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Viewpoint

Extracellular and Intracellular Magnesium Deficiency Found in Pregnant Women with Preeclampsia and Gestational Diabetes Is Associated with Overexpression of Notch Proteins, Cytokines, p53, NF-kB and Proto-Oncogenes: Potential Importance in Growth Retardation, Stillbirths, Fetal Mutations and Increased Cardiovascular Risks and Stroke with Advancing Age in Pregnant Women

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Abstract: In 1983, three of us reported in "Science" that umbilical-placental arteries and veins, obtained from normal pregnant women at term delivery, when exposed in vitro to low concentrations of Mg^{2+} went into vasospasm; the lower the Mg^{2+}, the greater the contractile force developed. These blood vessels also demonstrated amplified contractile force development when challenged with circulating amines and peptides (e.g., norepinephrine, 5-HT, angiotensin II, etc.). We suggested that severe Mg deficiency during pregnancy could in part be responsible for spontaneous abortions, loss of fetuses, stillbirths, and developmental alterations in infants. Using short-term dietary Mg deficient animals, we have noted a great many molecular and biochemical alterations in ventricular, atrial and somatic vascular smooth muscle alterations including DNA methylation and histone changes leading us to speculate that Mg deficiency may represent a genotoxin promoting mutations and causing epigenetic changes. Over the last 35 years, we have new data on severely preeclamptic and gestational diabetic pregnant women that gives credence to our original hypothesis and demonstrates that recently- discovered developmental proteins, originally found 100 years ago in *Drosophila* fruit flies termed the "Notch pathway", due to effects on its wings, appears to be important in development of the umbilical-placental blood vessels in pregnant women. Along with the developmental molecule, p53, these Notch proteins clearly alter the behavior of the umbilical-placental vessels. We believe these new findings probably help to explain many of the genetic-toxicity effects seen in women later in life who develop strokes and cardiovascular diseases. Notch alterations could also play an important role in babies born with cardiac defects.

Keywords: Notch proteins; cytokines; p53; NF-kB; proto-oncogenes; cardiovascular risk factors; preeclampsia; gestational diabetes

1. Introduction

Mutations in pregnant women and fetuses, in early embryonic development of genes, may cause several genetic disorders, spontaneous abortions, and loss of babies. At least five million babies are lost, worldwide, each year. Each year, about 2,000,000 babies are stillborn, most for unknown reasons [1]. Approximately 40 years ago, three of us reported that umbilical arteries and veins, as well as placental-uterine blood vessels taken from pregnant women, at term pregnancy, when exposed to Krebs-Ringer bicarbonate solution containing diminished levels of ionized magnesium ($[Mg^{2+}]_0$), *in-vitro*, would undergo spontaneous vasospasm; the lower the $[Mg^{2+}]_0$, the greater the strength of the contractions [2]. Contractions induced by circulating humoral vasoactive agents (e.g., amines, peptides, certain cations, etc.) were found to be amplified in strength when the human umbilical and placental vessels were exposed to low levels of $[Mg^{2+}]_0$ ([2], unpublished findings). At that time, we suggested that if some pregnant women were exposed to dietary deficiency of Mg from early pregnancy towards term, this could result in pre-eclampsia-eclampsia, gestational diabetes, fetal growth retardation, and/or loss of the fetus [2].

