Growth factors in the fetus and pre-adolescent offspring of hyperglycemic rats

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Abstract
Background: Maternal hyperglycemia influences childhood metabolic syndrome, including obesity and hyperglycemia. We tested the hypothesis that the maternal hyperglycemia influences growth factors in the fetal and pre-adolescent offspring.

Methods: Hyperglycemia was induced in pregnant rats on embryonic day (E)16 using streptozocin followed by implantation with insulin or placebo pellets at embryonic day 18 (E18). Fetuses at E20 and pre-adolescent pups at postnatal day 14 (P14) were studied: (1) normal untreated controls (CTL) at E20; (2) hyperglycemic placebo-treated (HPT) at E20; (3) hyperglycemic insulin-treated (HIT) at E20; (4) CTL at P14; and (5) HIT at P14. Fetal and pre-adolescent growth factors were determined.

Results: Biomarkers of hypoxia were elevated in the HPT group at E20. This group did not survive to term. Maternal insulin improved fetal survival despite lower fetal body weight at E20, however, at normal birth (postnatal day 0 (P0)) and at P14, body weights and blood glucose were higher than CTL. These high levels correlated with aberrant growth factors. Maternal hyperglycemia influenced glucose-6-phosphate dehydrogenase, glucagon, insulin, interleukin-10, and leptin genes.

Conclusions: The impact of maternal hyperglycemia on pre-adolescent glucose and body weight was not a consequence of maternal overnutrition. This suggests an independent link which may affect offspring metabolic health in later life.

Keywords
Fetus, glucose, growth factors, insulin, maternal hyperglycemia

Introduction
Maternal hyperglycemia is an important risk factor for developing metabolic syndrome in children. Type 1 diabetes mellitus (T1DM), and increasingly type 2 diabetes mellitus (T2DM), in pregnancy continue to cause significant maternal as well as perinatal morbidity and mortality1–4 with incidence as high as 5%–8% of all pregnancies in the United States and in Europe; and 15%–20% in some developing countries.5 Maternal glycemic status can influence fetal and postnatal gastrointestinal development and vasculature6–8.

Vascular endothelial growth factor (VEGF) is a mitogen for vascular endothelial cells derived from arteries, veins, and lymphatics, induced by hypoxia. It elicits a pronounced angiogenic response9,10 and is important for migration and proliferation of endothelial cells during vasculogenesis and angiogenesis.11 The action of VEGF is mediated by its two membrane receptors, vascular endothelial growth factor receptor-1 (VEGFR-1) and vascular endothelial growth factor receptor-2 (VEGFR-2). The soluble form of VEGFR-1 (sVEGFR-1) has been identified in amniotic fluid and in serum of pregnant women, where it binds to circulating VEGF acting as an endogenous inhibitor.12–14 VEGF is highly induced in hypoxia, and by high levels of glucose and advanced glycation end products.15–22 Hypoxia also induces erythropoietin (Epo), a 34 kDa glycoprotein hormone mainly involved in the regulation of red blood cell production...
during physiological and pathological conditions. Like VEGF, Epo is induced in utero via activation of hypoxia-inducible factor (HIF). High amniotic fluid levels of Epo were shown to be associated with fetal hypoxia and intrauterine growth restriction.

Insulin-like growth factor-1 (IGF-I) is maternally-derived and is important in the regulation of placental growth. The placenta also produces IGF-I which initiates its biological effects by binding to insulin-like growth factor-1 receptor (IGF-IR). IGF-I is important for regulating placental and fetal growth and development; trophoblast proliferation, differentiation, and invasion; as well as placental nutrient transport. Studies show that inhibition of prostaglandin (PG) E2 suppresses VEGF-induced angiogenesis, confirming a link between PGE2 and VEGF signaling. PGE2 is the main prostanoid in the intestines induced primarily by cyclooxygenase (COX)-2. At physiological levels COX-2 contributes to intestinal homeostasis and barrier maintenance. At high levels, COX-2 induces inflammation, and promotes angiogenesis, enterocyte proliferation, gut barrier failure, inflammatory bowel disease, necrotizing enterocolitis, angiogenesis, and nitric oxide (NO). NO modulates insulin sensitivity, and like PGE2, interacts with VEGF to promote endothelial cell proliferation, migration, differentiation, and interaction with the extracellular matrix.

