Embryonic malformations in rats, resulting from maternal diabetes: preliminary observations

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SUMMARY

Diabetes mellitus was induced in female Wistar rats by injections of either alloxan or streptozotocin, and their embryos were found to have significantly higher incidences (7.5%) of brain and heart abnormalities (non-closure of neural folds, and deformities of heart chambers) at mid-gestation than controls (2.2%). There were also increased numbers of resorptions (25% in diabetic animals: 7.2% in controls). Both drugs produced similar abnormalities. External and X-ray examination of 488 foetuses from streptozotocin-treated animals at 20 days showed eight cases of exomphalos, two cases of micrognathia with tongue protrusion, and 34 cases of incomplete sacral ossification. This last deformity occurred also in foetuses of mildly diabetic animals, and has been seen occasionally in infants of human diabetic mothers. Other evidence suggests that skeletal deformities may be due to hyperinsulinism in the foetuses of diabetic mothers. Even a mild or pre-diabetic condition may set the foetus at risk.

INTRODUCTION

Clinical data accumulated in the past 40 years have shown clearly that there is a higher incidence of congenital abnormalities in infants of women who have diabetes mellitus during pregnancy than in the general population (Pedersen, 1967). This has become more apparent since improved management of diabetic pregnancies has reduced the risks of foetal or perinatal death. There appears however to be no one malformation specific to infants of diabetic mothers, except for very rare cases of hypoplasia of the lower limbs and sacrum (Pedersen, Tygstrup, Villumsen & Pedersen, 1971; Pedersen, Pedersen & Andersen, 1974). Studies on animals have also so far failed to highlight any particular malformation associated with maternal diabetes. Horii, Watanabe and Ingalls (1966) reported a wide range of abnormalities in litters of diabetic mice. Studies on rats (Golob & Becker, 1969; Golob, Rishi, Becker & Moore, 1970; Kim, Runge, Wells & Lazarow, 1960; Lazarow, Kim & Wells, 1960; Van Assche, 1975) have reported changes in weight and in the pancreatic islet activity of foetuses, but no other abnormalities except foetal death, as a result of maternal diabetes induced by streptozotocin.

As a prelude to investigating the causes of embryonic abnormality in diabetic rodents, the present pilot study has been made on the incidence of embryonic

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abnormalities in Wistar rats, after maternal diabetes mellitus has been induced by the drugs alloxan and streptozotocin, which attack the beta-cells of the pancreas (Rerup, 1970). Some consistencies in the types of malformation occurring have been found.

**MATERIAL AND METHODS**

Female Wistar rats weighing 200–250 g were injected via the tail vein with either alloxan monohydrate (Koch-Light Laboratories) dissolved in sterile 0·9 % NaCl, or streptozotocin (Upjohn Ltd) dissolved in sterile, ice-cold 0·01 m citrate buffer, pH 4·7. Controls were injected with saline or buffer only. Blood glucose levels of injected animals were monitored each week by Dextrostix tests (Ames Co. Ltd) and hexokinase assays (Boehringer test kit). They were mated with normal males, and killed at 11, 13 or 20 days’ gestation. Early embryos were fixed in Bouin’s fluid, and 20-day foetuses were fixed in formol-saline (1 part 40 % HCHO: 3 parts 0·9 % NaCl) for 48 h, then transferred to 70 % alcohol. For histology, Bouin-fixed embryos were dehydrated in alcohols, cleared in methyl benzoate, embedded in paraffin and 8 μm sections stained with haematoxylin and eosin.

**RESULTS**

(a) *Alloxan-induced diabetes*

Seventeen females were injected with 60 mg/kg of alloxan on Day 9 (Day 0 = day on which a vaginal plug was found and sperm were present in the smear, after caging a proestrous female with a male overnight). Fifteen control females were injected with 0·9 % NaCl only. Embryos were examined 2 days later. Those

| Table 1. Frequencies of abnormalities in rat embryos at 11 days: alloxan experiments |
|-----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| No. of rats | No. of embryos alive | Dead embryos (11 days) | Resorptions | CNS abnormal | Heart abnormal | Total abnormal or dead |
| Experimentals | 17 | 120 | 4 | 30 | 7 | 2 | 13 |
| Controls | 15 | 136 | 0 | 10 | 3 | 0 | 3 |

of the alloxan-treated rats showed significantly higher incidences of abnormalities, and many more had been resorbed, than in the controls (see Table 1: \( P < 0·05, P < 0·01 \), respectively, for the differences in total numbers of abnormalities and of resorptions in experimental and controls, from a \( \chi^2 \) test). The abnormalities observed in the central nervous system were all non-closures of the neural folds, ranging from complete anencephaly to small gaps in the brain roof or at the caudal end of the spinal cord. The heart malformations were
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distortions or reductions in size of one or more chambers. All the experimental animals had blood glucose levels of 250 mg/100 ml or higher, whereas the levels in the controls lay between 90 and 130 mg/100 ml.

(b) Streptozotocin-induced diabetes

Streptozotocin is now preferred to alloxan for producing chronic diabetes mellitus in rats, since it can be given in mild doses which allow prolonged survival of the animals in a stable diabetic state (Rudas, 1969; Sybulski & Maughan, 1971). In the first of the present experiments, 20 females were injected with 40 mg/kg streptozotocin and 16 controls injected with the buffer only. The injections were given either to virgin females (group (i) of Table 2) or on the day after mating (Day 0 as defined above: group (ii) of Table 2), and embryos were examined on Day 13. As the Table shows, the results observed were essentially similar to those seen with alloxan. In the experimental animals there were far more resorptions, as well as a higher incidence of brain and heart abnormalities, than in controls. (Taking both groups together, $P < 0.001$ for the differences between experiments and controls). Blood glucose levels of $> 175$ mg/100 ml, as well as other usual symptoms such as thirst and polyuria, confirmed that the experimental animals were diabetic.

