Published in final edited form as:

Am J Obstet Gynecol. 2012 August ; 207(2): 140.e7–140.e19. doi:10.1016/j.ajog.2012.05.007.

Effects of Alcohol, Lithium, and Homocysteine on Nonmuscle Myosin-II in the Mouse Placenta and Human Trophoblasts

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Abstract

Mouse embryonic exposure to alcohol, lithium, and homocysteine results in intrauterine growth restriction (IUGR) and cardiac defects. Our present study focuses on the placental effects.

Objectives—We analyzed the hypothesis that expression of nonmuscle myosin (NMM)-II isoforms involved in cell motility, mechanosensing, and extracellular matrix assembly, are altered by the three factors in human trophoblast (HTR8/SVneo) cells in vitro and in the mouse placenta in vivo.

Study Design—After exposure during gastrulation to alcohol, homocysteine, or lithium, ultrasonography defined embryos exhibiting abnormal placental blood flow.

Results—NMM-IIA /NMM-IIB are differentially expressed in trophoblasts and in mouse placental vascular endothelial cells under pathological conditions. Misexpression of NMM-IIA/ NMM-IIB in the affected placentas continued stably to mid-gestation, but can be prevented by folate and myo-inositol supplementation.

Conclusions—It is concluded that folate and myo-inositol initiated early in mouse pregnancy can restore NMM-II expression, permit normal placentation/embryogenesis, and prevent IUGR induced by alcohol, lithium, and homocysteine.

Keywords

mouse; placenta; human trophoblasts; nonmuscle myosin II; alcohol; lithium; homocysteine; folate

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Disclosure: None of the authors have a conflict of interest.

Presentation: This work will be presented as a poster at the Research Society on Alcoholism annual meeting in June 2012 in San Francisco, CA

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Introduction

Our previous studies using the mouse model demonstrated that an acute embryonic exposure to the drug lithium (Li^{++}), to an elevated serum level of the natural metabolite homocysteine (HCy), or to a binge-drinking level of alcohol (ethanol, EtOH) during gastrulation on embryonic day (ED) 6.75, results in similar fetal cardiac and placental anomalies, including intrauterine growth restriction (IUGR) ^{1–3}. This timing of exposure extrapolates to 16–19 days of human pregnancy. Before the four-chambered heart forms, a beating, tubular human heart with extra- and intra-embryonic circulations is present on day 21 after fertilization bringing blood to the embryo. Given that 49% of pregnancies are unintended ⁴, the timing and effects of early exposure to environmental factors as alcohol, Li^{++} , or HCy, together with prophylactic mechanisms are critical to define. Placental blood flow represents a large fraction of cardiac output at early stages of development⁵. Because nonmuscle myosin-II_s (NMM-II_s) are important in processes associated with placentation ⁶, eg., hemodynamic mechanosensing and cell migration ⁷, we analyzed possible expression alterations in this class of proteins in placentas associated with IUGR in the mouse model.

Class II myosins comprise a protein family that includes the sarcomeric, smooth muscle, and the nonmuscle myosins (NMM-II_s). Each NMM-II isoform is composed of two identical heavy chains (NMHCs) and two pairs of light chains. NMM-II is a cytoskeletal protein that interacts with actin contributing to cell motility, cell polarity, chemotaxis, cell adhesion, cytoskeletal tension sensing, and cytokinesis. In mammals there are three NMM II isoforms, NMM-IIA, -IIB, and -IIC ⁸. Three separate genes MYH9, MYH10, and MYH14 encode the NMHCs, NMHC-IIA, -IIB, and IIC, respectively.

