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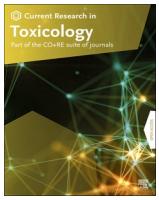

Mono-(2-ethylhexyl) phthalate induces trophoblast hypoxia and mitochondrial dysfunction through HIF-1α-miR-210-3p axis in HTR-8/SVneo cell line

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ABSTRACT

The exposure to the ubiquitous phthalate metabolite mono-(2-ethylhexyl) phthalate (MEHP) is connected to dysregulated trophoblast function and placenta health; however, the underlying mechanisms preluding this scenario remain to be elucidated. In this study, we explored the hypoxemic effects of MEHP on a human placental first-trimester trophoblast cell line (HTR-8/Svneo). MEHP-treated trophoblast cells displayed significantly increased levels of oxidative stress and hypoxia-inducible factor-1 alpha (HIF-1α) attributed by the induction of hypoxia. Further, HIF-1α exhibited higher DNA binding activity and upregulated gene expression of its downstream target vascular endothelial growth factor A (VEGFA). The hypoxia-induced microRNA miR-210-3p was also significantly increased upon MEHP treatment followed by disrupted mitochondrial ATP generation and membrane potential. This was identified to possibly be facilitated by lowered mitochondrial DNA copy number and inhibited expression of electron transport chain subunits, such as mitochondrial complex-IV. These results suggest potential adverse effects of MEHP exposure in a trophoblast cell line mediated by HIF-1α and the epigenetic modulator miR-210-3p. Chronic placental hypoxia and oxidative stress have long been implicated in the pathogenesis of pregnancy complications such as preeclampsia. As we've revealed genetic and epigenetic factors underscoring a potential mechanism induced by MEHP, this brings to light another significant implication of phthalate exposure on maternal and fetal health.

Introduction

Phthalates are a widely used class of plasticizers that can be found in various consumer products including daily use plastics and personal care items (Shree et al., 2022). The pervasive presence of phthalates has begun to raise concern due to their potentially detrimental effects on human health (Shree et al., 2022; Wang and Qian, 2021). It is also proposed that women are more highly exposed to phthalates due to the frequent use of feminine hygiene products (Gao et al., 2020). Epidemiological studies have indicated that several phthalate metabolites were significantly associated with an increased risk of pregnancy complications including preeclampsia (PE) and preterm birth (Hirke et al., 2023; Kolan and Hall, 2023). Among these metabolites, mono-(2-ethylhexyl) phthalate (MEHP), a metabolite of di(2-ethylhexyl) phthalate (DEHP), emerges as a significant detriment to reproductive health, especially pertaining to placental function (Martínez-Razo et al., 2021; Wang et al., 2016). Research on phthalate exposure has consistently revealed elevated levels of MEHP in the urine of patients with preterm birth and fetal growth restriction (Martínez-Razo et al., 2021; Ferguson et al., 2014). Moreover, a study employing various cell models has demonstrated MEHP-induced alterations in trophoblast transcriptomic profiles (Lapehn et al., 2023). Collectively, evidence strongly suggests a correlation between MEHP exposure and placental health.

The placenta is a transient organ that plays a pivotal role during pregnancy (Serman and Serman, 2011). It is responsible for the exchange of nutrients, oxygen, and waste products between the mother

Abbreviations: **DEHP**, **di(2-ethylhexyl) phthalate**; **MEHP**, **mono-(2-ethylhexyl) phthalate**; **EVT**, **Extravillous trophoblasts**; **HIF-1α**, **Hypoxia-inducible factor-1 alpha**; **PE**, **preeclampsia**; **ROS**, **reactive oxygen species**; **MiR**, **microRNA**; **VEGFA**, **vascular endothelial growth factor A**; **DMSO**, **dimethyl sulfoxide**; **PBS**, **phosphate buffered saline**; **MtDNA**, **mitochondrial DNA**; **nDNA**, **nuclear DNA**; **NDUFB8**, **NADH: Ubiquinone Oxidoreductase Subunit B8**; **SDHB**, **Succinate Dehydrogenase Complex Iron Sulfur Subunit B**.

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and the fetus. During placentation, a subgroup of trophoblasts differentiates from a proliferative subtype into an invasive subtype known as extravillous trophoblasts (EVTs) (Fujiwara et al., 2005). EVTs facilitate proper anchoring and vascularization of the placenta by invading the decidua and remodeling spiral arteries (Fujiwara et al., 2005). Compromised placental health can have profound consequences on the development of the fetus, potentially leading to adverse pregnancy outcomes (Burton et al., 2016). Among the many potential complications, PE is the most daunting by threatening the lives of both mother and child, affecting 5–8 % of pregnancies worldwide (Rana et al., 2019). PE is typically associated with increased placental hypoxia or ischemia and the release of mediators that damage the maternal endothelium (Tal, 2012). During the early stages of pregnancy, before placental vasculature is established, hypoxia-inducible factor-1 alpha (HIF-1α) is a transcription factor that plays a crucial role in facilitating the adaptation to a low oxygen environment (Caniggia et al., 2000). HIF-1α accomplishes this by regulating pivotal trophoblast functions such as angiogenesis and invasion, further promoting survival and growth of both the embryo and placenta in this hypoxic environment (James et al., 2006; Hayashi et al., 2004). However, in preeclamptic pregnancies, this finely tuned balance is disrupted, leading to unattenuated, increased transcriptional activity of HIF-1α (Tal, 2012). This imbalance of HIF-1α expression in the placenta can induce apoptosis, bearing detrimental effects on trophoblast development and function, ultimately contributing to the pathophysiology of PE.

