Diabetes in Pregnancy

Skeletal Malformations in the Offspring of Diabetic Rats After Intermittent Withdrawal of Insulin in Early Gestation

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SUMMARY

Precise timing of the teratogenic period in diabetic pregnancy is of clinical importance since correction of the glucose intolerance during this period may protect the offspring from malformations. An experimental approach to elucidate this problem with regard to skeletal development was made in groups of pregnant streptozotocin-diabetic rats (MDI), which were treated with daily insulin injections except for a 2-day period in the first half of pregnancy. The degree of metabolic derangement was estimated by measurements of serum glucose concentrations. During the insulin-free period, the rats showed severe hyperglycemia (>20 mM) while during ongoing insulin treatment, only brief periods of hyper- or hypoglycemia were observed. Insulin treatment was withdrawn successively between gestational days 3 and 12. Control groups consisted of normal pregnant rats (N) or pregnant rats with manifest diabetes (MD) without insulin treatment. The serum glucose levels of the N animals were below 6 mM while those of the MD animals were above 25 mM throughout pregnancy. Skeletal malformations in the viable offspring were recorded on gestational day 20 after Alizarin staining of calcified ossification centers, which also allowed an estimate of skeletal development as a whole.

Untreated diabetes in the MD rats induced a high rate of fetal resorptions, a decrease in fetal weight and viability, as well as retardation of skeletal development. Intermittent insulin treatment in the MDI rats ameliorated, but did not abolish, these changes. In the MD group 9 of 48 viable fetuses showed severe malformations of either the lower jaw (micrognathia) or of the lumbosacral region (caudal dysgenesis). No skeletal malformations were observed in 151 offspring of the N group. Among the MDI animals, skeletal malformations occurred in 2 of 52 viable fetuses with interruption of maternal insulin treatment on gestational days 2–3, in 7 of 118 fetuses with interruption of insulin on days 4–5, and in 4 of 77 fetuses with interruption on days 6–7. No skeletal malformations were seen in a total of 75 MDI fetuses after interruption of insulin treatment on either days 8–9 or 10–11. However, a high rate of resorptions was also seen in these two groups.

These observations strongly suggest that in rats, the teratogenic impact of maternal diabetes on embryonic skeletal development manifests itself at an unexpectedly early embryonic age and that correction of metabolism with insulin during this critical period may protect the offspring from malformations. DIABETES 32:1141–1145, December 1983.

The persistently high rate of congenital malformations in diabetic pregnancy represents a serious clinical problem that calls for urgent therapeutic action.[1] Recent studies indicate that diabetic mothers with increased HbA_{1c} values in early pregnancy are particularly prone to give birth to malformed babies,[2,3] and it has been suggested that these malformations are likely to be induced before the seventh week of gestation.[4] Teratogenesis during a diabetic pregnancy may therefore be specifically linked to glucose intolerance in early gestation, but the precise relationship remains unknown.

Previous studies indicate that malformations due to diabetic pregnancy can be reproducibly induced in the rat and mouse and that this pathologic fetal development can be rectified by insulin treatment of the mother.[5–13] This would allow a more detailed experimental study of the teratogenic period in diabetic pregnancy with particular emphasis on the early stage, which often goes unnoticed by the pregnant diabetic woman and her doctor. The present study is an attempt to define the teratogenic period for embryonic skeletal development by intermittent withdrawal of insulin from insulin-treated pregnant and diabetic rats.

MATERIALS AND METHODS

Female virgin Sprague-Dawley rats (Anticimex AB, Sollentuna, Sweden) weighing 220–280 g were made diabetic by a single i.v. injection of streptozotocin (SZ, kindly donated

