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ORIGINAL RESEARCH

Placental programmed cell death: insights into the role of aquaporins

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STUDY HYPOTHESIS: Are the placental aquaporins (AQPs) involved in the apoptosis of human trophoblast?

STUDY FINDING: The general blocking of placental AQPs with $HgCl_2$ and, in particular, the blocking of AQP3 activity with $CuSO_4$ abrogated the apoptotic events of human trophoblast cells.

WHAT IS KNOWN ALREADY: Although apoptosis of trophoblast cells is a natural event involved in the normal development of the placenta, it is exacerbated in pathological processes, such as pre-eclampsia, where an abnormal expression and functionality of placental AQPs occur without alterations in the feto-maternal water flux. Furthermore, fluctuations in O_2 tension are proposed to be a potent inducer of placental apoptotic changes and, in explants exposed to hypoxia/reoxygenation (H/R), transcellular water transport mediated by AQPs was undetectable. This suggests that AQPs might be involved in processes other than water transport, such as apoptosis.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: Explants from normal term placentas were maintained in culture under conditions of normoxia, hypoxia and H/R. Cell viability was determined by assessing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide incorporation. For the general or specific inhibition of AQPs, 0.3 mM $HgCl_2$, 5 mM $CuSO_4$, 0.3 mM tetraethylammonium chloride (TEA) or 0.5 mM phloretin were added to the culture medium before explants were exposed to each treatment. Oxidative stress parameters and apoptotic indexes were evaluated in the presence or absence of AQPs blockers. AQP3 expression was confirmed by western blot and immunohistochemistry.

MAIN RESULTS AND THE ROLE OF CHANCE: First, we observed that in H/R treatments cell viability decreased by $20.16 \pm 5.73\%$ compared with those explants cultured in normoxia ($P = 0.009$; $n = 7$). Hypoxia did not modify cell viability significantly. Both hypoxia and H/R conditions induced oxidative stress. Spontaneous chemiluminescence and thiobarbituric acid reactive substance levels were significantly increased in explants exposed to hypoxia ($n = 6$ per group, $P = 0.0316$ and $P = 0.0009$, respectively) and H/R conditions ($n = 6$ per group, $P = 0.0281$ and $P = 0.0001$, respectively) compared with those cultured in normoxia. Regarding apoptosis, H/R was a more potent inducer of trophoblast apoptosis than hypoxia alone. Bax expression and the number of apoptotic nuclei were significantly higher in explants cultured in H/R compared with normoxia and hypoxia conditions ($n = 12$, $P = 0.0135$ and $P = 0.001$, respectively). DNA fragmentation was only observed in H/R and, compared with normoxia and hypoxia, the activity of caspase-3 was highest in explants cultured in H/R ($n = 12$, $P = 0.0001$). In explants exposed to H/R, steric blocking of AQP activity with $HgCl_2$ showed that DNA degradation was undetectable ($n = 12$, $P = 0.001$). Bax expression and caspase-3 activity were drastically reduced ($n = 12$, $P = 0.0146$ and $P = 0.0001$, respectively) compared with explants cultured in H/R but not treated with $HgCl_2$. Similar results were observed in explants exposed to H/R when we blocked AQP3 activity with $CuSO_4$. DNA degradation was undetectable and the number of apoptotic nuclei and caspase-3 activity were significantly decreased compared with explants cultured in H/R but not treated with $CuSO_4$ ($n = 12$, $P = 0.001$ and $P = 0.0001$, respectively). However, TEA and phloretin treatments, to block AQP1/4 or AQP9, respectively, failed in abrogate apoptosis. In addition, we confirmed the expression and localization of AQP3 in explants exposed to H/R.

LIMITATIONS, REASONS FOR CAUTION: Our studies are limited by the number of experimental conditions tested, which do not fully capture the variability in oxygen levels, duration of exposure and alternating patterns of oxygen seen *in vivo*.

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WIDER IMPLICATIONS OF THE FINDINGS: Our results suggest that any alteration in placental AQP expression might disturb the equilibrium of the normal apoptotic events and may be an underlying cause in the pathophysiology of placental gestational disorders such as pre-eclampsia. Furthermore, the dysregulation of placental AQPs may be one of the crucial factors in triggering the clinical manifestations of pre-eclampsia.

LARGE SCALE DATA: n/a.

STUDY FUNDING AND COMPETING INTEREST(S): This study was supported by UBACyT 20020090200025 and 20020110200207 grants and PIP-CONICET 11220110100561 grant, and the authors have no conflict of interest to declare.

Key words: apoptosis / trophoblast / aquaporins / human placenta / pre-eclampsia / intermittent hypoxia

Introduction

Placental development is critical to achieving a successful pregnancy. It requires the precise regulation of cellular events such as proliferation, differentiation and apoptosis.

Apoptosis of trophoblast cells is a natural event that plays a major role in the physiological development of human placenta (Sharp *et al.*, 2010). It is well known that the amount of apoptosis in placental villi increases progressively throughout normal pregnancy (Smith and Baker, 1999; Athapathu *et al.*, 2003). Apoptotic events are involved in the fusion of mononuclear villous cytotrophoblast and contribute to its differentiation into a multinucleate syncytium, allowing continuous regeneration of the syncytiotrophoblast throughout gestation (Huppertz *et al.*, 2006).

In other tissues, it was observed that an imbalance of this orderly process can often result in various disease states (Reed, 1998; Yuan and Yankner, 2000).

Furthermore, emerging evidence establishes that O_2 is a key regulator of trophoblast differentiation, and failure of the oxygen-associated developmental events contributes to placental disease (Genbacev *et al.*, 1997; James *et al.*, 2006). It was established that the human placenta is exposed to profound changes in oxygenation during normal pregnancies and those with pathological conditions such as pre-eclampsia. Such fluctuations in O_2 tension could provide the basis for a hypoxia/reoxygenation (H/R) type injury, mainly because of the generation of reactive oxygen species (ROS) (Hung *et al.*, 2001). In addition, intermittent placental perfusion was proposed to be a potent stimulus for trophoblast apoptosis via the mitochondrial pathway, taking place through the alteration of the equilibrium between pro-oxidant and antioxidant defenses (Hung and Burton, 2006).

Although the mechanisms underlying apoptosis-induced cell death during H/R injury in human placenta are not fully understood, increased apoptosis in the syncytiotrophoblast disrupts its homeostasis and, furthermore, alters its synthetic and transport functions (Myatt and Cui, 2004). Moreover, accumulated evidence suggests that in pathological placentas characterized by shallow trophoblast invasion and poor remodeling of the maternal spiral arteries, the expression of a variety of syncytiotrophoblast transporters is reduced or abnormal (Damiano *et al.*, 2006; Castro-Parodi *et al.*, 2009; del Mónaco et al., 2006; Dietrich *et al.*, 2013). In this regard, we have previously identified aquaporin (AQP)3 and AQP9 in normal syncytiotrophoblast (Damiano *et al.*, 2001) and in subsequent experiments we have observed that AQP9 expression was increased in pre-eclamptic placentas (Damiano *et al.*, 2006). Even though we assumed that these aquaglyceroporins could participate in transcellular water transport between the mother and the fetus, the hypotheses concerning their functions are still speculative.

