Preeclampsia (PE) represents a major factor for maternal and perinatal morbidity and mortality [1] and it affects 7–10% of pregnancies worldwide [2]. PE is characterized by maternal syndromes such as gestational hypertension, proteinuria, oedema and, in 30% of cases, intrauterine growth retardation (IUGR). The precise factors involved in the pathogenesis of PE remain unclear and it is considered as a multisystem disorder [1]. PE is principally characterized by impaired placenta and would be the result of an impaired differentiation of villous trophoblasts, which leads to an abnormal remodelling of the spiral arteries [3]. Diabetes, obesity, hypertension, renal disease, primiparity, maternal age, sperm and oocyte donation are all known predisposing factors for PE [1]. The oxidative stress, resulting from deficient remodelling of spiral arteries, is an important consequence of PE. It induces the placenta...
to release various factors, as inflammatory cytokines, apoptotic wastes and anti-angiogenic factors [4]. These secreted soluble factors are then thought to alter endothelial metabolic status, mitochondrial integrity and vascular functions [5]. Oxidation of DNA bases by endogenously generated and environmental reactive oxygen species (ROS) leads to mutations. Guanine is particularly prone to oxidation by ROS. 8-Oxo-deoxyguanine (8-oxo-dG) is a quantitatively major form of oxidative DNA damage [6, 7], inducing mainly G (guanine) to T (thymine) and A (adenine) to C (cytosine) substitutions [8]. Human 8-oxoguanine-DNA glycosylase 1 (hOGG1) is the main enzyme that excises 8-oxo-dG from damaged DNA via the base-excision repair pathway [9]. It was suggested that cells under oxidative stress may require increased expression of hOGG1 to protect them from oxidative damage-induced mutations [10].

In addition, during PE, the oxidative stress exposes the placenta to fluctuating oxygen concentration [11] and this periodic hypoxia will deplete placental cells from ATP. More precisely, it was observed that lower ATP levels in PE placental cells are due to an important change in expression of ATP synthase gene [12], an important mitochondrial enzyme synthesizing ATP from ADP.

The most effective treatment for PE is delivery itself; however, several randomized trials reported the effective use of various methods to reduce the rate or severity of PE [1], such as calcium (Ca²⁺) supplementation, but results showed minimal to no benefit. Several alterations in maternal Ca²⁺ homeostasis were identified in PE, such as low urinary Ca²⁺ excretion and low circulating level of 1,25-dihydroxyvitamin D₃, parathyroid hormone-related peptide and calcitonin gene related peptide [13, 14]. Epidemiological data suggest an inverse correlation between dietary Ca²⁺ and calcitonin gene related peptide [13, 14]. In a previous experiment, we quantified secretion of hCG, a well-known marker of this differentiation process for a period of 7 days [25], and interestingly, we observed that maximal hCG secretion was at day 4 of culture and paralleled the peak in Ca²⁺ transport [26]. Intracellular Ca²⁺ signalling pathway can be briefly described as extracellular stimulations of tyrosine kinase and G-protein-coupled receptors launching cascade reactions leading to the production of the second messenger IP3 (inositol1–4, 5-triphosphate) [27, 28]. IP3 will bind to its receptor (IP3R) and Ca²⁺ will be released from the endoplasmic reticulum (ER), triggering Ca²⁺ entry through the different plasma membrane store-operated channels. Ryanodine receptors (RyRs) are multimeric Ca²⁺ channels in the ER membrane, which also participate in the release of Ca²⁺ from the ER [29, 30]. Ca²⁺ release from ER must be followed by Ca²⁺ replenishment via sarcoendoplasmic reticulum Ca²⁺ ATPases (SERCAs) [31, 32]. In addition, the mitochondria constitutes another Ca²⁺ storage organelle which can control the amplitude, length, localization and propagation of cytosolic calcium elevations, and the Ca²⁺ recycling back to the ER. Ca²⁺ accumulation by the mitochondria implicates VDACs (voltage-dependent anion channels) [33]. Roles of VDACs and SERCAs are essential in maintaining a low resting intracellular calcium concentration [Ca²⁺ᵢ] [34].

The Ca²⁺ transepithelial transfer through the ST is a passive-transport and requires various proteins. The Ca²⁺ diffuses from the maternal blood to the cytoplasmic compartment of the syncytium through specific channels named ‘transient receptor potential vanilloid’ (TRPV) on the brush border membrane using the electrochemical gradient. Then, calcium-binding proteins (CaBP) bind Ca²⁺ and shuttle it to the foetal face of the ST where it is actively excreted by pumps (PMCA) [35, 36]. Ca²⁺ signalling pathways in the placenta are still under investigation and there is very little information concerning the expression of these proteins in PE placental tissues.

