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NIFEDIPINE PREVENTS APOPTOSIS OF ALCOHOL-EXPOSED FIRST TRIMESTER TROPHOBLAST CELLS

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Abstract

Background—Maternal alcohol abuse leading to fetal alcohol spectrum disorder (FASD) includes fetal growth restriction (FGR). Ethanol induces apoptosis of human placental trophoblast cells, possibly disrupting placentation and contributing to FGR in FASD. Ethanol facilitates apoptosis in several embryonic tissues, including human trophoblasts, by raising intracellular Ca^{2+} . We previously found that acute ethanol exposure increases trophoblast apoptosis due to signaling from both intracellular and extracellular Ca^{2+} . Therefore, nifedipine, a Ca^{2+} channel blocker that is commonly administered to treat preeclampsia and preterm labor, was evaluated for cytoprotective properties in trophoblast cells exposed to alcohol.

Methods—Human first trimester chorionic villous explants and the human trophoblast cell line HTR-8/SVneo were pre-treated with 12.5 - 50 nM of the Ca^{2+} channel blocker nifedipine for 1 hour before exposure to 50 mM ethanol for an additional hour. Intracellular Ca^{2+} concentrations were monitored in real-time by epifluorescence microscopy, using fluo4-AM. Apoptosis was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), accumulation of cytoplasmic cytochrome c, and cleavage rates of caspase 3 and caspase 9.

Results—The increase in intracellular Ca^{2+} upon exposure to ethanol in both villous explants and HTR-8/SVneo cells was completely blocked ($p < 0.05$) when pretreated with nifedipine, accompanied by inhibition of ethanol-induced release of cytochrome c, caspase activities, and TUNEL.

Conclusion—This study indicates that nifedipine can interrupt the apoptotic pathway downstream of ethanol exposure, and could provide a novel strategy for future interventions in women with fetuses at risk for FASD.

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INTRODUCTION

Alcohol readily crosses the placenta and accumulates in the fetus at levels proportionate to maternal blood alcohol concentrations (Idanpaan-Heikkila et al., 1972). Ethanol exposure causes cell death in neural regions of the human embryo and is attributed to the etiology of neurodevelopmental disability. (Hannigan and Armant, 2000). Prevalent features of fetal alcohol spectrum disorder (FASD) include a continuum of anomalies that occur in children exposed to alcohol during pregnancy (Wilhoit et al., 2017). Facial and cranial anomalies, and impaired behavioral and cognitive ability are associated with FASD (Hannigan and Armant, 2000). Antenatal ethanol can cause fetal growth abnormalities, which has been attributed to the progenitor cytotrophoblast cell population and reduced proliferation of the syncytiotrophoblast within the chorionic villi, causing fetal growth restriction (FGR) (Axt et al., 1999b, Barrio et al., 2004, Erel et al., 2001, Levy et al., 2002, Smith, 1997).

Ethanol alters the physiological transformation of maternal spiral arteries and disrupts trophoblastic cell motility in a dose-dependent manner (Gundogan et al., 2008, Gundogan et al., 2010, Gundogan et al., 2015). Pregnant rats exposed to chronic ethanol feeding demonstrated disruption of placentation, significantly lower levels of AAH, and intrauterine growth development (Gundogan et al., 2008). This can be shown by a Doppler interrogation of uterine vessels in humans. In this same study, damage occurred in the placenta and fetus leading to reduced trophoblast motility and invasiveness, smaller pups, and increased fetal demise (Gundogan et al., 2008). The development of FGR associated with FASD can be linked to in-utero ethanol exposure and poor trophoblast survival and dysfunction.

While apoptosis is essential for the architecture of placental development and invasion of trophoblasts into the spiral arteries, elevated apoptosis within placental cell populations is clearly associated with the pathophysiology of placental mediated disorders, including fetal growth restriction (FGR) and preeclampsia (Sharp et al., 2010, Nishizawa et al., 2011). Apoptosis, together with mitosis, functions to control cell populations. The process is influenced by ethanol, causing increased cell death (Cartwright et al., 1998, Cheema et al., 2000, Ikonomidou et al., 2000), which occurs in placentas of pregnancies complicated by FGR and preeclampsia (Smith et al., 1997, Axt et al., 1999a, Erel et al., 2001, DiFederico et al., 1999, Allaire et al., 2000). Exposure of the human first trimester cytotrophoblast cell line HTR-8/SVneo (Graham et al., 1993) to ethanol causes apoptosis demonstrated by DNA fragmentation (TUNEL) and phosphatidylserine externalization (Annexin V binding) with a linear dose response between 25 and 100 mM ethanol (Wolff et al., 2007). Trophoblast apoptosis is induced by ethanol through the endogenous pathway using caspase 9 and caspase 3. Apoptosis within the trophoblast population could disrupt placentation, leading to a reduction in nutrient transport, producing endothelial dysfunction (Sharp et al., 2010). The biological pathways disrupted by ethanol that induce trophoblast apoptosis are not completely understood, and would add light on the etiology of FASD.

