The 1986 McCollum award lecture. Fuel-mediated teratogenesis during early organogenesis: the effects of increased concentrations of glucose, ketones, or somatomedin inhibitor during rat embryo culture

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ABSTRACT Whole rat embryos were explanted at head-fold, late pre-somite stage (day 9.5 of gestation) and cultured in rat sera varyingly supplemented with glucose (3, 6, 9, or 12 mg/mL), D,L-sodium beta-hydroxybutyrate (2, 4, 8, or 16 mM), or both (6 mg/mL D-glucose plus 8 mM beta-hydroxybutyrate). During 48 h culture, increasing glucose alone or beta-hydroxybutyrate alone effected growth retardation and faulty neural and extraneural organogenesis in dose-dependent fashion. Synergistic dysmorphogenic effects occurred when minimally teratogenic concentrations of glucose and beta-hydroxybutyrate were combined. Sera from diabetic animals containing somatomedin inhibitor bioactivity were also able to produce growth retardation and major developmental lesions in presence of amounts of glucose and ketones which of themselves were not teratogenic. Thus, aberrant fuels and fuel-related products can impair growth and organogenesis in early post-implantation embryo. Such fuel-mediated teratogenesis may be multifactorial and include possibilities for synergistic and additive interactions. Am J Clin Nutr 1986;44:986-95.

KEY WORDS Rat embryo culture, birth defects, diabetes, hyperglycemia, hyperketonemia, somatomedin inhibitors

Introduction

It is a well-recognized truism in clinical endocrinology that giantism, once established, never regresses. Elmer Verner McCollum conforms to that dictum. Fully 19 years after his death in 1967, he still casts a giant shadow over the nutritional world scene and his contributions continue to exert gigantic impact. Thus, it is an extraordinary honor to be able to join the distinguished list of McCollum awardees who, since 1965, have been privileged to reaffirm that in nutrition, as in clinical endocrinology, giantism once established never regresses.

McCollum's classical studies with Vitamins A and D established a very special role for them in developmental biology. In the context of developmental events, some of the actions of Vitamins A and D may be viewed as pharmacological effects. In 1980, we suggested that...
normal nutrients during intrauterine development may also act as pharmacological agents with a potential for developmental actions above and beyond their simple nutritive functions (1). That suggestion was based on our earlier proposal that pregnancy constitutes a tissue culture experience (2, 3). We pointed out that most major fuels in the mother gain access to the conceptus in a concentration-dependent fashion. Thus, the concentration of maternal fuels delimits the incubation medium in which the cells of the conceptus arise de novo and undergo organization, differentiation, and functional maturation. Because of this tissue-culture relationship, we postulated that maternal fuels may influence genetic expression of terminally differentiated, poorly replicating cells during critical phases of embryonic or fetal life and thereby impact on the progeny in permanent fashion (1–3).

We designated these potentially pharmacological actions of maternal fuel fuel-mediated teratogenesis (1). We hypothesized that the phenomenon could occur at any time during pregnancy and that the subsequent manifestations would depend on the developmental events that are going on at the time of the disturbance in maternal fuel metabolism. For example, inappropriate stimulation by maternal hyperglycemia or hyperaminoacidaemia or both during the third trimester when fetal adipocytes, muscle cells, pancreatic beta cells, and neuroendocrine axes are undergoing maximal proliferation and differentiation might result in a greater propensity to later obesity or noninsulin-dependent diabetes (anthropometric or metabolic teratogenesis); abnormal fuel mixtures during the second trimester when brain cells are being formed might find expression in altered intellectual or psychological patterns (behavioral teratogenesis); and disturbances in ambient fuels during the early part of the first trimester might compromise ongoing organogenesis (organ teratogenesis) (1). We have been studying all of these possibilities (4–8). However, today we should like to focus on the emerging evidence that fuels per se (or certain products of disturbed fuel interactions or both) may act as pharmacological modifiers of normal organogenesis in the early postimplantation embryo—that is, fuel-mediated organ teratogenesis.