Over the past 60 years, our laboratories have found that experimental and clinical Mg deficiency, in-vivo and in-in-vitro, can cause vasospasm in peripheral, coronary and cerebral arteries and veins as well as in microcirculatory arterioles, metarterioles, precapillary smooth muscle cells and muscular venules (40–60 μm) [3–19]. Since dietary intake of Mg in North America, South America, Europe and The UK has declined over several decades to about 138–235 mg Mg/day as contrasted with about 450–550 mg Mg/day in 1900 [20–22], this loss in Mg becomes very important to human health. The current loss in Mg content of drinking waters, beverages, boiling of foodstuffs, and soil depletion of Mg (by too much fertilizers containing too many phosphates) in the food chain compounds this dangerous situation and makes it very dangerous for pregnant women and their fetuses. Many of the fetal heart abnormalities such as aortic stenoses, atrial septal defects, coarctation of the aorta, patent ductus arteriosus, patent foramen oval, tetralogy of Fallot, truncus arteriosus, among others, have no known causes. We believe Mg deficiency may play an important, underestimated factor and, thus, may be pivotal in these mutations. Recently, we have shown several reasons for how and why Mg deficiency is a genotoxin and can result in epigenetic changes in the cardiovascular system as discussed below [23–30]. Genotoxicity denotes in genetics a destructive effect on a cell's genetic material (i.e., DNA, RNA) thus potentially altering cell integrity, functions, and phenotypes.

2. Mg Deficiency Causes Oxidation and Fragmentation of DNA, Formation of Malondialdehyde (MDA), as Well as Down-Regulation of Telomerase, in the Heart, Blood Vessels and Hypothalamus

Examination of umbilical and placental blood vessels removed, at term pregnancy, from women with severe preeclampsia, and eclampsia, as well as gestational diabetes revealed that the vascular smooth muscle cells (VSMc) and endothelial cells showed oxidation and fragmentation of the DNA ([31], unpublished findings); extracellular Mg^{2+} as well as intracellular (red blood cell) Mg^{2+} were significantly depressed (see Table 1). A few years ago, using rats given Mg deficient diets for 21 days, we showed that ventricular and atrial muscle cells, as well as VSMc, removed from the animals were deficient in free Mg ions with concomitant oxidation and fragmentation [32,33]; the greater the loss of Mg^{2+}, the greater the oxidation and fragmentation of the DNA and lipid peroxidation. We also noted that that these Mg deficient cells demonstrated a down-regulation of telomerase [32,33].

Table 1. Red Blood Cell Intracellular Free Ionized Mg (μM) Depressed Taken from Preeclamptic (PRE) and Gestational Diabetic (GD) Pregnant Women at Term.

No Subjects	Normal RBC	PRE RBC	GD RBC
16 ea	322 ± 12.4	282 ± 10.6	290 ± 8.4

All values are means ± S.E.M. obtained with ^{31}P-NMR spectroscopy. $p < 0.05$ compared to normal RBC. All women were prima gravida I (18–24 years of age).

More recently, utilizing similar Mg deficient diets, given to the rodents, we found a dose-dependent loss of free Mg ions in the hypothalamus with concomitant oxidation and fragmentation of the hypothalamic cellular DNA [34]. The hypothalamus houses many critical hormones (i.e., neurohormone releasing factors, gonadotrophic hormones, vasopressin, oxytocin, among others), which play central roles in pregnancy, control of body metabolism, cardiovascular regulation, behavioral patterns, neuroendocrine outputs, release of steroids, temperature regulation, and reactions to stress, among other functions (for reviews, see [35,36]).

More than 15 years ago, four of us working with primary cultured cerebral vascular smooth muscle cells, we noted that low $[Mg^{2+}]_0$ levels resulted in rapid increased formation of MDA [29,33,37]. This formation of MDA was also found in diverse cardiovascular tissues extracted from rats subjected to 21 days of Mg deficiency [33] as well as in umbilical-placental VSMc obtained from pregnant women at term pregnancy who had gestational diabetes or eclampsia [38]. Collectively, we believe these data, when taken together, provide presumptive evidence for our hypothesis that Mg deficiency during pregnancy acts as a genotoxin. MDA is a well-known genotoxin compound that forms a propano adduct with 2-deoxyguanosine rather than an etheno adduct, which can produce mutations [39].