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by Dr. W. E. Dulin, The Upjohn Co., Kalamazoo, Michigan) in a dose of 45–50 mg/kg body wt. One week after the SZ injection the blood glucose concentration of each animal was measured by means of a Beckman Glucose Analyzer 2 (Beckman Instruments, Fullerton, California). Only rats with a serum glucose concentration exceeding 20 mM were included in the study. At 1–3 wk after induction of diabetes, treatment with insulin was begun in a dose of 4–6 IU of Novo Ultralente insulin (Novo A/S, Copenhagen, Denmark) given as a single daily s.c. injection. These insulin-treated rats are designated MDI rats. A further SZ-injected group of rats received no insulin treatment at all and remained manifestly diabetic throughout pregnancy (MD rats). Noninjected rats of the same age and weight served as normal controls (N rats). Mating of the animals was accomplished by caging them with normal male rats between 5 p.m. and 7 a.m. Mating commenced about 1 wk after the beginning of the insulin treatment in the MDI rats and about 2 wk after the SZ injection. Conception was confirmed by examination of vaginal smears for the presence of sperm immediately after separation from the male. The day when a positive vaginal smear was obtained was denoted as gestational day 0.

The MDI rats were randomly divided into five groups, each of which was left without insulin treatment on gestational days 2–3, 4–5, 6–7, 8–9, and 10–11, respectively. On the first day after the insulin-free period, the dose of insulin was increased threefold (12–18 IU) while on the second day, the dosage was increased twofold (8–12 IU) in order to rapidly normalize the maternal blood glucose concentration. During the insulin-free period and the subsequent 3 days, nonfasting serum glucose levels were determined daily in the pregnant animals. Additional serum glucose values were measured in all pregnant animals at least twice a week.

All pregnancies were interrupted on gestational day 20, and the litters were examined with respect to fetal and placental weights, viability, and number of resorptions. Dead fetuses were not prepared for further study, since the time of death could not be accurately ascertained. After being removed from the uterus and visually inspected, fetuses were ether anesthetized, then fixed in 70% alcohol for at least 1 wk. To detect skeletal anomalies and malformations, the calcified parts of the fetal skeleton were then stained with Alizarin Red S.[14] For this purpose, the fixed fetuses were placed in a 1% (w/v) solution of KOH for 24 h and then transferred to a solution of Alizarin Red S (30 mg/L of 1% KOH) for another 24-h period. Clearing of the tissue was performed in a mixture of benzyl alcohol:glycerol:70% ethanol (1:2:2) for an additional 24-h period. The stained and cleared specimens were stored in glycerol:70% ethanol (1:1) until the skeleton was examined and the calcified zones were enumerated and evaluated using a stereomicroscope.

The skeletal development was assessed as described in detail before.[13] Briefly, the number of ossification centers were counted in six different locations as given in Table 3. Minor deviations from the normal ossification pattern in these locations were designated as anomalies. Extra ribs and bifocal calcification centers of the sternum and vertebrae were included in this category. Gross structural defects of the skeleton, which would inflict permanent structural and functional damage, were classified as malformations. There were two major skeletal alterations that conformed to this definition, namely, micrognathia (defined as partial lack of the lower jaw) and caudal dysgenesis (defined as lack of the tail and failing ossification of the caudal vertebrae). Fetuses displaying only lack of the tail or failing caudal ossification were classified as anomalous but not malformed. The probability (P) of chance differences between groups was calculated by *t* statistics, Student's *t* test or Fisher's exact test for 2 × 2 tables.[15] The application of the various methods is given in the legends to the tables.

RESULTS

Figure 1 shows the variations in mean serum glucose concentrations of the different MDI groups during pregnancy. It is evident that the insulin treatment failed to completely normalize blood sugar levels, and in each experimental group, occasional brief excursions up to and above 10 mM glucose occurred during ongoing treatment. However, it is also evident that cessation of treatment for a 2-day period resulted in a rapid and more marked elevation of the blood sugar level that lasted for 2 successive days and could be efficiently lowered again by renewed insulin administration. In each of the insulin-treated groups some brief episodes of hypoglycemia (serum glucose levels below 3.5 mM) were also encountered throughout pregnancy. On the whole, these episodes were more frequent than the hyperglycemic ones and occurred mainly in the first half of the gestation (Figure 1). The blood sugar levels of the nontreated manifest diabetic animals remained above 25 mM throughout pregnancy, whereas those of the normal rats were below 6 mM (data not shown).