Because PE is potentially associated with an impaired maternal Ca²⁺ homeostasis, we suggested that transplacental Ca²⁺ exchange by ST is disturbed in PE. Our objectives were to: (i) characterize the placential Ca²⁺ transfer in PE; (ii) identify altered gene expression of Ca²⁺ handling proteins involved in transplacental Ca²⁺ transport, such as Ca²⁺ channels TRPV5 and 6, CaBP-9K, CaBP-28K and PMCA1 to 4; (iii) investigate Ca²⁺ intracellular signalling in PE placentas, involving ER and mitochondria, by studying the expression pattern of IP3Rs, RyRs, SERCAs and VDACs and (iv) to find what may be the cause of such changes in PE, as inadequate ATP and ROS levels.
Materials and methods

Placental tissues

Placental tissues were obtained from human placentas in accordance with the established guidelines of the ethical committee of St. Luc Hospital of the Centre Hospitalier Universitaire de Montréal and of Université du Québec à Montréal, (Montréal, QC, Canada). Placentas were obtained immediately after vaginal delivery, from normal term pregnancies (n = 16) and from PE pregnancies (n = 8). In order to avoid inter-ethnic variability of 1,25(OH)2D3 and Ca2+ metabolism, only Caucasian women were invited to participate to this study. The sample contained women without pre-existing clinical risk factors of gestational hypertension. The following conditions were excluded from this study: pre-pregnancy diabetes mellitus, gestational diabetes, foetal physical or chromosomal abnormalities, untreated hypo/hyper thyroidism, tobacco and alcohol use, renal diseases with altered renal function, active and chronic liver disease, cancer, collagen diseases, congenital rickets and other malformations. The control conditions were excluded from this study: pre-pregnancy diabetes mellitus, gestational diabetes, foetal physical or chromosomal abnormalities, untreated hypo/hyper thyroidism, tobacco and alcohol use, renal diseases with altered renal function, active and chronic liver disease, cancer, collagen diseases, congenital rickets and other malformations. The control group was represented by women without diagnosed pathologies, with no drugs intake. PE was diagnosed by a clinician and classified according to strict criteria recommended by the Society of Obstetricians and Gynaecologists of Canada [37]: a systolic blood pressure of 140 mmHg or higher or a diastolic blood pressure of 90 mmHg or higher on two occasions at least four hours apart, occurring after 20 weeks of gestation in a pregnant woman with previously normal blood pressure, and detectable proteinuria (>0.3 g/24 hrs). This group also includes the late onset of PE characterized by the late manifestation of PE and excludes pregnancy with IUGR, SGA (small for gestational age) foetus or HELLP (haemolysis, elevated liver enzymes, low platelet count) syndrome. Four of our PE patients were treated with magnesium sulphate (MgSO4), safe for both mother and foetus [38], which prevents seizures by altering membrane permeability and increasing seizure threshold [39]. Interestingly, MgSO4 infusion caused significant increases in ionized Mg2+ levels, while serum ionized Ca2+ concentrations were unchanged [40], suggesting that the effect of MgSO4 is not exerted through modulations of ionized calcium levels [41]. Also, it was observed that in the normal pregnant women, ionized Ca2+ levels were higher in foetal blood and that this same condition was found in PE women treated with MgSO4 [42], suggesting that this treatment has no effect on Ca2+ transplacental transfer. Characteristics of pregnancies are summarized in Table 1. From the placentas, maternal and foetal membranes were removed and small pieces of tissue from several cotyledons (5–10) were collected to have a good representation of the total placenta [43]. For RNA sample, tissues were embedded in RNA later stabilization reagent (Qiagen, Mississauga, Ontario, Canada), frozen in liquid nitrogen and stored at −80°C. Tissue samples for protein extraction were directly frozen into liquid nitrogen and subsequently transferred into sterile tubes for storage at −80°C.

RNA extraction and cDNA synthesis

RNA was isolated from total placenta (30 mg) using RNeasy mini kit extraction columns (Qiagen) and reverse-transcribed. After RNA isolation, 1 μg of RNA was reverse transcribed into cDNA at 37°C for 1 hr using 10 μM poly(dT) (Roche Applied Science, Laval, QC, Canada), 0.5 mM dNTP and 4 U of Omniscript reverse transcriptase (Omniscript RT kit) (Qiagen) in a 20 μl final volume. Exceptionally for the CaBP-28K gene, after reverse transcription, cDNA were first preamplified with the LightCycler RNA Pre-Amplification Kit (Roche Applied Science) before real-time PCR analyses.

Real-time PCR

Quantification of the mRNA coding for TRPV5, TRPV6, CaBP-9K, CaBP-28K, PMCA1, PMCA2, PMCA3 and PMCA4 was performed by real-time PCR using the Roche LightCycler 480 PCR (Roche Applied Science). All primer sequences (Table 2) were generated through Primer3 input (http://fokker.wi.mit.edu/primer3/input.htm), verified for specificity by BLAST analyses and designed to span from two different exons. PMCA2 and PMCA3 primers were purchased from Qiagen, Inc. Real-time PCR reactions were performed in a volume of 10 μl containing 1 μl of cDNA, 1 μM of each primer and 5 μl of SYBR Green I Master (Roche Applied Science). Quantification of the endogenous ribosomal gene hypoxanthine phosphoribosyltransferase (HPRT)1 was performed to normalize variations in cDNA content among different samples. The expression of HPRT1 remained stable under our experimental conditions. Results were analysed with the LightCycler 480 software version 1.5, and melting curves were used to verify the specificity of the amplified products. Quantification of PCR products was performed through a standard curve generated by simultaneously amplifying serial dilutions of the corresponding cDNA. Values obtained for each gene were normalized to HPRT1 in the same sample.