In addition, we have recently reported that O_2 tension may modulate AQP9 expression. We found a significant protein increase in trophoblast tissue exposed to H/R. However, contrary to expectation, water uptake was significantly reduced and was not blocked by $HgCl_2$ (a general blocker of AQPs) suggesting that water was not passing through AQPs (Castro-Parodi *et al.*, 2013). Our results bring up the idea that the role of AQPs exclusively on water transport should be revised.

Increasing data show that AQPs may participate in the movement of water across the plasma membrane in dying cells during the apoptotic volume decrease (AVD) (Jablonski *et al.*, 2004a,b; Chen *et al*, 2008). It was proposed that this critical event occurs via AQPs, and the inhibition of these proteins may abrogate the apoptotic response (Jablonski *et al.*, 2004a). In addition, it was reported that AQP overexpression increased the rate of apoptosis (Jablonski *et al.*, 2004b). So far, the association between AQPs and apoptosis in human placenta has not been explored.

In this study, we aimed to determine the role of placental AQPs in human trophoblast apoptosis.

Materials and Methods

Tissue collection

This study was approved by the local ethics committee of the Hospital Nacional Dr Prof. Alejandro Posadas, Buenos Aires, Argentina, and written informed consent was obtained from the patients before the collection of samples.

Full-term normal ($n = 15$) placental tissues were obtained after Cesarean section. Clinical data are summarized in Table I. All women belong to the white hispanic ethnic group.

All placentas came from pregnant women with no diseases or previous history of disease who gave birth to a newborn without anomalies.

Tissue culture

Placental tissue was gently separated by sterile dissection from different cotyledons, excluding chorionic and basal plates, minced with scalpel blades and washed repeatedly with 0.9% sodium chloride to remove blood from the intervillous space. Explants were cultured as we previously described (Damiano *et al.*, 2006). Briefly, whole villous tissue (~50 mg/well) was preincubated in 24-well polystyrene tissue culture dishes in 2 ml of serum-free Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc. BLR, Grand Island, NY, USA) containing 100 IU/ml penicillin, 100 mg/ml streptomycin and 32 mg/ml gentamicin at 37°C for 2 h under standard tissue culture conditions (5% CO_2/95% air) to allow explants to recover from the isolation procedures.

After changing the medium, some plates were incubated at 37°C for 18 h under standard conditions (or 'normoxia') in the cell culture incubators.

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Hypoxic exposures (2% O_2/5% CO_2/93% N_2) were carried out in a hypoxic chamber/glove box (Billups-Rothenberg Inc., Del Mar, CA, USA) for 18 h. At the end of the first 15 h incubation period, some explants were again exposed to standard conditions for 3 h (Castro-Parodi et al., 2013).

For the general or specific inhibition of AQPs, the blockers shown in Table II were added to the culture medium before explants were exposed to each treatment (Tsukaguchi et al., 1998; Zelenina et al., 2004; Detmers et al., 2006; Haddoub et al., 2009).

To confirm that explants were subject to hypoxia conditions, the expression of the hypoxia inducible factor 1-alpha (HIF-1α) was checked by western blot.

Biochemical assays

Tissue viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich Corp., San Luis, MO, USA) assay as described previously (Castro-Parodi et al., 2013). Tissue samples (200–300 mg, unfrozen) were collected in triplicate at different times or different O_2 conditions, exposed to MTT, and the formation of the formazan product of MTT was measured by monitoring relative absorbance at 595 nm. The experiments were independently conducted in triplicate at least three times. MTT assays were performed for seven consecutive days.

Table I Clinical characteristics of the women with uncomplicated pregnancies in the study of trophoblast apoptosis.

	Pregnant women
Number of pregnant women	15
Parity	
Primiparous	8
Multiparous	7
Maternal age, years	22.3 ± 1.5
Gestational age, weeks	38.7 ± 1.0
Mean blood pressure, mmHg	
Systolic	110 ± 3.9
Diastolic	63 ± 2.3
Proteinuria	Negative
BMI, kg/m^2	24 ± 3
Birth weight, g	3090 ± 240
Fetal sex	
Male	9
Female	6

Values are mean ± SD.

Oxidative stress parameters

Spontaneous chemiluminescence

Spontaneous chemiluminescence is a method to evaluate signals of oxidative metabolism. This assay is specific and allows evaluation of peroxidative breakdown of lipids. The termination reaction of peroxyl radicals and singlet oxygen yields excited states and chemiluminescence in parallel with malondialdehyde production and conjugated lipid dienes. Spontaneous chemiluminescence of each explant was measured with a photon counter (Johnson Research Foundation University of Pennsylvania, Philadelphia, PA, USA). A photomultiplier (EMI 9658; EMI-Gencom, Plainview, NY, USA) responsive from 300 to 900 nm and cooled at −20°C with an applied potential of −1.4 kW was used. The phototube output was connected to an amplifier-discriminator adjusted to a single photon counting that was, in turn, connected to both a frequency counter and a recorder. Photoemission was expressed as counts per second per cm^2 of tissue surface (cps/cm^2) (Musacco Sebio et al., 2014; Semprine et al., 2014).

Thiobarbituric acid reactive substances

The concentration of thiobarbituric acid reactive substances (TBARS) was determined using a spectrophotometric method based on the 2-thiobarbituric acid reaction. Each sample of placental explants exposed to different treatments was homogenized in 100 mM sodium phosphate plus 120 mM potassium chloride buffer (pH 7.40) and centrifuged at 900 *g* for 5 min at 4°C. TBARS were detected at 532 nm, using a spectrophotometer (Hitachi, Tokyo, Japan). The molar extinction coefficient (1.54.105 M^{-1} cm^{-1}) allows calculation of the concentration of malondialdehyde. Results were expressed in μmol g^{-1} protein.

Apoptotic indexes

Bax expression

Placental explants were processed according to the method previously described (Castro-Parodi et al., 2013). Briefly, explants were kept in a buffer containing 10 mM HEPES–KOH, 0.1 mM EGTA, 250 mM sucrose, pH 7.40, with protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 25 mg/ml *p*-aminobenzamidine, 20 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mg/ml pepstatin), homogenized and centrifuged at 3100 *g* for 10 min. The supernatants were collected, and protein concentration of each sample was measured by the BCA protein assay kit (Pierce, Thermo Fisher Scientific Inc., Waltham, MA, USA).

For immunoblotting studies, 100 μg of protein were loaded and resolved on a 15% polyacrylamide gel, and electrotransferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech Ltd, Pittsburgh, PA USA). After blocking with 1% bovine serum albumin in sodium phosphate buffer (PBS), membranes were incubated overnight with the primary antibody anti-Bax (Sigma-Aldrich Corp., San Luis, MO, USA; 1:500) and then with a goat anti-rabbit immunoglobulin G ([IgG]; Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA; 1:10,000) conjugated to peroxidase.