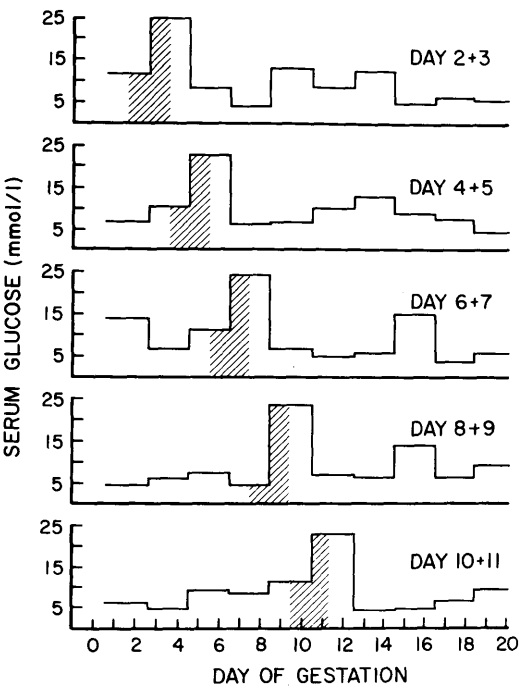

FIGURE 1. Maternal serum glucose concentrations in the MDI animals. The period of interrupted insulin administration in the various groups is given in numbers to the upper right above and marked with a stippled area within each graph. Each graph represents the mean serum glucose concentration per 2-day period throughout pregnancy. The total numbers of pregnant rats in each group are given in Tables 1–3.

TABLE 1
Number of implantations and resorptions in the normal (N), manifest diabetic (MD), and insulin-treated diabetic (MDI) groups on gestational day 20

Group	No. of litters	Implantation sites (total no.)	Resorption	
			No.	Percent
N	12	153	2	1
MDI (2–3)	5	76	24[a]	32
MDI (4–5)	10	140	22[a]	16
MDI (6–7)	7	98	21[a]	21
MDI (8–9)	6	82	41[a]	50
MDI (10–11)	3	45	10[a]	23
MD	6	74	26[a]	36

The figures in parentheses (left margin) denote the 2 gestational days when no insulin therapy was given to the MDI animals. Significances: [a]$P < 0.001$ versus N. Statistical probabilities (P) were assessed by Fisher's exact test for 2 × 2 tables.[15]

As can be seen in Table 1 the average number of detectable implantation sites per pregnant mother were as numerous in the MDI group as in the N group. There was also no difference in this respect in comparison with the MD group. The frequency of resorptions, however, was much higher in all diabetic groups irrespective of whether or not they were insulin-treated. As seen in Table 2 the weights of the viable fetuses were significantly lower than normal in all diabetic groups, but in the MDI groups the fetal weights were significantly above those of the MD animals. In contrast to the fetuses the placentas of the MD animals were heavier than those of the N animals. The placental weights of the MDI animals, again, were either intermediate between the two other groups or close to the N group.

The maturation of the fetal skeleton on gestational day 20 in the different groups is given in Table 3. A pronounced retardation of skeletal development was found in the MD group, in which only half the number of calcified ossification centers was found in comparison to the N group. Although a decreased number of ossification centers was also present in all the MDI groups, this decrease was less pronounced than in the MD group. The restoring effect of maternal insulin treatment on skeletal development in the offspring was most pronounced in those MDI animals that received no treatment either on day 2–3 or 6–7. In these diabetic animals the total number of calcified ossification centers in the offspring was significantly above that of the MD group (Table 3).

The incidence of skeletal anomalies and malformations is shown in Table 4. It is evident that anomalies were frequently encountered in the offspring of both normal and diabetic rats. A significantly higher incidence was nevertheless seen in the diabetic groups although there was a somewhat lower frequency in the offspring of MDI than of the MD animals. Skeletal malformations were absent in the N group. By contrast, gross skeletal malformations (micrognathia and caudal dysgenesis) were found in 18.8% of the fetuses in the MD group (4 fetuses with caudal dysgenesis and 5 with micrognathia). However, among the offspring of MDI animals skeletal malformations were encountered only in those groups in which the insulin treatment had been interrupted early in pregnancy, which means between days 2 and 7. In this group 8 fetuses with caudal dysgenesis and 5 with micrognathia were found. In each of these treatment groups the frequency of skeletal malformations was nevertheless significantly below that of the MD animals. In those MDI groups in which insulin treatment was abolished between gestational days 8 and 11 skeletal malformations were completely absent. These two MDI groups tended to show a different skeletal malformation rate (0 malformed out of 75 fetuses) when compared with the three other MDI groups (13 malformed out of 247 fetuses, $0.05 < P < 0.10$).