Recently, studies indicate that epigenetic modulators such as non-coding RNAs, including microRNAs (miRs), also contribute to normal placental development (Choudhury and Friedman, 2012). A hypoxia-induced miR, miR-210-3p, has previously been demonstrated to be upregulated in the placentas of women experiencing PE (Chan and Loscalzo, 2010; Jaszczuk et al., 2022). This miR was further discovered to modulate various cellular mechanisms involved in oxidative stress including mitochondrial metabolism, angiogenesis, DNA repair, and cell survival (Bavelloni et al., 2017; Wen et al., 2023; Anton et al., 2019). MiR-210-3p is essential in modulating mitochondrial metabolism by negatively regulating mitochondrial respiration activity and increasing the generation of reactive oxygen species (ROS) (Dang and Myers, 2015). MiR-210-3p exerts this role by directly inhibiting key genes of the electron transport chain such as Iron-sulfur cluster assembly enzyme and Cytochrome *c* oxidase 10 (Chan et al., 2009; Chen et al., 2010). Several studies have provided evidence that the expression of miR-210-3p can also be induced by HIF-1α, providing a possible relationship between a miR and a mediator of PE (Huang et al., 2009; Zhao et al., 2022; Cicchillitti et al., 2012).

The influence of the phthalate metabolite MEHP on the underpinning regulations of trophoblast development and function remains uncertain. Several rodent models have provided a closer simulation of the pivotal role that trophoblasts play in placental development and pregnancy complications such as PE (Alexander et al., 2001; Abbott et al., 1987; Maynard et al., 2003). In this study, we aimed to investigate the effects of MEHP on trophoblast dysfunction mediated by hypoxia induction and ROS production. Therefore, we examined this scenario in HTR-8/SVneo cells, a widely-used EVT model for *in vitro* studies (Ding et al., 2016; Msheik et al., 2020; Sun et al., 2018). We demonstrated that MEHP increased oxidative stress and induced hypoxia in treated cells in a dose-dependent manner. We also identified that the activation of HIF-1α is a critical contributor to the induction of hypoxia. Furthermore, we determined that MEHP exposure promotes the expression of miR-210-3p that regulates downstream genes involved in placental oxidative stress and mitochondrial respiration, such as succinate dehydrogenase complex iron sulfur subunit B (SDHB, mitochondrial complex-II) and mitochondrial complex-IV (Cicchillitti et al., 2012; Ramakrishnan et al., 2014). Our findings reveal a potential mechanistic link where MEHP functions as a mimic of hypoxia, activating the HIF-1α-miR-210-3p pathway to induce trophoblast oxidative stress, which can compromise placental health predeceasing pregnancy complications such as PE.

Materials and methods

Chemicals

MEHP (Cat#CDS010608) was purchased from Sigma-Aldrich (St. Louis, MO). Anti-HIF-1α antibody (Cat#3716) and goat anti-rabbit IgG (Cat#7047) were purchased from Cell Signaling (MA, USA). Anti-actin (Cat#sc-47778) and anti-tubulin (Cat#sc-8432) polyclonal antibodies were acquired from Santa Cruz, Ltd. (CA, USA).

Cell culture and MEHP treatment

The experiments were carried out in HTR-8/SVneo cell line, which are immortalized cells derived from first-trimester human trophoblasts. These cells exhibit markers of extravillous invasive trophoblasts, including invasion and migration capabilities, and has been widely used in trophoblast studies (Ding, Chen et al. 2016, Sun, Na et al. 2018, Msheik, Azar et al. 2020). HTR-8/SVneo cells were cultured in 75 cm^2 culture flasks in RPMI 1640 media with L-glutamine (Corning Cellgro, VA) supplemented with 10 % fetal bovine serum (Corning Cellgro, VA) and 1 % penicillin/streptomycin. The cells were maintained in a tissue culture incubator with a humidified atmosphere of 5 % CO_2 and a temperature of 37 °C. The dimethyl sulfoxide (DMSO) concentration was maintained at 0.1 % for the treatment and control groups. Cells were treated with 50, 100, 180, 250, or 360 μM MEHP for 4, 24, or 48 h. The concentrations were selected based on an environmentally relevant range and described in our previous articles (Am et al., 2005; Tetz et al., 2013).

Hypoxia/Oxidative stress Assay

A hypoxia/oxidative stress detection kit (Cat#ENZ-51042, Enzo Life Sciences) was used for detecting hypoxia and oxidative stress in the placental cells treated with MEHP. HTR-8/SVneo cells were treated with DMSO (negative control), 125 μM cobalt chloride ($CoCl_2$), an inducer of hypoxia-related responses (positive control), or varying concentrations of MEHP for 48 h. Cells were incubated with hypoxia or oxidative stress probes according to the manufacturer's protocol. The fluorescent signals were detected with a microplate reader (Infinite® M1000 Pro, TECAN, NC). The experiments were repeated three times with three technical replicates.

HIF-1α transcription factor Assay

HIF-1α stability in MEHP-treated placental cells was detected using a HIF-1α transcription factor assay kit according to the kit directions (Cat#10006910, Cayman Chemical). In brief, nuclear proteins were extracted from HTR-8/SVneo cells treated with various concentrations of MEHP or negative control DMSO for 48 h. Approximately 10–15 μg of protein was used for the assay. HIF-1α complexes bound to a specific double-stranded DNA sequence containing the HlF-1α response element (5′-ACGTG-3′) were then detected using an anti-HIF-1α antibody. A secondary antibody conjugated to horseradish peroxidase was added to provide a sensitive colorimetric readout at 450 nm. Nuclear extracts of deferoxamine-treated HeLa cells were used as the positive control as deferoxamine is an established HIF-1α stabilizer. The experiments were repeated three times with three technical replicates.

