Research Article

Oxidative Stress Alters miRNA and Gene Expression Profiles in Villous First Trimester Trophoblasts

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The relationship between oxidative stress and miRNA changes in placenta as a potential mechanism involved in preeclampsia (PE) is not fully elucidated. We investigated the impact of oxidative stress on miRNAs and mRNA expression profiles of genes associated with PE in villous 3A first trimester trophoblast cells exposed to H₂O₂ at 12 different concentrations (0-1 mM) for 0.5, 4, 24, and 48 h. Cytotoxicity, determined using the SRB assay, was used to calculate the IC₅₀ of H₂O₂. RNA was extracted after 4 h exposure to H₂O₂ for miRNA and gene expression profiling. H₂O₂ exerted a concentration- and time-dependent cytotoxicity on 3A trophoblast cells. Short-term exposure of 3A cells to low concentration of H₂O₂ (5% of IC₅₀) significantly altered miRNA profile as evidenced by significant changes in 195 out of 595 evaluable miRNAs. Tool for annotations of microRNAs (TAM) analysis indicated that these altered miRNAs fall into 43 clusters and 34 families, with 41 functions identified. Exposure to H₂O₂ altered mRNA expression of 22 out of 84 key genes involved in dysregulation of placental development. In conclusion, short-term exposure of villous first trimester trophoblasts to low concentrations of H₂O₂ significantly alters miRNA profile and expression of genes implicated in placental development.

1. Introduction

Preeclampsia (PE), which affects 3% to 8% of pregnant women, remains a major cause of short- and long-term maternal and neonatal morbidity and mortality. It is a medical condition characterized by de novo hypertension in pregnancy (diastolic > 90 mm Hg) after 20-week gestation with high proteinuria (>300 mg) [1]. PE is thought to result from a combination of many factors including shallow trophoblast invasion, failed maternal spiral artery remodeling, and an increase in endothelial activation leading to placental hypoxia, reactive oxygen species (ROS) generation, apoptosis and necrosis of trophoblasts, and systemic activation of inflammatory processes in the mother [1]. Superoxide anions generated endogenously or exogenously can rapidly be converted to hydrogen peroxide (H₂O₂) [2] and studies showed a significant elevation of H₂O₂ levels in the bloodstream of women with PE [3, 4]. H₂O₂ levels are also significantly higher in preeclamptic placentas compared to normotensive placentas at term [5]. Moreover, evidence supports an early increase in oxidative stress in the placenta by the end of the first trimester before the clinical development of PE [6–8]. Oxidative stress can induce endothelial dysfunction and vasoconstriction [9]. Therefore, the relationship between oxidative stress and PE is a vicious cycle where increased oxidative stress can induce PE and the occurrence of PE also exacerbates oxidative stress. The age-adjusted incidence of PE in the United States increased almost 25% from 1987 to 2004 [10]. With disease risk on the rise but no effective way to predict its development, it is crucial to understand the early etiologic mechanisms of PE to develop early detection biomarkers and possible preventive measures for the disease.

MicroRNAs (miRNAs) are short 20–22 single strand regulatory RNAs that function by inhibiting translation of their
targets or promoting target RNA degradation [11, 12]. There is a
temporal and placental-specific pattern of miRNA expres-
sion [13]. This pattern includes two large imprinted miRNA
clusters, one located at chromosome 19q13.41 (C19MC) and
another at 14q32 (C14MC). Expression of C14MC decreases
while expression of C19MC increases as pregnancy progresses
[14]. Women with PE, eclampsia, and HELLP (Hemolysis, Elevated Liver enzymes, Low Platelets) syndrome have signif-
icaent alterations in the placental miRNA profile. For example,
dysregulation of C19MC expression is seen in preeclamptic placentas [15]. In addition, miR-210, which regulates hypoxia-
inducible factor 1, is commonly found to be upregulated in PE
women [16]. Dysregulated expression of a number of other
miRNAs, such as the oncogenic miR-17 family, occurs in
preeclamptic placentas but the temporal change is unknown
[17–20]. Given the reported changes in miRNAs observed in
PE, early detection, protection, and regulation of miRNAs
may help decrease the impact of this disease.

The relationship between oxidative stress and miRNA
changes in placenta as a potential mechanism involved in PE
is not fully elucidated. In the present study, we investigate the
impact of oxidative stress on miRNA and mRNA expression
profiles, with specific emphasis on miRNAs of genes known
to be associated with PE, in villous first trimester 3A cytotro-
phoblast cell line.

2. Materials and Methods

2.1. Chemicals and Reagents. The villous 3A cytotrophoblast
first trimester placental cell line (CRL-1584) was purchased
from American Type Culture Collection (ATCC) (Manassas,
VA). The miRNA and mRNA kits with appropriate real-
time reagents and miRNEasy were purchased from Qiagen
(Valencia, CA). CytoScan SRB cell cytotoxicity assay kit was
purchased from G-biosciences (St. Louis, MO). Eagle’s Min-
imum Essential Medium (EMEM) and 0.25% trypsin were
Gibco brand (Life technologies, Grand Island, NY). Fetal
bovine serum (FBS) was acquired from Atlanta Biologicals
(Lawrenceville, GA). All other chemicals were purchased
from Sigma-Aldrich Co. (St. Louis, MO, USA) and were of
the highest purity available.