DISCUSSION

In order to define the teratogenic period for skeletal development the authors attempted to normalize the metabolism of pregnant diabetic animals by insulin administration with the notable exception of well-defined periods in the first half of gestation. This goal was, however, only partly fulfilled since episodes of metabolic derangement reflected in both hyper- and hypoglycemia occurred in all insulin-treated groups throughout pregnancy. During ongoing treatment the hyperglycemic episodes were nevertheless of both shorter duration and milder than the severe hyperglycemia seen after the complete cessation of insulin administration. In addition, these episodes occurred at approximately equal frequency in each experimental group, which means that they did not correlate with the different rates of skeletal malformations and therefore presumably lack teratogenic significance. Likewise, occasional hypoglycemic episodes were noted in all the insulin-treated groups and, for the same reason, teratogenic effects would not be ascribed to these episodes. This view is further supported by previous observations showing that a similar insulin treatment, but without free intervals, in rats made diabetic before conception was able to almost completely abolish the occurrence of skeletal malformations in the offspring.[13] This does not exclude the possibility that other malformations, which might have gone unnoticed both in the previous and in the present study, would have been caused by hypoglycemia.

The present use of Alizarin Red S for staining the calcified fetal skeleton allowed a detailed mapping not only of skeletal malformations but also of the development of the skeleton as a whole. On the other hand, malformations of other organ systems were not included in the present material and even the figures for skeletal malformations represented only those

TABLE 2
Average fetal and placental wet weight (g) in viable offspring of normal (N), manifest diabetic (MD), and insulin-treated diabetic (MDI) rat mothers

Group	No. of litters	Weight of	
		Fetus	Placenta
N	12	3.63 ± 0.11	0.55 ± 0.02
MDI (2–3)	5	2.81 ± 0.07[3a,2b]	0.54 ± 0.02[1b]
MDI (4–5)	10	2.93 ± 0.17[2a,1b]	0.64 ± 0.09
MDI (6–7)	7	2.89 ± 0.11[3a,2b]	0.54 ± 0.03[1b]
MDI (8–9)	6	2.82 ± 0.11[3a,2b]	0.59 ± 0.02
MDI (10–11)	3	2.83 ± 0.11[2a,1b]	0.59 ± 0.03
MD	6	2.31 ± 0.10[3a]	0.65 ± 0.03[2a]

The figures in parentheses (left margin) denote the 2 gestational days when no insulin therapy was given to the MDI animals. Significances: [1a/2a/3a]$P < 0.05/0.01/0.001$ versus N; [1b/2b/3b]$P < 0.05/0.01/0.001$ versus MD. Means ± SEM. Comparisons between groups were made by Student's two-tailed t test.[15]

TABLE 3
Number of calcified ossification centers per litter in the different experimental groups on gestational day 20 in sternum (ST), metacarpus (MC), metatarsus (MT), anterior and posterior proximal phalanges (APP and PPP), and caudal vertebrae (CV)