Western blots analysis

Tissues were initially washed with lymphocyte lysis buffer containing 1.7 mM Tris-HCl (pH 7.3), 1.44 mM NaCl and protease inhibitor cocktail in tablets and then proteins were purified by the addition of the extraction buffer containing 125 mM Tris-HCl (pH 8.0), 2 mM CaCl2, 1.4% Triton X-100 (v/v) and protease inhibitor cocktail in tablets (Roche Applied Science). Protein concentrations were evaluated with BCA (bicinchoninic acid) assay (Pierce, Brookville, Ontario, Canada). Cellular proteins (100 μg) were solubilized in Laemmli buffer and denatured by heating at 95°C for 5 min. Lysates were resolved in 10% SDS-PAGE and proteins were electro-blotted onto a polyvinyldene difluoride (PVDF) membrane (Millipore, Cambridge, Ontario, Canada) at 17 V for 30 min. Membranes were blocked for 1 hr at room temperature in TBS-T (20 mM Tris (pH 7.6),

### Table 1 Clinical characteristics of mothers and babies

| Characteristics          | Normal group (n = 16) | PE group (n = 8) |
|--------------------------|----------------------|-----------------|
| Maternal age (years)     | 33.7 (±5)            | 34 (±4)         |
| Gestational age (weeks)  | 37.9 (±1.7)          | 36.7 (±2)       |
| Body mass index (kg/m²)  | 21.2 (±3)            | 26.9 (±4)       |
| Placental weight (g)     | 533 (±115)           | 518 (±130)      |
| Babies birth weight (kg) | 3.1 (±0.36)          | 2.9 (±0.5)      |
| Mode of delivery         | Vaginal              | Vaginal         |
| Smoking                  | No                   | No              |
Table 2  Real-time PCR primer sequences

| Gene      | Primer sequence sense antisense | Product size (bp) |
|-----------|---------------------------------|------------------|
| CaBP-9k   | 5’ ATA TGC AGCCAA AGA AGG TG 3’ 5’ TGG ACC TTT GAG TAA ACT GGG 3’ | 94               |
| CaBP-28k  | 5’ TCA GGA CGG CAA TGG ATA CA 3’ 5’ AAG AGC AAG ATC CTT TCG GT 3’ | 166              |
| TRPV5     | 5’ AGA CAA GGA GGA TGG CCA GGA 3’ 5’ CCC AGG GTG TTT TGA CAA AG 3’ | 168              |
| TRPV6     | 5’ TCT GCG AGC GGA AGT ATG G 3’ 5’ CCT GTG CGT AGC GTT GGA T 3’ | 96               |
| PMCA1     | 5’ CAG CAG GAG AAC CAG AAC CA 3’ 5’ ATT CCA GCC CTC TGA CAC TT 3’ | 139              |
| PMCA2     | Hs_ATP2B2_1_SG QuantiTect Primer Assay (Qiagen, Mississauga, Ontario, Canada) | 220              |
| PMCA3     | Hs_ATP2B3_1_SG QuantiTect Primer Assay (Qiagen) | 149              |
| PMCA4     | 5’ TCGAAGATCCCAACGTTG 3’ 5’ TCATGACAGTGGCTACC3’ | 148              |
| ATPase    | 5’ CGC CAC CCT AGC AAT ATC AA 3’ 5’ TTA AGG CGA CAG CGA TTT CT 3’ | 98               |
| IP3R1     | 5’ TGA CGA GAA CCT GGC CTA T 3’ 5’ TCC TTT GCG CAT CTT GCT 3’ | 430              |
| IP3R2     | 5’ GCA GTC GTG TCT GTT CCA 3’ 5’ TCT TCA AGT CTC AGC ATC G 3’ | 332              |
| IP3R3     | 5’ GCC TAC TAT GAG AAC CAC ACG 3’ 5’ CAG AAG AGC AAT GAG ATG AGA G 3’ | 389              |
| RyR1      | 5’ TGA CTA CCA TCA CGA CCA CA 3’ 5’ AGG AAG AGC GGA AAT A 3’ | 297              |
| RyR2      | 5’ TTT ACC AGC ACC CTA ATC TC 3’ 5’ CAG CCA AAT AAC CAA CTA CC 3’ | 348              |
| RyR3      | 5’ TCT GCT GTC TGG GTC TAT CTC 3’ 5’ CCA AAT GTG TTA TGC GTC AC 3’ | 262              |
| SERCA1    | 5’ GTG ATC CAG CAC CTA ATG 3’ 5’ CGA ATG TCA GGT CTT CAC TCT 3’ | 361              |
| SERCA2    | 5’ CGC TAC CTC ATC TCG TCC A 3’ 5’ TCG GGT ATG ATG GGG ATT CAA 3’ | 406              |
| SERCA3    | 5’ GAT GGA GTG AAT GCA GCA 3’ 5’ CCA GTT ATC AGA AGA AGA G 3’ | 409              |
| VDAC1     | 5’ CAC CAC GTA TGC CGA TCG TCT GT 3’ 5’ TGC TTG AAA AAC TCC CAC TG 3’ | 340              |
| VDAC2     | 5’ CAC CTC ATG TGC TTA TGC TCC C 3’ 5’ AGC CTC CAA CTC CAG GGC 3’ | 340              |
| VDAC3     | 5’ CAC CAC ACC AAC GTA CTA ATG TG 3’ 5’ AGC TTC CTC TAA CAC TCC 3’ | 340              |
| hOGG1     | 5’ TGG AGG AAC AGG GGG GCC TA 3’ 5’ ATG GAC ATC CAC GCC CAG AC 3’ | 164              |
| HPRT1     | 5’ GAC CAG TCA ACA GGG GAC ATA A 3’ 5’ AGG CTT GGC ACC TTG ACC 3’ | 149              |