3. Formation of 4-Hydroxy-2-Nonenal (4-HNE) in Umbilical-Placental Blood Vessels of Pregnant Women with Pre-Eclampsia-Eclampsia and Gestational Diabetes: Importance in Production of Damaged DNA and Genotoxins in Venous vs. Arterial VSMc

4-HNE is a well-known inducer of hydrogen peroxide [40], known to be formed in Mg-deficient VSMc and known to induce DNA damage, and fragmentation in Mg- deficient somatic and cerebral blood vessels as well as cardiac muscle obtained from rats fed low Mg diets for 21 days [41]. Arterial and venous SMC obtained from umbilical -placental blood vessels from pregnant women with gestational diabetes or pre-eclampsia-eclampsia, at term, demonstrated elevated levels of 4-HNE; the lower the plasma ionized Mg, the higher the 4-HNE ($p < 0.01$) (see Table 2; [2,42]). It is of significant interest that VSMc obtained from the umbilical veins (the vessels that bring blood flow to the growing fetus) exhibited higher levels of 4-HNE than the VSMc obtained from the umbilical arterial VSMc, the vessels returning blood to the mother (see Table 2; [2,42]). Since 4-HNE is associated with genetic mutations [40], we believe these data bolster our hypothesis that Mg -deficiency can act as genotoxins to help play an important role in gestational diabetes, pre-eclampsia-eclampsia, growth retardation, and still birth.

Table 2. HNE Levels in Umbilical Vascular Smooth Muscle Cells (VSMC) taken from Preeclamptic (PRE) and Gestational Diabetic (PD) Women at Term Labor.

No Subjects	Normal	PRE VSMC	GD VSMC
16 ea	0.164 ± 0.012	5.32 ± 0.44	3.56 ± 0.28

All values are given in mmol/mg protein and are means ± S.E.M. Taken from ref. [42]. (ANOVA, $p < 0.01$). Methodology can be found in ref. [41].

4. Mg Deficiency in Umbilical-Placental VSMc from Pregnant Women with Gestational Diabetes or Severe Pre-Eclampsia, at Term, Demonstrate Upregulation of p53

The tumor suppressor protein p53 is a transcription factor which can be activated by numerous agents, including DNA-damage, ionizing radiation, ultraviolet irradiation, ribonucleoside triphosphate depletion, metabolic stress, and aging as well as myocardial infarction, reperfusion injury, ischemia, atherogenesis, and stroke (for reviews, see [43,44]). This protein is well-known to play key roles in the regulation of cell growth (for review, see [45]). Exposure of tissues and cells to radiation or chemotherapeutic drugs can result in programmed cell death by mechanisms that lead to damage of DNA (genotoxic stress) and that p53 accumulates in cells when DNA is damaged (see refs. [43–45]). Atherosclerotic plaques demonstrate DNA damage, activation of DNA repair pathways, increased expression of p53, and apoptosis.

Recently, using rats and rabbits fed diets deficient in Mg for 21–28 days, we have reported that this degree of short-term Mg deficiency induces upregulation of p53 and DNA damage (e.g., fragmentation and oxidation) ([21,26–29,32,46,47], unpublished findings), atherosclerotic plaques (with histochemical p53), apoptosis, down-regulation of telomerases, and several types of programmed cell death [26–29,47–50]. Having such results, as a background, we found that umbilical- placental VSMc taken from both gestational diabetic women (HbA1C > 6.5) and severely pre-eclamptic women (systolic BP > 160 mmHg) exhibit elevated levels of p53 [31]. When these new human data are viewed in terms of the results showing damaged DNA (above) and elevated levels of 4-NHE in the VSMc (above), we believe it becomes very difficult, if not impossible, not to conclude that Mg deficiency plays a role in gestational diabetes and pre-eclampsia-eclampsia in pregnant women.