NMMII-A/II-B are essential during embryogenesis and placentation ^{9–11}. Little is known of their role and expression in the placenta. When NMM-IIA is deleted by germline ablation, early embryonic death results from altered cell-cell adhesion, lack of visceral endoderm and placental defects ⁹. NMM-IIA has an important role in placental development which appears different from its function in cell adhesion⁶. In NMM-IIA mutated embryos displaying embryonic lethality, placental vascularization was abnormal and placentas were more compact. Although NMM-IIB is present in the placenta, it cannot compensate for the novel function of NMM-IIA. In contrast when NMM-IIB is deleted, embryos die around ED14.5 due to heart and brain defects ¹⁰. We demonstrated that NMM-IIB is important in cardiac myofibrillogenesis, left-right asymmetry and heart looping^{11–13}. It has a role in cardiac trabeculation and mechanotransduction of blood flow forces¹⁴. NMM-II isoforms and smooth muscle myosin were defined biochemically to be present in the human placenta ¹⁵ suggesting our studies are relevant to human pregnancy. This may be of specific importance for individuals that have mutations in NMHC-IIA that comprise a spectrum of human diseases^{16–18}.

Invasion of human trophoblasts is promoted through activation of Wnt signaling, indicating this pathway has a role in placental development and morphogenesis ^{19, 20}. Lithium mimics the canonical Wnt pathway by inhibiting glycogen synthase kinase-3 ²¹. Lithium also has been shown to deplete phosphoinositides that act within the Wnt second messenger phosphatidylinositol signaling pathway ²². Importantly, phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) and phosphatidylinositol-anchored proteins have a role in trophoblast cell differentiation ^{23–25}. Thus, we analyzed whether addition of myo-inositol (MI) to the media used for culturing the human first trimester trophoblast cell line (HTR-8/SVneo cells) would show any additional benefit than use of folate alone. MI supplementation was expected to stabilize normal phosphatidylinositol signaling, preventing depletion by environmental factors and thus normalizing multiple intermediaries and processes important in placentation. Interestingly, during embryonic development *a plateau*

seemingly has been reached in FA reduction of neural tube defects (NTDs) and a portion of NTDs are *resistant* to FA prevention. With these resistant forms as well as in diabetic embryopathy, the embryo is protected by *myo-inositol supplementation*²⁶.

Our objectives were to define in trophoblast HTR-8/SVneo cells changes in cell proliferation and migration that may occur with Li, HCy and EtOH exposure and alterations in the expression of NMM-IIA or NMM-IIB. Importantly, NMM-IIA and -IIB are associated with mechanotransduction^{27, 28}, cell motility²⁹, and extracellular matrix (ECM) assembly³⁰, all integral to placenta development. We subsequently addressed the changes in the NMM-II isoform expression patterns that occur in vivo in mouse placentas of embryos exposed to Li, elevated HCy, and alcohol and analyzed specifically those embryos displaying IUGR, abnormal hemodynamics, and cardiac anomalies. Lastly, we compared the abnormal NMM-II expression patterns from above fetuses with placentas of folate (FA) rescued mouse embryos in which pregnancy complications were prevented. Although the mouse placenta is considered an imperfect model for studies relating to the human placenta, many similarities exist on the molecular and cellular levels³¹. The different cell types of the murine placenta described in our study all have their counterparts in the human placenta. The analyses of placental alterations experimentally induced in the mouse are expected to enable characterization of mechanisms involved in development of human placental abnormalities.

Materials and Methods

A human first trimester trophoblast cell line HTR-8/SVneo has been well characterized³² and was used in this study. This cell line was established from explant culture of first-trimester human placenta and was immortalized by transfection with the gene encoding simian virus 40 large T antigen. The resulting cell line (HTR-8/SVneo) shares phenotypic similarities with the progenitor cells and proliferation, migration and invasiveness were shown to be regulated by the same signaling molecules that modulate extravillous trophoblast cells. Generally, this cell line has been considered a highly useful model to study mechanisms regulating normal human trophoblast proliferation, migration, and invasiveness in response to environmental factors³³. The cells were cultured in an atmosphere of 5% CO₂ / 95% air in RPMI 1640 medium containing 5% fetal bovine serum (FBS), 1% penicillin and streptomycin. The medium was changed every 3 days.