Although it is well-known that infants of diabetic mothers are prone to obesity and type 2 diabetes in later life, no previous studies have assessed the effects of maternal hyperglycemia on fetal and pre-adolescent factors that influence growth, hypoxia, and angiogenesis. We therefore tested the hypothesis that the maternal hyperglycemia alters fetal growth factors and predisposes the pre-adolescent offspring to obesity and subsequent hyperglycemia. Our hypothesis was tested with the following objectives: (1) to examine whether maternal hyperglycemia influences growth factors in the fetal compartment, and large bowels of fetuses and pre-adolescent offspring; and (2) to determine whether maternal hyperglycemia control with insulin mitigates the responses in the fetal and adolescent offspring. Our primary outcome was maternal and offspring glucose levels, and our secondary outcomes were fetal growth parameters and biomarkers of growth, hypoxia, and angiogenesis.

Material and methods
All experiments were approved by the State University of New York, Downstate Medical Center Animal Care and Use Committee (protocol # 09-393-08). Animals were cared for according to the guidelines of the United States Department of Agriculture, and the Guide for the Care and Use of Laboratory Animals.

Experimental design
Certified infection-free Sprague Dawley timed pregnant rats were purchased from Charles River Laboratories (Wilmington, MD) at 15 days gestation. Rats were fed commercially available pelleted diet with free access to food and water. Fetuses (embryonic day 20, E20) and adolescent offspring (postnatal day 14, P14) from three groups of pregnant rats (n=4 pregnant rats/group) were studied: (1) normal untreated controls (CTL); (2) hyperglycemic with placebo treatment (HPT); and (3) hyperglycemic with insulin treatment (HIT). Baseline values glucose and ketones were determined in the urine and blood samples (tail vein) in all groups. For groups 2 and 3, hyperglycemia was induced on embryonic day (E)16 by injection of a single dose of 65 mg/kg streptozocin (Sigma-Millipore, St. Louis, MO, USA) into the tail vein. Streptozocin destroys beta cells of the pancreas and is most commonly used to induce hyperglycemia in animal models. Hyperglycemia was confirmed on E18 with daily monitoring of urinary glucose and ketosis using appropriate urine strips. Once diabetes was confirmed, the rats from groups 2 and 3 were implanted with placebo (group 2) or insulin (group 3) pellets purchased from LinShin Canada, Inc., Toronto, Canada. Upon implantation, gradual erosion of the implant starts immediately with the effects of released insulin (1 U/day) on blood glucose detected <1h post implantation. The placebo control implants were made from palmitic acid micro crystals of the same dimensions.

Implantation procedure
Once diabetes was confirmed, the rats from groups 2 and 3 were anesthetized with ketamine (40 mg/kg) and xylazine (10 mg/kg) for subcutaneous implantation of placebo (group 2) or insulin (group 3) pellets purchased from LinShin Canada, Inc., Toronto, Canada. Upon implantation, the upper abdominal area of the rats was shaved and cleansed with 10% povidone-iodine solution. Implantation was made with the use of a trocar inserted subcutaneously.

Harvesting of fetuses
Two pregnant rats from each group were anesthetized with ketamine (40 mg/kg) and xylazine (10 mg/kg) at E20. A C-section was performed and the two uterine horns were isolated under sterile conditions. The amniotic fluid was aspirated using a 19-gauge needle attached to a 3.0cc syringe, following which the fetuses and placentas were expelled and weighed. The fetal organs were removed and weighed, and the large bowels collected. Placentas and fetal membranes and large bowels were rinsed in ice-cold phosphate buffered saline (PBS) on ice, snap frozen in liquid nitrogen, and stored at −80°C until analysis. Amniotic fluid was placed in sterile Eppendorf tubes and frozen at −20°C until analysis. Samples contaminated with blood were discarded.

Pre-adolescent pups
All pups from group 2 (hyperglycemic, placebo-treated) died in utero. To determine outcomes in the adolescent...
pups, the rat pups born at term from groups 1 and 3 were left with their mothers until P14. At P14, the pups were weighed and euthanized for glucose levels and harvesting and weighing of their organs.

**Blood and urine glucose and ketone levels**

Each day post diabetes induction, the rats were placed in a decapicone and urine samples were collected. Rats usually void urine frequently when handled. Urinary glucose and ketones were measured using a dipstick. For urinary ketones, levels <20 mg/dL were considered low; levels of 30–40 mg/dL were considered moderate; and levels >80 mg/dL were considered high. Maternal blood glucose levels were determined from the tail vein, and fetal and adolescent blood glucose was determined at the time of euthanasia. Glucose levels were determined immediately upon withdrawal using an Accu-Check glucose meter.