Immunofluorescence microscopy

HTR-8/SVneo cells (1x10^4 per well) were seeded in a chamber slide (Falcon) and incubated overnight at 37 °C and 5 % CO_2. Cells were then treated with MEHP (180 μM) for 48 h. After treatment, the cell culture media was removed and the cells were then fixed with 4 % paraformaldehyde (pH 7.4) for 30 min at room temperature. After washing the cells with phosphate buffered saline (PBS), cells were permeabilized

with 0.1 % Triton X-100 in PBS and incubated at room temperature for 30 min. Cells were blocked using a blocking solution (2 % bovine serum albumin in PBS) for 60 min at room temperature. Anti-HIF-1α (10 μg) was diluted in blocking solution and added to the cells for overnight incubation at 4 °C. Cells were washed and labeled with Alexa Fluor 594-labeled antibody (Invitrogen) and DAPI for 45 min at room temperature, protected from light. The cells were washed, dried, and mounted in SlowFade antifade reagent (Cat#S36917, ThermoFisher Scientific, TX), and visualized by fluorescence microscopy on an Olympus BX1 microscope equipped with a 60x objective (OLYMPUS UPlanFL N 60X oil) with NA=1.25. Images were acquired using Olympus CellSens Standard 1.18 software. All images were acquired under the same conditions, three technical replicates were in each group.

ATP measurement

The CellTiter-Glo® Assay (Cat#G7570, Promega, WI) was used to quantify ATP levels in the cells treated with MEHP. Briefly, HTR-8/SVneo cells were plated in 96-well plates and treated for 48 h with various concentrations of MEHP. 100 μl of CellTiter-Glo Reagent was used per well and the contents were then mixed for 2 min to induce cell lysis. After the plate was incubated at room temperature for 10 min to stabilize the luminescent signal, luminescence was recorded using a microplate reader (Infinite® M1000 Pro, TECAN, NC). The experiments were repeated three times with three technical replicates.

Mitochondrial DNA content

Total DNA was extracted from the cells using a DNA extraction kit (Cat# 56304, Qiagen, Germany), according to the manufacturer's recommendations. Template DNA (2 μL) was assessed with 2 μL of specific primer pairs (400 nM final concentration each) for mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). The detection of a mtDNA copy number utilized a 107 bp amplicon of mtDNA tRNALeu (UUR), and nDNA was detected with an 86 bp amplicon of β2-microglobulin as an internal control (Venegas et al., 2011). The cycling parameters used in the ABI 7300 Real-Time PCR System were: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and annealing temperature (primer specific) for 60 s. A dissociation curve was calculated for each sample to ensure the presence of a single PCR product. mtDNA and nDNA Ct values from triplicate reactions were used to determine the mtDNA content relative to nDNA using the following equations: ΔCT = (nDNA CT – mtDNA CT); Relative mtDNA content = $2 \times 2^{\Delta CT}$. The sequences of primers used are: tRNA-Leu(UUR)-forward: 5′-CAC CCA AGA ACA GGG TTT GT-3′, reverse: 5′-TGG CCA TGG GTA TGT TGT TA-3′; β2-microglobulin-forward: 5′-TGC TGT CTC CAT GTT TGA TGT ATC T-3′, reverse: 5′-TCT CTG CTC CCC ACC TCT AAG T-3′. The experiments were repeated three times with three technical replicates.

Real-Time PCR

HTR-8/SVneo cells were seeded at a density of 80,000 cells/well in a 24-well plate and allowed to adhere for 20 h. Cells were treated with DMSO or MEHP (50, 100, and 180 μM) for 4, 8, 24 and 48 h. MiRNA and mRNA were extracted using an E.Z.N.A. miRNA Kit (Omega Bio-Tek, GA) according to the manufacturer's protocol. Respectively, for mRNA Real-Time PCR, reverse transcription (RT) was carried out using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, NY) according to the manufacturer's instructions, and PCR was performed with SYBR Green PCR Master Mix (Life Technologies, NY). Relative gene expression was normalized to 18S. MiR Real-Time PCR, cDNA synthesis, and miR-specific quantitative real-time PCR were performed as described previously (Meruvu et al., 2016). Relative miRNA expression was normalized to U6. The primer sequences are listed in the Supplementary Table S1. Data was analyzed using the $2^{-\Delta\Delta Ct}$ method and represented as fold change. The experiments were repeated three times with three technical replicates.

Western blot analysis

HTR-8/SVneo cells (9 x 10^4 cells/well) were plated in 6-well plates and treated with different concentrations of MEHP. The cells were then lysed after 24 h for protein extraction and used for Western blot as previously described (Ding et al., 2024). Briefly, Pierce™ BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA) was used to determine protein concentration. Protein lysate (10 μg) was separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane. Membranes were imaged using a LiCor Odyssey scanner and blots were analyzed by ImageStudio software (LiCor Lincoln, NE). Primary antibodies included UQCRC2 (sc-390378) and SDHB (sc-271548) from Santa Cruz Biotechnology (Dallas, TX), and COX-IV (#11967) from Cell Signaling Technology (Danvers, MA). Goat anti-mouse IRDye 680 secondary antibodies (Cat# 926–68070) from LiCor (Lincoln, NE) were used for the detection and quantification of immunoblots. The experiments were repeated two times with three technical replicates.