Cell growth curve experiments

HTR-8/SVneo cells (5×10^3 cells/well) were seeded into 96 well plates (Corning 3610). Cells were treated with the following substances added to the culture medium: ethanol (100 mM), FA (75 μ M), myo-inositol (MI; 50 μ M), HCy (50 μ M), and LiCl (50 μ M). The control group was HTR-8/ SVneo cells incubated in culture medium without test substances. The cell culture media was replaced on day 3 and assayed on day 4.

The number of viable HTR-8 cells was determined colorimetrically using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). After 6 hours of treatment, the CellTiter 96® AQueous solution (20 μ L) was added to each well and incubated for an additional 2 hours. The absorbance (490 nm) was recorded using Gen 5 Biotek Synergy 2 Microplate Data Collection. These procedures were conducted daily for 5 days to obtain the proliferation curves. The experiment was repeated 3 times for a total of 10 samples for the following groups: EtOH, FA, EtOH plus FA, MI, EtOH plus MI, EtOH plus combination of MI/FA, and FA alone; quadruplicates were obtained for HCy, HCy plus FA, and duplicates for LiCl and LiCl plus FA.

Correlation between cell numbers and color formation was determined using cell concentrations (1×10^4 , 1.2×10^5 , 2.3×10^5 , 3.4×10^5 , 5.6×10^5 , 6.7×10^5 , 7.8×10^5 , 8.9×10^5 , 1×10^6) in 96 well plates with RPMI medium containing FBS and antibiotics. After one-hr equilibration, the Cell Titer 96® Aqueous solution was added to each well. After 2 hours, the absorbance (490 nm) was recorded as above.

HTR-8/SVneo-Scratch Wound Assay for Cell Migration and Immunostaining

Cells (5×10^5 /mL) were directly cultured using treated glass culture slides, 8 chambers, in RPMI 1640 medium as described above and incubated until 90% confluence. The analysis was carried out in triplicate for each experimental condition. Culture medium was changed to expose the cells to HCY, HCY/ FA, EtOH, EtOH/FA, or FA alone and incubated for 2 hrs. The monolayer of cells then was scratched once from the left to the right wall of the well with a sterile plastic tip, resulting in a cell-free cleft. The cells were fixed at 8 hrs post-“wounding” and at 24 hrs. This scratch assay permitted following specific cell markers during the early steps of directed migration of the trophoblasts at the cell-free boundary as cells begin to invade the gap at 8 hrs and to analyze the same markers after a 24 hr incubation in the different experimental media. Results are shown after 24 hr, as differences were most notable. Mitotic cells were immunostained with Histone 3 antibody (Cell Signaling Technology, Inc., Beverly, MA) and Cy-3 conjugated- secondary antibody. *Other antibodies used* included a rabbit polyclonal antibody for fibronectin (Sigma, St. Louis, MO), affinity purified rabbit antibodies for NMM-IIA and -IIB (Covance, Emeryville, CA) characterized by Western blotting^{11, 34}. An antibody for active β -catenin was purchased from Millipore (Temecula, CA). The horseradish peroxidase-conjugated secondary goat anti-rabbit antibody was from Vector Laboratories (Burlingame, CA) and the Cy3-conjugated secondary goat anti-rabbit antibodies from Jackson Lab (West Grove, PA).

Ultrasound Analysis of Placental Blood Flow

The protocol for use of animals was approved by the USF Institutional Animal Care and Use Committee. On ED 6.75, pregnant mice were given a single intraperitoneal (i.p.) injection of lithium, homocysteine, or a binge drinking level of alcohol (the latter by two i.p. injections at 3 and 6 PM)^{1, 3}. This dose was used in our previous study¹ and was based on the well-described and generally used model of binge alcohol dose for pregnant mice using two i.p. injections^{35–38}. Echocardiography on each ED15.5 embryo was carried out noninvasively using Vevo 770 instrumentation (VisualSonics, Inc., Toronto, Canada) to analyze embryonic cardiac function and umbilical artery blood flow^{39, 40}.