**Food and water intake**

Food intake was measured by weighing the food before, and every 24h. For the P14 groups, this process was repeated every 5 days. Total water intake was calculated by measuring the volume of water in a graduated cylinder at the beginning, and every 24h. The amount of food consumed and the total water intake during the experiment were determined by subtracting the remainder from the amounts given. The average number of days for the E20 groups was 5 (embryonic day 16 (E16)–embryonic day 20 (E20)) and the average number of days for the P14 groups is 15 (P0–P14).

**Growth factors**

Levels of growth factors (IGF-I, VEGF, sVEGFR-1, Epo, and nitric oxide stable metabolites, nitrate and nitrite, NOx) were determined in the fetal compartment (placenta, amniotic fluid, and fetal membranes). In the large bowels of fetuses and pre-adolescent rats at P14, VEGF, sVEGFR-1, NOx, and PGE₂ were determined. All samples were analyzed on the same day. The samples were homogenized using the Fast-Prep system (MP Biomedical, Santa Ana, CA, USA), and the homogenates were centrifuged at 4°C at 10,000 rpm for 20 min. The supernatant was filtered, and the filtrate was used for the assays. A portion of the filtrate was used for total cellular protein levels. IGF-I, VEGF, sVEGFR-1, Epo, and NOx (R&D Systems, Minneapolis, MN, USA), and PGE₂ (Enzo Life Sciences, Farmingdale, NY, USA) levels were determined using commercially available assay kits. All samples were processed and assayed according to the manufacturer’s protocol and standardized using total cellular protein levels.

**Total cellular protein levels**

A 10 µL portion of the tissue homogenates was utilized for total cellular protein levels using the Bradford method (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The protein assay was carried out on the same day as the assays for growth factors.

**Real-time PCR**

Large bowels were harvested in situ, washed in ice-cold PBS on ice, snap frozen in liquid nitrogen, and stored at −80°C until analysis. Total ribonucleic acid (RNA) was extracted as previously described. To identify genes that are affected by diabetes with and without insulin treatment, real-time polymerase chain reaction (PCR) was carried out using the rat angiogenesis arrays (Qiagen, USA) using a BioRad IQ5 real-time instrument (BioRad, Hercules, CA) per manufacturer’s instructions.

**Statistical analyses**

One-way analysis of variance (ANOVA) was used to determine differences among the groups for normally-distributed data, and Kruskal-Wallis test was used for non-normally-distributed data following Bartlett’s test for equality of variances. Post hoc analysis was performed using the Tukey and Student-Newman-Keuls tests for significance. Unpaired t-test was used for comparison between the two groups, following Levene’s test for equality of variances. For non-normally distributed data, the Mann-Whitney U test was used. Significance was set at \( p < 0.05 \) and data are reported as mean ± SEM, where applicable. All analyses were two-tailed and performed using the IBM Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL).

**Results**

**Maternal**

The general maternal outcomes are presented in Table 1. In addition to blood glucose, urine glucose and ketones, daily food and water intake were monitored at baseline, implantation and at the time of fetal harvesting at E20 or spontaneous delivery at P0. Food and water intake were averaged over 5 days (from E16 to E20) for fetal groups and over 15 days (P0–P14) for pre-adolescent groups. Data showed comparable food and water intake over the 5-day period for the fetal groups. In contrast, animals in the HIT groups ate significantly more than the CTL group. Blood glucose levels were similar at baseline and diabetes was confirmed at the time of implantation (E18) in the two hyperglycemic groups. Despite insulin treatment, blood glucose levels remained elevated at the time of fetal harvesting at P14 compared to the corresponding CTL group. Urinary
Table 1. Maternal data.