2.2. Cell Culture and \( H_2O_2 \) Treatment. Villous 3A cytotro-
phoblast cells were maintained in 75 cm\(^2\) flasks at 37°C/5%
CO\(_2\) in complete medium consisting of EMEM supplemented
with 10% fetal bovine serum and 1% penicillin/streptomycin.
Cells were passaged at ~90% confluency. \( H_2O_2 \) exposures
were performed in complete medium for the times and
concentrations specified in a humidified incubator at 37°C
supplemented with 5% CO\(_2\).

2.3. Determination of \( H_2O_2 \) IC\(_{50}\). Cells were seeded in 12-well
plates at a density of 0.25 \( \times 10^5 \) cells/well and were allowed to
attach for 24 hours. Growth media were replaced either with
fresh media for mock exposure or with one of 12 different con-
centrations of \( H_2O_2 \) (0 to 1 mM) in complete medium for 0.5,
4, 24, or 48 h without further media replacement. Cytotoxic-
ity was assessed at the end of \( H_2O_2 \) exposure using the SRB
assay as previously described [21]. Briefly, after exposure, cells
were fixed with 10% trichloroacetic acid for one hour and
then stained with 0.4% SRB dissolved in 1% acetic acid. Excess
dye was washed and wells were air-dried. Bound dye was solu-
bilized in 10 mM Tris (pH 7.4) and absorbance was measured
at 560 nm using a microplate reader (TECAN GENios Pro,
Männedorf, Switzerland). Results were expressed as the rela-
tive percentage of absorbance compared to control. All exper-
iments were performed in triplicate. Half-maximal inhibitory
concentration (IC\(_{50}\)) was calculated using SigmaPlot, version
12.3 (Systat Software Inc., San Jose, CA, USA).

2.4. Assessment of Oxidative Stress Markers. Reduced glu-
thathione (GSH) levels and superoxide dismutase (SOD)
activity were assessed in the cell lysates as markers for
oxidative stress [22, 23]. To determine the level of GSH, an
aliquot (0.2 mL) of cell lysate was added to a tube containing
1.7 mL phosphate buffer and 0.1 mL Ellman’s reagent; then the
absorbance was read at 412 nm within 5 min [24]. The results
were expressed as mg/μg protein. SOD activity was assessed
in the cell lysates using an assay that relies on the ability
of SOD enzyme to inhibit the phenazine methosulphate-
(PMS-) mediated reduction of nitroblue tetrazolium dye. The
change in absorbance over 5 min was measured at 560 nm
[25]. SOD activity was expressed as U/mg protein. Protein
levels were determined using the BCA protein assay (Pierce
Biotechnology, Rockford, IL, USA). Student’s t-test was used
for statistical comparisons and significant differences were
established at \( P < 0.05 \).

2.5. Determination of Effects of \( H_2O_2 \) Exposure on miRNA and
mRNA Expression Profiles. Cells were grown to 90% conflu-
ency in 75 cm\(^2\) flasks and exposed to 25 μM \( H_2O_2 \) (equivalent
to 5% of the IC\(_{50}\)) in complete medium for 4 h. Total RNA
was isolated using the Qiagen miRNEasy Mini Kit and quality/
quantity was measured in the Molecular Genomics Core at
UTMB. RNA was quantitated spectrophotometrically using a
NanoDrop ND-1000 (NanoDrop Technologies, DE). Quality
of the purified RNA was assessed by visualization of 18S and
28S RNA bands using an Agilent Bioanalyzer 2100 (Agilent
Technologies, CA). Resulting electropherograms were used
in the calculation of the 28S/18S ratio and the RNA Integrity
Number. Reverse transcription was carried out using either the
miScript II RT kit or RT\(^2\) First Strand and subsequent
SYBR green based real-time PCR on a Bio-Rad Chromo4
Real-Time PCR Detector per the manufacturer’s recom-
endation. The miRNA profile screening was performed using
miScript Human miRNome PCR Array (MIHS-3216Z, Qiagen,
Valencia, CA). RT\(^2\) Profiler Human Preeclampsia PCR Array
(PAHS-163Z, Qiagen, Valencia, CA) was used for gene
expression profiling. Data was analyzed using the \( \Delta\Delta CT \)
method with either the miScript miRNA PCR Array Data
Analysis version 3.5 or with the RT\(^2\) Profiler PCR Array Data
Analysis (SABiosciences, Valencia, CA).