Ethanol increases free cytoplasmic Ca^{2+} and induces apoptosis in numerous models including neural crest progenitors (Debelak-Kragtorp et al., 2003) and cerebellar granule neurons (Kouzoukas et al., 2013). Cytoplasmic Ca^{2+} is regulated by entry of extracellular Ca^{2+} , and its release from the endoplasmic reticulum through capacitative calcium entry

(Putney and Bird, 2009, Huang et al., 2014). In a study of embryonic neural crest development, ethanol-induced apoptosis was specifically blocked by chelation of intracellular Ca^{2+} , or depletion of extracellular Ca^{2+} , prior to ethanol exposure (Garic-Stankovic et al., 2005). Ethanol-dependent intracellular Ca^{2+} release requires phosphoinositide-specific phospholipase-C (PLC). Several blockers of PLC-mediated phosphoinositide production, including IP3 receptor antagonists, block apoptosis of ethanol-exposed fetal cells (Garic-Stankovic et al., 2005). In each model, cell death is linearly associated with ethanol concentration or time of exposure (Debelak-Kragtorp et al., 2003, Flentke et al., 2014, Kilburn et al., 2006, Pantazis et al., 1993). We established a similar mechanism of apoptosis in HTR-8/SVneo cytotrophoblast cells acutely exposed to ethanol, and found cell death to be dependent on both intracellular and extracellular Ca^{2+} signaling (Bolnick et al., 2014). In elucidating the apoptotic mechanism induced by ethanol, new intervention strategies for pregnant women exposed to alcohol might be devised.

Nifedipine is a dihydropyridine Ca^{2+} channel blocker with strong arterial vasodilatory activity (Toal et al., 2012). Nifedipine acts directly on vascular smooth muscle cells and peripheral resistance vessels, relaxing the cells, and thereby producing vasodilation to reduce systemic blood pressure (Chen et al., 2012). However, there is growing evidence to suggest that Ca^{2+} channel blockers, including nifedipine, also have direct positive effects on endothelial function (Chen et al., 2012). Studies have suggested that the action of Ca^{2+} channel blockers on vascular endothelial cells could suppress cellular damage caused by reactive oxygen species, and that nifedipine can reduce the response of endothelial cells to exogenous activators and prevent apoptosis (Chen et al., 2010). Ca^{2+} channel blockers can prevent the morbidities associated with placental mediated disorders and improve pregnancy outcomes in women with chronic hypertension (Jiang et al., 2015). Nifedipine causes haemodynamic changes in maternal-fetal unit, including a maternal cardiac afterload reduction as measured by Doppler studies. Physiological increase in cardiac output maintains the blood pressure; however, studies have shown no impact on the uteroplacental and fetal circulation, or fetal heart rate (Lurie et al., 1990, Scardo et al., 1996, Moretti et al., 1990, Cornette et al., 2011). The available evidence suggests that oral nifedipine can be considered the first line therapy for management of acute onset of hypertension in pregnancy, as well as in the postpartum period (Vermillion et al., 1999, Raheem et al., 2012, Shekhar et al., 2013). Calcium channel blockers have been associated with an increase in maternal tachycardia and subsequent hypotension due to vasodilation of the blood vessels secondary to alterations of the cardiac output. Since nifedipine has been used successfully in pregnant women, it might provide an intervention to reduce placental damage from consumption of alcohol by limiting trophoblast apoptosis due to ethanol-induced Ca^{2+} signaling. As an initial step to evaluate this concept, we have investigated whether nifedipine can rescue human trophoblast cells from apoptosis due to alcohol exposure during *in vitro* culture. Here, we examined the effect of ethanol and nifedipine on intracellular Ca^{2+} concentration, and correlations to ethanol-induced apoptosis in HTR-8/SVneo cells, as well as first trimester placental villous explants.

MATERIALS AND METHODS

Human Cytotrophoblast Cell Culture

The HTR-8/SVneo (HTR) cell line was cultured in a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (1:1; DMEM/F12; Life Technologies, Grand Island, NY) media containing 10% fetal bovine serum (Life Technologies). Culture medium was changed every two to three days and cells were passaged with trypsin-EDTA solution (Life Technologies). Before experimentation, culture medium was replaced and cells were cultured serum-free in medium containing 5 mg/ml BSA for 18–24 h.

Villous Explant Culture

Placental tissues (n=4; mean gestational age 8.5 weeks) were obtained with Wayne State University Institutional Review Board approval and patient informed consent. Specimens were collected from first trimester terminations at a Michigan family planning facility from otherwise healthy patients. Fresh tissue was placed on ice in PBS and immediately taken to the lab for processing. The chorionic villi were dissected and cut into approximately 5 mg wet weight pieces, and transferred individually into DMEM/F12 culture medium supplemented with 10% donor calf serum and 1% antibiotic-antimycotic solution (Gibco, Grand Island, NY) in a 24-well culture plate (Costar, Corning, NY) for 24 hours of culture, as previously described (Bolnick et al., 2015). Chorionic villi were cultured for 1–2 hours during all experimental procedures. Villous explants in each well were gently rinsed 3 times with PBS at the conclusion of culture, and fixed for 30 min in 10% neutral buffered formalin. Fixed villous explants were embedded in paraffin, and 5 μ m sections were cut and mounted on glass slides. Paraffin sections were deparaffinized with xylene, and rehydrated into Tris-buffered saline before immunocytochemical labeling or cell death assays.