Birth defects in pregnancies complicated by diabetes

Our work on dysmorphogenesis evolved as a consequence of our long-standing interest in diabetes in pregnancy (1). By 1980, perinatal mortality in the offspring of insulin-treated diabetic mothers in the major US teaching centers had declined from 33.3% in the 1920s to 6.5% during 1976–1979 (9). To some extent, these proud achievements reflected greater emphasis on team care, improved obstetrical monitoring capabilities, and development of facilities for intensive perinatal care. However, for the most part they attested to the increasing commitment to tight metabolic regulation of the mother's diabetes. Yet, in 1980, amidst the general satisfactions about the improved expectations for pregnancies complicated by diabetes, a disconcerting totally enigmatic finding surfaced. Between 1950 and 1980, the heightened incidence of birth defects in the offspring of mothers with diabetes mellitus had not been reduced to any extent. Congenital lesions continued to occur at least three times more frequently than the 2–3% background incidence in the general population (1, 10–13).

Penetrating insights soon became available. On the basis of embryological timetables and the proposition that malformations of an organ cannot occur after the organ has been completely differentiated, Mills et al (14) suggested that the common congenital malformations in infants of diabetic mothers occur between the 3rd and 6th wk of gestation. Therefore, the failure to attenuate their incidence might simply reflect the fact that most congenital anomalies are established before there is an awareness of pregnancy and before the all-out attempts at diabetes regulation that have become the standard approach to antepartum management are instituted.

The possibility that disturbances in maternal metabolism during early pregnancy might be implicated was supported by experiences with glycosylated hemoglobin. Several groups noted higher levels of glycosylated hemoglobin during the first trimester in diabetic women delivering infants with major congenital anomalies than in those with normal offspring (15–17). Since glycosylated hemoglobin is a valid index of the integrated values for blood
glucose during the preceding 6 to 8 wk, the findings implied that congenital lesions are more likely when maternal diabetes is less well-regulated during the periconceptual period. A number of animal studies strengthened the link to maternal metabolism during early gestation: Horii (18), Baker (19), Eriksson (20, 21), and their coworkers observed that early treatment with insulin diminished the frequency of birth defects in the pups of rats rendered experimentally diabetic. However, the invocation of faulty metabolism as the determinant of dysmorphogenesis still failed to identify the mediating factors. Accordingly, within the framework of fuel-mediated teratogenesis, our laboratory initiated a series of studies in early 1980 to assess whether diabetes-related fuels and fuel-derived products could directly affect organogenesis in the early post-implantation rat embryo in culture.

Effects to ambient fuels on morphogenesis during culture of rat embryos

Materials and methods

For our efforts, we employed minor modifications (6) of the whole-rodent embryo culture technique pioneered by New in Cambridge, England (22). Briefly, 250 ± 25 g virgin rats of an outbred Sprague-Dawley strain (Crl:CD®(SD) BR) obtained from Charles River Laboratories, Wilmington, MA, are mated by exposing them from 1800 h to 0800 h to males from the same strain. Pregnancy is timed from midnight preceding the morning on which vaginal plug is observed; that midnight is designated day 0. Mothers are killed by cervical dislocation on day 9.5 of gestation when embryos are at the early head-fold, late pre-somite stage and contain ~5 μg of protein. Conceptuses are excised and freed of decidua; Reichert's membrane is opened and intact embryo units (that is, embryos together with visceral yolk sac, amnion, and ectopleural cone) are explanted into culture medium, which consists of immediately centrifuged, heat-inactivated (23) serum from normal female rats supplemented with 100 units penicillin and 100 μg streptomycin per mL, and combined in the proportion of 3:1 (vol/vol) with isotonic (0.85% wt/vol) saline (to obtain final glucose concentration of 1.2 mg/mL). Control cultures with 3:1 serum:saline medium containing 1.2 mg glucose/mL are included with every experiment; dysmorphogenic lesions have not exceeded a frequency of 2% in control vessels (6). In the present series of experiments, the embryos from individual culture tube (usually 6) were pooled after visual inspection and analyzed for mean protein content (27). For acute incubations, intact embryo units were harvested from culture tubes on day 10.4 or 11.4 and incubated in 1 mL fresh 75% immediately centrifuged (23) normal rat serum that contained 1.2 mg/mL D-glucose and 3-hydroxy[3-14C]-butyrate (Amersham Corp, Arlington Heights, IL). Incubations were performed in rubber-stoppered vessels at a shaking rate of 80 cycles/min and with an atmosphere of 5% O2, 5% CO2, and 90% N2 on day 10.4 and 20% O2, 5% CO2, and 75% N2 on day 11.4. Duplicate vessels for each time contained 3 embryo units on day 10.4 and 2 embryo units on day 11.4. Collection of 14CO2 after incubation was initiated by acidifying the mediums after introducing hydroxide of hyamine into cups which were suspended in the sealed incubation vials. Comparative timetables in rat and man for embryogenesis during the above interval (28) are as follows: in the rat implantation occurs on day 6–7 of pregnancy, the neural plate is established on day 9.5, the anterior neuropore closes on day 10.5, and closure of the posterior neuropore occurs on day 11.3. In humans, establishment of the neural plate takes place on day 18–20, the anterior...
neuropore is closed on day 24–25; and closure of the posterior neuropore occurs on day 26–27. Thus, our 48-h period of rat embryo culture (ie, from day 9.5 to 11.5 of development) roughly corresponds to the interval between day 18 to 28 of human pregnancy and encompasses the period during which such devastating consequences of faulty neural tube closure as anencephaly, meningomyelocele, or spina bifida may be engendered.