3. Results

3.1. \( H_2O_2 \)-Induced Cytotoxicity in Villous 3A Trophoblasts. In
order to determine the appropriate \( H_2O_2 \) concentration for
further experiments, cells were exposed to varying micromolar concentrations of H$_2$O$_2$ in complete medium for up to 48 h and cytotoxicity was determined using the SRB assay. Exposure of 3A placental cells to H$_2$O$_2$ resulted in a time- and concentration-dependent cytotoxic effect. The IC$_{50}$ values for H$_2$O$_2$ were 592, 487, 90, and 15 μM after 30 min, 4, 24, and 48 h exposures, respectively (Figure 1). A concentration equivalent to 5% of the IC$_{50}$ concentration was used for the miRNAs and mRNA expression profiling experiments. The 4-hour exposure was selected for short-term exposure studies of the effect of H$_2$O$_2$ on miRNA and mRNA expression profile. The levels of GSH as well as SOD activity were assessed after exposure of the cells to 25 μM H$_2$O$_2$ for 4 h in complete medium and total RNA (including miRNA) was isolated using the Qiagen miRNeasy Mini Kit. The Qiagen v16 miRNA Array was used to evaluate the expression of 1008 miRNAs after H$_2$O$_2$ exposure compared to unexposed control. In our study, 417 miRNAs were not expressed (unevaluable) in the tested cell line. Out of the 591 evaluable miRNAs, 195 were up- or downregulated by at least twofold after H$_2$O$_2$ exposure (Supplemental Table 1 available online at http://dx.doi.org/10.1155/2015/257090). The majority of altered miRNAs (95.5%) were upregulated by at least 2-fold and only 4% were downregulated by at least 2-fold (Figure 2). Mir-21, -770, and -596 were downregulated by more than 5-fold, while mir-3907 was downregulated by more than 50-fold. Mir-637, mir-1911, mir-26b, mir-615, let-7a, and let-7f were upregulated more than 50-fold (Supplemental Table 1).

3.2. H$_2$O$_2$ Alters Normal miRNA Expression Profile in Villous 3A Trophoblasts. To investigate the potential effect of oxidative stress on miRNA expression profile in villous 3A trophoblasts, cells were exposed to 25 μM H$_2$O$_2$ for 4 h in complete medium and total RNA (including miRNA) was isolated using the Qiagen miRNeasy Mini Kit. The Qiagen v16 miRNA Array was used to evaluate the expression of 1008 miRNAs after H$_2$O$_2$ exposure compared to unexposed control. In our study, 417 miRNAs were not expressed (unevaluable) in the tested cell line. Out of the 591 evaluable miRNAs, 195 were up- or downregulated by at least twofold after H$_2$O$_2$ exposure (Supplemental Table 1 available online at http://dx.doi.org/10.1155/2015/257090). The majority of altered miRNAs (95.5%) were upregulated by at least 2-fold and only 4% were downregulated by at least 2-fold (Figure 2). Mir-21, -770, and -596 were downregulated by more than 5-fold, while mir-3907 was downregulated by more than 50-fold. Mir-637, mir-1911, mir-26b, mir-615, let-7a, and let-7f were upregulated more than 50-fold (Supplemental Table 1).
miRNA profiling

![Pie chart representation for miRNome array results after 4h exposure to H₂O₂ in villous 3A trophoblasts.](image)

TABLE 1: Clusters and families of miRNAs significantly altered by H₂O₂ in villous 3A trophoblasts.

| Cluster      | Count | Percent | P value |
|--------------|-------|---------|---------|
| hsa-let-7e cluster | 3     | 100     | 0.0418  |
| hsa-let-106b cluster | 3     | 100     | 0.0418  |
| hsa-let-23b cluster | 3     | 100     | 0.0418  |
| let-7 family   | 9     | 100     | 6.40e-5 |
| mir-15 family  | 4     | 100     | 0.0444  |
| mir-17 family  | 6     | 75      | 0.0237  |
| mir-181 family | 4     | 100     | 0.0444  |
| mir-29 family  | 3     | 100     | 0.0418  |
| mir-329 family | 3     | 100     | 0.0418  |
| mir-368 family | 3     | 100     | 0.0418  |
| mir-99 family  | 3     | 100     | 0.0418  |

Analysis of overexpressed miRNAs was performed by TAM (tool for annotations of microRNAs, version 2). Size of miRNA category was set as 1–100.

Percent of miRNA changed between treated and controls cells within the cluster or family.

P < 0.05 indicates a significant number of miRNAs altered within a cluster/family.

Using TAM (tool for annotations of microRNAs, version 2; http://202.38.126.151/hmdd/tools/tam.html) analysis, our data indicate that the altered miRNAs fall into 43 clusters and 34 families, with 41 functions identified. Three clusters of miRNAs were significantly altered (P < 0.05) after 4h exposure to 25 µM H₂O₂, including hsa-let-7e, -let-106b, and -let-23b clusters (Table 1). Eight miRNA families (let-7, mir-15, mir-17, mir-181, mir-29, mir-329, mir-368, and mir-99) were significantly altered (P < 0.05) after H₂O₂ challenge (Table 1). Significant alterations (P < 0.05) occurred after exposure to H₂O₂ in miRNAs involved in critical cellular functions such as angiogenesis, apoptosis, cell proliferation, epithelial-mesenchymal transition, folliculogenesis, granulopoiesis, hormone regulation, human embryonic stem cell regulation, immune response, inflammation, anticell proliferation as well as miRNA tumor suppressors, and onco-miRNAs which can affect cell proliferation and invasion (Table 2). Notably, 21 out of 30 (70%) of evaluable miRNAs of the maternally imprinted miRNA cluster on chromosome 14 (C14MC) that is predominantly expressed in placenta and developing embryonic tissues were altered. Expression of the paternally imprinted chromosome 19 cluster (C19MC) was too low to be evaluated in this study.