JC-1 assay

Mitochondrial membrane potential was measured with JC-1 – Mitochondrial Membrane Potential Assay Kit (ab113850, Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions. Briefly, HTR-8/SVneo cells were seeded and incubated with JC solution for 10 min and were treated with DMSO (negative control), Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (positive control), or MEHP for 4, 24, 48 h. The fluorescent signals of aggregate excitation (590 nm) and monomer excitation (530 nm) were detected with a microplate reader (Infinite® M1000 Pro, TECAN, NC, USA). The membrane potential was calculated as the ratio of aggregate/monomer signals. All data were normalized to the DMSO group.

Statistical analysis

All data are represented as a mean ± standard error (SEM). Comparison between groups was performed by one-way ANOVA followed by Tukey's post hoc test unless mentioned otherwise. $P<0.05$ was considered statistically significant in all experiments.

Results

MEHP induces hypoxia and oxidative stress in HTR-8/SVneo cells

Hypoxia during the first trimester can induce oxidative stress, disrupting normal trophoblast development and resulting in impaired placental function (Schoots et al., 2018). This, in turn, may give rise to pregnancy complications such as PE (Schoots et al., 2018). Therefore, we investigated whether MEHP can induce hypoxia and oxidative stress in first-trimester trophoblast cell line HTR-8/SVneo. MEHP treatment in the range of 100–360 μM induced significant hypoxia compared to the control group ($p < 0.005$) (Fig. 1A). This induction is similar to the effect of $CoCl_2$, a known hypoxia mimetic agent. Furthermore, MEHP also induced oxidative stress in the placental cells with a significant increase ($p < 0.005$) in cellular ROS generation when treated with 50–360 μM of MEHP (Fig. 1B). Interestingly, $CoCl_2$ treatment did not induce oxidative stress in these cells. These results demonstrate that MEHP significantly induces hypoxia and oxidative stress in placental cells at environmentally relevant doses.

MEHP promotes HIF-1α expression and activity in HTR-8/SVneo cells

HIF-1α is a key mediator of hypoxia (Hu et al., 2003). To investigate the underlying causal pathway that induces hypoxia and oxidative stress, we evaluated the transcriptional expression of HIF-1α in MEHP-

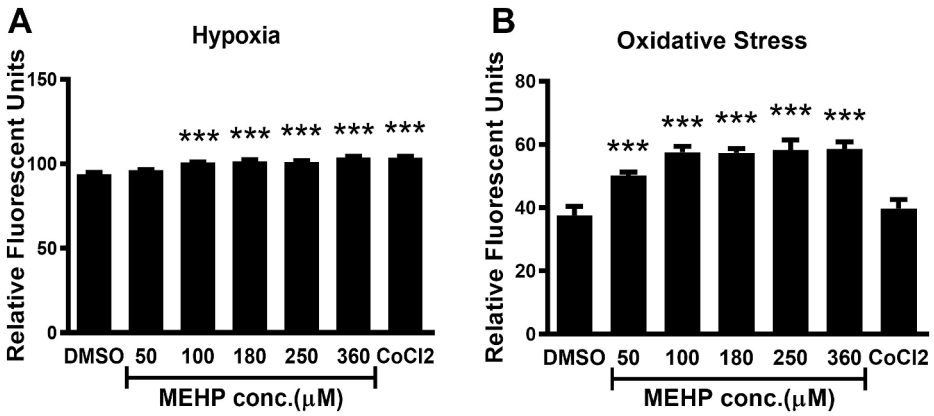

Fig. 1. MEHP induces hypoxia and oxidative stress in HTR-8/SVneo cells. The relative fluorescent units of cells treated with different concentrations of MEHP, DMSO, or $CoCl_2$ (hypoxemic positive control) for 48 h and treated with hypoxia probe (**A**) and oxidative stress probe (**B**). Data are presented as means ± SEM from 3 independent experiments (n = 3). DMSO vs treatment. ***$p < 0.005$.

treated placental cells at earlier time points, including 4 h and 24 h. A significant upregulation of HIF-1α was detected at 24 h and 48 h when treated with 180 μM MEHP ($p < 0.05$) while increasing trends were exhibited at 4 h or when treated with 100 μM MEHP (Fig. 2A). Additionally, 100 μM and 180 μM MEHP also significantly increased HIF-1α DNA-binding capacity (100 μM: $p < 0.05$; 180 μM: $p < 0.01$) in a dose-dependent manner compared to the DMSO control, indicating higher HIF-1α activity (Fig. 2B). Moreover, HIF-1α nuclear translocation was significantly increased under the 180 μM dose of MEHP treatment (Fig. 2C). To validate the increased activity of HIF-1α, we measured the expression level of vascular endothelial growth factor A (VEGFA), a known downstream target of HIF-1α, through real-time PCR. As expected, VEGFA exhibited a dose-dependent increase of expression with 50 μM ($p < 0.01$), 100 μM ($p < 0.005$), and 180 μM ($p < 0.005$) MEHP treatment at 48 h (Fig. 2D), supporting the effect of increased HIF-1α activity. These results suggest a potential mechanistic link between MEHP exposure and HIF-1α activation, highlighting its role in mediating downstream effects related with hypoxia in trophoblast cells.