Experiences with increased concentrations of D-glucose

High concentrations of D-glucose were tested in our initial efforts. In accordance with the prior observations in rat-embryo culture by Cockroft and Coppola (29) with 12 and 15 mg/mL D-glucose and the subsequent experiences with 9.9 mg/mL of exogenous D-glucose by Garnham et al (30), 6–9 mg/mL by Cockroft (31), and 7.5 mg/mL by Reece et al (32), we found that isosmotic supplementation of the culture medium with 12 mg/mL D-glucose during the 48-h incubations effected a generalized retardation of rat-embryo growth and lesions such as microencephaly, exencephaly, open neural tube, and pericardial edema (6). We documented specificity by demonstrating that the findings are not replicated with isosmotic equimolar additions of certain other hexoses, such as sorbitol, fructose, inositol, or galactose (6). Teratogenic potentialities of high glucose concentrations have also been demonstrated with cultured mouse embryos. Sadler elicited dysmorphogenic effects with increasing frequency by adding 5 mg/mL or 8 mg/mL D-glucose to the suspending rat serum during mouse-embryo culture (33). Goldman et al also obtained teratogenesis in cultured mouse embryos with 8 mg/mL D-glucose and ascribed these effects to a functional deficiency of arachidonic acid since they could be offset by supplementation with arachidonic acid (34).

To define the quantitative relationships between ambient glucose and dysmorphogenesis in greater detail, we augmented basal (control) incubation media with 3, 6, 9, or 12 mg/mL D-glucose during the 48-h incubation in our rat-embryo system. Results are summarized in Figure 1. During the period of these studies in 1980–1981, isosmotic additions of 12 mg/mL elicited a 49% incidence of minor and a 23% incidence of major lesions. By contrast isosmotic additions of 3 mg/mL D-glucose to the incubation medium did not evoke any discernible lesions during 48 h of culture, 6 mg/mL resulted in only a 2.2% incidence of minor and no major lesions, and 9 mg/mL D-glucose were required to elicit 5.1% major and 17.8% minor lesions in the cultured intact embryos from our outbred strain of Charles River Sprague-Dawley rats (Fig 1). Thus, in confirmation of earlier reports (29, 33), the dysmorphogenic potentialities of ambient glucose are clearly concentration dependent although the precise relationships may be quantitatively different in various species or in different strains from the same species.