Table 3  Antibodies information

| Names      | Companies                           | Dilution |
|------------|-------------------------------------|----------|
| TRPV5      | Alpha Diagnostic International, San Antonio, TX, USA | 1/1000   |
| TRPV6      | Alomone Labs, Jerusalem, Israel     | 1/1000   |
| CaBP-9k    | Santa Cruz, Santa Cruz, CA, USA     | 1/1000   |
| CaBP-28k   | Sigma-Aldrich, Oakville, Ontario, Canada | 1/1000 |
| PMCA1/4    | Santa Cruz                          | 1/1000   |
| Antimouse-IgG HRP-conjugated | Millipore, Billerica, MA, USA | 1/3000   |
| Anti-rabbit-IgG HRP-conjugated | Cell Signaling, Danvers, MA, USA | 1/2500   |
| Cytokeratin-7 FITC-conjugated  | Abcam, Cambridge, MA, USA  | 1.25 μg per 10^6 cells |

137 mM NaCl and 0.1% Tween-20) containing 5% skimmed milk. Membranes were then incubated with the appropriate primary antibody (see Table 3 for details on antibodies) in TBS-T–3% bovine serum albumin (BSA) overnight at 4°C, washed three times with TBS-T and probed with horseradish peroxidase-conjugated secondary antibodies for 2 hrs at room temperature. Blots were washed three times with TBS-T and the detection was performed with the BM Chemiluminescence (POD) system (Roche Applied Science) and visualized on Kodak(tm) X-omat™ Blue XB-1 autoradiography films from Perkin Elmer (Waltham, MA, USA). PVDF membranes were stripped with a stripping solution containing 25 mm glycine-HCl (pH 2) and 1% SDS at room temperature for 10 min., rinsed twice with PBS (10 mM sodium phosphate (pH 7.2) and 0.9% NaCl) and blocked for 1 hr before reprobing with another antibody. Precisely, we loaded eight samples/gel (eight normal NaCl) and blocked for 1 hr before reprobing with another antibody. Precisely, we loaded eight samples/gel (eight normal NaCl) and blocked for 1 hr before reprobing with another antibody. Precisely, we loaded eight samples/gel (eight normal NaCl) and blocked for 1 hr before reprobing with another antibody. Precisely, we loaded eight samples/gel (eight normal NaCl) and blocked for 1 hr before reprobing with another antibody. Precisely, we loaded eight samples/gel (eight normal NaCl) and blocked for 1 hr before reprobing with another antibody.
Cell cultures

For Ca\(^{2+}\) transport assay, primary cytotrophoblast cells were prepared from placentas obtained immediately after vaginal delivery, from normal term pregnancies (\(n = 5\)) and from PE pregnancies (\(n = 4\)) (see characteristics in Table 1). Cytotrophoblast cells were isolated by the trypsin (Sigma)-DNase (Roche Applied Science)/Percoll (Sigma) method as described by Kliman et al. [25], with some modifications [44]. Following cell isolation, cytotrophoblast cells were seeded at a density of \(1.5 \times 10^{5}\) cells per well in 24-well plate (Corning, Acton, USA) and maintained in Dulbecco’s modified Eagle’s medium (high glucose) (DMEM-HG) (Sigma) containing 10% FBS (Cansera International Inc, Etobicoke, Ontario, Canada), 2 mM glutamine (Gibco-Invitrogen, Burlington, Ontario, Canada), 25 mM HEPES (Sigma) and PSN, a penicillin-streptomycin-neomycin antibiotic mixture (Gibco-Invitrogen). The medium was refreshed daily. The purity of the cytotrophoblast cell preparation was evaluated by flow cytometry using FITC-conjugated monoclonal antibody against cytokeratin-7 as previously described [44].

MTT Assay

The cells viability was measured by the MTT assay (Sigma). Cells were cultured with DMEM-HG without phenol red and were incubated with 100 \(\mu\)l MTT per well each day. Plates were incubated for 1 hr at 37°C and 200 \(\mu\)l of DMSO were added to each well. After mixing, the optical density was measured at 570 nm.