MiR-210-3p is induced in MEHP-treated HTR-8/SVneo cells

To explore a possible non-coding connection by which MEHP impacts trophoblast hypoxia, we examined the level of the hypoxia-related microRNA miR-210-3p (Ivan and Huang, 2014). Upregulation of miR-210-3p was observed in the HTR-8/SVneo cells treated with MEHP in a dose- and time-dependent manner (Fig. 3). There was a significant

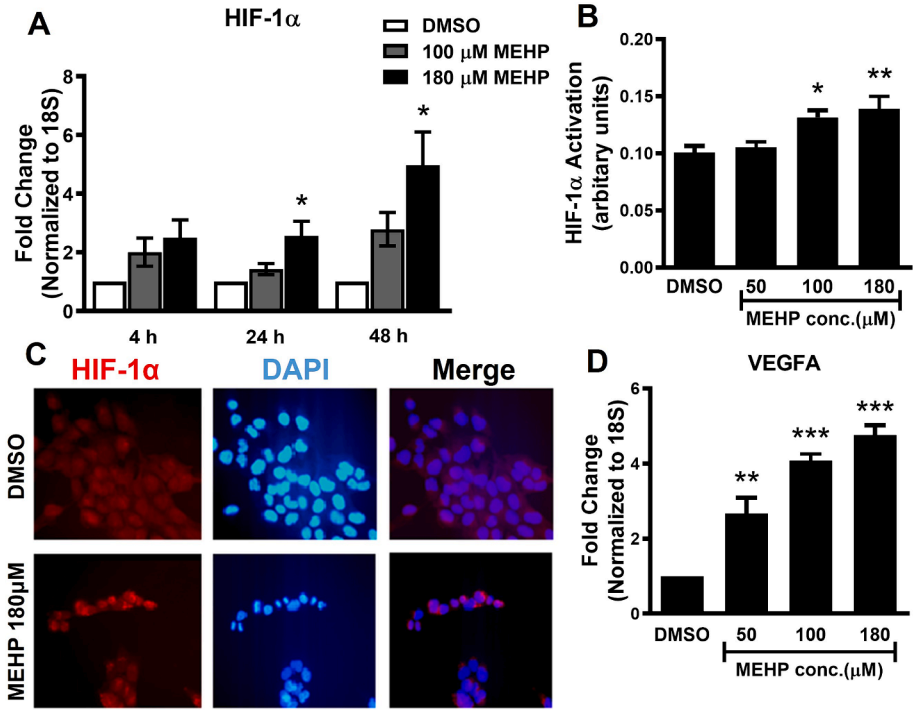

Fig. 2. MEHP increases the expression level and activity of HIF-1a in HTR-8/SVneo cells. A. mRNA expression levels of HIF-1α in cells treated with different concentrations of MEHP at 4, 24, and 48 h. **B.** The relative HIF-1 α activation (arbitrary units) of cells treated with different concentrations of MEHP for 48 h. Data are presented as means ± SEM from 3 independent experiments (n = 3). *$p < 0.05$; **$p < 0.01$. **C.** Representative images with 60X magnification of cells stained with HIF-1α (red) and DAPI (Blue) in 180 μM MEHP treated and DMSO (control) treated groups. **D.** mRNA expression levels of VEGFA in cells treated with different concentrations of MEHP at 48 h. Data are presented as means ± SEM from 3 independent experiments (n = 3). **$p < 0.01$; ***$p < 0.005$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

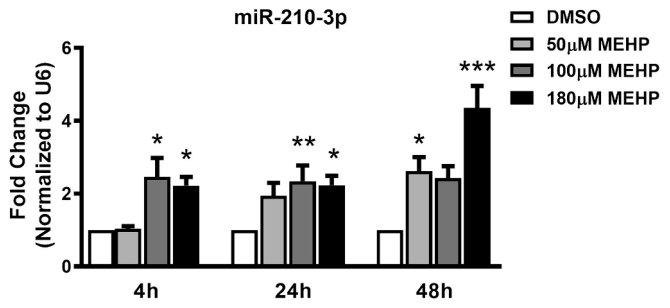

Fig. 3. Upregulation of miR-210-3p under MEHP treatment in HTR-8/SVneo cells. The relative expression levels of miR-210-3p in cells treated with different concentrations of MEHP for 4, 24, and 48 h. Data are presented as means ± SEM from 3 independent experiments (n = 3). *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

increase in miR-210-3p levels at 4 h with doses of 100 and 180 μM of MEHP treatment ($p < 0.05$). After the initial induction of miR-210-3p at 4 h, the upregulation was maintained for the next 48 h reaching a maximum of 4.5-fold increase with the 180 μM of MEHP ($p < 0.005$). These suggested a potential role of epigenetic regulation in the MEHP-induced trophoblast hypoxia.

Effect of MEHP on mitochondrial functions in HTR-8/SVneo cells

Studies have revealed that oxidative stress induced by hypoxia impairs mitochondrial function, and results in a diminished capacity for ATP production (Wheaton et al., 2011). Thus, we measured the ATP levels in cells treated with different concentrations of MEHP where hypoxia was induced. Cellular ATP levels were reduced by 15 % ($p < 0.05$), 23 % ($p < 0.005$), and 31 % ($p < 0.005$) with the respective doses of 50, 100, and 180 μM MEHP, indicating that MEHP alters the energy status of the placental cells (Fig. 4A). Evidence has established that mtDNA replication and transcription are essential for maintaining cellular energy requirements (Fukuoh et al., 2014) and decreased mtDNA copy number is shown to inhibit mitochondrial ATP synthesis (St. John, J., , 2014). To further investigate if the source of the inhibited ATP levels was associated with decreased mtDNA copy number, we measured the relative mtDNA content. Following a 48 h treatment of MEHP, a decreasing trend (10 % in the 50 μM MEHP group, 23 % in the 100 μM MEHP group, and 21 % in the 180 μM MEHP group) in mtDNA/nDNA was observed (Fig. 4B). Mitochondrial dysfunction can also be induced through the inhibition of mitochondrial complexes (Gomez-Lazaro et al., 2007; Jha et al., 2000). Therefore, we examined the expression levels of mitochondrial complex-related genes, NADH: Ubiquinone Oxidoreductase Subunit B8 (NDUFB8) and SDHB. We discovered a significant downregulation of NDUFB8 and SDHB ($p < 0.05$) when cells were treated with 100 μM MEHP for 24 h (Fig. 4C). These findings suggest that MEHP may induce mitochondrial stress by disrupting NDUFB8 and SDHB.