Immunoreactivity was detected using the Enhanced Chemiluminescence (ECL) Western Blotting Analysis System (ECL plus; Amersham

Table II AQP inhibitors used in the study of human placental explants *in vitro*.

Aquaporin	Inhibitor	Site of inhibition	References
AQPs (except AQP4 and AQP7)	$HgCl_2$ (Hg^{2+}) 0.3 mM	Cys189	Haddoub et al. (2009)
AQP3	$CuSO_4$ (Cu^{2+}) 5.0 mM	Trp128, Ser152, His154	Zelenina et al. (2004), Haddoub et al. (2009)
AQP1 and AQP4	Tetraethyl ammonium chloride 0.3 mM	Tyr 186	Detmers et al. (2006), Haddoub et al. (2009)
AQP9	Phloretin 0.5 mM	Asg216, Asp69, His151	Tsukaguchi et al. (1998), Haddoub et al. (2009)

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Pharmacia Biotech Ltd, Pittsburgh, PA USA) according to the manufacturer's instructions.

The densitometry of the bands was quantified by the ImageJ 1.45s software package (Bethesda, MD, USA).

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling assay

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) staining on histological sections was performed with a fluorescein-based cell death detection kit (*In Situ* Cell Death Detection kit, Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Nuclear counterstain was performed with Hoechst 33 342 (Sigma-Aldrich Corp., San Luis, MO, USA) fluorescent dye. Data were documented by an epifluorescent microscope (Nikon, Eclipse E:200).

To quantify the number of apoptotic cells, TUNEL positive cells were counted in three images each from three separate, non-adjacent sections of each treatment group. Two independent, blinded counters were used, and totals were averaged for each treatment. The counts were then averaged over the total area counted in mm^2.

DNA fragmentation assay

After treatments, explants were incubated with a Lysis buffer (50 mM Tris–HCl, 50 mM EDTA, 1% sodium dodecyl sulfate, 50 mM NaCl, pH 8.0) and Proteinase K (10 mg/ml; Sigma-Aldrich Corp., San Luis, MO, USA) for 2 h at 55°C. Samples were centrifuged at 21 000 *g* for 15 min. After that, 5 M NaCl was added to supernatants and they were centrifuged at 21 000 *g* for 15 min. Ice-cold absolute ethanol was then added to supernatants, and samples were incubated overnight at −20°C. Finally, the samples were centrifuged at 21 000 *g* for 15 min, DNA pellets were resuspended in DNAase-free water and run in 1.5% agarose gels with 1× TBE buffer (45 mM Tris-borate and 1 mM EDTA). GelRed™ (Biotium Inc., Hayward, CA, USA) was used to visualize the fragments of DNA.

Caspase-3 activity

The CaspACE™ Assay System colorimetric kit (Promega, USA) was used according to the manufacturer's protocol. Briefly, explants were cultured with and without the caspase-3 inhibitor Z-VAD-FMK in normoxia, hypoxia and H/R. Later on, explants were homogenized in the lysis buffer in the kit. After 10 min centrifugation at 9000 g, total protein amount was measured in the supernatant. The volume corresponding to 80 µg protein of each sample was used to measure caspase-3 activity. After 4 h incubation with Ac-DEVD-pNA (caspase-3 substrate), the released *p*-nitroaniline (pNA) was measured at 405 nm.

AQP3 expression

Western blot

Treated and untreated explants were processed according to the method previously described (Castro-Parodi *et al.*, 2013). Briefly, explants were kept in a buffer containing 10 mM HEPES–KOH, 0.1 mM EGTA, 250 mM sucrose, pH 7.4, with protease inhibitors (0.2 mM PMSF, 25 mg/ml *p*-aminobenzamidine, 20 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mg/ml pepstatin), homogenized (Ultra-Turrax homogenizer) and centrifuged at 3100 *g* for 10 min. The supernatants were collected, and protein concentration of each sample was measured by the BCA assay (Pierce).

For immunoblotting studies, 100 µg of protein were loaded and resolved on a 15% polyacrylamide gel, and electrotransferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech Ltd.). After blocking with 1% bovine serum albumin in PBS, membranes were incubated overnight with the primary antibody anti-AQP3 (Alpha Diagnostic International Inc.; 1:1000) and then with a goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.; 1:10 000) conjugated to peroxidase. Immunoreactivity was detected using the ECL Western Blotting Analysis System (ECL plus, Amersham Pharmacia Biotech Ltd.) according to the manufacturer's instructions. The densitometry of the bands was quantified by the ImageJ 1.45s software package.

Immunoperoxidase assays

Treated and untreated explants were fixed overnight in 10% formaldehyde/0.1 mol/l in PBS, pH 7.4, dehydrated and embedded in paraffin as previously described (Damiano *et al.*, 2001). Thin sections (4–5 mm) were cut, dewaxed, rehydrated and incubated in 3% hydrogen peroxide/methanol for 5 min to block endogenous peroxidase. After blocking nonspecific binding

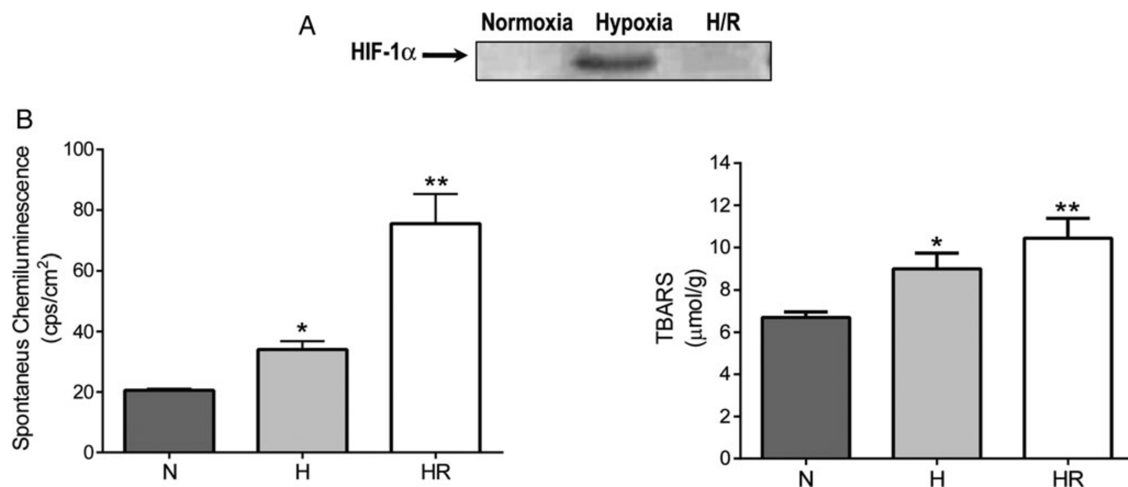

Figure 1 Characteristics of human placental explants maintained *in vitro* under conditions of normoxia (N), hypoxia (H) and hypoxia/reoxygenation (HR). (**A**) We observed that the HIF-1α protein was present only in those explants cultured under hypoxic conditions. (**B**) Oxidative stress parameters. (i) We observed an increased spontaneous chemiluminescence only in explants in H and H/R conditions compared with those cultured in *N* ($n = 6$ per group, *$P = 0.0316$, *$P = 0.0001$). (ii) We found that the concentrations of TBARS were significantly increased in H ($n = 6$ per group, *$P = 0.0281$) and in H/R ($n = 6$ per group, *$P = 0.0009$) compared with *N*. One-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test was applied. Error bars are SEM.