Experiences with increased concentrations of D,L β-hydroxybutyrate

Many fuels besides glucose are disturbed in even the mildest forms of diabetes in pregnancy (35). We evaluated ketones for dysmorphogenic capabilities in vitro (36) because of the close relationship between circulating ketones and ongoing insulinization in the mother. Preliminary acute incubations with 14C-labelled β-hydroxybutyrate indicated that cultured embryo units can oxidize ketones on day 10.4 as well as 11.4 of development (36) so that ketones can subserve nutrient functions in some portions of the conceptus at both times (Fig 2).
FIG 2. Oxidation of D-3-hydroxy[3-14C]-butyrate by rat-embryo units on days 10.4 and 11.4 of development. Intact rat-embryo units were incubated acutely for 1, 2, or 3 h in fresh media which contained 1.2 mg of D-glucose/mL and 0.2 (closed circles) or 10.0 mM (open circles) D,L β-hydroxybutyrate labeled with 1 × 10^5 DPM/mL D-3-hydroxy[3-14C]-butyrate. Values for 14C02 evolved at the end of the timed incubation were expressed per µg conceptus protein.

What about the effects of ketones on embryogenesis during these intervals? As summarized in Figure 3, isosmotic additions of 2 or 4 mM buffered D,L sodium β-hydroxybutyrate during 48-h culture of rat conceptus from day 9.5 to 11.5 of development did not elicit any discernible dysmorphogenesis. However, with 8 mM, 24.5% of the embryos developed minor lesions, and the inclusion of 16 mM D,L β-hydroxybutyrate was associated with a 71% frequency of minor and 45% incidence of major lesions (36). Studies by Sheehan et al have disclosed similar dose dependencies for teratogenesis in the presence of added β-hydroxybutyrate during culture of embryos from random-bred Wistar rats (37). On the other hand, during early concurrent studies by Horton and Sadler (38), slightly higher concentrations of D,L β-hydroxybutyrate appear to have been necessary to elicit dysmorphogenesis in cultured mouse embryos. Thus, there may be some species differences in the vulnerability to ambient ketones during organogenesis. Nonetheless, in the mouse as in the rat, these effects of ketones seem to correlate with the absolute level of ketones in the culture medium.

Experiences with glucose-ketone combinations

In view of the above concentration dependencies, we examined whether the interactions between potentially dysmorphogenic fuels are additive or synergistic (36). We combined minimally teratogenic amounts of glucose (6 mg/mL) with minimally teratogenic amounts of ketones (8 mM D,L β-hydroxybutyrate). The combination produced significantly greater reductions of somite number, crown-rump length, and mean embryo-protein content than either fuel supplement alone (Table 1). In this setting of retarded growth and development, a devastating synergy of dysmorphogenesis supervened during the 48-h incubations (Table 1 and Fig 4). Whereas neither fuel effected any major lesions when tested separately at these concentrations, 66% of the embryos displayed minor lesions following 48-h culture with the two fuels in combination and major lesions were present in 27.7% (Table 1). A number of the lesions, such as microencephaly, malformed brain spheres, persistent opening of the anterior neuropore in the presence of a closed posterior neuropore, and abnormal somite development could not be explained by simple retardation of normal growth (Fig 4).

Experiences with somatomedin inhibitor

Faulty fuel interactions can also be responsible for more subtle possibilities. Phillips (38) demonstrated that factors which can impair the growth promoting properties of various insulin-like principles, ie, somatomedin inhibitors, may appear in the circulation during impaired glucose utilization (39). We at-
FIG 4. Effects of combining D-glucose and β-hydroxybutyrate during 48 h of rat-embryo culture between days 9.5 and 11.5 of development. These are four sets of embryos from one experiment on day 11.5 of development after 48-h culture. The two embryos on the extreme left were grown in normal culture medium (6). The other sets of two embryos (from left to right) were grown in normal medium supplemented with 6 mg/mL D-glucose, 8 mM D,L β-hydroxybutyrate, or both, respectively. Marked growth retardation and multiple developmental abnormalities (such as open neural tubes, microcephaly, malformed brain spheres, abnormal somites, pericardial edema, incomplete axial rotation, etc), which occur during 48-h culture in the presence of both fuels, are demonstrated by the two embryos on the extreme right.