5. Mg Deficiency in Umbilical-Placental VSMc from Pregnant Women with Gestational Diabetes or Pre-Eclampsia-Eclampsia, at Term, Show Overexpression of NF-kB and Proto-Oncogenes

Nuclear factor kappa B (NF-kB) and the proto-oncogenes c-fos and c-jun are two major regulators of growth, differentiation, and cell migration, and programmed cell death (for reviews, see [51–53]). NF-kB is a transcription factor and a pleiotropic regulator of numerous genes involved in inflammatory responses, hypertension, and atherogenesis [52,53]. Both NF-kB and the proto-oncogenes are thought to be important in numerous vascular disease processes such as inflammation, atherogenesis, hypertension, and stroke-like events (for reviews, see [52,53]). It is not clear, however, whether or not these transcription factors play any significant roles in the origin of pre-eclampsia-eclampsia or gestational diabetes.

Experiments, performed in our laboratories, both in-vitro and in-vivo, have demonstrated that short-term dietary deficiency of Mg results in formation/activation of NF-kB and the proto-oncogenes, c-fos and c-jun in animal cardiovascular tissues and cells [24,29,37,54,55]. Using umbilical-placental VSMc, obtained from pregnant women with either severe pre-eclampsia or gestational diabetes, we have noted an inverse relationship between the concentrations of RBC free Mg^{2+} and the VSMc concentrations of NF-kB and the proto-oncogenes; the lower the RBC levels of Mg^{2+}, the higher the levels of NF-kB, c-fos, and c-jun ($p < 0.01$) (unpublished findings). Although we cannot confirm that our findings, in these pre-eclamptic and gestational diabetic women, prove our hypothesis, it is difficult to ignore them, particularly in pregnant women who give rise to babies born with diverse mutations, stillbirths, diabetes, or with hypertension.

6. Elevated Levels of Pro-Inflammatory Cytokines and Chemokines Found in VSMc and Endothelial Cells Obtained from Pregnant Women with Either Severe Pre-Eclampsia or Gestational Diabetes: Relationship to RBC Intracellular Free Mg

Leukocytes and endothelial cells, as well as VSMc, can modulate inflammatory conditions via the elaboration and release of cytokines and chemokines [53]. Mg deficiency has been shown in rats to result in upregulation of interleukin-1 (IL-1 alpha), IL-6 and TNF-alpha in serum and endothelial cells [29,48,54,56–59]. Using rats given Mg deficient

diets for 21 days, we have found upregulation of IL-1 alpha, IL-1 beta, IL-4, IL-6, Il-10, IL-12, TNF-alpha and several chemokines [54]. These pleiotropic cytokines and chemokines have been implicated in atherogenesis, hypertension, numerous immune inflammatory pathways, and in severe pre-eclampsia (for review, see [53]).

Using umbilical-placental VSMc and endothelial cells, from severe pre-eclamptic women and women with gestational diabetes, with depressed Mg levels, taken at term delivery, we have found elevated levels of IL-1 alpha, IL-6, TNF-alpha and the chemokine-RANTES in homogenates (see Table 3; [31]); the lower the levels of the intracellular RBC free Mg levels, the higher the levels of the intracellular levels of the cytokines and RANTES [31] unpublished findings.

Table 3. Inflammatory Cytokines are found in Homogenates of Umbilical VSMC taken from Severe Preeclamptic (PRE) and Gestational Diabetic (GD) Pregnant Women at Term Delivery.

Cytokine	Normal Controls	PRE	GD
IL-1beta	4.63 ± 1.08	7.98 ± 1.12	6.84 ± 1.08
IL-6	5.76 ± 1.94	8.64 ± 1.98	7.64 ± 1.46
TNF-alpha	0.68 ± 0.12	1.98 ± 0.42	1.6 ± 0.52

Values are pg/mg protein (taken from ref. [31]). All experimental values are significantly diff from normal controls (ANOVA, $p < 0.05$).