Ethanol Exposure

Ethanol (Mid-West Grain Company, Perkin, IL) was prepared at 50 mM in serum-free medium immediately before use in cell culture. Chorionic villous explants, and HTR cytotrophoblast cells were also treated in certain experiments with 12.5 – 50 nM of the Ca^{2+} channel blocker, nifedipine (Sigma, St. Louis, MO) for 1 hour before exposure to 50 mM ethanol. Ethanol treatment was for 1 hour.

Intracellular Ca^{2+} Measurement

HTR cytotrophoblast cells were grown to 50% confluence and cultured overnight in serum-free medium in 96 well strip plates (2500 cells/well). Cells were loaded with 4 μ M fluo-4-AM (Life Technologies) for 30 min at 37°C, followed by two rinses with modified BWW medium (Sigma). Intracellular Ca^{2+} transients were monitored cells by illuminating at 10-second intervals for fluorescence evaluation. Images were taken with a Leica (Wetzlar, Germany) DM IRB epifluorescence microscope interfaced with a Hamamatsu Orca Digital camera (Hamamatsu City, Japan). Fluorescence intensities were analyzed using Simple PCI imaging software (Hamamatsu). Mean fluorescence intensity was evaluated over an entire field of cells, and intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was calculated using the following formula:

$$([Ca^{2+}]_i) = K_d (F - F_{min}) / (F_{max} - F),$$

where K_d (345 nM) is the dissociation constant of the Ca^{2+} indicator, F is the fluorescence intensity, F_{min} is the relative background fluorescence, and F_{max} is the maximum fluorescence intensity obtained after equilibrating intracellular and extracellular Ca^{2+} with 5 nM ionomycin at the end of each experiment.

Cell Death Assay

HTR cells were cultured and treated in 96-well plates, and villous explants were cultured and treated in 24-well plates. Cell death, measured as DNA fragmentation, was detected by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL), using a fluorescein-based kit from Roche Applied Science (Indianapolis, IND). Cells were counterstained with 5 μ g/ml 4,6-diamidino-2-phenylindole, HCl (DAPI; EMD Biosciences). Fluorescent nuclei were imaged at 40 $\times$ magnification, and Simple PCI imaging software was used to count total DAPI labeled and TUNEL positive nuclei for each field. The percentage of TUNEL/DAPI-labeled nuclei (TUNEL index) was calculated by averaging triplicate fields in each well.

Measurement of Caspase Activity

The activation of caspase 3 and 9 were measured by fluorometric assays, as previously described (Marino et al., 2013). Briefly, treated cells were lysed for 30 min at 4°C in 130 μ L of lysis buffer (1% NP-40 (IGEPAL), 10 mM TRIS-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM PMSF, 1 mM leupeptin, 0.3 mM aprotinin). Cell lysates were centrifuged at 16,000 $\times$ g for 15 min and total protein concentration was determined using the Bradford protein assay. Protein (35 μ g) was diluted in assay buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4 , pH 7.4, 20% glycerol, 5 mM DTT), and incubated at 37°C for 2 h with 50 μ M of either Ac-DEVD-AMC or Ac-LEHD-AMC as substrates for caspase 3 and caspase 9, respectively. The release of AMC due to the cleavage of DEVD or LEDH by active caspase 3 or 9 were read at 355 nm excitation and 460 nm emission wavelengths using an H1 microplate reader (BioTek, Winooski, VT).

MitoTracker and Cytochrome c Staining

HTR cells grown in 8-well cell culture slides were rinsed with PBS and fixed using freshly prepared 4% paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature. Fixative was removed, cultures were washed in PBS, and permeabilized with 0.1% Triton X-100, for 10 min, at room temperature. The cells were then dual labeled by incubating overnight at 4 °C with 100 nM MitoTracker Green FM (Invitrogen Corp., Carlsbad, CA) and a rabbit monoclonal antibody against cytochrome *c* [EPR1327] (Abcam; Cambridge, MA). After overnight incubation, cells were washed three times with PBS. For cytochrome *c* staining, primary antibody was visualized with 0.3 μ g/ml Texas Red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and counterstained with 5 μ g/ml DAPI. Fluorescence was imaged and captured using an epifluorescence microscope and digital camera.

Statistics

Assays were conducted in triplicate, and all experiments were repeated at least three times and on separate days with different cell preparations. Data were analyzed using SPSS Version 21.0 (IBM Corp., 2012). For experiments in which time was the variable, one-way ANOVA was conducted to examine TUNEL index. Following significant ANOVA, Tukey's method was used for mean separations. For assessment of Ca^{2+} blocker on ethanol-induced cell death, independent sample *t*-tests were used to detect the effects of ethanol. One-way ANOVA with Tukey's post hoc test was performed across the treatments. Differences were considered significant at $p < 0.05$.