Mouse Placental Tissue

Control placentas and placentas from embryos displaying abnormal blood flow patterns and cardiac defects were fixed in para-formaldehyde, embedded in paraffin, and sectioned. Mouse placental tissue was immunostained as published⁴¹.

Statistical analysis of ultrasound parameters showing significant values of experimental exposure in comparison with control group was based on nonparametric Kruskal Wallis test. Significance is based on probability values of <0.05 .

Results

Using HTR-8/SVneo trophoblast cells, cell proliferation was not altered after EtOH, Li, or HCY exposure, with and without FA/myo-inositol supplementation of media

Figure 1A shows that EtOH, FA only, EtOH plus FA, EtOH plus MI, or MI only, did not significantly affect cell proliferation in vitro of this trophoblast cell line. Similarly HCY or

Li, with or without FA supplementation, by day 5 did not affect proliferation (Figure 1B). The decline in figure 1B appearing on day 4 in our assay may be due to change of culture medium on day 3. The decrease was within the standard deviation. For detection of mitotic cells after HTR-8/SVneo cell exposure to EtOH, HCy, or Li, with and without FA, we immunostained cells using histone 3 antibody (Figure 2). Confirming above proliferation assay results, no significant difference in numbers of mitotic cells was observed. We next analyzed effects on cell migration.

HTR-8/SVneo Cell Migration Scratch Assay Model

Using an in vitro scratch model of wounding, wound-induced cell migration was assayed at 24 hrs as cells migrate to close the wound gap. β -catenin an important intermediary within the canonical Wnt pathway, showed nuclear localization in cells both near the edge of the wound, as well as away from the edge (Figures 3A–C), indicating active Wnt signaling. The ECM protein FN is highly expressed and assembled by the HTR-8/SVneo trophoblast cells (3D–F). Twenty-four hrs post-wounding, FN is expressed (white arrows) in all cells >106 μ m (green line) away from the wound edge, and also present near the free edge (3F). Similarly, NMHC-IIA and -IIB are expressed by HTR-8/SVneo cells. Cells near the free edge express higher levels of NMHC-IIA and -IIB as cells migrate to fill the gap (3G–I and 3J–L, respectively).

Scratch Assay Model with HCy Exposure and FA/MI Supplementation: Upregulation of FN, NMHC-IIA and NMHC-IIB

FN Localization (Figures 4A–E)—After exposure of the human HTR-8/SVneo extravillous trophoblast cells to an elevated HCy concentration, FN is synthesized by untreated control cells at the wound edge (Figure 4A; cell nuclei in blue shown by overlay of DAPI staining). FN localization shows relatively low level of expression by cells at the wound edge with HCy exposure (4B). With FA supplementation (**HCy/FA**; 4C), FN expression is slightly elevated ~100 μ m away from gap edge with little change at cell free edge at gap. With the addition of MI to the incubation medium, with or without HCy and FA, there is a notable upregulation of FN (4D,E) with high levels 89 μ m to 110 μ m away from wound edge and slightly higher expression seen near the gap.

NMM-IIB Localization (Figures 4F–J)—NMHC-IIB localization is at a relatively high level in untreated control cells after 24 hrs near edge of wound gap (blue DAPI stained nuclei shown as an overlay in Fig. 4F). With HCy exposure, NMHC-IIB is seen generally at a low level of expression (Fig. 4G); an elevation of NMM-IIB is apparent with FA supplementation of media Figs 4H, J). With the addition of myo-inositol (MI) to the incubation medium, as well as with FA, NMM-IIB is expressed at control levels (Fig 4I,J). Therefore, addition of MI and FA upregulated FN synthesis and NMHC-IIB expression in the human HTR-8/SVneo cells, to levels apparent in saline-treated control cultures without HCy.