<table>
<thead>
<tr>
<th></th>
<th>CTL-E20</th>
<th>HPT-E20</th>
<th>HIT-E20</th>
<th>CTL-P14</th>
<th>HIT-P14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average daily food intake (g)</td>
<td>55 ± 11</td>
<td>39 ± 8</td>
<td>76 ± 15</td>
<td>45 ± 12</td>
<td>54 ± 18</td>
</tr>
<tr>
<td>Average daily water intake (mL)</td>
<td>68 ± 20</td>
<td>117 ± 24</td>
<td>76 ± 15</td>
<td>60 ± 23</td>
<td>186 ± 45**</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>107 ± 3.2</td>
<td>133 ± 5.4</td>
<td>101 ± 5.6</td>
<td>97 ± 6.9</td>
<td>120 ± 5.0</td>
</tr>
<tr>
<td>E20/P14</td>
<td>90 ± 5.0</td>
<td>&gt;2000**</td>
<td>362 ± 43.3**</td>
<td>108 ± 10.0</td>
<td>232 ± 33.6**</td>
</tr>
<tr>
<td>Urine glucose (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>Implantation</td>
<td>Neg</td>
<td>&gt;1000**</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>E20/P14</td>
<td>Neg</td>
<td>&gt;2000**</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
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<tr>
<td>Urine ketones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>Implantation</td>
<td>Neg</td>
<td>80 ± 31.3**</td>
<td>160 ± 23.8**</td>
<td>Neg</td>
<td>160 ± 33.5**</td>
</tr>
<tr>
<td>E20/P14</td>
<td>Neg</td>
<td>160 ± 25.6**</td>
<td>Neg</td>
<td>160 ± 25.3**</td>
<td>Neg</td>
</tr>
</tbody>
</table>

Food and water intake are averaged over 5 days (E16–E20) for fetal groups, and over 15 days (P0–P14) for pre-adolescent groups. For fetal groups, data are analyzed using one-way ANOVA, and for pre-adolescent groups, data are analyzed using unpaired t-test. CTL: normal controls, untreated; HPT: hyperglycemic, placebo-treated; HIT: hyperglycemic, insulin-treated; E20: embryonic day 20; P14: postnatal day 14. *p < 0.01 versus CTL-E20. **p < 0.05. ***p < 0.01 versus CTL-P14.

Table 2. Fetal and pre-adolescent data.

<table>
<thead>
<tr>
<th></th>
<th>CTL-E20 (n = 22)</th>
<th>HPT-E20 (n = 10)</th>
<th>HIT-E20 (n = 20)</th>
<th>CTL-P14 (n = 21)</th>
<th>HIT-P14 (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placental Wt. (g)</td>
<td>0.60 ± 0.03</td>
<td>0.52 ± 0.01</td>
<td>0.51 ± 0.01**</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Body Wt. (g)</td>
<td>2.8 ± 0.06</td>
<td>2.1 ± 0.03**</td>
<td>2.5 ± 0.07**</td>
<td>27.7 ± 0.49</td>
<td>40.7 ± 0.22**</td>
</tr>
<tr>
<td>Brain Wt. (g)</td>
<td>0.14 ± 0.006</td>
<td>0.13 ± 0.002</td>
<td>0.13 ± 0.003</td>
<td>1.2 ± 0.02</td>
<td>1.2 ± 0.05</td>
</tr>
<tr>
<td>Heart Wt. (g)</td>
<td>0.18 ± 0.001</td>
<td>0.016 ± 0.0007</td>
<td>0.017 ± 0.0006</td>
<td>0.17 ± 0.006</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>Lungs Wt. (g)</td>
<td>0.11 ± 0.007</td>
<td>0.12 ± 0.006</td>
<td>0.13 ± 0.007</td>
<td>0.54 ± 0.02</td>
<td>0.6 ± 0.02#</td>
</tr>
<tr>
<td>Liver Wt. (g)</td>
<td>0.24 ± 0.01</td>
<td>0.29 ± 0.011**</td>
<td>0.31 ± 0.01**</td>
<td>0.84 ± 0.02</td>
<td>1.0 ± 0.04##</td>
</tr>
<tr>
<td>Kidneys Wt. (g)</td>
<td>0.02 ± 0.002</td>
<td>0.016 ± 0.001**</td>
<td>0.027 ± 0.0007**</td>
<td>0.4 ± 0.01</td>
<td>0.55 ± 0.22##</td>
</tr>
<tr>
<td>Glucose levels (mg/dL)</td>
<td>30.8 ± 0.06</td>
<td>468.4 ± 20.9**</td>
<td>369.1 ± 35.7**</td>
<td>145.0 ± 3.7</td>
<td>170.4 ± 3.1##</td>
</tr>
</tbody>
</table>

P14 data are compared using unpaired t-test. Placental weights were not determined for the P14 groups due to maternal consumption of placenta during delivery of the pups. CTL: normal control, untreated; HPT: hyperglycemic, placebo-treated; HIT: hyperglycemic, insulin-treated; E20: embryonic day 20; P14: postnatal day 14. *p < 0.01 versus CTL-E20. **p < 0.05. ***p < 0.01 versus CTL-P14.