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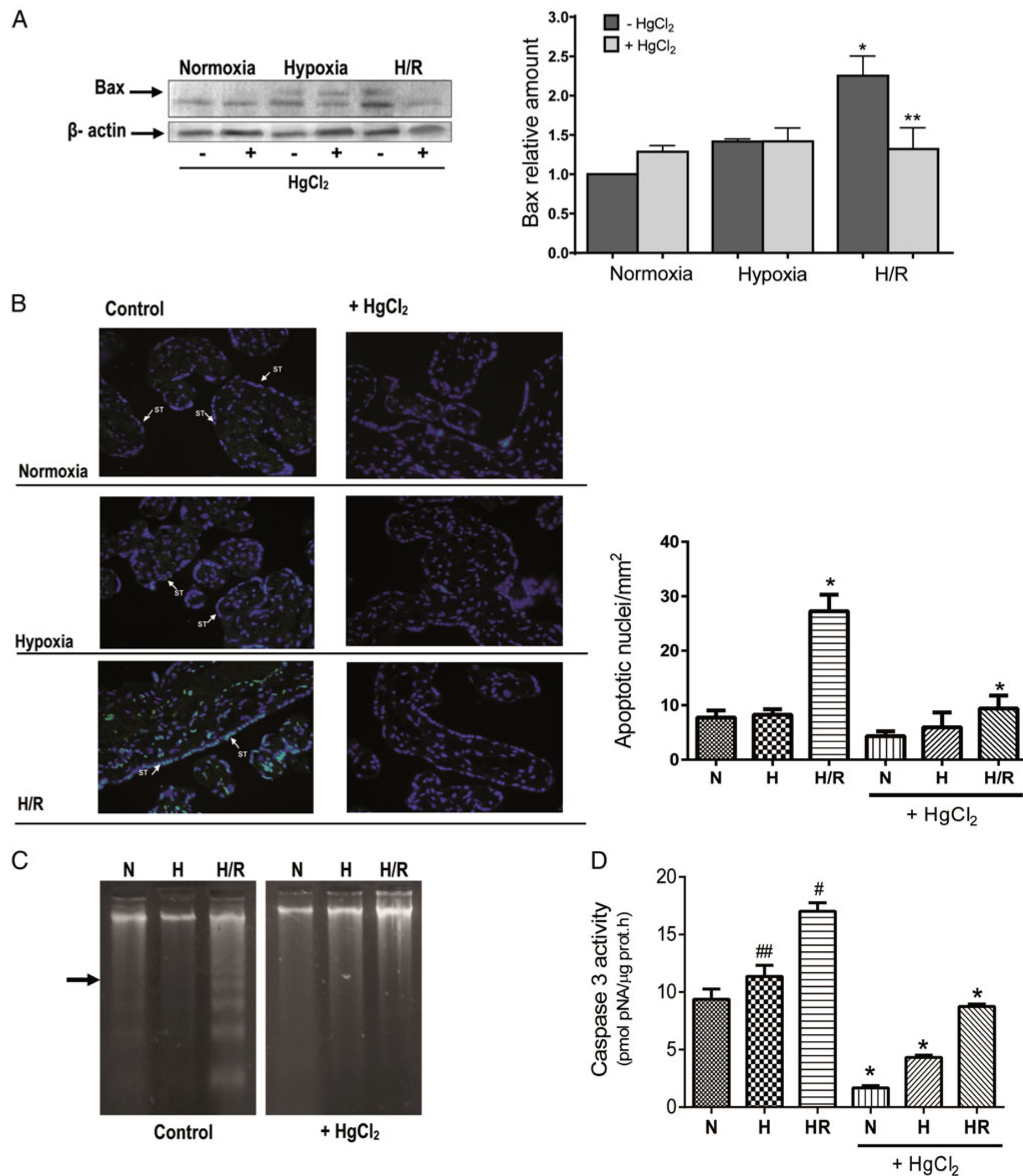

Figure 2 Apoptotic indexes in human placental explants after general inhibition of AQPs by $HgCl_2$. (**A**) Bax expression was significantly increased in explants cultured in H/R compared with explants cultured in N ($n = 12$ per group, $*P = 0.0135$) and dramatically decreased after treatment with $HgCl_2$, ($n = 12$ per group, $**P = 0.0146$). ANOVA followed by Fisher's LSD test was applied. Error bars are SEMs. (**B**) Apoptotic cells were detected by TUNEL assay. TUNEL positive cells, green; Hoescht, blue. ST, syncytiotrophoblast cells. Quantification of TUNEL positive cells per mm^2 showed that the number of apoptotic nuclei was significantly higher in explants cultured in H/R compared with N and H conditions ($n = 12$ per group, $*P = 0.001$). In explants cultured in H/R, the incubation with $HgCl_2$ reduced significantly the number of apoptotic nuclei ($n = 12$ per group, $*P = 0.001$). ANOVA followed by Fisher's LSD test was applied. Error bars are SEM. Magnification: ×400. (**C**) DNA fragmentation was observed in H/R. However, it was undetectable after treatment with $HgCl_2$. (**D**) The highest activity of caspase-3 was observed in explants cultured in H/R ($n = 12$ per group, $^{\#}P = 0.0001$ compared with *N* and $^{\#\#}P = 0.0265$ compared with *H*). After treatment with $HgCl_2$, caspase-3 activity was reduced in N, H and H/R conditions compared with the untreated groups ($n = 12$ per group, $*P = 0.0001$). ANOVA followed by Fisher's LSD test was applied. Error bars are SEMs.

sites with DAKO reagent (DAKO LSAB kit, Dako Corp.), tissue slices were incubated overnight (4°C) with anti-AQP3 (1:100). Later, the samples were placed in prediluted link antibody and incubated in a solution of streptavidin-conjugated horse-radish peroxidase. Staining was conducted with a Vectastain kit (Vector Laboratories), and labeling was visualized by reaction with diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.

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Statistical analysis

Statistical analysis of data was performed by GraphPad Prism v5 software (GraphPad Software, Inc., La Jolla, CA, USA). Data were compared by one-way analysis of variance followed by Fisher's Least Significant Difference test. A *P*-value of <0.05 was considered to be statistically significant.

Results

Biochemical assays

We found that MTT incorporation, a measure for mitochondrial dehydrogenase enzymatic activity, was similar over the first 24 h in culture ($P = 0.72$; $n = 7$). However, when explants were exposed to H/R treatments we observed that cell viability decreased $20.16 \pm 5.73\%$ compared with those explants cultured in normoxia ($P = 0.009$; $n = 7$). Hypoxia treatment did not modify cell viability significantly ($P = 0.074$; $n = 7$; data not shown). None of the blockers of AQPs modified cell viability in the concentrations used (data not shown).

