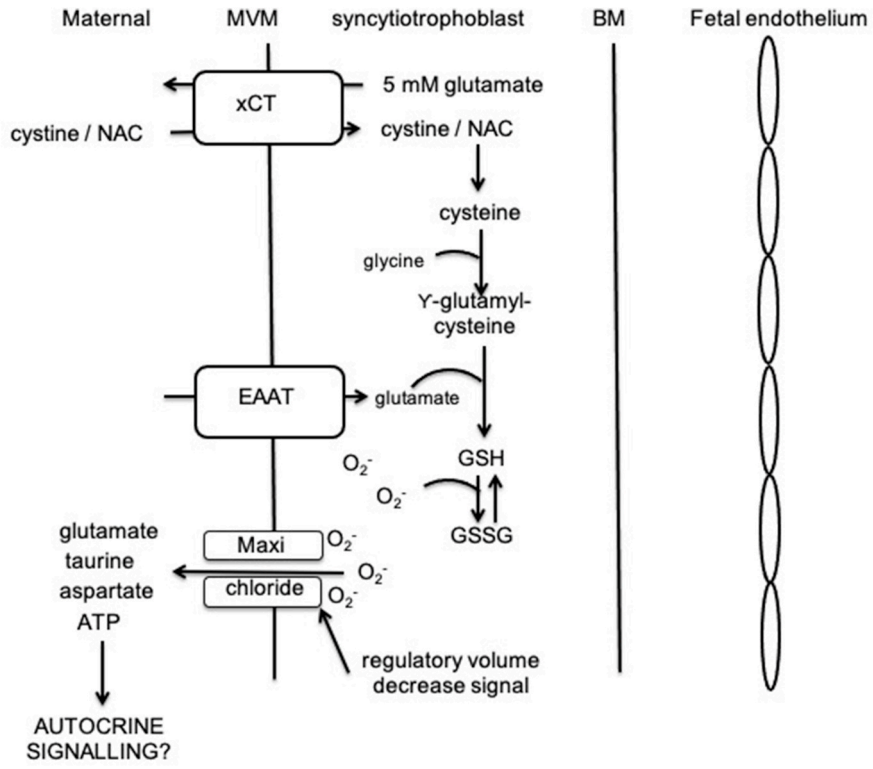

Fig. 5. Proposed interaction between xCT and Maxi-chloride channel. The transporters xCT and the excitatory amino acid transporters (EAATs) provide NAC/cystine and glutamate for the cell. In the presence of free radicals, the Maxi-chloride channel opens, mediating glutamate efflux. We propose that NAC presence contributes to free radical scavenging either directly (A) or by contributing to glutathione synthesis (B) and a reduction in Maxi-channel activation. As the glutathione inhibitor BSO did not inhibit urea stimulated glutamate efflux, we hypothesise that it more likely that NAC has a direct effect upon the channel rather than contributing to glutathione synthesis. The efflux of glutamate, taurine, aspartate and ATP via the Maxi-chloride channel may then participate in autocrine signalling mechanisms.

NAC was also shown to regulate the redox sensitive Maxi-chloride channel, inhibiting regulatory volume decrease. Taken together, these findings suggest that NAC reduces placental oxidative stress suggesting it may be beneficial to the placenta. NAC is known to act through an increase in glutathione production but this study suggests that NAC alters redox status, independently from glutathione. While further work is required, the use of NAC may promote a better physiological balance, normalising redox status, without inhibiting physiological redox signalling.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2021.05.009.

Data availability statement

The data that support the findings of this study are openly available in ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011425 (Reviewer account details: Username: reviewer25729@ebi.ac.uk Password: VIH27nwJ).

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