Glucose and ketones remained elevated in the hyperglycemic groups at the time of implantation, but by E20 or P14, were negative in the HIT group.

Fetal and pre-adolescent data show that glucose and ketones remained elevated in the hyperglycemic groups at the time of implantation, but by E20 or P14, were negative in the HIT group. Between the adolescent groups, mean total body weight at birth was 8.3 ± 0.13 (CTL) and 9.2 ± 0.08, p < 0.01 (HIT). Total body weight, blood glucose levels, as well as lung, liver and kidney weights at P14 were higher in the HIT compared to CTL group. It was not possible to collect placental weight in these groups because the dams consumed the placentas during spontaneous delivery of the pups. Furthermore, no P14 data were collected for the HPT group because all of the pups died in utero.

Fetal growth factors

Table 2 presents growth parameters for the fetal and pre-adolescent offspring. Among the fetal groups, placental weights were lower in the hyperglycemic groups than controls. However, total body weight was lower and liver weights were higher in both hyperglycemic groups. Conversely, kidney weights were lower in the HPT and higher in the HIT group than controls. As expected, fetal blood glucose levels were higher in the hyperglycemic groups regardless of insulin treatment, although the HIT group had lower insulin levels than the HPT group. VEGF levels in the fetal compartment is presented in...
Figure 2. VEGF levels in the placenta (panel A) and fetal membranes (panel C) were lower in the hyperglycemic groups. However, in the amniotic fluid (panel B), VEGF levels were higher in the HPT group and lower in the HIT group. Similarly, sVEGFR-1 levels (Figure 3) were lower in the placenta (panel A) from hyperglycemic pregnancies. In contrast, levels were higher in the amniotic fluid (B) and fetal membranes (C) in the HPT, and lower in the amniotic fluid and fetal membranes from the HIT group compared to CTL. Epo levels in the fetal compartment is presented in Figure 4. Epo was lower in the placenta (panel A), amniotic fluid (panel B), and fetal membranes (panel C) from the HIT group, and higher in the placenta and amniotic fluid from the HPT group, than CTL. NOx levels are presented in Figure 5. NOx was higher in all fetal samples from the HPT group and lower in the placenta (panel A) and fetal membranes (panel C) from the HIT group.

Large bowel growth factors

Growth factors in the large bowel homogenates from fetal and pre-adolescent (P14) rat offspring of hyperglycemic mothers are shown in Figure 6, panels A to D. A marked elevation in VEGF was noted the HPT fetuses, and HIT pre-adolescent groups compared to their respective controls (panel A). A similar response was noted for fetal sVEGFR-1, but a reduction in sVEGFR-1 was seen in the pre-adolescent HIT group (panel B). NOx was also reduced in the pre-adolescent HIT group compared to controls (panel C), and PGE₂ levels were elevated in both fetal and pre-adolescent HIT groups (panel D).

Gene expression of growth factors in large bowels

Of the 88 genes in angiogenesis PCR arrays, we found that in the fetal large bowels, glucose-6-phosphate dehydrogenase (G-6-PD) was upregulated in the HIT versus HPT groups (24.3-fold vs 5.2-fold). In contrast, glucagon (15-fold vs 11.3-fold), insulin (28.5-fold vs 2.7-fold), interleukin (IL)-10 (25.2-fold vs 2.5-fold), leptin (17.3-fold vs 1.1-fold), and VEGF (8.0-fold vs −1.3-fold) were upregulated in the HPT group. At P14, G-6-PD (~2.0-fold), glucagon (~8.0-fold), and VEGF (~1.1-fold) were downregulated in the HIT versus CTL groups while insulin (6.0-fold), interleukin-10 (IL-10) (6.3-fold), and leptin (2.8-fold) were upregulated.
Discussion