HIF-1α expression

We also determined whether low-oxygen conditions affected HIF-1α protein levels in our experimental system. Only explants cultured under hypoxic treatments showed an increase in HIF-1α protein compared with those cultured under normoxia or H/R condition (Fig. 1A).

Oxidative stress parameters

To evaluate signals of oxidative metabolism, we tested spontaneous chemiluminescence in placental explants incubated in normoxia, hypoxia and H/R. This assay is specific and allows evaluation of peroxidative breakdown of lipids. The termination reaction of peroxyl radicals and singlet oxygen yields excited states and chemiluminescence in parallel with malondialdehyde production and conjugated lipid dienes. As it was expected, we observed an increased chemiluminescence in explants exposed to hypoxia and H/R conditions compared with those cultured in normoxia (Fig. 1B).

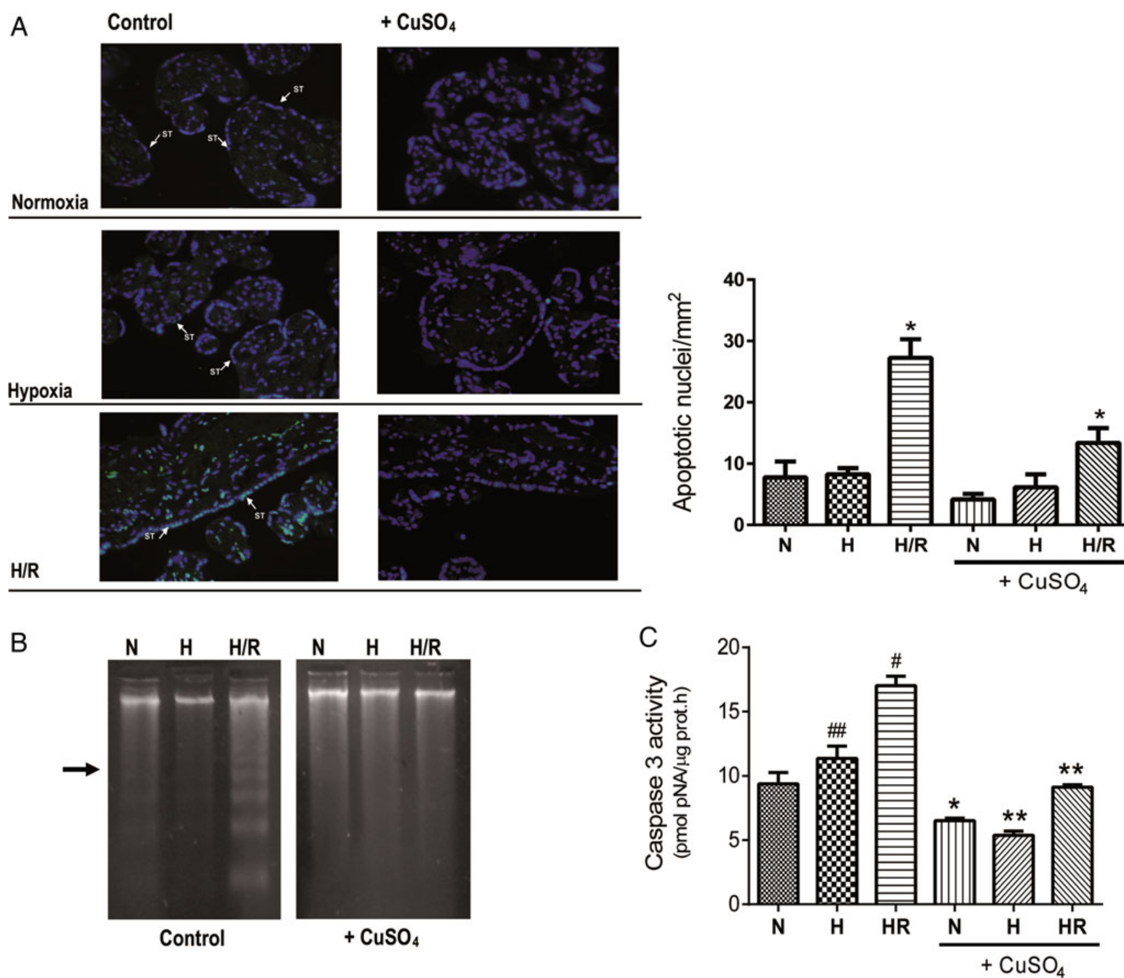

Figure 3 Apoptotic indexes in human placental explants after inhibition of AQP3 by $CuSO_4$. (**A**) Quantification of TUNEL positive cells per mm^2 showed that incubation with $CuSO_4$ reduced the number of apoptotic nuclei in explants cultured in H/R ($n = 12$ per group; $*P = 0.001$). ANOVA followed by Fisher's LSD test was applied. Error bars are SEMs. *Magnification*: $\times 400$. (**B**) DNA fragmentation was undetectable after treatment with $CuSO_4$ in H/R condition. (**C**) After treatment with $CuSO_4$, caspase-3 activity was reduced in N, H and H/R conditions compared with the untreated groups ($n = 12$ per group, $*P = 0.001$, $**P = 0.0001$). ANOVA followed by Fisher's LSD test was applied. Error bars are SEM.

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TBARS levels were also determined as an index of lipid peroxidation and, accordingly with our spontaneous chemiluminescence results, we found an increase in the concentrations of TBARS in hypoxia and H/R, which correlates with the induced oxidative stress in this condition (Fig. 1C).

Apoptosis indexes and inhibition of AQPs

First, we investigated if the general blockage of AQPs by $HgCl_2$ suppressed the apoptotic response in normal placental explants exposed to the different concentrations of O_2.

Thus, we evaluated the expression of the pro-apoptotic protein Bax, the nuclear features by TUNEL, DNA fragmentation plus Caspase-3 activity in the presence and in the absence of $HgCl_2$.

As we expected, Bax protein was significantly increased in placental explants cultured in H/R. However, after treatment with $HgCl_2$, Bax protein dramatically decreased compared with control explants (Fig. 2A).

We also found that the number of apoptotic nuclei was significantly higher in explants cultured in H/R compared with those cultured in normoxia or hypoxia. In addition, the incubation with $HgCl_2$ reduced the number of apoptotic nuclei of explants cultured in H/R (Fig. 2B).

Regarding DNA fragmentation, we clearly observed the typical ladder pattern of DNA bands in explants cultured in H/R conditions. However, after treatment with $HgCl_2$ the DNA fragmentation was undetectable (Fig. 2C).

Finally, we tested caspase-3 activity. Caspase-3 plays a crucial role as a final execution enzyme in both intrinsic and extrinsic pathways of apoptosis. We observed the highest activity of caspase-3 in explants cultured in H/R. After blocking AQPs with $HgCl_2$, we found that caspase-3 activity was significantly reduced in all conditions (Fig. 2D).