Group	No. of litters	Localization of ossification centers					
		ST	MC	MT	APP	PPP	CV	Total
N	12	5.5 ± 0.1	3.8 ± 0.1	4.0 ± 0.01	0.8 ± 0.3	0.1 ± 0.04	6.0 ± 0.1	20.0 ± 0.5
MDI (2–3)	5	3.6 ± 0.4	3.1 ± 0.04	3.8 ± 0.1	0.1 ± 0.1	0	5.7 ± 0.1	16.1 ± 0.5[3a,2b]
MDI (4–5)	10	3.6 ± 0.6	3.1 ± 0.3	3.6 ± 0.3	0.2 ± 0.1	0	5.0 ± 0.4	13.8 ± 1.7[2a]
MDI (6–7)	7	2.7 ± 0.6	3.2 ± 0.2	3.6 ± 0.1	0.2 ± 0.1	0	4.9 ± 0.3	14.8 ± 1.5[3a,1b]
MDI (8–9)	6	2.7 ± 0.9	2.9 ± 0.3	3.5 ± 0.2	0.04 ± 0.04	0	4.9 ± 0.4	13.9 ± 1.6[3a]
MDI (10–11)	3	3.1 ± 0.6	3.0 ± 0.03	3.6 ± 0.2	0	0	5.3 ± 0.1	13.7 ± 1.6[3a]
MD	6	1.8 ± 0.4	3.0 ± 0.2	2.7 ± 0.3	0.1 ± 0.1	0	3.0 ± 0.4	10.4 ± 1.1[3a]

Data are given as means ± SEM. The figures in parentheses (left margin) denote the 2 gestational days when no insulin therapy was given to the MDI animals. Significances for comparison of total number of ossification centers: [1a/2a/3a]$P < 0.05/0.01/0.001$ versus N; [1b/2b/3b]$P < 0.05/0.01/0.001$ versus MD (Student's two-tailed t test[15]).

fetuses that were still viable on day 20 of gestation. The use of a well-defined and easily detectable group of malformations as an index of the teratogenic effect of maternal diabetes would, however, offer such practical advantages that it should be preferred to a more tedious, albeit complete, investigation. It is conceivable, however, that malformations of other organ systems in offspring of diabetic rats and mice would be caused by teratogenic mechanisms different from those operating in the induction of skeletal malformations.[5–13]

An abnormal fetal development with regard to body and placental weights, number of resorptions, and occurrence of calcified ossification centers was found in the offspring of both MD and MDI rats, but, as a rule, the aberrations were less severe in the latter groups. This conforms to our previous observations in offspring of manifest diabetic rats that were treated with insulin continuously during gestation.[13] It therefore seems that in the MDI group these alterations correlated to a persisting mild glucose intolerance during insulin therapy rather than the brief, although severe, hyperglycemia following complete cessation of insulin administration. In further support of this notion there was no apparent correlation between the time of interruption of insulin treatment and the severity or type of developmental aberration. With regard to skeletal malformations a 48-h period of insulin withdrawal at any time during gestational days 3–8 in the rat was sufficient to precipitate severe skeletal malformations identical to those recorded in the MD group. Thus, among 247 embryos exposed to untreated manifest maternal diabetes during this period, 13 exhibited skeletal malformations, while among 75 embryos exposed to hyperglycemia at later periods, no skeletal malformations could be discovered. These observations strongly suggest that the teratogenic influence of diabetes in pregnancy becomes manifest at early stages of embryonic development and that insulin treatment during this critical period may prevent such developmental perturbations. It should, however, also be pointed out that the absence of gross skeletal malformations when insulin was withdrawn on days 8–9 and 10–11 does not necessarily exclude the fact that malformations would have occurred in a larger group of observations. In support of this was a persistently high rate of resorptions and retarded skeletal development among these litters. It is noteworthy in this context that rodent embryos of approximately this stage of development have shown malformations after in vitro exposure to high glucose concentrations.[16,17]

TABLE 4
Incidence of skeletal anomalies and malformations in the offspring of normal (N), manifest diabetic (MD), and insulin-treated diabetic (MDI) rats on gestational day 20

Group	No. of fetuses	Skeletal anomalies		Skeletal malformations	
		No.	Percent	No.	Percent
N	151	48	32	0	0
MDI (2–3)	52	39[3a,1b]	75	2[1b]	3.8
MDI (4–5)	118	68[3a,3b]	58	7[2a,1b]	5.9
MDI (6–7)	77	36[1a,3b]	47	4[1a,1b]	5.2
MDI (8–9)	41	26[3a,3b]	63	0[2b]	0
MDI (10–11)	34	28[3a]	82	0[2b]	0
MD	48	46[3a]	96	9[3a]	18.8