MEHP inhibits membrane potential and mitochondrial protein levels in HTR-8/SVneo cells

To comprehensively evaluate mitochondrial function, we first measured mitochondrial membrane potential which is an essential driving force for ion and protein transport as well as mitochondrial bioenergetics (Zorova et al., 2018). We observed a significant decrease in the membrane potential when the cells were treated with 100 μM and 180 μM MEHP for 4, 24, and 48 h. However, no effects in the 50 μM MEHP treated group were observed (Fig. 5A). Mitochondrial respiratory

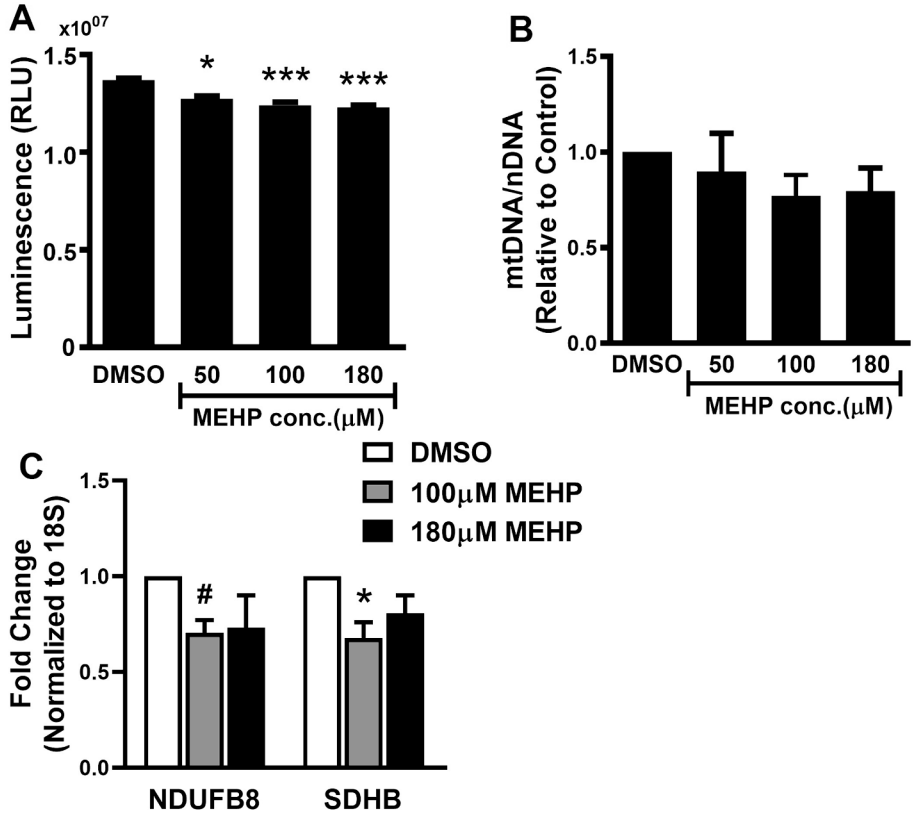

Fig. 4. MEHP inhibits ATP levels and mitochondrial gene expressions in HTR-8/SVneo cells. A. The ATP levels in cells treated with different concentrations of MEHP for 48 h. **B.** MtDNA/nDNA ratio in cells treated with different concentrations of MEHP for 48 h. **C.** Gene expression of NDUFB8 and SDHB in cells treated with different concentrations of MEHP at 24 h. Data are presented as means ± SEM from 3 experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$. #$p < 0.05$ by Student's *t*-test vs DMSO group.