Altered umbilical and intraembryonic blood flow observed by doppler ultrasound analysis after exposures to alcohol, Li⁺⁺ or HCy

Of these three environmental factors assayed, a binge level of alcohol exposure had the most severe effect on embryonic cardiac function¹ when compared to Li⁺⁺ exposure or to HCy, the latter having the most moderate effect³. This was reflected also in the pulsatility indices of the ductus venosus (DV; 5A), umbilical artery (UA; 5B), descending aorta (DA; 5C), and in the cardiac outflow velocity (OFT; 5D). The morphometric and cardiovascular parameters are summarized in Table I.

Mouse embryos exposed acutely during gastrulation to the specified environmental factors, also demonstrated significant IUGR and reduced placental weights, when compared to control group. The placentas of mouse embryos displaying IUGR, cardiac valve regurgitation and abnormal Doppler waveforms were analyzed for changes in NMHC-IIA and NMHC-IIB expression to compare with control tissue.

A single exposure of the pregnant mouse on ED6.75 to ethanol, Li⁺⁺, or HCy differentially altered placental NMM-IIA and NMM-IIB into mid-gestation (ED 15.5) of IUGR embryos

Comparing to control placentas (Figure 6A–D), a single exposure to EtOH during gastrulation on ED6.75 *enhanced* NMHC-IIA expression (brown horseradish peroxidase localization) within the maternal decidua and labyrinth layers and to a lesser extent in the fetal side of the chorionic plate. This was detectable over a week later (6E–H). There was little signal in the spongiotrophoblast cells. The placentas were smaller than those of control embryos. Dietary supplementation with FA initiated the morning after conception rescued normal embryonic heart development¹ from alcohol exposure effects, and led to relatively normal control levels of NMHC-IIA in the maternal decidua and labyrinth layers. With FA, however, maternal decidua layer continued to be smaller than in control placentas. With and without environmental exposure or FA supplementation, the fetal chorionic plate NMHC-IIA expression remained stable.

Comparing control placental tissue (Figure 7A–D), an acute EtOH exposure induced a significant *upregulation* of NMHC-IIB expression (brown signal) in all regions of the placenta (7 E–H). Dietary supplementation with FA, allowed normal NMHC-IIB expression in the maternal decidua (7I), spongiotrophoblast layer (7J), as well as in the fetal side of the placenta within the chorionic plate (7L): In all cell populations expression levels were closer to that observed in the control tissue. Some patches of cells in the labyrinth layer, however, showed a higher expression of NMHC-IIB (arrow; 7K). Although these pockets of NMM-II misexpression may be noted, they generally are prevented by FA supplementation.

NMHC-IIA in FA supplemented tissue (Figure 8A–C; i.e., no environmental exposures), as in saline exposed control placentas displayed a low level of positive (brown) expression in the maternal decidua/spongiotrophoblast layers (8A), some signal in the labyrinth layer (8B), as well as little on the fetal side of the labyrinth (8C). Lithium exposure significantly induced an *upregulation* of NMHC-IIA expression in all placental cell populations (8D–F). FA supplementation prior to Li exposure did not decrease the effects of Li significantly (8G–I). HCy exposure *increased* NMHC-IIA slightly above control levels in the maternal decidua/spongiotrophoblast layers (8J), but not to the extent that Li did. In the labyrinth layer or on the fetal side of the placenta there was no noticeable increase above control level. As with Li, FA supplementation had little effect on the change in NMHC-IIA expression after HCy exposure.