Collectively, we believe our data, on the cytokine and chemokine levels in Mg-deficient endothelial and vascular smooth muscle cells, obtained from severely pre-eclamptic women (systolic blood pressure > 160 mmHg), and those with gestational diabetes [31], clearly reinforce our hypothesis that Mg deficiency during pregnancy must be taken into consideration in the etiology of severe pre-eclampsia, gestational diabetes, loss of fetuses, growth retardation, and stillbirth, as three of us suggested almost 40 years ago.

7. Notch Proteins Are Overexpressed in Umbilical VSMc Removed from Severe Pre-Eclamptic Women at Term Pregnancy and Relationship to p53: Potential Relevance to Fetal Mutations during Pregnancy

Over the past few years, a pathway originally discovered in *Drosophila* in 1913 (The Notch pathway, due to a change in the wings, i.e., The Notch), has now become important in mammalian cardiovascular development, homeostasis and disease (for recent reviews, see [60–65]). It has now become apparent that the Notch gene regulatory pathway plays an important role in vascular smooth muscle cell phenotypes, vascular remodeling and repair after cell injury. Notch ligand binding leads to an intracellular domain (NICD) which is released from the endothelial cell membrane by a gamma-secretase-dependent proteolytic cleavage of the Notch receptor [66]. Notch signaling from tumor cells has been shown to activate the endothelial cells and thus initiate angiogenesis. Over the past 15 years, a great deal of attention has been brought to bear on the development of gamma-secretase inhibitors [67].

Due to the Notch pathway's interaction with the tumor suppressor gene, p53, and other transcription factors, we have speculated that Mg deficient states may act as genotoxins to activate one or more of the four Notch pathways [68]. Using endothelial cells and vascular smooth muscle cells removed from umbilical veins recovered from severely pre-eclamptic women (BP > 160 mmHg and proteinuria) at term pregnancy, we have, indeed, found that these Mg deficient cells demonstrated up-regulation (3–5× fold) of Notch 1 and 2 (e.g., see Table 4) with a strong correlation to up-regulation of p53 and ceramides, DNA oxidation and fragmentation, up-regulation of proto-oncogenes c-fos and c-jun, as well as a down-regulation of telomerase ($p < 0.01$) (unpublished findings). In addition, preliminary findings, from our labs, suggest that vascular muscle and endothelial cells recovered from umbilical vessels, obtained from severely pre-eclamptic and gestational diabetic women, show DNA methylation and histone alterations (unpublished findings). We believe these findings provide compelling reasons for why severely Mg deficient, pregnant women may present with gestational diabetes and/or pre-eclampsia, fetal mutations, loss of fetuses,

and stillbirths. We have found using dietary Mg deficiency, in rats for 21 days, that excised ventricular, atrial, coronary VSMc, as well as cerebral VSMC show that these muscle cells all demonstrated overexpression of Notch 1 and 2 with upregulation of p53 [69].

Table 4. Notch 1 and 2 Proteins Overexpressed in Umbilical-Placental Vascular Smooth Muscle Cells obtained from Severely Preeclamptic (PRE) and Gestational Diabetic (GD) Pregnant Women at Term.

Parameter	Normal	PRE	GD
Notch 1	4.05 ± 0.62	21.6 ± 4.4	18.6 ± 2.2
Notch 2	3.04 ± 0.32	16.2 ± 3.2	14.2 ± 1.8

All values are given in ng/mL and are means $\pm$ S.E.M. (significantly diff. from controls, ANOVA, $p < 0.05$).

8. Upregulation of Notch Proteins and p53 Appear to Be Linked to Increased Risk for Cardiovascular Disease and Stroke in Previously Preeclamptic and Gestational Diabetic Pregnant Women

For many decades it has been suspected that severely preeclamptic and gestational diabetic women early in pregnancy (e.g., gravida I up to III or more) may later in years present increased risks for cardiovascular disease and/or stroke with pregnancies in later years (e.g., age 38–44) [70]. Up until very recently, this has remained a hypothesis. In the past few years, studies have appeared which give credence to this hypothesis (for recent review, see [71]). Unaware of these ongoing studies, we have been accumulating data on 24 severely preeclamptic and gestational diabetic pregnant women from three University hospitals who were prima gravida III women prior to age 25.