TABLE 1
Effects of combining subteratogenic amounts of D-glucose and β-hydroxybutyrate during 48 h of rat-embryo culture between days 9.5 and 11.5 of development *

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Normal culture medium plus:</th>
<th>Embryo</th>
<th>Somite</th>
<th>Crown-rump</th>
<th>Protein mean µg/embryo</th>
<th>Minor</th>
<th>Major</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>#</td>
<td>mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>91</td>
<td>26.9 ± 0.1</td>
<td>3.59 ± 0.03</td>
<td>188.5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D,L BHOB (8 mM)</td>
<td>53</td>
<td>24.9 ± 0.2</td>
<td>3.25 ± 0.05</td>
<td>141.3</td>
<td>24.5%</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D-Glucose (6 mg/mL)</td>
<td>54</td>
<td>25.7 ± 0.2</td>
<td>3.35 ± 0.04</td>
<td>167.1</td>
<td>1.8%</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D,L BHOB (8 mM) and D-Glucose (6 mg/mL)</td>
<td>47</td>
<td>22.6 ± 0.4</td>
<td>2.82 ± 0.07</td>
<td>105.1</td>
<td>66.0%</td>
<td>27.7%</td>
<td></td>
</tr>
</tbody>
</table>

* n denotes total number of rat embryos examined. Values for somite number (#) and crown-rump length are presented as mean ± SEM. Lesions denotes frequency of major (ie, neural) and minor (ie, extraneural) abnormalities observed after 48-h culture. BHOB is sodium β-hydroxybutyrate.

tempted to evaluate whether some of the reported dysmorphogenic properties of the sera from untreated diabetic rats (40-42) are correlated with the net serum content of somatomedin-inhibitor bioactivity (43).

Sprague-Dawley rats weighing ~170 g were rendered diabetic with a single tail-vein injection of 250 mg/kg streptozotocin (provided by the National Cancer Institute). After treatment with insulin during the subsequent 2 days and
withholding of insulin for the 2 days thereafter, sera were harvested (23) from these manifestly diabetic rats. Bioassay of their sera disclosed predominant somatomedin-inhibitor bioactivity. Embryo culture was then performed for 48 h with 1) somatomedin-inhibitor containing sera from diabetic rats; 2) sera from normal rats of the same sex; and 3) sera from normal rats which had been supplemented with glucose (up to 5 mg/mL) and ketones (up to 5 mM β-hydroxybutyrate) to replicate the levels in the diabetic sera (43). As shown on Table 2, amounts of glucose and ketones which were without discernible effect on somite number, crown-rump length, or organogenesis when added to normal sera were associated with a profound growth retardation and a 67% incidence of major lesions when present in diabetic sera that contained net somatomedin inhibitor bioactivity (43). Thus, it would appear that factors such as somatomedin inhibitors related to the adequacy of fuel disposition may further enhance the dysmorphogenic potencies of aberrant fuel mixtures (43). Because more purified preparations of somatomedin inhibitors were not available for these early experiments, we could not examine for the type of dose-response relationships which Sadler and Phillips subsequently reported (44).

Moreover, the possible developmental effects resulting from the relative absence or the inappropriate presence of other bioactive factors in diabetic sera were not evaluated. It does not seem likely that the absence of insulin or the increase of glucagon contributed meaningfully to the dysmorphogenic actions of diabetic sera since treatment of streptozotocin-diabetic rats with insulin in vivo does not completely abolish these effects of their sera in vitro (41, 42), and additions of insulin or glucagon to sera during rodent embryo culture do not appear to influence morphogenesis [Lewis NJ, Gorman L, and Freinkel N, unpublished observations, and (42)].

Conclusions about fuel-mediated teratogenesis during organogenesis

From all the foregoing, what can one say in 1986 about the pathogenesis of the heightened incidence of birth defects in pregnancies complicated by diabetes mellitus? The in vitro studies with embryo culture in combination with prior clinical correlations and animal experiments (15–21) provide solid evidence for a metabolic mediation which appears to be multifactorial and directly linked to fuel metabolism. Fuels such as glucose and ketones and fuel-related principles such as somatomedin-inhibitors have been implicated so far. A number of other metabolic aberrations that occur in poorly regulated diabetes, such as the abnormal concentrations of other fuels besides glucose and ketones (eg, certain amino acids, certain fatty acids, etc) remain to be examined. On the basis of our present findings, the possibility for synergistic as well as additive interactions should be incorporated into any in vitro testing of such fuel-related risk factors. Moreover, genetic factors must also be considered insofar as several laboratories have now reported that exposure to the same aberrant fuel mixtures need not elicit similar amounts of dysmorphogenesis in different inbred strains from the same species (34, 45, 46). Indeed, as a unifying hypothesis, it may