NMHC-IIB *localization* in FA only supplemented tissue (Figure 9A–C) was similar to control tissue (not depicted), and expression was noted in all three regions of the mouse placenta. With Li exposure, NMHC-IIB expression was *decreased* in all three layers (9D–F). Folate supplementation before Li exposure (9G–I) maintained NMHC-IIB expression at control levels (compare with (9A–C)). With HCy exposure, as with Li, NMHC-IIB was *decreased* in the maternal decidua/spongiotrophoblast (9J) and the fetal side of the labyrinth (9L) layers, and remained at control levels within the labyrinth layer with only some cells showing increased expression (9K; arrows). FA supplementation increased NMHC-IIB closer to control levels (9M–O). Thus, NMHC-IIA and NMHC-IIB are differentially regulated in the IUGR mouse placentas after exposure to each of the three environmental factors. Folate supplementation in general helps to protect control levels of the two NMM-II isoforms.

Comments

We demonstrated that three environmental factors, Li mimicking canonical Wnt signaling^{42, 43} and a drug used therapeutically for bipolar disorder, Hcy a normal metabolite in the FA cycle and elevated in patients with mutations in methyltetrahydrofolate reductase (Mthfr), and alcohol, induce similar cardiac anomalies and altered embryonic blood flow^{1, 3}. The results demonstrate these factors induce higher placental resistance and IUGR. The factors differentially misexpressed NMM-IIA and NMM-IIB in the affected mouse placentas, as well as in human HTR-8/SVneo trophoblast cells in culture. In the HTR-8/SVneo extravillous trophoblast cells, exposure to Li, Hcy or ethanol did not affect cell proliferation, but rather altered expression of NMHC-IIs and FN associated with cell migration. Supplementation of cell culture media with FA or MI, enhanced expression of molecules associated with cell migration. It should be noted that our directed migration study is not the classic trophoblast migration assay in which cells migrate through a filter insert and number of migrated cells are quantitated on the underside of the filter⁴⁴. Rather we chose the scratch wound assay to follow immunohistochemically specific cell markers over time during the directed trophoblast migration process under the different experimental conditions. In addition in the in vivo environment, providing pregnant mice with high FA as a dietary supplement the morning after conception as defined by a vaginal plug and exposing the dams to the three experimental factors during gastrulation on ED 6.75, normal placentation occurred, protein marker expression was similar to control placentas, embryonic weights normalized, and cardiac defects were prevented.

NMMII-A and II-B are essential during mouse and avian embryogenesis^{9–11}. Although NMM-II isoforms have been identified biochemically in the human placenta¹⁵, the cells expressing these isoforms and their involvement with specific cellular processes were not defined. Our studies provide cellular data on an importance of NMM-IIs in the placenta and that their expression can be altered by even a single exposure of mouse embryos during gastrulation to teratogens as alcohol, lithium, and elevated homocysteine. These above in vitro results using an extravillous trophoblast cell line and in vivo results taken together suggest a possible relevancy to human pregnancy before three weeks of gestation in which pregnancy is generally unrecognized. These results demonstrate early folate and myo-inositol supplementation can protect normal cell migration-related processes in the human HTR-8/SVneo cell line and mouse placentation to prevent IUGR.

The present study points to new directions for analyses. Lithium by potentiating Wnt signaling and alcohol have the potential to lead to inositol depletion²² and repress phosphatidylinositol (PI) signaling⁴⁵, a second messenger of Wnt signaling. Evidence indicates that phosphoinositide 3-kinase (PI3K) plays a crucial role in growth factor-mediated trophoblast migration⁴⁶. The relationship between alcohol/Li/Wnt/ β -catenin signaling and epigenetic regulation is important to define⁴⁷. It is recognized that Wnt signaling be considered not as a linear cell signaling pathway, but as having multiple inputs, not only at the level of Wnt-receptors, but also at downstream intracellular responses taking place concomitantly⁴⁸. We suggest a balancing of canonical Wnt signaling exists in placental development and part of the regulation involves epigenetic mechanisms supported by FA one-carbon metabolism and methylation. Multiple Wnt ligands and their frizzled receptors localize in placental tissue^{19, 20, 49}. The expression patterns vary with gestational age and different trophoblast subtypes. Our FA protection findings substantiate that an epigenetic component exists in protection of normal trophoblast differentiation, invasion, and formation of the placenta relating to Wnt signaling⁵⁰. Although our acute environmental exposure during mouse gastrulation occurs during trophoblast differentiation stages, we analyzed effects at ED15.5 to observe that placental changes are stably maintained to mid-gestation, and possibly into later stages of gestation resulting in the observed IUGR. These

interacting pathways are necessary new areas for future research to elucidate teratogenic effects on placental development.