The figures in parentheses (left margin) denote the 2 gestational days when no insulin therapy was given to the MDI animals. Significances: [1a/2a/3a]$P < 0.05/0.01/0.001$ versus N; [1b/2b/3b]$P < 0.05/0.01/0.001$ versus MD. Statistical probabilities (P) were assessed by Fisher's exact test for 2 × 2 tables.[15]

In a recent study, Baker et al.[12] showed that a high rate of lumbosacral defects could be observed in the offspring of rats made diabetic with SZ on day 6 of gestation. These animals developed hyperglycemia from day 7 and may therefore correspond roughly to the MDI (6–7) animals of the present study. Our data agree with those of Baker et al. in that the teratogenic period in the diabetic rats seemed to last up to 8 days after conception. In the present study, however, it was a surprise to note that a period of hyperglycemia even before the fifth day of pregnancy was clearly teratogenic. This may suggest that the embryo is sensitive to teratogenic influences even before implantation and that these agents may thus be present in the uterine secretion. The implanting blastocyst is able to utilize glucose and it has been shown that the glucose content of the uterine secretion is dramatically increased in diabetic animals.[18] This indicates that diabetes in the mother is reflected also in the immediate environment of the nonimplanted blastocyst and therefore may already initiate an abnormal development before implantation. Provided these observations also have relevance for the human situation, they strongly underline the importance of early blood sugar control in diabetic pregnancy.

It should be kept in mind, however, that the incidence of skeletal malformations observed in each of the individual MDI groups was always less than one-third of that in the MD

group. This would mean that in these MDI groups the insulin-deficient periods were not sufficiently long to produce a maximal number of malformed fetuses. Indeed, the total incidence of skeletal malformations in the individual MDI groups approximated that observed in the MD group, suggesting that the number of malformed fetuses may increase continuously during a teratogenic period lasting altogether for 6–7 days. This finding suggests that the teratogenic impact of diabetic pregnancy on skeletal development in the offspring is a function of both the degree of glucose intolerance and the length of time during which the embryo is exposed to this condition.

Although the precise cause of malformations in the offspring of diabetic rats is still an enigma, some factors of potential relevance for the present observations may be considered. It seems unlikely that SZ itself exerted any teratogenic effects, since the drug was injected at least 2 wk before conception and previous studies have shown that it is eliminated from the body within 6 h after i.v. administration.[19] Likewise, neither insulin nor hypoglycemia would be teratogenic since malformations are virtually abolished by maternal insulin administration[8,9,12,13] and the fetal B-cell of the rat does not produce insulin before day 12 of intrauterine life.[20] By contrast, hyperglycemia per se or other metabolic disturbances related to the diabetic state would be more likely candidates for teratogenic agents. For instance, severe fluctuations in maternal blood glucose values may be teratogenic by themselves.[21] Fetal hyperglycemia may thus lead to glycosylation of proteins resulting in perturbations of organogenesis. It is of interest in this context that malformations of rodent embryos in whole-embryo culture have been induced merely by increasing the glucose concentration of the culture medium,[16,17] or by using media supplemented with serum from diabetic rats.[22,23] The latter observation indeed suggests that factors other than, or in addition to, hyperglycemia per se could be teratogenic in diabetic pregnancy. Another notable observation in both the present and in previous studies was the growth retardation of the offspring of MD and MDI rats.[12,13,24] Pedersen and Mølsted-Pedersen[25,26] recently reported that an early growth delay in the fetuses of diabetic pregnant women may predispose to congenital malformations. Although this hypothesis could not be directly verified in the present investigation, we have observed in separate studies a tendency toward lower body weights in malformed rat fetuses in comparison with their littermates (Eriksson, unpublished data). It should furthermore be noted that the growth-retarded fetuses of diabetic rats have been shown to exhibit a marked deficiency of zinc.[27] Deficiency of this trace metal is known to retard fetal growth and to induce skeletal malformations similar to those observed in the offspring of diabetic rats.[28] Although the exact levels of trace metals in the fetus during implantation and early organogenesis remain to be determined, the rat model offers opportunities for further studies of the complex etiology of the teratogenicity of diabetic pregnancy. Furthermore, the present and previous studies[2–4,8,12,13] underline the supreme importance of strict metabolic control of the diabetic mother during the initial part of pregnancy.

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