### TABLE 2

<table>
<thead>
<tr>
<th>Serum</th>
<th>Embryo</th>
<th>Somite</th>
<th>Crown-rump</th>
<th>Protein</th>
<th>Major lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>17</td>
<td>27.4 ± 0.3</td>
<td>3.44 ± 0.06</td>
<td>169.0</td>
<td>0</td>
</tr>
<tr>
<td>Normal plus Glucose and</td>
<td>12</td>
<td>27.6 ± 0.4</td>
<td>3.55 ± 0.07</td>
<td>173.5</td>
<td>0</td>
</tr>
<tr>
<td>Ketonets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic with Somatomedin</td>
<td>18</td>
<td>18.3 ± 2.2</td>
<td>1.73 ± 0.11</td>
<td>39.0</td>
<td>66%</td>
</tr>
<tr>
<td>Inhibitors</td>
<td></td>
<td></td>
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</table>

Normal serum plus glucose and ketones denotes sera from normal rats supplemented with glucose and ketones to replicate diabetic serum. *n* denotes total number of embryos examined. Values for somite number (#) and crown-rump length are presented as mean ± SEM; however, somite number could not be estimated with confidence in 12 of the 18 embryos following 48-h culture in diabetic serum.
well be that the intra- and interspecies differences in teratogenic vulnerability may be due, at least in part, to genetically determined differences in the pathways for fuel disposition or in the patterns for responding to fuel-related insults (6, 47).

Translated into palpable clinical relevance, these recent developments may justify certain therapeutic extrapolations. In the least, they suggest that serious attempts to normalize fuel metabolism should be instituted in every woman with diabetes whenever pregnancy is contemplated. The proposition that good periconceptual regulation of diabetes may diminish the frequency of congenital lesions is being tested at present in an NIH-sponsored, multicenter prospective study (48). Some clinical evidence has already surfaced to support the putative relationship between abnormal fuel metabolism during early organogenesis and congenital lesions in pregnancies complicated by diabetes mellitus. Fuhrman (49) has reported a 0.8% incidence of anomalies in 128 infants delivered from insulin-dependent diabetic subjects with metabolic control started before conception and continued during the first critical weeks of pregnancy vis-à-vis frequencies of 7.5% in 292 subjects who began metabolic control after 8 wk gestation and 1.4% in 420 infants of nondiabetic women delivered at the same time in the same center in the German Democratic Republic.

However, the precise endpoint for such metabolic control has not yet been defined (50) and full normalization of fuel metabolism may not be necessary to eliminate birth defects as judged by some of the published experiences with glycosylated hemoglobin (16, 17). Recent studies with the intact rat (51) have confirmed the proposition (50) that even mild and relatively brief episodes of hypoglycemia can impair embryogenesis during the period in which development of the postimplantation conceptus is wholly dependent upon uninterrupted glycolysis (6). If these findings may be extrapolated to early human pregnancy, they could suggest that intensive insulin therapy, with the inevitably increased risk for hypoglycemia, may not be without danger. For the moment therefore, and until more data become available, near normalization of plasma glucose profiles and glycosylated hemoglobin levels may constitute the most judicious target for insulin therapy during the first 2 mo of pregnancy complicated by diabetes (50, 51).

Thus, as of 1986, the thesis that fuels can exert direct pharmacological effects on the development of the early post-implantation conceptus [ie, fuel-mediated organ teratogenesis (1)] has substantial experimental support. As a consequence, a knowledgeable assault on birth defects in pregnancies complicated by diabetes may be underway. Like so many developments in diabetes, the felicitous outcome appears to reflect a successful fusion of laboratory and clinical investigation. In the translation of these recent findings into standard clinical practice, we may yet witness the elimination of the last enigmatic aspect of diabetes in pregnancy.

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