3.3. H₂O₂ Alters mRNA Expression in Villous 3A Trophoblasts. In order to investigate the effect of oxidative stress on mRNA expression profile, we determined mRNA expression levels of 84 genes potentially involved in preeclamptic pregnancies. Villous 3A cells were mock-exposed in complete medium or exposed to 25 µM H₂O₂ in complete medium for 4 hours and total RNA was isolated using the Qiagen miRNeasy Mini Kit. The Qiagen Rt² Preeclampsia Array was used to evaluate the expression of these 84 genes after H₂O₂ exposure. As shown in Figure 3, of these 84 genes, 22 were up- or downregulated by at least twofold after H₂O₂ exposure. Of these, only

TABLE 2: Functional pathways regulated by miRNAs altered by H₂O₂ in villous 3A trophoblasts.

| Function          | Count | Percent | P value |
|-------------------|-------|---------|---------|
| Angiogenesis      | 14    | 61      | 7.9e-3  |
| Apoptosis         | 22    | 56      | 3.10e-3 |
| Bone regeneration | 17    | 61      | 3.41e-3 |
| Cell cycle related| 29    | 50      | 7.50e-3 |
| Cell proliferation| 16    | 62      | 3.76e-3 |
| Epithelial-mesenchymal transition | 24 | 63 | 1.72e-4 |
| Folliculogenesis  | 7     | 100     | 5.67e-4 |
| Granulopoiesis    | 9     | 90      | 4.48e-4 |
| Hormones regulation| 29  | 54      | 1.77e-3 |
| Human embryonic stem cell (hESC) regulation | 36 | 51 | 1.80e-3 |
| Immune response   | 29    | 73      | 3.36e-7 |
| Inflammation      | 22    | 65      | 2.02e-4 |
| Adipocyte differentiation | 16 | 64 | 2.08e-3 |
| Anticell proliferation | 11 | 100 | 1.07e-6 |
| Brain development | 12    | 63      | 9.45e-3 |
| Cell death        | 31    | 62      | 2.54e-5 |
| Cell division     | 11    | 73      | 2.29e-3 |
| Cell fate determination | 12 | 55 | 0.0415 |
| Hematopoiesis     | 18    | 62      | 1.77e-3 |
| Lipid metabolism  | 11    | 58      | 0.0304  |
| miRNA tumor suppressors | 24 | 65 | 9.08e-5 |
| Onco-miRNAs       | 19    | 61      | 1.61e-3 |

Analysis of altered miRNAs was performed by TAM (tool for annotations of microRNAs, version 2). Size of miRNA category was set as 1–100.

Percent of mRNA changed between treated and controls cells within a pathway/function.

P < 0.05 indicates a significant number of miRNAs altered within a pathway/function.
In the current study, H₂O₂ exerted a cytotoxic effect in 3A trophoblasts in a concentration- and time-dependent manner. These data are consistent with previous reports [5, 26]. However, the median inhibitory concentration (IC₅₀) of H₂O₂ in our study after 48 h exposure was 15 μM which is 10 times lower than the IC₅₀ reported by Zhou et al. using HTR-8/SVneo cells [5]. In a separate study, Moll et al. [26] used H₂O₂ concentrations up to 1000 μM, which is 66 times higher than our IC₅₀ concentration, to evaluate apoptosis and proliferation in human term placentas. Murata et al. [27] used a concentration of 100 μM H₂O₂ to determine apoptotic and invasion rates in term extravillous trophoblasts at 24 hours. This concentration is comparable to our IC₅₀ of 90 μM at 24 hours. This variability in results can be attributed to the variation in sensitivity of the cells studied to determine H₂O₂ toxic effects. The villous trophoblast cell line 3A used in our study, which is a first trimester placental cell line, seems to be more sensitive to H₂O₂ damage compared to HTR-8/SVneo cells or term primary cells used in these investigations [5, 26]. Our studies were carried out using 5% O₂ and 5% CO₂ to mimic in vivo conditions and to enhance oxidative stress [22, 23]. Both GSH and SOD are of major importance in intracellular redox regulation [29, 30]. Our results revealed a significant reduction in GSH level and SOD activity indicating oxidative stress at this exposure level.

Reports indicate that miRNAs regulate migration, invasion, apoptosis, and proliferation of trophoblasts as well as angiogenesis within the placenta, although the functions of only a few miRNAs have been characterized [18]. Expression changes of miRNAs that regulate these functions have been reported in PE. Wang et al. [20] demonstrated an increase in miR-17, -20a, and -20b in PE. These miRNAs target the HBEGF gene was found to be upregulated while the other 21 genes were downregulated (Table 3).

We used the Qiagen online RT² Profiler PCR Array Data Analysis v3.5 to determine the correlation between the altered miRNAs and mRNAs in our study. Our analysis indicates that 53 of the overexpressed miRNAs in our study putatively target 11 of the downregulated genes (Table 4).