RESULTS

Nifedipine Attenuates Ethanol-Induced Elevation of Cytoplasmic Free Ca^{2+}

The effect of nifedipine on Ca^{2+} signaling in trophoblast cells during ethanol exposure was evaluated. Intracellular Ca^{2+} was monitored in real time after exposing HTR cells pretreated with 0, 12.5, 25 or 50 nM nifedipine to 50 mM ethanol. Fluorescence images of nearly confluent cells pre-loaded with fluo-4-AM were captured at 10 s intervals before and after addition of vehicle or ethanol (Fig. 1). Intracellular Ca^{2+} concentrations increased synchronously in trophoblast cells within 10 s of exposure to 50 mM ethanol, rising from 95.6 ± 12.8 nM to 1230.8 ± 319.4 nM after 90 s of exposure, followed by a return to baseline over the next 5 minutes. The increase in intracellular Ca^{2+} was significantly blocked in cells pretreated with 50 nM nifedipine (Fig. 1), but not at lower concentrations.

Nifedipine Prevents Ethanol-Induced Apoptosis

HTR cells and first trimester chorionic villous explants pretreated with vehicle displayed an increase in apoptosis after exposure to 50 mM ethanol, as assessed by TUNEL. Cell death (mean $\pm$ SEM) increased significantly from $4.3 \pm 0.4\%$ to $11.8 \pm 0.6\%$ in HTR cells (Fig. 2A), and from $16.2 \pm 0.5\%$ to $29.1 \pm 1.3\%$ in villous explants (Fig. 2B) after exposure to 50 mM ethanol ($p < 0.05$). Pretreatment of the cells with nifedipine prevented the ethanol induced cell death detected by TUNEL ($p < 0.05$; Fig. 2A–H). The apoptotic pathway is mediated by a cascade of cysteine proteases (Riedl and Shi, 2004), including the initiator caspases, 8 and 9, and the effector caspase, caspase 3. Caspase 3 and 9 proteolytic activity was measured in HTR cells using fluorogenic substrate for caspase-3 (Ac-DEVD-AMC) and caspase-9 (Ac-LEHD-AMC). Cleavage of the substrates was monitored by measuring the fluorescence of released AMC, and revealed elevated activity of both caspase 9 (Fig. 3A) and caspase 3 (Fig. 3B) after cells were exposed to 50 mM ethanol for 1 h ($p < 0.05$). Furthermore, we found that the significant elevation of both caspase (3 and 9) activities in ethanol-exposed cells was attenuated with nifedipine pretreatment (Fig. 3 A, B). Based on these findings, it appears that nifedipine can prevent the activation of the apoptotic pathway in human cytotrophoblast cells exposed to ethanol.

Nifedipine Prevents Release of Cytochrome C from Mitochondria During Ethanol Exposure

To investigate whether cytochrome *c* is released during ethanol-induced apoptosis, cytochrome *c* localization was analyzed in HTR cells using immunofluorescence. HTR cells

pretreated with vehicle displayed a strong peri-nuclear co-localization of Mitotracker green and cytochrome *c* staining (Fig. 3C). After 1 hour of exposure to 50 mM ethanol, HTR cells released cytochrome *c* into the cytosol, indicated by the diffuse expression pattern (Fig. 3C). These observations suggest that the release of cytochrome *c* is an early event in ethanol-induced apoptosis. Pretreatment of the cells with nifedipine blocked the ethanol-induced release of cytochrome *c* (Fig. 3C).

DISCUSSION

Human trophoblast functions are important for proper implantation and fetal development. These cells control hormone production and secretion and are imperative for fetal survival because they regulate the maternal-fetal interface (Novakovic et al., 2011). Anomalies in the formation of the fetal-maternal interface along with cytotoxicity-induced trophoblast dysregulation contribute to abnormal pregnancy complications (McAleer and Tuan, 2004, Red-Horse et al., 2004). Abnormal placenta trophoblast proliferation and apoptosis is related to the development of placental mediated disorders and could be involved in the pathogenesis of Obstetrical adversities including preeclampsia, FGR, abruption, and fetal demise (He et al., 2013). Exposure to ethanol initiates cell death in chick and mouse embryos during gastrulation (Debelak-Kragtorp et al., 2003, Kilburn et al., 2006) therefore we have investigated the mechanism of cell death in ethanol-exposed human trophoblast cells. Ethanol-induced cell death is also contingent on elevation of intracellular Ca^{2+} concentrations (Debelak-Kragtorp et al., 2003, Garic-Stankovic et al., 2005). Therefore, nifedipine, a commonly administered Ca^{2+} blocker used to treat pregnancy complications was also investigated.