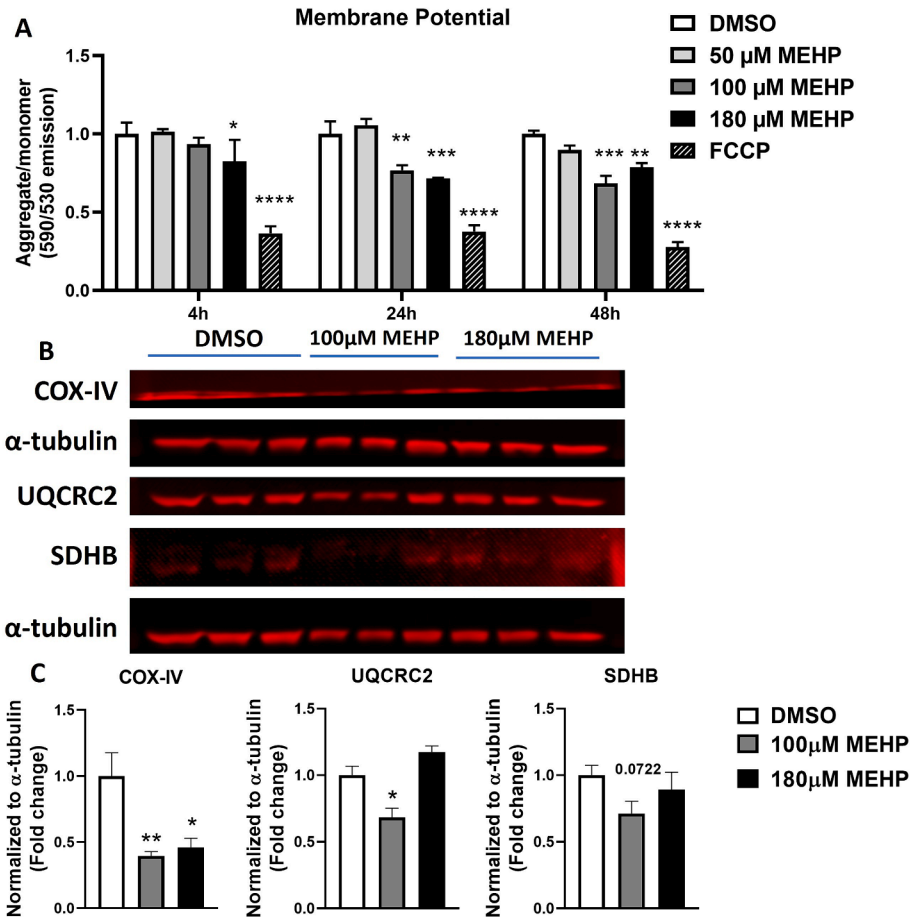

Fig. 5. MEHP inhibits membrane potential and mitochondrial protein expressions in HTR-8/SVneo cells. A. Membrane potential is presented by the ratio of aggregate and monomer in cells treated with DMSO, FCCP (depolarization positive control), or different concentrations of MEHP at 4, 24, and 48 h. Data are presented as means ± SEM (n = 3). *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; ****$p < 0.001$ compared to DMSO group. **B.** Representative protein expression of COX-IV, UQCRC2, SDHB, and α-tubulin in cells treated with DMSO or different concentrations of MEHP at 24 h. **C.** Densitometry analysis of COX-IV, UQCRC2, SDHB protein expression. Expression was normalized to α-tubulin (n = 3)., *$p < 0.05$; **$p < 0.01$ compared to DMSO group.

complexes play a crucial role in maintaining mitochondrial membrane potential (Zorova et al., 2018; Hawkins et al., 2010). To investigate the underlying molecular mechanisms leading to membrane potential disruption, we evaluated the levels of mitochondrial complex proteins including complex II (SDHB), complex III (UQCRC2), and complex IV (COX-IV) that are essential components of the electron transport chain and regulate the process of oxidative phosphorylation (Adlimoghaddam et al., 2022; Chandel, 2010; Pfanner et al., 2019). We observed a significant decrease in COX-IV protein levels in both 100 μM ($p < 0.01$) and 180 μM MEHP ($p < 0.05$) groups as well as a significant decrease of UQCRC2 in the 100 μM MEHP group ($p < 0.05$) (Fig. 5B-C). However, no changes were seen in the 180 μM MEHP treated group. We also observed a decreasing trend with SDHB in the 100 μM MEHP group ($p = 0.0722$). These results confirmed our findings that MEHP treatment induced mitochondrial dysfunction in trophoblast cells.

Discussion

MEHP exposure has been linked to increased risks of preterm birth, PE, and other pregnancy complications (Ferguson et al., 2014; Zhang et al., 2023); however, the underlying mechanism of how it affects trophoblast function in these contexts remains unclear. In this study, we reveal that MEHP induces hypoxia and oxidative stress to subsequently impact energy generation in a trophoblast cell line. This disruption of energetics may thus contribute to pregnancy complications such as PE, thereby highlighting the potential implications of MEHP exposure on placental function (Armistead et al., 2020). Under normal conditions, hypoxia is essential for directing physiological responses that are necessary during the critical first-trimester window for placental development by regulating angiogenesis and trophoblast function (Colson et al., 2020). Excessive oxidative stress via unattenuated hypoxia can disrupt trophoblast invasion, proliferation, and differentiation thereby disturbing the delicate balance required for normal trophoblast development and function (Phoswa and Khaliq, 2021). Simultaneously, the heightened oxidative stress introduces the potential for cellular and mitochondrial damage that is linked to placental complications (Phoswa and Khaliq, 2021; Poston and Raijmakers, 2004). The concurrent over-induction of hypoxia and oxidative stress in response to MEHP exposure underscores a precarious relationship and their potential role in placental dysfunction. In addition, our lab has previously discovered that MEHP triggers the expression of miR-16 in trophoblast cell line to increase apoptosis, which is associated with oxidative stress (Meruvu et al., 2016; Wu et al., 2015). This connection emphasizes the capacity of MEHP to modulate the expression of a MiR to trigger apoptosis through mitochondrial dysfunction via excessive production of ROS and inhibition of ATP production. These findings imply that MEHP may serve as a significant contributor to epigenetic disruption underlying the maintenance of placental energy homeostasis. As contact with phthalates such as MEHP is nearly unavoidable in daily life, this prompts a demand for further investigation into the mechanism of how MEHP can contribute to pregnancy-related complications.

A manner through which hypoxia finetunes the adaptation to a low

oxygen environment is by coordinating the regulation of hypoxia-inducible factors and epigenetic modifications. There is the triggering of expression of specific miRs to facilitate these processes (Nallamshetty et al., 2013; Lacedonia et al., 2018). Among these, miR-210-3p is recognized for its role in modulating functions such as mitochondrial function, angiogenesis, DNA repair, and cell survival in response to oxidative stress and hypoxia (Chan et al., 2012; Qin et al., 2014). It has been reported that miR-210-3p can act as a downstream target of HIF-1α under hypoxia (Huang et al., 2009). In this study, we reveal a simultaneous induction of miR-210-3p and HIF-1α under treatment with environmentally relevant doses of MEHP in a trophoblast cell line. The induction of miR-210-3p hints at an epigenetic mechanistic link between MEHP exposure and trophoblast oxidative stress. This also puts into perspective the potential impact of MEHP exposure on the cellular functions that miR-210-3p is engaged with.