To evaluate the contribution of each placental AQP to the apoptotic events, we assessed different AQPs blockers.

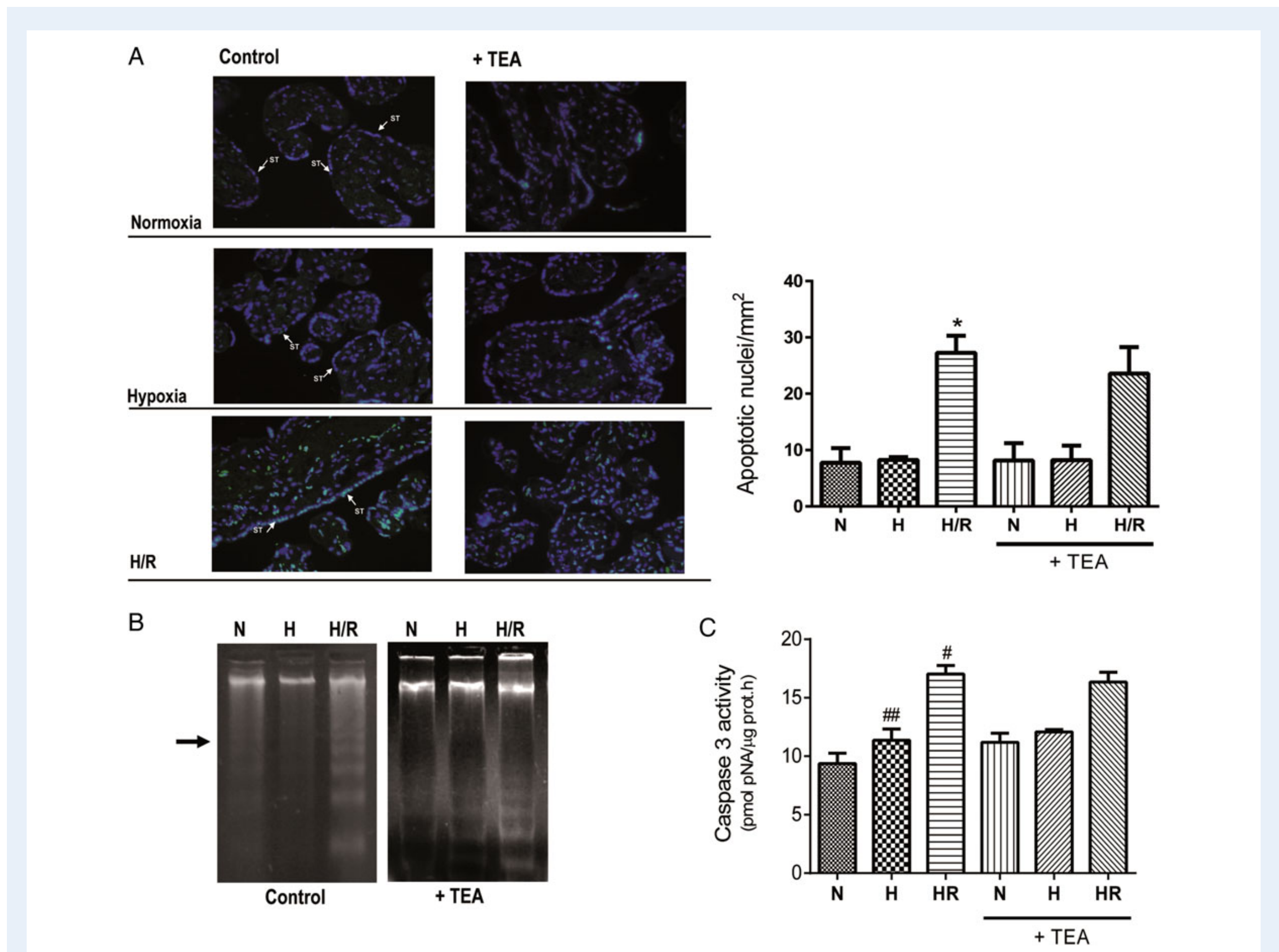

Figure 4 Apoptotic indexes in human placental explants after inhibition of AQP4 by tetraethylammonium chloride (TEA). (**A**) Incubation with TEA showed that there were no statistically significant differences in the number of TUNEL positive cells between all three groups treated with TEA versus all three untreated groups ($n = 12$ per group). ANOVA followed by Fisher's LSD test was applied. Error bars are SEMs. *Magnification*: ×400. (**B**) DNA fragmentation was present after treatment with TEA in H/R conditions. (**C**) There was no change in caspase-3 activity after TEA incubation between all three groups treated with TEA versus all three untreated groups ($n = 12$ per group). ANOVA followed by Fisher's LSD test was applied. Error bars are SEM.

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We observed that only incubation with $CuSO_4$, used to block AQP3, reduced the number of apoptotic nuclei, the DNA fragmentation and caspase-3 activity (Fig. 3).

No changes were observed on the apoptotic indexes studied after TEA treatment to inhibit AQP1 and AQP4 (Fig. 4) or phloretin incubation used to block AQP9 (Fig. 5).

Effect of oxygen tension on AQP3 expression

AQP3 immunoblotting analysis showed a 30% decrease when explants were cultured under hypoxic conditions. The following reoxygenation partially restored AQP3 expression (~80%) (Fig. 6A). Immunolocalization experiments showed that AQP3 was present in the apical membrane of syncytiotrophobast in explants cultured in normoxia. However, in explants exposed to hypoxia, the label for AQP3 was mainly found in the cytosol. After reoxygenation, AQP3 was again localized in the apical membrane of syncytiotrophoblast cells (Fig. 6B).

Discussion

It is well known that O_2 plays an important role in placental development and function. Several data suggest that human placenta is exposed to profound changes in oxygenation because of the intermittency of perfusion within the intervillous space during normal and pathological pregnancies. Thus, fluctuations in O_2 tension are proposed to be a potent inducer of apoptotic changes in human placenta and provide the basis for an H/R type injury (Hung *et al.*, 2001; Hung and Burton, 2006). Apoptosis is an important mechanism of cell death necessary for the normal development of human placenta (Smith and Baker, 1999; Sharp *et al.*, 2010). The amount of apoptosis in placental villi changes throughout normal pregnancy and it is considerably increased in pathological placentas, such as in pre-eclamptic (Athapathu *et al.*, 2003). Therefore, the exacerbation of apoptotic events may lead to alterations in syncytiotrophoblast function altering the expression of a variety of transporters including AQPs. Five AQPs (AQP1, AQP3, AQP4, AQP8 and AQP9) have been