4. Discussion

In the current study, H₂O₂ exerted a cytotoxic effect in 3A trophoblasts in a concentration- and time-dependent manner. These data are consistent with previous reports [5, 26]. However, the median inhibitory concentration (IC₅₀) of H₂O₂ in our study after 48 h exposure was 15 μM which is 10 times lower than the IC₅₀ reported by Zhou et al. using HTR-8/SVneo cells [5]. In a separate study, Moll et al. [26] used H₂O₂ concentrations up to 1000 μM, which is 66 times higher than our IC₅₀ concentration, to evaluate apoptosis and proliferation in human term placentas. Murata et al. [27] used a concentration of 100 μM H₂O₂ to determine apoptotic and invasion rates in term extravillous trophoblasts at 24 hours. This concentration is comparable to our IC₅₀ of 90 μM at 24 hours. This variability in results can be attributed to the variation in sensitivity of the cells studied to determine H₂O₂ toxic effects. The villous trophoblast cell line 3A used in our study, which is a first trimester placental cell line, seems to be more sensitive to H₂O₂ damage compared to HTR-8/SVneo cells or term primary cells used in these investigations [5, 26]. Our studies were carried out using 5% O₂ and 5% CO₂ to mimic in vivo conditions and to enhance oxidative stress [22, 23]. Both GSH and SOD are of major importance in intracellular redox regulation [29, 30]. Our results revealed a significant reduction in GSH level and SOD activity indicating oxidative stress at this exposure level.

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| Layout | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A      | ABCC1 | ABCG2 | ADM | AGTR1 | ANGPT2 | APLN | ATP1B1 | ATP2A2 | BCL6 | BHLHE40 | C3  | CAV1 |
|        | 1.46 | -1.58 | -1.07 | -1.01 | -2.49 | -1.87 | -1.87 | 1.96 | 1.89 | 1.34 | -1.04 | -1.25 |
| B      | CCL2 | CD40LG | CDH13 | CFD | CLU | COL1A4A1 | CP | CRH | CRHBP | CXCL10 | CXCL9 | CYP26A1 |
|        | -2.38 | -2.21 | -1.35 | -1.71 | 1.05 | -1.94 | -2.27 | -2.1 | 1.24 | -1.87 | -1.62 | -1.87 |
| C      | DCN | DUSP1 | EDN1 | ENG | F5 | FABP4 | FLT1 | FLT4 | FSTL3 | HBEGF | HGF | HIF1A |
|        | -2 | 1.64 | 1.01 | 1.19 | -1.87 | -1.7 | 1.62 | -1.69 | -1.61 | 5.76 | -1.94 | 1.57 |
| D      | HLA-G | HP | HSD17B1 | HSP90AA1 | HTR3A | HTRA1 | IFNG | IGF1 | IGF2B3 | IL10 | IL11 | IL15 |
|        | -1.37 | -2.41 | -1.87 | 1.38 | -1.8 | -1.06 | -1.87 | -2.6 | 1.07 | -1.18 | -1.63 | -1.38 |
| E      | IL18 | IL1A | IL2 | IL6 | IL8 | INHA | INHBA | ITGB3 | KIT | KRT19 | LEP | LPL |
|        | -2.64 | -2.02 | -1.87 | -2.02 | -1.64 | -1.87 | -1.04 | -1.98 | -1.22 | -1.87 | -8.42 | -2.22 |
| F      | MAI | MMP12 | MMP9 | NCA1 | NDRG1 | NO3S | NTRK2 | PAPPA2 | PDGFD | PGF | PGR | QPCT |
|        | -1.87 | -1.8 | -2.07 | -1.42 | -2.59 | -1.76 | -2.67 | -2.31 | -1.87 | 1.21 | -1.87 | -2.54 |
| G      | SERPINA3 | SOD1 | SPP1 | STAT1 | TAC1 | TAC3 | TEK | TGFBI | TNF | TREM1 | VCAN | VEGFA |
|        | -2.17 | -1 | -1.64 | -1.08 | -2.54 | -1.87 | -1.87 | 1.26 | -5.13 | -1.71 | -1.87 | 1.22 |

**Figure 3:** Heat map representation for the differential expression of genes associated with PE after 4 h exposure to H₂O₂ in villous 3A trophoblasts. Gene expression is represented in the heat map in the color scale of −3.073–3.073 in green-red color scheme (n = 2). Genes evaluated and their locations on the heat map are depicted in the associated table above the heat map.
the angiogenesis factors EPHB4 and ephrin-B2 and could be responsible for the decreases in angiogenesis seen in PE [20]. Upregulation of miR-29b results in increased apoptosis with a corresponding decrease in invasion and angiogenesis in trophoblast cells [31]. MiR-155 targets cyclin D1 and the angiogenic regulating factor CYR61; both are downregulated in PE [32, 33]. Overexpression of miR-210 in extravillous trophoblasts in culture results in decreased cell invasion [34]. MiR-376c, which is lower in both placenta and serum of preeclamptic women, reduces protein levels of ALK5 and ALK7, thus increasing cell proliferation [35].