The present study confirms our previous finding that the intracellular Ca^{2+} concentration increased synchronously in trophoblasts within 10 s of ethanol exposure, followed by activation of the caspases 3 and 9, and an increase in cell death within 1 h (Bolnick et al., 2014). A major caspase activation pathway is the cytochrome *c*-initiated pathway. In this pathway, apoptotic stimuli cause cytochrome *c* release from mitochondria, which induces a biochemical cascade that activates caspase 3 prior to cell death (Jiang and Wang, 2004). This study revealed ethanol exposure induced mitochondrial damage and initiated release of cytochrome *c* into the cytosol, implicating this pathway as an early event in ethanol-induced apoptosis. Ethanol-induced apoptosis in human first trimester trophoblast cells is dependent upon both intracellular and extracellular Ca^{2+} signaling (Bolnick et al., 2014). We found that nifedipine prevented the production of an ethanol-induced Ca^{2+} transient and rescued first trimester trophoblast cells from apoptosis. There is evidence suggesting that Ca^{2+} channel blockers have direct positive effects on endothelial dysfunction, which is common in placenta-mediated obstetric disorders (Rey et al., 1997, Brown et al., 2000, Magee et al., 2005, Raheem et al., 2012). While the effect of nifedipine on chronic ethanol exposure is not fully understood, the short-term effects of nifedipine in trophoblasts acutely exposed to ethanol, expands the knowledge of the underlying mechanisms of ethanol-induced apoptosis. Reduction of Ca^{2+} signaling by nifedipine in pregnancies compromised by ethanol exposure, or in other etiologies that induce trophoblast cell death, could benefit patients at risk for FGR.

There is a strong association between the ethanol dose-dependency of apoptosis and intracellular Ca^{2+} concentration in cytotrophoblast cells. Cells exposed to 50 mM (229 mg/dL) or higher ethanol show a significant increases in both apoptosis and intracellular Ca^{2+} concentration. Similar cell culture studies have shown that these values are similar to blood alcohol levels in pregnant alcoholics (Kouzoukas et al., 2013). The increase in cell death and reduction in proliferation of human cytotrophoblasts treated with ethanol (Wolff et al., 2007) is consistent with previous studies conducted with animal models (Kilburn et al., 2006, Smith, 1997). In this investigation, ethanol concentrations were chosen representing social and binge alcohol consumption.

Previous studies investigating the signaling pathways responsible for the effects of ethanol in human trophoblast cells were conducted with an immortalized trophoblast cell line (Wolff et al., 2007, Bolnick et al., 2014). We included primary first trimester chorionic villi to validate the mechanism. Primary tissue responded similarly to cell lines. All experiments using the HTR cell line were conducted in serum-free medium, which while not optimal for cell growth, does not induce increased apoptosis.

The findings of this study support the hypothesis that maternal alcohol consumption during pregnancy could induce apoptosis of trophoblasts and interfere with trophoblast accumulation in the developing placenta. Prenatal alcohol exposure affects all organ systems, and can cause cell death, vasoconstriction of blood vessels, oxygen deprivation to the placenta, and neurological impairment (Hannigan and Armant, 2000). Alcohol related abnormalities cause learning and attention deficits, mental impairment, microcephaly, and FGR (Ornoy and Ergaz, 2010).

The effectiveness of nifedipine in preventing cell death, and its current use therapeutically in pregnant women suggest that it could provide a safe and effective pharmacological intervention to reduce risk of FGR in women using alcohol during pregnancy. However, clinical and preclinical studies are required to validate the implications of this *in vitro* investigation and establish the effective timing for nifedipine usage relative to alcohol intake. The findings of this study support the hypothesis that alcohol consumption during pregnancy could jeopardize trophoblast expansion in the early developing placenta due to increased apoptosis. Apoptosis among the invasive extravillous trophoblasts, represented by the HTR-8/SVneo cell line, would be particularly devastating as those cells are responsible for remodeling the uterine arteries to high volume, low resistance vessels that permit maximal perfusion of the intervillous space by maternal blood (DiFederico et al., 1999). In the chorionic villi, apoptosis increases the release of trophoblastic debris into the maternal circulation, that could activate endothelial cells, which contributes to the occurrence of preeclampsia (Allaire et al., 2000, Redman and Sargent, 2005). Intracellular Ca^{2+} signaling plays a central role in the underlying mechanism, and Nifedipine or other drugs that interrupt the apoptotic pathway downstream of ethanol exposure could provide a novel strategy for intervention in women with fetuses at risk for FASD.