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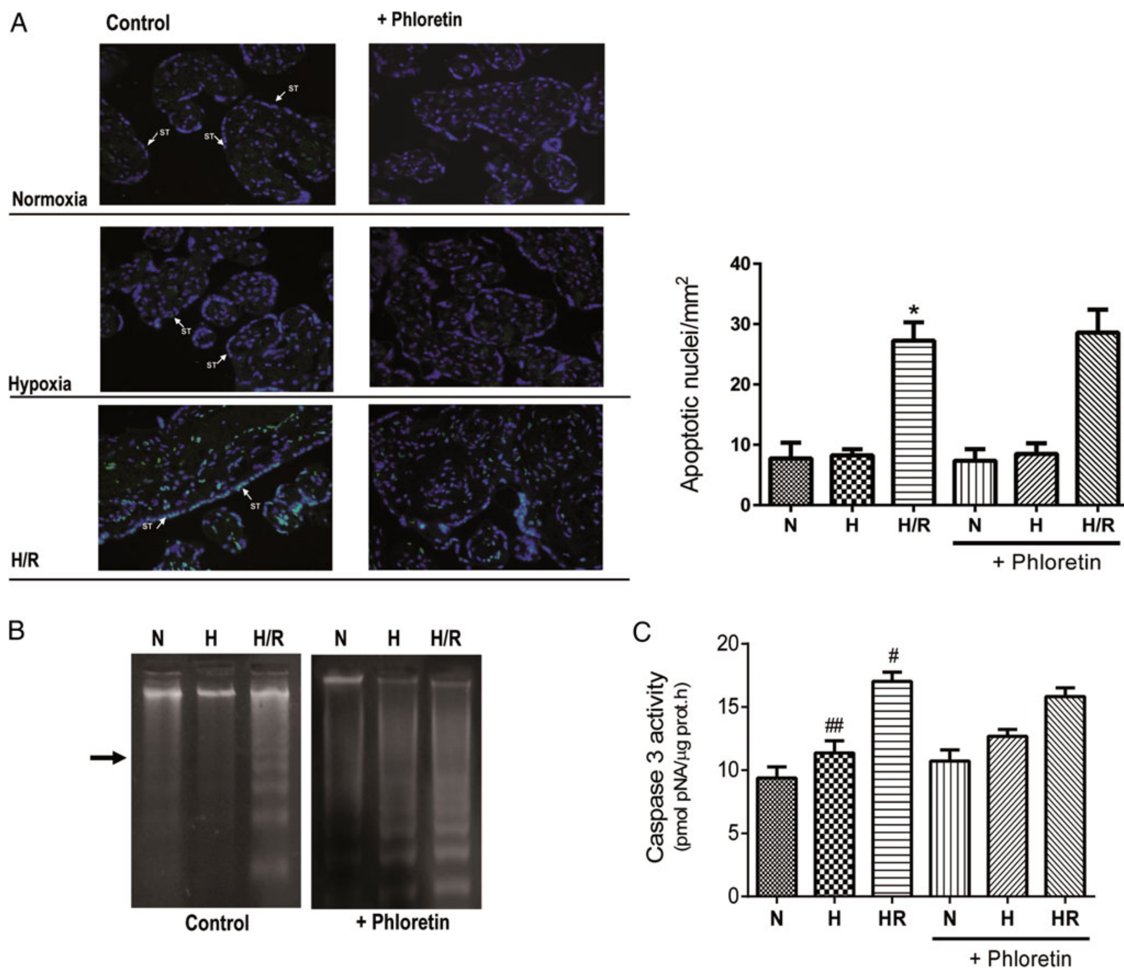

Figure 5 Apoptotic indexes in human placental explants after inhibition of AQP9 by phloretin. (**A**) Incubation with phloretin showed that there were no statistically significant differences in the number of TUNEL positive cells between all three groups treated with phloretin versus all three untreated groups ($n = 12$ per group). ANOVA followed by Fisher's LSD test was applied. Error bars are SEM. *Magnification*: ×400. (**B**) DNA fragmentation was present after treatment with phloretin in H/R conditions. (**C**) There were no differences in caspase-3 activity after phloretin treatment between all three groups treated with phloretin versus all three untreated groups ($n = 12$ per group). ANOVA followed by Fisher's LSD test was applied. Error bars are SEM.