In our study, after 4 h of H₂O₂ exposure, expression of 195 of evaluable miRNAs (CT < 35) was altered by at least twofold. Of these, only seven were downregulated. We chose to focus on the short-term exposure to H₂O₂ for the determination of miRNA profile as other studies show a rapid (<4 h) miRNA expression response and subsequent downregulation of protein targets [36]. Our data are in partial agreement with published studies that examined miRNA changes in PE, with some conflicts. There were 6 miRNAs reported to be either up- or downregulated in PE that were not altered in our system. These include miR-210 [37], -34a [38], -149 [19], -19b [39], -92b, and -197 [17]. Two miRNAs, miR-194 and 195, were upregulated in our system in contrast to reported downregulation in the literature [19, 40]. The discrepancy in results is not surprising since most studies were conducted on term placental tissue while we used a first trimester cytotrophoblast cell line. Expressions of placental miRNAs are known to be cell- and trimester-specific [14, 41]. In addition, PE often occurs concurrently with IUGR (intrauterine growth restriction) and preterm labor, both of which have their own miRNA pattern which can thus produce conflicting data. The placental mammal specific miRNA cluster on chromosome 14 (C14MC) predominantly expressed in placenta and embryonic tissues and maternally imprinted had 21 out of 30 (70%) of evaluable miRNAs altered in our study. Meanwhile, expression of the paternally imprinted C19MC cluster was not detected. This expression pattern is in agreement with that found by Morales-Prieto et al. [14], showing higher expression of the C14 cluster in the first trimester with very little expression of C19 miRNAs. Although there are some data indicating an increase in C19MC miRNAs in PE, this increase is most likely due to a loss of methylation as this miRNA cluster is paternally imprinted [15]. Evidence suggests that hypomethylation of multiple genes contributes to early onset PE [42]. Data is mostly lacking for the role of the C14MC cluster in PE. MiR-483-5p was not altered while miR-377 was significantly upregulated in one study of preeclamptic women [16]. Yet, neither of these miRNAs could be evaluated in our study due to the high cycle number in our assay (CT > 35).

The expression profile of 84 genes known or suspected to be altered in PE was examined in this study (Figure 3). These genes are involved in different pathways including pregnancy maintenance, oxidative stress, hormones, growth factors, endothelial cell signaling, and signal transduction. As shown in Table 3, of these 84 genes, 22 were up- or downregulated

### Table 3: miRNAs altered by at least twofold after 4 h H₂O₂ exposure in villous 3A trophoblasts.

| Gene symbol | Gene description                                      | Fold changeb |
|-------------|-------------------------------------------------------|--------------|
| HBEGF      | Heparin-binding EGF-like growth factor                | 5.7637       |
| ANGPT2     | Angiopoietin 2                                        | -2.4932      |
| CCL2       | Chemokine (C-C motif) ligand 2                        | -2.3834      |
| CD40LG     | CD40 ligand                                           | -2.2084      |
| CP          | Ceruloplasmin (ferroxidase)                           | -2.2705      |
| CRH         | Corticotropin releasing hormone                       | -2.1038      |
| DCN         | Decorin                                               | -2.0042      |
| HP          | Haptoglobin                                           | -2.4083      |
| IGF1        | Insulin-like growth factor (somatomedin C)            | -2.5991      |
| IL18        | Interleukin 18 (interferon-gamma-inducing factor)     | -2.6445      |
| IL1A        | Interleukin 1, alpha                                   | -2.0748      |
| IL6         | Interleukin 6 (interferon, beta 2)                    | -2.0811      |
| LEP         | Leptin                                                | -8.4152      |
| LPL         | Lipoprotein lipase                                     | -2.2238      |
| MMP9        | Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase) | -2.0748      |
| NDRG1       | N-myc downstream regulated 1                          | -2.5901      |
| NTRK2       | Neurotrophic tyrosine kinase, receptor, type 2         | -2.6721      |
| PAPPA2      | Pappalysin 2                                          | -2.3102      |
| QPCT        | Glutaminyl-peptide cyclotransferase                    | -2.5368      |
| SERPINA3    | Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 | -2.1705      |
| TAC1        | Tachykinin precursor 1                                | -2.5368      |
| TNF         | Tumor necrosis factor                                 | -5.1266      |

aUpregulated gene is in bold font.
bFold change in H₂O₂ treated cells compared to nontreated control.
Table 4: Altered miRNAs with putative altered mRNAs targets after 4 h exposure to H₂O₂ in villous 3A trophoblasts.