Acknowledgments

Financial Sources: Support to KKL from NHLBI, American Heart Association, the Suncoast Cardiovascular Education and Research Foundation founded by Helen Harper Brown, and from the Mason Endowment of USF Foundation is gratefully acknowledged.

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**Comparison of cell proliferation of HTR-8/SVneo trophoblast cells
with different environmental exposures**

Figure 1.

Figure 1A. Cell proliferation of HTR-8/SVneo trophoblast cells incubated in the presence of ethanol and with folate (FA) or myo-inositol (MI) supplementation in comparison to normal media.

Figure 1B. Cell proliferation of HTR-8/SVneo trophoblast cells incubated in the presence of homocysteine (Hcy) or LiCl and with FA supplementation in comparison to normal media.

Figure 2.

Cy-3 (bright red) immunolocalization of Histone 3 in cells undergoing mitosis in control HTR-8/SVneo trophoblast cells (A; negative control shown in B); in panels C–H, HTR-8/SVNEO cells are shown that were incubated in the presence of the three specified environmental factors, with and without folate (FA).

Magnification bar = 100 μ m in panel G and for all panels

Figure 3.

Scratch Assay of HTR-8/SVneo trophoblast cells grown to confluency. Bright red, Cy3-immunolocalization (see arrows) shown for β -catenin (A–C); for fibronectin (FN, D–F): Green line in panel F represents 106 μm distance at wound edge where little FN is being synthesized. Panels G–I depict NMHC-IIA and J–L, NMHC-IIB. The latter two NMM-IIs are important in cell migration. Each row represents a lower to higher magnification of the edge of the wound. Magnification bar in M= 200 μm ; in N= 100 μm ; and in L=100 μm . M,N are negative controls. Brightfield images of left wound edge only at 8 hrs (O) and of same cells migrating in at 24 hrs (P) taken at same magnification (4X). Magnification bar =100 μm for all panels in M, N and L and for the upper panels in the respective columns.

Figure 4.

Scratch assay of HTR-8/SVneo trophoblast cells incubated for 24 hrs after wounding in the presence of untreated controls (A,F; blue DAPI labeled nuclei shown in overlay); homocysteine only (HCy, B,G); supplementation with HCy and FA (HCy/FA, C,H); with HCy and the FA/MI combination (D,I), or with MI only (E) or FA only (J). Column on left shows bright red Cy-3 immunolocalization for fibronectin (FN) and on the right for NMHC-IIB. All vertical magnification bars on right side of images equal 100 μ m.

Figure 5. Pulsatility indices (PI) of the the ductus venosus (DV), umbilical circulation (umbilical artery, UA, 5B), of the arterial Doppler of the descending aorta (DA, 5C), and the cardiac outflow velocity (OF, 5D) after experimental embryonic exposures to ethanol, Li, and homocysteine. Mean and one standard deviation of pulsatility index values and peak outflow velocities of Doppler blood flow parameters are shown.

Figure 6.

Placental NMHC-IIA localization (brown horseradish peroxidase) on ED 15.5 after acute **ethanol** (EtOH) exposure on ED 6.75. Columns represent four cell populations (maternal decidua, spongiotrophoblast, labyrinth, and chorionic plate on fetal side) of the placenta; rows represent control saline exposed placenta (6A–D); ethanol exposed (6E–H) showing a significant increase in expression in the maternal decidua layer and slight increase in labyrinth and chorionic plate; and folate supplementation with alcohol exposure (FA+EtOH; 6I–L) depicting comparatively control levels of expression. Magnification bar in C and for all panels is 100µm.