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References

- Allaire AD, Ballenger KA, Wells SR, McMahon MJ, Lessey BA. Placental apoptosis in preeclampsia. *Obstet Gynecol.* 2000; 96:271–276. [PubMed: 10908776]
- Axt R, Kordina AC, Meyberg R, Reitnauer K, Mink D, Schmidt W. Immunohistochemical evaluation of apoptosis in placentae from normal and intrauterine growth-restricted pregnancies. *Clin Exp Obstet Gynecol.* 1999a; 26:195–198. [PubMed: 10668155]
- Axt R, Meyberg R, Mink D, Wasemann C, Reitnauer K, Schmidt W. Immunohistochemical detection of apoptosis in the human term and post-term placenta. *Clin Exp Obstet Gynecol.* 1999b; 26:56–59. [PubMed: 10459437]
- Barrio E, Calvo MT, Romo A, Alvarez R, Gutierrez JI, Naval J, Ferrandez Longas A. Intrauterine growth retardation: study of placental apoptosis. *J Pediatr Endocrinol Metab.* 2004; 17(Suppl 3): 451–456. [PubMed: 15134306]
- Bolnick JM, Karana R, Chiang PJ, Kilburn BA, Romero R, Diamond MP, Smith SM, Armant DR. Apoptosis of alcohol-exposed human placental cytotrophoblast cells is downstream of intracellular calcium signaling. *Alcohol Clin Exp Res.* 2014; 38:1646–1653. [PubMed: 24889927]
- Bolnick JM, Kilburn BA, Bolnick AD, Diamond MP, Singh M, Hertz M, Dai J, Armant DR. Sildenafil stimulates human trophoblast invasion through nitric oxide and guanosine 3',5'-cyclic monophosphate signaling. *Fertil Steril.* 2015; 103:1587–1595 e1582. [PubMed: 25813289]
- Brown MA, Hague WM, Higgins J, Lowe S, McCowan L, Oats J, Peek MJ, Rowan JA, Walters BN, Australasian Society of the Study of Hypertension in P. The detection, investigation and management of hypertension in pregnancy: full consensus statement. *Aust N Z J Obstet Gynaecol.* 2000; 40:139–155. [PubMed: 10925900]
- Cartwright MM, Tessmer LL, Smith SM. Ethanol-induced neural crest apoptosis is coincident with their endogenous death, but is mechanistically distinct. *Alcohol Clin Exp Res.* 1998; 22:142–149. [PubMed: 9514299]
- Cheema ZF, West JR, Miranda RC. Ethanol induces Fas/Apo [apoptosis]-1 mRNA and cell suicide in the developing cerebral cortex. *Alcohol Clin Exp Res.* 2000; 24:535–543. [PubMed: 10798591]
- Chen Q, Guo F, Liu S, Xiao J, Wang C, Snowise S, Stone PR, Chamley LW. Calcium channel blockers prevent endothelial cell activation in response to necrotic trophoblast debris: possible relevance to pre-eclampsia. *Cardiovascular research.* 2012; 96:484–493. [PubMed: 22933321]
- Chen XN, Xu J, Feng Z, Fan M, Han JY, Yang Z. Simvastatin combined with nifedipine enhances endothelial cell protection by inhibiting ROS generation and activating Akt phosphorylation. *Acta Pharmacol Sin.* 2010; 31:813–820. [PubMed: 20562903]
- Cornette J, Duvekot JJ, Roos-Hesselink JW, Hop WC, Steegers EA. Maternal and fetal haemodynamic effects of nifedipine in normotensive pregnant women. *BJOG.* 2011; 118:510–540. [PubMed: 21219592]
- Debelak-Kragtorp KA, Armant DR, Smith SM. Ethanol-induced cephalic apoptosis requires phospholipase C-dependent intracellular calcium signaling. *Alcoholism, clinical and experimental research.* 2003; 27:515–523.
- DiFederico E, Genbacev O, Fisher SJ. Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. *Am J Pathol.* 1999; 155:293–301. [PubMed: 10393861]
- Erel CT, Dane B, Calay Z, Kaleli S, Aydinli K. Apoptosis in the placenta of pregnancies complicated with IUGR. *Int J Gynaecol Obstet.* 2001; 73:229–235. [PubMed: 11376669]