\(\text{Ca}^{2+}\) transport assay

\(\text{Ca}^{2+}\) transport studies were performed on trophoblast cells after 4 days of culture. Briefly, cells were washed twice with the \(\text{Ca}^{2+}\) uptake buffer (HBSS [Hanks’ balanced salt solution from Sigma] containing 1.26 mM CaCl\(_2\), 10 mM HEPES, and 0.1% BSA [from Roche Applied Science]) and allowed to equilibrate in the same buffer (250 \(\mu\)l) for 10 min. Thereafter, cells were incubated at 37°C for different intervals of time after the addition of 250 \(\mu\)l of uptake buffer containing \(^{45}\text{CaCl}_2\) (5 \(\mu\)Ci/well) (MP Biomedicals, Inc., Irvine, CA, USA). The incubation was stopped by aspiration of the uptake buffer. The cells were washed three times with 1 ml of ice-cold PBS containing 4 mM ethylene glycol tetraacetic acid (EGTA) (to eliminate the nonspecific component of the uptake), then solubilized in 0.1 M NaOH. The cell-associated radioactivity was measured by a \(\beta\)-scintillation counting (Roche Applied Science) with some modifications [44]. Following cell isolation, cytotrophoblast cells were seeded at a density of \(1.5 \times 10^{5}\) cells per well in 24-well plate (Corning, Acton, USA) and maintained in Dulbecco’s modified Eagle’s medium (high glucose) (DMEM-HG) (Sigma) containing 10% FBS (Cansera International Inc, Etobicoke, Ontario, Canada), 2 mM glutamine (Gibco-Invitrogen, Burlington, Ontario, Canada), 25 mM HEPES (Sigma) and PSN, a penicillin-streptomycin-neomycin antibiotic mixture (Gibco-Invitrogen). The medium was refreshed daily. The purity of the cytotrophoblast cell preparation was evaluated by flow cytometry using FITC-conjugated monoclonal antibody against cytokeratin-7 as previously described [44].

Statistical analyses

Data were expressed as the mean ± S.E.M. and were analysed using GraphPad Prism software for windows, version 5.0. One-way ANOVA followed by the Tukey’s test was used to analyse \(\text{Ca}^{2+}\) transport and nonparametric Mann-Whitney U-test was used to determine differences in mRNA and protein levels between normal and PE placentas. A \(P\)-value < 0.05 was considered statistically significant.

Results

\(\text{Ca}^{2+}\) transport is less efficient in ST from PE placentas than in normal ST

To assess whether \(\text{Ca}^{2+}\) homeostasis is affected in PE, we first examined the \(\text{Ca}^{2+}\) transport in primary ST, obtained from normal and PE placentas. The \(\text{Ca}^{2+}\) transport assay was performed by means of a radioactive form of calcium (\(^{45}\text{CaCl}_2\)) for different time intervals (10, 20, 30, 40, 50 sec., 1, 2, 3, 4, 5, 10, 15, 20, 30 min.). As we described earlier, isolated trophoblasts undergo morphological changes during culture to form the functional multinucleated syncytiotrophoblast, which is properly developed after 96 hrs of culture. Therefore, we measured the cell-associated radioactivity after 4 days of culture, based on \(\beta\)-scintillation. The \(\text{Ca}^{2+}\) transport was expressed as nmole of \(\text{Ca}^{2+}\) (from specific activity) per milligram of cellular proteins. The \(\text{Ca}^{2+}\) uptake from normal and PE ST is rapid and there is no difference during the first 2 min. with an initial velocity (\(V_i\)) of 4.39 ± 0.73 nmol/mg/min for PE and 3.78 ± 1.04 nmol/mg/min for normal group. Then, the \(\text{Ca}^{2+}\) transport reaches a plateau, which represent a balance between the \(\text{Ca}^{2+}\) entry and exit, showing a decrease of 60% in PE group (3.75 ± 0.31 nmol/mg protein) compared to normal group (8.99 ± 0.48 nmol/mg protein) at 10, 20 and 30 min. The plateau is reached earlier for the PE group (after 2 min. of treatment) than the normal group (after 10 min. of treatment). The plateau is generally known to reflect cells \(\text{Ca}^{2+}\) entry and exit (Fig. 1A). To make sure that the \(\text{Ca}^{2+}\) transport in PE cultures was not affected by a probable higher rate of apoptosis, we measured the cells viability in the two cellular populations and there was no difference between normal and PE trophoblast cells (Fig. 1B). These observations suggest strongly that in PE, \(\text{Ca}^{2+}\) transfer by ST is compromised.

To study whether the decreased calcium transport in placentas from PE women is due to a decrease of calcium-handling protein in ST, we extracted mRNA and proteins from total placental tissues and we compared the changes in protein and mRNA levels, by Western blot and real-time PCR, of several genes implicated in placental \(\text{Ca}^{2+}\) transport in normal and PE placentas.