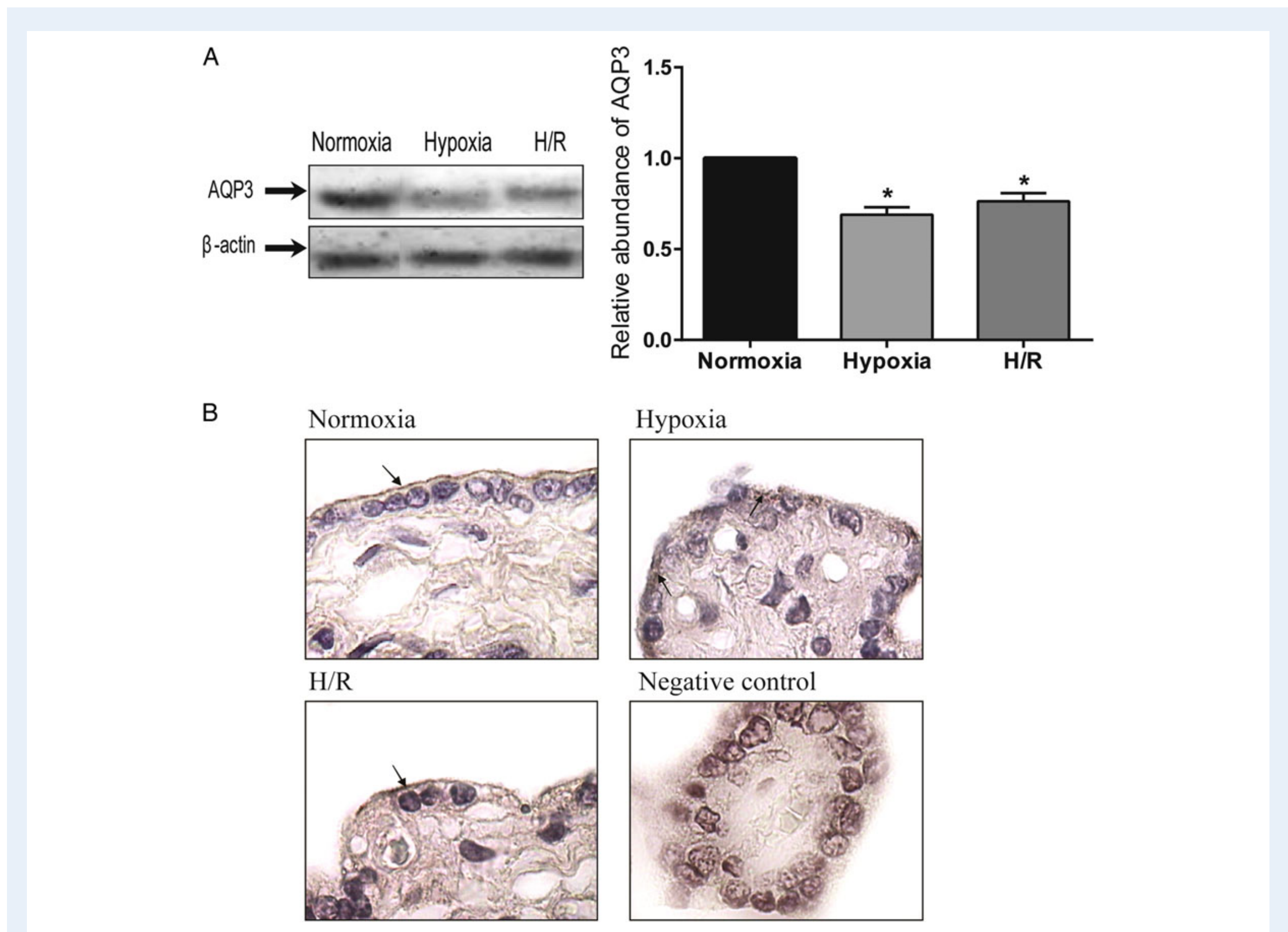

Figure 6 AQP3 expression in human placental explants exposed to different O_2 tensions. (**A**) A representative immunoblot for AQP3 in explants exposed to N, H and H/R. A band of 37 kDa corresponding to the glycosylated form of the AQP3 was detected in the three cases. Densitometry of immunoblots containing AQP3 protein level expression was performed, and after normalization for β-actin, the values were plotted as AQP3/β-actin relative ratio. H treatment showed a 30% decrease of AQP3 expression ($n = 6$ per group, $P = 0.0002$ compared with N) while the following reoxygenation restored partially AQP3 expression ($n = 6$ per group, $P = 0.0014$ compared with N). ANOVA followed by Fisher's LSD test was applied. Error bars are SEM. (**B**) Immunostaining with an anti-AQP3 antibody revealed specific labeling in the apical membrane of syncytiotrophoblast in explant cultured in *N*. However, in explants exposed at low O_2 tension, the label of AQP3 was mainly found in the cytosol. After reoxygenation, AQP3 was again localized in the apical membrane of syncytiotrophoblast cells. Negative controls were performed by omitting the primary antibody and replaced by a non-immune rabbit serum. *Magnification*: ×1000.

described in trophoblast tissue, but their role in human placenta still remains uncertain (Damiano, 2011).

Based on previous reports, ROS are proposed to induce programmed cell death in various cell types including trophoblast cells. In the present work, evidence of oxidative stress in explants exposed to hypoxia and H/R was provided by the increased levels of spontaneous chemiluminescence and lipid peroxidation (Payne *et al.*, 1995; Hung and Burton, 2006). It is well known that ROS up-regulate the production of pro-apoptotic proteins Bad and Bax, resulting in the subsequent intracellular translocation of these proteins from cytosol to mitochondria (Ishikawa *et al.*, 2003). These events trigger cytochrome C release from mitochondria and lead to the activation of caspases, which, in turn, initiate cell destruction by activating DNAses (Kaufmann and Earnshaw, 2000; Herrera *et al.*, 2001). Here, we evaluated the amount of apoptotic nuclei using the TUNEL assay, DNA fragmentation, Bax protein levels and caspase-3 activity. Our results showed that increased oxidative stress correlates with the increased apoptosis when placental explants were exposed to changes in O_2 tension. Furthermore, in line with previous reports by Hung and coworkers, we found that H/R is a more potent inducer of syncytiotrophoblast apoptosis than hypoxia alone (Hung and Burton, 2006).

Accumulated evidence suggests that AQPs may be involved in the movement of water across the plasma membrane during apoptosis (Jablonski *et al.*, 2004a). Morphologically, one of the earliest and most conserved events in apoptosis is water loss and subsequent cell shrinkage. Previous studies demonstrated that loss of water from the cell during the AVD is critical to the progression of apoptosis and that water loss in apoptotic cells occurs via AQPs (Jablonski *et al.*, 2004b). In addition, it was observed that, while inhibition of these proteins abrogates the apoptotic response, their overexpression increases the rate of apoptosis (Jablonski et al., 2007).

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Our previous findings showed that transcellular water transport mediated by AQPs was undetectable in explants exposed to H/R (Castro-Parodi *et al.*, 2013). The discrepancy between the expression and the functionality of these proteins led us to propose that the role of placental AQPs exclusively on water transfer between the mother and the fetus should be revised.

Jabloski and coworkers postulated that the loss of intracellular K^+ concentration establishes an osmotic gradient that draws water out of the cell and forces the cell to shrink (Jablonski *et al.*, 2004a, b). Furthermore, K^+ efflux must be greater than water loss allowing apoptotic events to continue after AVD. The mechanism they propose is that after AVD, AQPs might be inactivated producing changes in the plasma membrane, which become significantly less permeable to water.

Inactivation of these water channels and the continued efflux of ions K^+ would lead to a decrease in ionic strength of the cytoplasm, which is necessary for the activation of caspases and apoptotic nucleases (Chen *et al.*, 2008).

Here, we explored the association between apoptosis and placental AQPs. We found that, by sterically blocking AQPs' activity with $HgCl_2$, DNA degradation, the number of apoptotic nuclei, Bax protein and caspase-3 activity were drastically reduced. The same result was observed after blocking AQP3 with $CuSO_4$. However, TEA and phloretin treatments failed in abrogate apoptosis.

In addition, we found that AQP3 protein expression decreased after oxygen deprivation, and the latter reoxygenation restored its expression on the apical membrane of syncytiotrophoblast. However, the subsequent reoxygenation fails to restore AQP3 to basal levels, possibly due to the oxidative damage of the plasma membrane of syncytiotrophoblast, which probably creates an unfavorable environment for AQP3 insertion in the plasma membrane. Despite this, our results confirmed the expression of AQP3 in explants exposed to H/R, suggesting that the specific block of this protein with $CuSO_4$ decreases the apoptotic events.

It is well established that alterations in placental function by external factors, such as fluctuations in O_2 tension and ROS, can lead to significant increases in placental apoptosis. In this study, we provide evidence that AQPs, and in particular, AQP3, may act as a critical factor in the regulation of placental apoptosis. Therefore, any alteration in AQPs expression or functionality may disturb the equilibrium of this orderly process and contribute to the pathophysiology of placental gestational disorders such as pre-eclampsia.

Taking into account the abnormal expression and functionality of AQPs in pre-eclamptic placentas (Damiano *et al.*, 2006) and the lack of evidence regarding any link between an altered feto-maternal water flux and pre-eclampsia, we propose that AQPs might be involved in other processes besides water transport. Furthermore, our studies strongly argue for a role of placental AQPs in apoptosis and suggest that, in particular, the blocking of AQP3 activity with $CuSO_4$ may prevent the apoptotic events of the trophoblast. Therefore, changes in the expression and function of placental AQPs in women with pre-eclampsia may be one of the crucial factors in triggering the clinical manifestations of this gestational hypertensive disorder.

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Authors' roles

N.S., M.C.-P., J.R. and M.R. carried out the experimental work and analysis of data; B.M. provided the samples and critically reviewed the manuscript; N.M. carried out data analysis and discussion and critically reviewed the manuscript. A.E.D. designed the study and wrote the manuscript. All authors contributed to the final version of the manuscript.

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Conflicts of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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