| miRNA name       | Number of genes targeted by this miRNA | Number of target sites identified in target genes | Range of strength scores | Target genes         |
|------------------|----------------------------------------|--------------------------------------------------|--------------------------|----------------------|
| hsa-miR-181a-5p  | 3                                      | 3                                                | −0.4593 to −0.234        | DCN, IL1A, TNF       |
| hsa-miR-181c-5p  | 3                                      | 3                                                | −0.4573 to −0.219        | DCN, IL1A, TNF       |
| hsa-miR-181b-5p  | 3                                      | 3                                                | −0.4593 to −0.234        | DCN, IL1A, TNF       |
| hsa-miR-181d-5p  | 3                                      | 3                                                | −0.4573 to −0.234        | DCN, IL1A, TNF       |
| hsa-miR-27b-3p   | 3                                      | 3                                                | −0.203 to −0.0872        | IGF1, LEP, LPL       |
| hsa-miR-29a-3p   | 3                                      | 4                                                | −0.4102 to −0.094        | IGF1, LEP, LPL       |
| hsa-miR-29c-3p   | 3                                      | 4                                                | −0.4102 to −0.09         | IGF1, LEP, LPL       |
| hsa-miR-875-3p   | 3                                      | 3                                                | −0.504 to −0.2443        | CD40LG, NDRG1, TNF   |
| hsa-miR-9-5p     | 3                                      | 3                                                | −0.453 to −0.0467        | IGF1, LEP, NDRG1     |
| hsa-miR-454-3p   | 2                                      | 3                                                | −0.3152 to −0.219        | IGF1, TNF            |
| hsa-miR-543      | 2                                      | 2                                                | −0.39 to −0.259          | ANGPT2, IL1A         |
| hsa-miR-30e-5p   | 2                                      | 2                                                | −0.452 to −0.1342        | IGF1, IL1A           |
| hsa-miR-30b-5p   | 2                                      | 2                                                | −0.452 to −0.1236        | IGF1, IL1A           |
| hsa-miR-30a-5p   | 2                                      | 2                                                | −0.452 to −0.1342        | IGF1, IL1A           |
| hsa-miR-30c-5p   | 2                                      | 2                                                | −0.452 to −0.1236        | IGF1, IL1A           |
| hsa-miR-30d-5p   | 2                                      | 2                                                | −0.452 to −0.1342        | IGF1, IL1A           |
| hsa-miR-424-5p   | 1                                      | 1                                                | −0.0766 to −0.0766       | IGF1                 |
| hsa-miR-152-3p   | 1                                      | 1                                                | −0.3972 to −0.3972       | IGF1                 |
| hsa-miR-148b-3p  | 1                                      | 1                                                | −0.3972 to −0.3972       | IGF1                 |
| hsa-miR-425-5p   | 1                                      | 1                                                | −0.4961 to −0.4961       | IGF1                 |
| hsa-miR-16-5p    | 1                                      | 1                                                | −0.0977 to −0.0977       | IGF1                 |
| hsa-miR-497-5p   | 1                                      | 1                                                | −0.0766 to −0.0766       | IGF1                 |
| hsa-miR-490-5p   | 1                                      | 1                                                | −0.197 to −0.197         | NTRK2                |
| hsa-miR-193a-5p  | 1                                      | 1                                                | −0.115 to −0.115         | NTRK2                |
| hsa-miR-151a-5p  | 1                                      | 1                                                | −0.401 to −0.401         | NTRK2                |
| hsa-miR-330-3p   | 1                                      | 2                                                | −0.05 to −0.01           | NTRK2                |
| hsa-miR-17-5p    | 1                                      | 2                                                | −0.149 to −0.148         | NTRK2                |
| hsa-miR-1271-5p  | 1                                      | 1                                                | −0.108 to −0.108         | NDRG1                |
| hsa-miR-20a-5p   | 1                                      | 2                                                | −0.07 to −0.148          | NTRK2                |
| hsa-miR-154-5p   | 1                                      | 1                                                | −0.263 to −0.263         | NTRK2                |
| hsa-miR-26b-5p   | 1                                      | 1                                                | −0.1205 to −0.1205       | IGF1                 |
| hsa-miR-766-3p   | 1                                      | 1                                                | −0.161 to −0.161         | IGF1                 |
| hsa-miR-190a-5p  | 1                                      | 1                                                | −0.1862 to −0.1862       | IGF1                 |
| hsa-miR-452-5p   | 1                                      | 1                                                | −0.3111 to −0.3111       | IGF1                 |
| hsa-miR-320a     | 1                                      | 1                                                | −0.1466 to −0.1466       | IGF1                 |
| hsa-miR-320b     | 1                                      | 1                                                | −0.1466 to −0.1466       | IGF1                 |
| hsa-miR-18b-5p   | 1                                      | 1                                                | −0.2185 to −0.2185       | IGF1                 |
| hsa-miR-374a-5p  | 1                                      | 1                                                | −0.511 to −0.511         | CCL2                 |
| hsa-miR-26a-5p   | 1                                      | 1                                                | −0.105 to −0.105         | IGF1                 |
| hsa-miR-222-3p   | 1                                      | 1                                                | −0.1612 to −0.1612       | IGF1                 |
| hsa-miR-221-3p   | 1                                      | 1                                                | −0.1612 to −0.1612       | IGF1                 |
| hsa-miR-192-5p   | 1                                      | 1                                                | −0.2211 to −0.2211       | IGF1                 |
| hsa-miR-196a-5p  | 1                                      | 1                                                | −0.0267 to −0.0267       | IGF1                 |
| hsa-let-7f-5p    | 1                                      | 1                                                | −0.1633 to −0.1633       | IGF1                 |
| hsa-let-7a-5p    | 1                                      | 1                                                | −0.1592 to −0.1592       | IGF1                 |
by at least twofold after H\textsubscript{2}O\textsubscript{2} exposure. While it is difficult to provide mechanistic explanations to all changes observed in the expression profiles of the 22 genes, it is possible to provide some explanations based on the putative function of these genes in PE and through the correlation of our miRNA and mRNA data. For example, expression of Heparin-binding EGF-like growth factor (HBEGF) was upregulated by 5.8-fold in cells challenged with H\textsubscript{2}O\textsubscript{2}. HBEGF is expressed in both villous and extravillous trophoblasts through the first trimester [43] and is known to act as a survival factor that hampers apoptosis [44] triggered by oxidative stress or other factors [26, 45]. Therefore, its induction under the current experimental conditions could be a cellular defense against oxidative stress known to be associated with PE.