TRPV5 and TRPV6 expression is decreased in PE

To establish if the entry of calcium in the placenta is disturbed during PE, we examined the protein and mRNA expression of TRPV5 and TRPV6, gatekeepers of \(\text{Ca}^{2+}\) transport. We found that the protein expression of TRPV5 was significantly decreased in PE compared to normal placental tissues (Fig. 2A), as its mRNA expression (Fig. 2B). Similarly, TRPV6 protein and mRNA expression decreases in PE compared to normal group (Fig. 2C, D). These findings suggest that the decrease in the \(\text{Ca}^{2+}\) transport in ST is partially due to an impaired entrance in the placenta.
CaBP-9K and CaBP-28K expression is decreased in PE

Immediately after its penetration in the ST, Ca\(^{2+}\) moves toward the foetal side, where it is extruded, or it is buffered to avoid reaching lethal concentration for the cell. Those tasks are accomplished by calcium binding proteins, as CaBP-9K and CaBP-28K. To verify if this system is altered in PE, we analysed their protein and mRNA expression. We found that the protein expression of CaBP-9K is decreased in the PE placental tissue compared to normal ones (Fig. 3A) as well as mRNA expression (Fig. 3B). For the gene CaBP-28K, the protein and mRNA expression is also decreased in PE placentas (Fig. 3C, D). These observations suggest that Ca\(^{2+}\) buffering is altered in PE placental cells.

PMCAs expression is decreased in PE

Once arrived on the other side of the cell, Ca\(^{2+}\) has to cross over a last membrane to reach the foetal circulation. This expulsion is mainly achieved by the ATPase pumps PMCAs, which respond to foetal specific needs in calcium. To determine if an impaired Ca\(^{2+}\) exit from ST could be responsible for the impaired placental transfer seen in Fig. 1, we studied protein and mRNA levels of 4 isoforms of PMCA (PMCA1–4). For the protein expression, we used an antibody that detects PMCA1 and PMCA4 together. Thus, we observed that the protein expression of PMCA 1/4 (Fig. 4A) decreases significantly in PE compared to normal. The viability was measured by the MTT assay and the results are shown as optic density measured at 570 nm of the blue formazan crystals formed.
findings indicate that the Ca\(^{2+}\) extrusion towards the foetal side is compromised in PE.

**ATP synthase level is decreased in PE**

PMCs are ATPase pumps, thus they need ATP to function properly. As it was observed that lower ATP levels in PE placental cells is due to an important change in expression of ATP synthase gene [12], we wanted to verify if that phenomenon was present in our tissue samples and if it could be the cause of the reduction of PMCs. Therefore, we analysed mRNA levels of the mitochondrial ATP synthase gene in normal and PE placental tissues (Fig. 5). We observed that its mRNA expression was drastically decreased PE compared to normal. This result suggests, as we suggested, that depletion of ATP by hypoxia affect PMCs expression in PE tissues.

**Ca\(^{2+}\) intracellular signalling implicating mitochondria and ER is altered in PE**

Following the decrease of CaBPs (Fig. 3) and PMCs (Fig. 4) expressions, respectively, responsible for Ca\(^{2+}\) buffering and extrusion, we suggested that placental cells may suffer of Ca\(^{2+}\) overload. Therefore, we analysed the mRNA expression of IP3R and RyR, molecules in charge of the Ca\(^{2+}\) release from the ER, increasing Ca\(^{2+}\) intracellular concentration. As we show in Figs 6 and 7, IP3R and RyR mRNA expressions are decreased in PE placentas compared to normal ones. In parallel, we also analysed
mRNA expression of SERCA, transferring Ca\(^{2+}\) from the cytosol of the cell to the lumen of the ER, to see if it responded to that impending Ca\(^{2+}\) overload by diminishing [Ca\(^{2+}\)]. Unsurprisingly, we obtained an increase in mRNA of SERCA gene in PE placentas (Fig. 8). Likewise, the mitochondria is another important organelle that stores Ca\(^{2+}\) and as its replenishment implicates VDAC gene, we analysed its mRNA expression and also observed a significant increase in PE placentas (Fig. 9). These results support our hypothesis about the alteration of intracellular Ca\(^{2+}\) signalling in placentas with PE, more precisely of Ca\(^{2+}\) overload.

**Presence of high hOOG1 mRNA expression: PE placentas undergo severe oxidative stress**

hOOG1 is an enzyme repairing DNA oxidation damages by excising oxidized-guanine. As the presence of elevated levels of hOOG1 correlates with elevated ROS levels, we analysed its mRNA expression to determine, as we suggested, if PE placentas are more affected by oxidative stress than normal ones. Interestingly, we observed a huge increase of hOOG1 mRNA expression in PE placentas (Fig. 10). This result confirms that in PE, placental cells suffer from severe oxidative stress, which surely alters many cellular mechanisms, as Ca\(^{2+}\) signalling.

**Discussion**

In this study, we demonstrated for the first time a significant decrease in Ca\(^{2+}\) transport in primary cultures of ST from PE placentas compared to uncomplicated ones. As we show in Fig. 1, Ca\(^{2+}\) transport is about 60% lower in primary PE ST cells than in normal. Earlier, we and others demonstrated that, after 4 days of culture, there was a significant reduction of 40% in cell fusion in trophoblasts isolated from PE placentas when compared to...
trophoblasts obtained from normal patients (unpublished data). Thus, the decrease in Ca\(^{2+}\) transport from ST is only partially due to the 40% reduction of syncytial volume seen in primary ST from PE (unpublished data). To explain what may also be responsible for the altered Ca\(^{2+}\) transfer and to clarify the mechanism involved, we investigated the expression of different proteins involved in Ca\(^{2+}\) homeostasis in placentas from PE pregnancies. Until now, there is no information on the variation of the Ca\(^{2+}\) handling proteins and the Ca\(^{2+}\) transfer in PE placentas. Importantly, for the first time, we successfully quantified the placental mRNA expression of CaBP-28K by real-time PCR. Herein, we demonstrated that the Ca\(^{2+}\) homeostasis is perturbed in PE placentas, most likely caused by a high oxidative stress level and lack of ATP.