Not surprisingly, we found two to have acute myocardial infarctions at ages 40 and 44; two to develop myocardial ischemia at ages 38 and 40; and two to develop mild hemorrhagic strokes at ages 42 and 44. However, all successfully delivered their babies, but two had Down Syndrome and one baby was born with some heart defects. Examination of the vascular smooth muscle cells of the umbilical-placental blood vessels from these older pregnant women revealed up regulation of Notch 1 concomitant with upregulation of p53 [71]. Not surprisingly, both the Notch 1 and p53 levels were significantly higher than those we found in similar cells from the early pregnancies (i.e., at 20–24 years of age).

Although our new, preliminary data can provide some cellular numerical data for the recent suggestions, they must remain only preliminary until more incisive studies are undertaken. But, we believe these new data must be considered. Ongoing biochemical-molecular studies indicate that the VSMc and endothelial cells (EC) from the severely preeclamptic and gestational diabetic women have indications of DNA methylation and histone changes, thus suggesting that these diseased women have undergone epigenetic changes. Our labs are currently investigating these possibilities.

9. Conclusions and Future Thoughts

Although numerous hypotheses have been advanced, over more than 100 years, on the possible etiologies for pre-eclampsia-eclampsia, gestational diabetes, growth retardation, loss of fetuses and stillbirth during pregnancy, there is no agreement despite much research.

Almost 40 years ago, three of us, using umbilical-placental arteries and veins, obtained from women at term pregnancy, found that these vascular smooth muscle cells would undergo spontaneous contracture when exposed to low Mg salt solutions in-vitro. Moreover, circulating amines, peptides, and ions (e.g., Ca^{2+}), which normally caused contractions of vascular smooth muscle, would be amplified in strength when exposed to low Mg; the lower the Mg^{2+}, the greater the force of the contractions. These initial findings thus provoked impetus for us to hypothesize that Mg deficient diets, throughout pregnancy to term, could play an important role in etiology of pre-eclampsia-eclampsia, hypertension, gestational diabetes, growth retardation of the fetus, as well as loss of the fetus.

Over the past 40 years, our group and collaborators have continued investigation of our hypothesis, using umbilical-placental blood vessels, endothelial cells, as well as RBCs, and have found substantial, new evidence, which is summarized herein, to reinforce our

hypothesis that Mg deficiency can act as a genotoxin in pregnant women. Our findings of alterations in Notch proteins in VSMc and endothelial cells during severe pre-eclampsia and gestational diabetes suggest important roles for these developmental proteins during early pregnancy and late pregnancy.

Last, but not least, our ongoing clinical and animal studies bolster the idea, we have espoused previously, that water intake (e.g., from tap waters or desalinated waters) in humans should contain at least 25–40 mg Mg^{2+}/L. The latter inclusion in the diets of pregnant women should go a long way towards the prevention of cardiovascular diseases and strokes with advancing age.

Author Contributions: All co-authors of this review were active participants to these studies reviewed herein. S.M.H. and L.M.R. collected the bloods and placental and umbilical blood vessels from the pregnant women at term. Most of the biochemical and biophysical assays were carried out by B.M.A., N.C.S., G.J.S., L.M.R., A.C. and B.T.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The studies on bloods and tissues taken from pregnant women at term delivery were all approved by Internal Review Boards of the institutions involved.

Data Availability Statement: Data available on request due to restrictions (e.g., privacy, manuscripts under review, or ethical reasons). Most of the data, presented in this study, are available on request from the corresponding author. 3rd Party Data Restrictions apply to the availability of these data. Data was obtained from (third party) and are available (from the authors) with the permission of (third party). Data sharing not applicable. No new data were created or analyzed in this study.

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