Our findings showed that within 2 days of streptozocin treatment, the rats developed severe hyperglycemia and while insulin treatment successfully reduced urinary glucose and ketones, it was not adequate for complete reduction of maternal blood glucose to be comparable with controls. This was reflective of the increased thirst response. The data showed that maternal hyperglycemia has long-lasting effects on blood glucose, growth, and growth factors in the pre-adolescent offspring resulting in hyperglycemia and obesity, thus proving our hypothesis, and confirming previous reports. Furthermore, these data showed that uncontrolled hyperglycemia and fetal mortality was associated with hypoxia, evidenced by inductions in amniotic fluid VEGF, sVEGFR-1, NOx, and Epo, all of which were lowered with insulin treatment, subsequently resulting in improved survival. These findings suggest that the effect of maternal hyperglycemia on growth factors in the fetal compartment may be an independent risk factor for the reported obesity and metabolic consequences in the offspring.

This study found that despite insulin treatment, maternal hyperglycemia caused reductions in fetal body and kidney weights, as well as placental weights, while the liver weights were elevated. In contrast, body weight was elevated in the HIT group compared to CTL at normal birth. These findings suggest that the time from implantation of insulin to fetal harvesting at E20 was insufficient to reverse the negative effects of hyperglycemia on fetal growth. This was also represented by lower IGF-I levels which, during pregnancy, is important for normal fetal growth. Higher body weights at normal birth in the HIT group compared to CTL and was consistent with previous reports.

Alternatively, factors other than glucose may participate in this process. The liver plays an important role in control of blood glucose as insulin released from the pancreas passes via the portal vein directly into the liver. The fetal liver also controls distribution of nutrients from the placenta and regulates fetal growth. Therefore, larger fetal size is associated with higher umbilical venous liver flow. Studies show that diabetes induces fetal liver size, consistent with our findings. Our data of lower fetal body weight and increased liver size in both hyperglycemic groups suggest compromised nutrient supply from the placenta.

IGF-I plays an important role in fetal growth and development, and it is secreted by the placenta. Lower fetal

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Effects of maternal hyperglycemia with and without insulin treatment on VEGF levels in the fetal compartment (A: placenta; B: amniotic fluid; C: fetal membranes) at E20. Groups are as described in Figure 1. Data are expressed as mean ± SEM. n=8 samples/group.
IGF-I was associated with lower fetal birth weight and placental weight (Table 2). It was interesting to note that despite insulin treatment, IGF-I levels were also lower in the fetal hyperglycemic groups. This finding contrasts with previous reports of higher IGF-I in human gestational diabetes. In that report, levels were lower very early in gestation at <15 weeks. The differences may be due to differences in the length of gestation. Insulin is the primary driver of fetal IGF-I, therefore, lower fetal IGF-I could be attributable to lower fetal insulin in the hyperglycemic groups. Whether IGF-I administration improves fetal outcomes in maternal hyperglycemia remains to be determined. However, studies show that IGF-I is deficient in preterm infants at risk for many morbidities, and its replacement has potential benefits.

Our study was also consistent with previous reports which showed that diabetes increases the risk of fetal death, which may be associated with placental dysfunction and/or hypoxia. Factors associated with hypoxia and endothelial dysfunction in maternal hyperglycemia include VEGF, Epo, and NO. Placental angiogenesis affects placental blood flow and normal angiogenesis is essential for successful pregnancy outcomes. However, in hypoxia, hypoxia inducible factor (HIF) is activated which causes upregulation of a number of genes, including VEGF and Epo. Soluble VEGFR-1 (sVEGFR-1) is a splice variant of the membrane type, and is present in amniotic fluid. It is secreted by the placenta to regulate VEGF as it acts by binding VEGF and preventing its signaling to the receptor. Its overexpression has been implicated in placental abnormalities. Our study showed decreased VEGF in the placenta and fetal membranes in both hyperglycemic groups, but higher amniotic fluid levels in the HPT group, and was correlated with higher amniotic fluid and fetal membranes sVEGFR-1 levels. Higher VEGF with correspondingly higher soluble VEGFR-1 suggests not only indicate a hypoxia response, but reduced VEGF action, as sVEGFR-1 is a known VEGF “trap.” Studies also show that VEGF is induced by high levels of glucose and advanced glycation end products, and in our study, high levels persisted in the large bowels of the HPT fetuses, further confirming tissue hypoxia and vascular compromise.