### Decreased expression of TRPVs is accompanied with a decreased expression of CaBPs

A recent study shown that the Ca\(^{2+}\) concentration in foetal blood and amniotic fluid is significantly lower in murine TRPV6 knockout (KO) foetuses than in wild-types [45]. The same team also demonstrated that the transport activity of radioactive Ca\(^{2+}\) (\(^{45}\)Ca\(^{2+}\)) from mother to foetuses was 40% lower in TRPV6 KO placentas.

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Fig. 4 Protein and mRNA expression of calcium pumps (PMCA) isoforms 1 and 4 in normal and PE placentas. (A) This graph shows that the protein expression of both PMCA1 and 4 is significantly decreased in placentas PE compared to normal placentas (\(P = 0.0256\)). (B) The mRNA expression of PMCA1 is significantly decreased in PE placentas compared to normal group (0.0007). (C) The mRNA expression of PMCA 4 is significantly decreased in PE compared to normal placentas (\(P = 0.0082\)). The number of placentas tested for the mRNA expression is shown in parentheses for (B) and (C). For protein and mRNA expression, data were normalized according to amido black total protein levels and to HPRT1 housekeeping gene, respectively. *\(P < 0.05\).

Fig. 5 mRNA expression of the ATP synthase gene in normal and PE placentas. The mRNA expression is significantly decreased in PE compared to normal placentas (\(P = 0.0098\)). The number of placentas tested for the mRNA expression is shown in parentheses. For mRNA expression, data were normalized according to HPRT1 housekeeping gene. *\(P < 0.05\).
foetuses than in wild-types, that TRPV6 and CaBP-9K co-localized in the yolk sac and that CaBP-9K mRNA expression was decreased in TRPV6 KO mice placentas [45]. Also, TRPV6 expression has been shown to increase with the induction of CaBP-9K [46]. These data suggest an important role for the TRPV6 channel in Ca\(^{2+}\) homeostasis and placental transfer, and suggest that TRPV6 and CaBP-9K are cooperatively involved in maternal-foetal Ca\(^{2+}\) transport. This matches with our results, as we obtained a decrease in transport of \(^{45}\)Ca\(^{2+}\) in ST from PE placentas (Fig. 1A), accompanied by a decrease of TRPV6 (Fig. 2D) and CaBP-9K (Fig. 3B) protein and mRNA expression. Mice TRPV5 KO have shown a defective Ca\(^{2+}\) homeostasis, limited Ca\(^{2+}\) transfer to the foetus, as well as a CaBP-28K mRNA down regulation [47]. Moreover, a previous report showed that CaBP-28K translocates towards the plasma membrane and directly associates with TRPV5, facilitating high calcium transport rates by preventing
channel inactivation [48]. In our study, we also observed a decreased mRNA and protein expression of TRPV5 (Fig. 2A, B) accompanied by a decreased mRNA and protein expression of CaBP-28K (Fig. 3C, D). It has been demonstrated that TRPV5 and TRPV6 are considered as gatekeepers of transcellular Ca^{2+}/H^{+} transport as they are inversely correlated with the intracellular Ca^{2+} concentration [49]. Effectively, it was demonstrated that in condition of high Ca^{2+} concentration, both TRPV5 and TRPV6 exhibit calcium-dependant inactivation to avoid elevation in intracellular calcium to toxic levels [46]. This is the first time that the correlation of the expression of TRPVs with CaBPs is observed in human placentas, suggesting a fundamental role of CaBPs in the regulation of TRPVs. Moreover, it has also been demonstrated that CaBP-28K binds to the amino and carboxyl end of both TRPV5 and TRPV6 in low calcium concentration and that the disruption of this
association results in a decrease of Ca\(^{2+}\) buffering at the entry gate, followed by impairment of transcellular Ca\(^{2+}\) transport \[48\]. Finally, a synchronized rise in the expression of both TRPV5/6 and intracellular CaBP-9K/28K is essential to achieve maximal Ca\(^{2+}\) influx at the apical side, which seems to be deficient in placenta with PE.