One overlapping target of miR-210-3p and a contributor to PE when disturbed is mitochondrial health. Maintaining proper energy homeostasis provided by the mitochondria is essential for sustaining the metabolic demands of developing trophoblast (Sferruzzi-Perri et al., 2019). Our study highlights how MEHP induces ATP depletion in HTR-8/SVneo cells, correlating with mitochondrial dysfunction. Potential consequences of diminished mitochondrial function in trophoblasts can impede their invasion capacity to promote vascularization and placental development (Hebert and Myatt, 2021). Additionally, studies have demonstrated that mitochondrial respiration and mitochondrial complex activity are decreased in PE (Yung et al., 2019; Muralimanoharan et al., 2012). Our results showing decreased relative mtDNA content and mitochondrial membrane potential further illustrate the detriments incurred by mitochondria upon MEHP exposure, corroborating the ATP depletion findings. To further elucidate the underlying mechanism, we demonstrated that key mitochondrial complex genes and proteins, such as SDHB and COX-IV, were significantly downregulated. Suppression of these electron transport chain subunits exposes a potentially reduced capacity for energy production in trophoblast cells (Aye et al., 2022). Studies have also indicated that NDUFB8 and SDHB expression are downregulated by miR-210 (Colleoni et al., 2013; Aggarwal et al., 2021). This reinforces our hypothesis of potential adverse effects MEHP can have on trophoblast mitochondrial function through a miR-210-3p dependent mechanism (Wu et al., 2015).

In summary, our study delineates the possible role of MEHP as a hypoxia mimetic in trophoblast function mediated through the HIF-1α/miR-210-3p axis, leading to increased oxidative stress, ATP reduction, and inhibition of mitochondria complex genes. Overall, MEHP affects trophoblast health by upregulating miR-210-3p, increasing expression and activity of HIF-1 α as well as its downstream effector VEGFA to induce oxidative stress and mitochondrial dysfunction that can be permissive of trophoblast apoptosis, inhibition of invasion and differentiation, ultimately contributing to pregnancy complications (Fig. 6). This research provides a novel perspective on how endocrine disrupting chemicals such as MEHP may impact the development of life-threatening pregnancy disorders such as PE. The use of the HTR-8/SVneo cell line facilitated the avoidance of potential complex interactions of different cell types within the placental environment. However, it is important to note that this first-trimester cell line model cannot fully replicate the complexity of the *in vivo* placental environment. Therefore, further research including *in vivo* and clinical investigations is imperative to validate the impact of phthalates on pregnancy complications and decipher the underlying mechanisms.

CRediT authorship contribution statement

Sunitha Meruvu: Investigation, Data curation, Methodology, Visualization, Writing – original draft. **Zehuan Ding:** Formal analysis, Visualization, Writing – original draft. **Mahua Choudhury:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

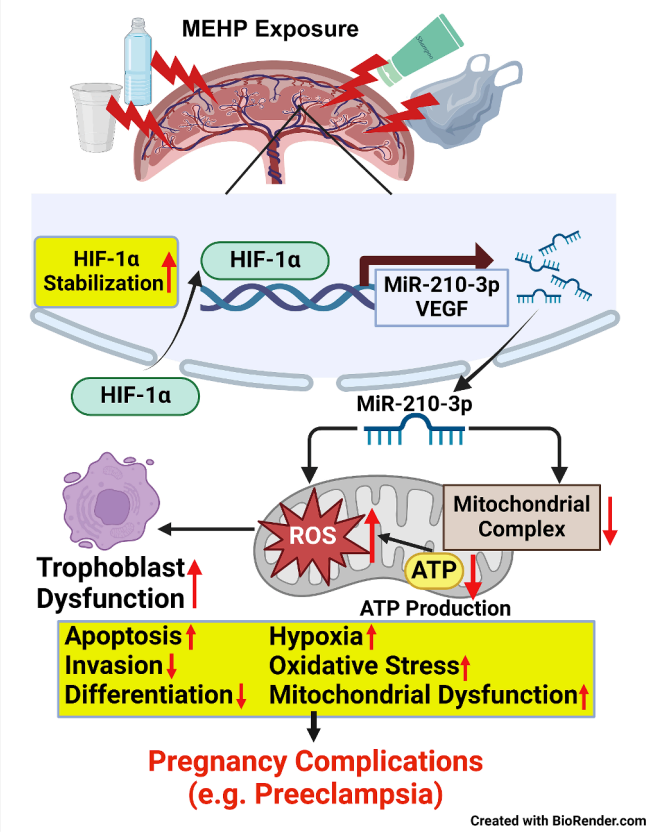

Fig. 6. Proposed model for the effects of MEHP-induced oxidative stress and mitochondrial dysfunction. The results demonstrate that MEHP induces hypoxia and oxidative stress in HTR-8/SVneo cells replicating conditions observed in the first trimester. MEHP treatment activates HIF-1α, triggering downstream effects such as increased miR-210-3p and VEGFA expression. miR-210-3p induction suggests a potential link between MEHP exposure and altered miR expression. Furthermore, miR-210-3p upregulation may lead to oxidative stress generation and ATP depletion, coupled with downregulated mitochondrial complex genes, indicating mitochondrial dysfunction. Genes related to apoptosis and cell invasion can also be affected in response to oxidative stress. The findings propose a mechanistic model wherein MEHP disrupts the HIF-1α/miR-210-3p axis, contributing to trophoblast oxidative stress and energy metabolism impairment, potentially influencing pregnancy complications, including PE.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2024.100188.

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