Our data showed that the expression of monocyte chemoattractant protein-1 (CCL2) was downregulated, while the expression of its putative regulator mir-374a-5p was upregulated. CCL2 is known to be expressed in first trimester trophoblasts [46] and is responsible for the activation and recruitment of macrophages to the developing placenta to aid in tissue remodeling after implantation [47]. The expression of CCL2 in first trimester trophoblasts was reported to be regulated by tumor necrosis factor (TNF-\(\alpha\)) [48]. In our study, expression level of TNF-\(\alpha\) was also downregulated as was angiopoietin-2 (ANGPT2) when exposed to increased oxidative stress. ANGPT2 is crucial in regulating vascular remodeling through its interaction with endothelial cell Tie-2 receptor [49]. The levels of ANGPT2 were found to be lower in PE compared to normal pregnancy [50]. Similarly, we found the insulin-like growth factor-1 (IGF-1) expression to be decreased. IGF-1 was reported to be decreased in placental tissues from women suffering PE [51]. Of note is that 38 miRNAs that putatively regulate IGF-1 (Table 4) were upregulated in our study. The pregnancy-associated plasma protein A2 (PAPP-A2) is an insulin-like growth factor-binding protein (IGFBP) protease expressed at high levels in the placenta. Increased levels of PAPP-A2 in PE suggest a compensatory response to abnormal placentalization, which might increase insulin-like growth factor (IGF) availability and promote fetoplacental growth [52]. However, PAPP-A2 was downregulated in our study, which might also explain the observed reduction in IGF-1 expression in the current work.

**Table 4: Continued.**

| miRNA name          | Number of genes targeted by this miRNA | Number of target sites identified in target genes | Range of strength scores\(^a\) | Target genes |
|---------------------|----------------------------------------|-----------------------------------------------|-------------------------------|--------------|
| hsa-miR-196b-5p     | 1                                      | 1                                             | −0.0267 to −0.0267            | IGF1         |
| hsa-let-7b-5p       | 1                                      | 1                                             | −0.1592 to −0.1592            | IGF1         |
| hsa-let-7i-5p       | 1                                      | 1                                             | −0.1592 to −0.1592            | IGF1         |
| hsa-let-7e-5p       | 1                                      | 1                                             | −0.1592 to −0.1592            | IGF1         |
| hsa-let-7d-5p       | 1                                      | 1                                             | −0.1592 to −0.1592            | IGF1         |
| hsa-let-7c-5p       | 1                                      | 1                                             | −0.1612 to −0.1612            | IGF1         |
| hsa-let-7g-5p       | 1                                      | 1                                             | −0.1592 to −0.1592            | IGF1         |
| hsa-miR-576-5p      | 1                                      | 1                                             | −0.1892 to −0.1892            | IGF1         |

\(^a\)Strength scores are the Z scores derived from the TargetScan algorithm. A more negative number indicates a stronger score and an increased likelihood that the gene is a bona fide target for the miRNA evaluated.

The levels of matrix metalloproteinase-9 (MMP9) were decreased in trophoblast cells exposed to H\textsubscript{2}O\textsubscript{2}. MMP9 is an important factor for extracellular matrix remodeling and is responsible for the invasiveness of trophoblasts [53]. Its deficiency induced phenotypic changes that mimic PE in mice [54] and its levels are reduced in pregnancies complicated with PE [55]. Moreover, the level of corticotrophin releasing hormone (CRH) was also downregulated by H\textsubscript{2}O\textsubscript{2} stress. CRH is released by trophoblasts to promote embryo implantation [56].

Leptin (LEP) gene expression was downregulated in our study. In silico analysis predicted the LEP to be a target of four miRNAs (mir-27b-3p, mir-29a-3p, mir-29c-3p, and mir-9-5p; Table 4). All of those miRNAs were overexpressed under the current experimental conditions. Our findings contrast other reports indicating that LEP is overexpressed in placental tissues at term in PE [57]. It should be noted however that most published data is derived from studies of term trophoblasts or placental tissues while our study focuses on first trimester trophoblasts, which mimics the early stage of pregnancy. This can explain some of the discrepancies in gene expression data observed between our study and others.

Our study has some limitations which we acknowledge. We examined only short-term effects of high H\textsubscript{2}O\textsubscript{2} exposure on cell viability and short-term effects of low H\textsubscript{2}O\textsubscript{2} exposure on miRNA and mRNA gene expression profiles. Gene expression changes can differ due to hypoxic versus normal growth conditions or short- versus long-term exposure. This study focuses only on short-term exposure and cytotoxicity due to the reported rapid changes in miRNA expression after initial exposure [36] but will be expanded in the future to include long-term genetic changes. In addition, a change in miRNA or mRNA expression does not necessarily lead to a change in encoded protein levels, protein modification, or protein function. Such information would be generated from specifically designed studies based on our findings. Lastly, we are using an SV40-transformed first trimester cell line rather than primary cells or choriocarcinoma cancer cells, which, to our knowledge, is one of the best available models for studies such as ours. Although the *in vitro* approach provides conformity of cell type over multiple experiments, *in vitro* response to ROS may not totally mimic the *in vivo* response.
In summary, our data indicate that short-term exposure of 3A villous first trimester trophoblasts to H$_2$O$_2$ significantly alters miRNA profile and mRNA expression of genes implicated in defective placental development. Our data, which indicate that oxidative stress alters miRNAs and RNAs expression, could partially explain some of the early changes in gene expression profiles and miRNA observed in PE.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Authors’ Contribution**

Courtney E. Cross and Mai F. Tolba contributed equally to this paper.

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