**PE placenta demonstrate a decreased expression of PMCA1 and 4 as well as an increase of ATP synthase and hOGG1 genes**

In Ca\(^{2+}\) transport, the importance of CaBPs is also highlighted by its co-expression with PMCA1 \[48\]. In human placentas, PMCAs are the principal proteins implicated in Ca\(^{2+}\) extrusion to the foetal side from the ST. Their function is to eliminate intracellular calcium in excess, and, at a resting state, maintain cytosolic calcium concentration to prevent toxic surcharge \[50\]. To be functional, PMCAs need the presence of ATP. It was shown that ATP level in PE placental cells is lower due to an important change in ATP synthase gene \[12\] and we demonstrated that the ATP synthase level in our tissue samples was significantly decreased in PE (Fig. 5). Moreover, it was shown that changes in PMCA expression was correlated with its activity and ATP content in rat cells \[51\]. This agrees with our results where we observed a significant decrease in mRNA and protein expression of PMCA1 and 4 (Fig. 4). This lack of ATP is possibly attributed to hypoxia in PE placenta, caused by oxidative stress, which would deplete cellular ATP. To determine if there was a real increased of ROS in our samples, we analysed the mRNA expression of hOGG1, a marker of oxidative stress. Effectively, mRNA expression on hOGG1 is extremely higher in PE placenttal tissue than in normal (Fig. 10). Indeed, oxidative stress, resulting of deficient spiral artery remodelling, is an element of PE acting as a key intermediary event in the pathology \[52\]. Thus, based on the reduced mRNA and protein expression of PMCA1 and 4 in PE placenta, we suggest that this could result in inefficient calcium exclusion from ST by PMCAs. Moreover, the oxidative stress in PE placenta promotes the formation of lipids peroxides, which alter cell membranes, where, as we known, are located the PMCAs \[52\]. It was recently demonstrated the PMCA activity is very sensitive to the level of lipid peroxidation of the plasma membrane \[53\] and that the reduced activity of PMCAs in PE women could not be associated with a defective enzyme but rather with a high level of lipid peroxidation \[54\]. It also has been demonstrated that oxidative stress induces a Ca\(^{2+}\) overload response and inhibition of PMCAs in rat pancreatic cells \[55\]. A recent study indicated that the inhibitory effect of both oxidative stress and mitochondrial inhibitors on PMCA activity was explained by ATP depletion due to the inhibition of mitochondria \[56\]. That information could suggest that PMCAs are negatively affected by the depletion of ATP caused by oxidative stress in PE, which in consequence will increase the intracellular calcium concentration. This rise in Ca\(^{2+}\) content could affect mitochondria and contribute to the rise of apoptotic events, accentuating the ATP depletion.

**Avoiding Ca\(^{2+}\) overload by the action of ER and mitochondria**

Besides the action of CaBPs, the major mechanisms known to avoid excessive calcium concentrations in the cell are the buffering action of the ER and the mitochondria. It is well known that ER, a specialized calcium store organelle, is intimately involved in regulating Ca\(^{2+}\) movements within cells. IP3Rs and RyRs participate in the release of Ca\(^{2+}\) from the ER \[29, 30\] and we observed that the mRNA expression of IP3R1,2 (Fig. 6) and RyR1,2,3 (Fig. 7) was decreased in PE placentas. Thus, we suggest that this decrease is a consequence of elevated \([\text{Ca}^{2+}]\). In contrast, we observed that SERCA1,2,3 (Fig. 8) mRNA expression was increased in PE placenta, which functions to pump Ca\(^{2+}\) from the cytosol into the ER store. Its action, in combination with Ca\(^{2+}\) efflux mechanisms, is essential in maintaining a low resting \([\text{Ca}^{2+}]\) \([34\]. In parallel, we also observed that the mRNA expression of VDCA3 (Fig. 9), channel implicated in mitochondrial Ca\(^{2+}\) accumulation, increased in PE placenta.

In conclusion, the important finding of this study is the alteration of the calcium transfer in PE placenta compared with normal placenta. Another important finding in this work was the successful quantification of the CaBP-28K mRNA by real-time PCR. Based on our results and recent works, we can hypothesize that oxidative stress in PE is a determinant factor affecting calcium transfer in ST. As we demonstrated, the decrease of PMCA expression could be attributed to the ATP depletion, probably caused by oxidative stress, and in consequence, elevates intracellular Ca\(^{2+}\) concentration, which in turn down-regulates CaBPs, TRPVs. We also propose that IP3Rs and RyRs expression decrease, while SERCAs and VDACs increase in order to avoid a Ca\(^{2+}\) overload. In perspectives, as placental insufficiency could lead to IUGR in 30% of the cases \[2\], which is associated with lower cord blood calcium concentration \[57\], it could be interesting to compare the...
mechanisms of calcium transfer in PE placenta with or without IUGR. Working on strategies to protect placentas against reduced expression of PMCA may be very useful to weaken symptoms related to oxidative stress and intracellular calcium overload, and to enhance \(Ca^{2+}\) transfer to the foetus. By elucidating factors involved in the placenta \(Ca^{2+}\) transport in pregnancy, we will be able to determine one of the possible mechanisms responsible for foetal predisposition to adult diseases related to PE. This fundamental approach will not have an immediate impact in clinical practice, but it will help to characterize the \(Ca^{2+}\) transfer process from mother to foetus in these pregnancies and evaluate if ultimately it will be possible to improve antenatal placental transfer using nutritional intervention, pharmacological or hormonal agents. This project is the first step of a project of great magnitude. Future studies are needed to investigate the mechanism of placentonal \(Ca^{2+}\) transport in PE and in several years, we shall perhaps be able to intervene to develop strategies before birth to improve foetal and neonatal health using nutritional, pharmacological or hormonal intervention.

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