Similarly, elevations in Epo were noted in the placenta and amniotic fluid, also confirming a hypoxia response, since Epo is a potent biomarker for intrauterine hypoxia. It does not cross the placenta and is fetal-derived. Studies show doubling of amniotic fluid Epo in severe fetal hypoxia, with the fetal membranes being the primary source. In our study,
Epo was reduced with insulin treatment, suggesting a relationship between insulin and Epo. Studies in streptozocin-induced hyperglycemia in rats showed that Epo treatment increased glucose utilization and reduced hyperglycemia. It is likely that insulin also has indirect effects on Epo, by reducing hypoxia and glucose. Although in normal pregnancy NO is generally increased, excessive NO produced via inducible nitric oxide synthase (NOS) is involved in inflammation, maternal hyperglycemia, and nitrosative stress in diabetes, and its principal target is the placenta. Indeed, in our study, we noted high NOx levels in the placenta, amniotic fluid, and fetal membranes but not in the large bowels. These high NO levels further support an inflammatory response to hyperglycemia and also contribute to fetal mortality in this group.

In the fetal large bowels, we also measured PGE$_2$ levels, which is predominantly formed by COX-2. Both COX-2 and PGE$_2$ have been linked to VEGF induction and angiogenesis, and is involved in inflammation and diabetes. High levels noted in our study may be reflective of low insulin and hyperglycemia. In the fetal large bowels, the genes that were mostly upregulated with maternal hyperglycemia were VEGF, glucose-6-phosphate dehydrogenase (G-6-PD), glucagon, insulin, IL-10, and leptin. G-6-PD is important for glucose metabolism and energy production. Studies show that patients with diabetes and G-6-PD deficiency have poor outcomes. Insulin induction of G-6-PD could lead to increased cellular energy and fetal survival. IL-10, an anti-inflammatory cytokine, its high levels in the HPT group suggest an inflammatory response. Leptin is produced by the adipose tissue and has a key role in energy balance and obesity control. Elevated leptin levels are associated with insulin resistance and diabetes. The high leptin levels in the HPT group may contribute to the low fetal body weight. In the pre-adolescent large bowels upregulation insulin and leptin genes correlate with hyperglycemia and obesity.

While our findings provide novel information regarding the influence of diabetes on fetal and adolescent growth and growth factors, there are some limitations. First, the subcutaneous insulin pellet did not reduce maternal glucose to comparable levels with controls, although urinary glucose or ketones were negative. Although fetal body weight was lower, birth weight was higher with insulin treatment, and overweight persisted until pre-adolescence. Second, we did not determine levels of IGFBP-1 for assessing bioavailability of free IGF-I, nor did we measure IGF-I levels at birth in the two surviving groups. We focused on IGF-I because it is a positive predictor of birthweight and postnatal growth, and because insulin

Figure 4. Effects of maternal hyperglycemia with and without insulin treatment on erythropoietin (Epo) levels in the fetal compartment (A: placenta; B: amniotic fluid; C: fetal membranes) at E20. Groups are as described in Figure 1. Data are expressed as mean ± SEM. n=8 samples/group.
Figure 5. Effects of maternal hyperglycemia with and without insulin treatment on nitric oxide stable metabolites, nitrate and nitrite (NOx) levels in the fetal compartment (A: placenta; B: amniotic fluid; C: fetal membranes) at E20. Groups are as described in Figure 1. Data are expressed as mean ± SEM. n = 8 samples/group.

Figure 6. Effects of maternal hyperglycemia with and without insulin treatment on VEGF (panel A), sVEGFR-1 (panel B), NOx (panel C), and PGE2 (panel D) levels in the large bowels from fetuses at E20 and pre-adolescent offspring at P14. All of the fetuses in the HPT group died in utero. Groups are as described in Figure 1. Data are expressed as mean ± SEM. n = 8 samples/group.
inhibits IGFBP-1 secretion.\(^6\)\(^4\) Nevertheless, our findings have clinical implications regarding the possible mechanisms associated with fetal growth, hypoxia, and fetal mortality associated with maternal hyperglycemia. Furthermore, although maternal insulin treatment improved birth weight and fetal survival, the long-term impact of maternal hyperglycemia on pre-adolescent glucose and body weight was not a consequence of maternal overnutrition. This suggests an independent link between maternal hyperglycemia and offspring obesity which may affect their metabolic health in later life.

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**Ethical statement**

All animal experiments were conducted following the national guidelines and the relevant national laws on the protection of animals.

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