- Flentke GR, Klingler RH, Tanguay RL, Carvan MJ 3rd, Smith SM. An evolutionarily conserved mechanism of calcium-dependent neurotoxicity in a zebrafish model of fetal alcohol spectrum disorders. *Alcoholism, clinical and experimental research*. 2014; 38:1255–1265.
- Garic-Stankovic A, Hernandez MR, Chiang PJ, Debelak-Kragtorp KA, Flentke GR, Armant DR, Smith SM. Ethanol triggers neural crest apoptosis through the selective activation of a pertussis toxin-sensitive G protein and a phospholipase Cbeta-dependent Ca²⁺ transient. *Alcoholism, clinical and experimental research*. 2005; 29:1237–1246.
- Graham CH, Hawley TS, Hawley RG, MacDougall JR, Kerbel RS, Khoo N, Lala PK. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp Cell Res*. 1993; 206:204–211. [PubMed: 7684692]
- Gundogan F, Elwood G, Longato L, Tong M, Feijoo A, Carlson RI, Wands JR, de la Monte SM. Impaired placentation in fetal alcohol syndrome. *Placenta*. 2008; 29:148–157. [PubMed: 18054075]
- Gundogan F, Elwood G, Mark P, Feijoo A, Longato L, Tong M, de la Monte SM. Ethanol-induced oxidative stress and mitochondrial dysfunction in rat placenta: relevance to pregnancy loss. *Alcoholism, clinical and experimental research*. 2010; 34:415–423.
- Gundogan F, Gilligan J, Qi W, Chen E, Naram R, de la Monte SM. Dose effect of gestational ethanol exposure on placentation and fetal growth. *Placenta*. 2015; 36:523–530. [PubMed: 25745824]
- Hannigan JH, Armant DR. Alcohol in pregnancy and neonatal outcome. *Seminars in neonatology* : SN. 2000; 5:243–254. [PubMed: 10956449]
- He G, Xu W, Chen Y, Liu X, Xi M. Abnormal apoptosis of trophoblastic cells is related to the up-regulation of CYP11A gene in placenta of preeclampsia patients. *PloS one*. 2013; 8:e59609. [PubMed: 23555723]
- Huang HM, Chen HL, Gibson GE. Interactions of endoplasmic reticulum and mitochondria Ca(2+) stores with capacitative calcium entry. *Metab Brain Dis*. 2014; 29:1083–1093. [PubMed: 24748364]
- Idanpaan-Heikkila J, Jouppila P, Akerblom HK, Isoaho R, Kauppila E, Koivisto M. Elimination and metabolic effects of ethanol in mother, fetus, and newborn infant. *American journal of obstetrics and gynecology*. 1972; 112:387–393. [PubMed: 5060384]
- Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovskaya V, Horster F, Tenkova T, Dikranian K, Olney JW. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science (Wash DC)*. 2000; 287:1056–1060.
- Jiang N, Liu Q, Liu L, Yang WW, Zeng Y. The effect of calcium channel blockers on prevention of preeclampsia in pregnant women with chronic hypertension. *Clin Exp Obstet Gynecol*. 2015; 42:79–81. [PubMed: 25864288]
- Jiang X, Wang X. Cytochrome C-mediated apoptosis. *Annu Rev Biochem*. 2004; 73:87–106. [PubMed: 15189137]
- Kilburn BA, Chiang PJ, Wang J, Flentke GR, Smith SM, Armant DR. Rapid induction of apoptosis in gastrulating mouse embryos by ethanol and its prevention by HB-EGF. *Alcohol Clin Exp Res*. 2006; 30:127–134. [PubMed: 16433740]
- Kouzoukas DE, Li G, Takapoo M, Moninger T, Bhalla RC, Pantazis NJ. Intracellular calcium plays a critical role in the alcohol-mediated death of cerebellar granule neurons. *J Neurochem*. 2013; 124:323–335. [PubMed: 23121601]
- Levy R, Smith SD, Yusuf K, Huettner PC, Kraus FT, Sadovsky Y, Nelson DM. Trophoblast apoptosis from pregnancies complicated by fetal growth restriction is associated with enhanced p53 expression. *Am J Obstet Gynecol*. 2002; 186:1056–1061. [PubMed: 12015537]
- Lurie S, Fenakel K, Friedman A. Effect of nifedipine on fetal heart rate in the treatment of severe pregnancy-induced hypertension. *Am J Perinatol*. 1990; 7:285–286. [PubMed: 2372339]
- Maggee LA, Miremadi S, Li J, Cheng C, Ensom MH, Carleton B, Cote AM, von Dadelszen P. Therapy with both magnesium sulfate and nifedipine does not increase the risk of serious magnesium-related maternal side effects in women with preeclampsia. *Am J Obstet Gynecol*. 2005; 193:153–163. [PubMed: 16021073]