Figure 7.

Placental NMHC-IIB localization (brown signal) on ED 15.5 after acute **ethanol** (EtOH) exposure on ED 6.75. Columns represent four cell populations (maternal decidua, spongiotrophoblast, labyrinth, and chorionic plate on fetal side) of the placenta. Rows represent control saline exposed placenta in panels 7A–D; ethanol exposed (7E–H) showing an upregulation of NMHC-IIB in all cell populations; and folate supplementation with alcohol exposure (FA/EtOH) returning expression close to control levels in 7I–L. 7M–P show the negative control sections treated with secondary antibody only. Magnification bar in A and for all panels is 100 μ m.

Figure 8.

Placental NMHC-IIA localization (brown signal) on ED 15.5 after acute **Li or HCy** exposure on ED 6.75, with and without FA supplementation. Columns represent four cell populations (maternal decidua/spongiotrophoblast, labyrinth, and chorionic plate on fetal side) of the placenta. Rows represent folate (FA) only supplemented placentas with control levels of NMHC-IIA expression (panels 8A–C); lithium (Li) exposed in 8D–F; Li exposure with FA supplementation (8G–I); HCy exposure (8J–L); and FA supplementation with HCy exposure (8M–O) did not show much change. Magnification bar in J and for all panels is 100 μ m.

Figure 9.

Placental NMHC-IIB localization (brown signal) on ED 15.5 after acute Li or HCy exposure on ED 6.75, with and without FA supplementation. Columns represent four cell populations (maternal decidua/spongiotrophoblast, labyrinth, and chorionic plate on fetal side) of the placenta. Rows represent folate (FA) only supplemented placentas with control levels of NMHC-IIB expression (panels 9A–C); lithium (Li) exposed in 9D–F; and Li exposure with FA supplementation (9G–I) returning expression to more control levels; HCy exposure (9J–L) and with FA supplementation (9M–O) returns expression levels closer to control levels. Magnification bar in A and for all panels is 100 µm.

Morphometric Analysis and Cardiovascular Parameters

Table 1

Mouse embryonic morphometric and cardiovascular parameters assessed on ED15.5 after exposure to ethanol, lithium, and homocysteine. Based on statistical analyses, a Doppler finding of an increased pulsatility index (PI) in the UA 1.75 was abnormal; in the descending aorta, a PI 2.1; and an outflow velocity <20 cm/sec were abnormal. In comparison to control group, venous flow showing an increased PI 1.2 was abnormal. Shaded cells show values significantly altered.

Parameters		Un-Injected n=24	Ethanol n=30	Lithium n=68	HCy n=59
Morphometric Measurements	CRL (mm)	15.33	12.60	13.80	13.08
	BW (gm)	0.44	0.37	0.37	0.35
	PW (gm)	0.13	0.10	0.11	0.11
Valvular Regurgitation	SLV (%)	0%	76.70	51.50	47.50
	AVVR (%)	0%	6.70	0.00	6.80
Myocardial Performance Index	MPI	0.47	0.65	0.60	0.44
	IRT%	12.04	17.20	16.82	11.93
Arterial Doppler	UA PI	1.31	1.80	1.75	1.54
	DA PI	1.53	1.76	2.10	1.98
	OFV (cm/s)	35.78	28.62	41.05	39.92
Venous Doppler	DV PI	0.86	1.19	0.97	0.97

Abbreviations: CRL, crown to rump length; BW, body weight; PW, placental weight; SLV, semilunar valve; AVVR, atrioventricular valve regurgitation; MPI, myocardial performance index; IRT, isovolemic relaxation time; UA, umbilical artery; DA, dorsal aorta; OFV, outflow velocity; DV, ductus venosus