- Marino J, Garcia Vior MC, Furmento VA, Blank VC, Awruch J, Roguin LP. Lysosomal and mitochondrial permeabilization mediates zinc(II) cationic phthalocyanine phototoxicity. *Int J Biochem Cell Biol*. 2013; 45:2553–2562. [PubMed: 23994488]
- McAleer MF, Tuan RS. Cytotoxicant-induced trophoblast dysfunction and abnormal pregnancy outcomes: role of zinc and metallothionein. *Birth defects research. Part C, Embryo today : reviews*. 2004; 72:361–370.
- Moretti MM, Fairlie FM, Akl S, Khoury AD, Sibai BM. The effect of nifedipine therapy on fetal and placental Doppler waveforms in preeclampsia remote from term. *American journal of obstetrics and gynecology*. 1990; 163:1844–1848. [PubMed: 2256493]
- Nishizawa H, Ota S, Suzuki M, Kato T, Sekiya T, Kurahashi H, Udagawa Y. Comparative gene expression profiling of placentas from patients with severe pre-eclampsia and unexplained fetal growth restriction. *Reprod Biol Endocrinol*. 2011; 9:107. [PubMed: 21810232]
- Novakovic B, Gordon L, Wong NC, Moffett A, Manuelpillai U, Craig JM, Sharkey A, Saffery R. Wide-ranging DNA methylation differences of primary trophoblast cell populations and derived cell lines: implications and opportunities for understanding trophoblast function. *Mol Hum Reprod*. 2011; 17:344–353. [PubMed: 21289002]
- Ornoy A, Ergaz Z. Alcohol abuse in pregnant women: effects on the fetus and newborn, mode of action and maternal treatment. *Int J Environ Res Public Health*. 2010; 7:364–379. [PubMed: 20616979]
- Pantazis NJ, Dohrman DP, Goodlett CR, Cook RT, West JR. Vulnerability of cerebellar granule cells to alcohol-induced cell death diminishes with time in culture. *Alcoholism, clinical and experimental research*. 1993; 17:1014–1021.
- Putney JW, Bird GS. Regulation of calcium entry in exocrine gland cells and other epithelial cells. *The journal of medical investigation : JMI*. 2009; 56(Suppl):362–367. [PubMed: 20224226]
- Raheem IA, Saaïd R, Omar SZ, Tan PC. Oral nifedipine versus intravenous labetalol for acute blood pressure control in hypertensive emergencies of pregnancy: a randomised trial. *BJOG*. 2012; 119:78–85. [PubMed: 21985500]
- Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M, Fisher SJ. Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. *J Clin Invest*. 2004; 114:744–754. [PubMed: 15372095]
- Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science (Wash DC)*. 2005; 308:1592–1594.
- Rey E, LeLorier J, Burgess E, Lange IR, Leduc L. Report of the Canadian Hypertension Society Consensus Conference: 3. Pharmacologic treatment of hypertensive disorders in pregnancy. *CMAJ*. 1997; 157:1245–1254. [PubMed: 9361646]
- Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol*. 2004; 5:897–907. [PubMed: 15520809]
- Scardo JA, Vermillion ST, Hogg BB, Newman RB. Hemodynamic effects of oral nifedipine in preeclamptic hypertensive emergencies. *American journal of obstetrics and gynecology*. 1996; 175:336–338. discussion 338–340. [PubMed: 8765250]
- Sharp AN, Heazell AE, Crocker IP, Mor G. Placental apoptosis in health and disease. *Am J Reprod Immunol*. 2010; 64:159–169. [PubMed: 20367628]
- Shekhar S, Sharma C, Thakur S, Verma S. Oral nifedipine or intravenous labetalol for hypertensive emergency in pregnancy: a randomized controlled trial. *Obstet Gynecol*. 2013; 122:1057–1063. [PubMed: 24104790]
- Smith SC, Baker PN, Symonds EM. Increased placental apoptosis in intrauterine growth restriction. *Am J Obstet Gynecol*. 1997; 177:1395–1401. [PubMed: 9423741]
- Smith TL. Regulation of glutamate uptake in astrocytes continuously exposed to ethanol. *Life sciences*. 1997; 61:2499–2505. [PubMed: 9416769]
- Toal CB, Meredith PA, Elliott HL. Long-acting dihydropyridine calcium-channel blockers and sympathetic nervous system activity in hypertension: a literature review comparing amlodipine and nifedipine GITS. *Blood pressure*. 2012; 21(Suppl 1):3–10.

- Vermillion ST, Scardo JA, Newman RB, Chauhan SP. A randomized, double-blind trial of oral nifedipine and intravenous labetalol in hypertensive emergencies of pregnancy. *American journal of obstetrics and gynecology*. 1999; 181:858–861. [PubMed: 10521742]
- Wilhoit LF, Scott DA, Simecka BA. Fetal Alcohol Spectrum Disorders: Characteristics, Complications, and Treatment. *Community Ment Health J*. 2017
- Wolff GS, Chiang PJ, Smith SM, Romero R, Armant DR. Epidermal growth factor-like growth factors prevent apoptosis of alcohol-exposed human placental cytotrophoblast cells. *Biol Reprod*. 2007; 77:53–60. [PubMed: 17392498]

Figure 1. Nifedipine blocks the intracellular Ca^{2+} increase induced by ethanol

HTR cells were preloaded with fluo-4-AM and intracellular Ca^{2+} signaling was monitored, as describe in the Materials and Methods section. After 90 sec HTR cells were exposed to 50 mM ethanol and monitored for an additional 270 seconds (A). HTR cells loaded with fluo-4-AM were pre-treated with 50 nM nifedipine, and intracellular Ca^{2+} signaling was monitored (B). After 90 sec, HTR cells were exposed to 50 mM ethanol and monitored for an additional 270 seconds. In all experiments, ionomycin was added after 300 seconds to equilibrate intracellular and extracellular Ca^{2+} in order to calculate the intracellular concentration.

Figure 2. Nifedipine prevents Ethanol induced cell death in HTR cells and first trimester villous explants

HTR cells (A) and villous explants (B) were pretreated with 0 or 50 nM nifedipine for 1 hour, and then exposed to 50 mM of ethanol for an additional hour. Cell death was assessed using TUNEL. TUNEL Index was calculated by dividing the number of TUNEL positive cells by total nuclei (assessed by DAPI) per field. *, $p < 0.05$ compared to vehicle. Columns and error bars represent mean $\pm$ SEM. Representative images of villi treated as indicated, and double labeled using fluorescent indicators to visualize DNA fragmentation by TUNEL (C–E) and nuclei labeled with DAPI (E–H).

Figure 3. Nifedipine Prevents Release of Cytochrome C from Mitochondria During Ethanol Exposure

HTR cells were treated with vehicle, ethanol, or pretreatment with 50 nM nifedipine for 1 hour and then exposure to 50 mM ethanol for an additional hour. Caspase 3 and 9 proteolytic activities were measured in HTR cells, using fluorogenic substrates for caspase-9 (A) and caspase-3 (B). Immunofluorescence images are shown for Mitotracker (green), cytochrome c (red), and merged fields, as indicated (C). DAPI nuclear counterstain (blue) is included in the merged images. Bars, 50 μ M.