Table 2. Frequencies of abnormalities in rat embryos at 13 days: streptozotocin experiments

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>No. of embryos alive</th>
<th>Resorptions</th>
<th>CNS abnormal</th>
<th>Heart abnormal</th>
<th>Total abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimentals</td>
<td>10</td>
<td>93</td>
<td>14</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Controls</td>
<td>10</td>
<td>125</td>
<td>8</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Group (i)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimentals</td>
<td>10</td>
<td>97</td>
<td>24</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Controls</td>
<td>6</td>
<td>51</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Group (ii)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Since clinical reports have been concerned mainly with late foetuses and newborn infants, further experiments were carried out in which virgin females were injected with 40–45 mg/kg streptozotocin at least 5 days before mating, and foetuses were examined on Day 20. Animals with blood glucose levels of 250 mg/100 ml or higher were classed as ‘diabetic’, while those with values of 130–175 mg/100 ml were classed as ‘mildly diabetic’, and the results (Table 3) have been grouped accordingly. It is clear that the main effect in the diabetic animals was a resorption of embryos. In fact, 9 of the 15 animals in this group had no implantations. The resorption sites observed among the other six animals were visible as small opacities after fixing and clearing the uterus by the method of
Table 3. Frequencies of abnormalities in 20-day rat foetuses: external and X-ray observations

<table>
<thead>
<tr>
<th>Blood glucose (mg/100 ml)</th>
<th>No. of animals</th>
<th>Total foetuses</th>
<th>Resorptions</th>
<th>Everted viscera</th>
<th>Protruding tongue and short lower jaw</th>
<th>Incomplete sacral ossification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetics</td>
<td>250–130</td>
<td>15</td>
<td>43</td>
<td>38</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Mild diabetics</td>
<td>130–175</td>
<td>24</td>
<td>262</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Controls</td>
<td>90–130</td>
<td>15</td>
<td>183</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Orsini (1962) and showed only loose masses of dead embryonic cells in histological section.

Among the surviving foetuses of diabetic animals, eight were found to have their viscera completely extruded ventrally (Fig. 1) – the condition known as ‘exomphalos’. Two others had protruding tongues (Fig. 2). X-rays of all the foetuses showed that the tongue protrusion was associated with a shortening of the lower jaw (Fig. 5). The most striking abnormality seen in X-rays, however, was incomplete ossification of the sacral vertebrae: this occurred in 21 of the foetuses from diabetic females and in 13 of those from mildly diabetic females, but not in controls. The affected foetuses showed from zero to three sacral vertebrae instead of the normal four (cf. Figs. 4 and 6).

There were no significant differences in length or weight, between experimental and control foetuses.

DISCUSSION

Although these are only preliminary observations (since the morphology and histology of organs in the 20-day foetuses has not yet been studied) they have shown clearly that certain specific types of malformation result in rat embryos when diabetes mellitus is induced in the mother, either before or during pregnancy. One can almost certainly rule out the possibility that the drugs themselves, rather than the maternal diabetes, were affecting the embryos. Alloxan is destroyed within 5 min after injection into mice (Horii et al. 1966). Streptozotocin is also eliminated very rapidly from rats, in a matter of 5–6 h (Karunanayake, Hearse & Mellows, 1976), and in the present experiments it was administered several days before implantation. Further evidence that the abnormalities were due to the maternal diabetes rather than to the drugs themselves, is the similarity in the types of malformation obtained at mid-gestation with alloxan and with streptozotocin (cf. Tables 1 and 2), as well as the higher frequencies of abnormalities observed in late foetuses of fully diabetic animals, compared with those of mildly diabetic animals (Table 3).
Figures 1–6 are photographs of foetuses on Day 20, linear magnification $\times 3$.

Fig. 1. Foetus from severely diabetic female, showing complete extrusion of viscera.

Fig. 2. Foetus with protruding tongue, also from severely diabetic female.

Fig. 3. Normal foetus from control female.

Fig. 4. X-ray of normal foetus. Note the presence of four sacral vertebrae (arrowed) caudal to the hind limb girdle.

Fig. 5. X-ray of foetus with protruding tongue and shortened lower jaw (both arrowed).

Fig. 6. X-ray of foetus with incomplete ossification of sacral region, from a diabetic female. Note the absence of any vertebrae in this region (arrowed), caudal to the hind limb girdle.
Apart from abnormalities of the nervous system, anomalies of the heart were the most common type observed in these early rat embryos, as in man (White, Titus, Joslin & Hunt, 1939; Rowland, Hubbell & Nadas, 1973). Central nervous system and heart are the main organs forming from 9–11 days in the rat. Late foetuses were remarkable for the two anomalies which have not previously been described in work on either human or animal diabetics: namely the exomphalos and the micrognathia with tongue protrusion. Even more interesting at these stages when the skeleton could be observed, was the presence of so many with incomplete or absent ossification of sacral vertebrae. This condition parallels the sacral hypoplasia observed very rarely in human infants of diabetic mothers (Pedersen et al. 1971). Landauer (1945) and Duraiswami (1950) found that high doses of insulin given to chicken embryos produced absence of sacral vertebrae, as well as other skeletal malformations. Evidence so far available on the responses of the foetal rat's pancreas to maternal diabetes (Kim et al. 1960; Van Assche, 1975) indicates hyperactivity of its beta-cells, which could lead to hyperinsulinism in the foetus at the time when the skeleton is ossifying. Whether foetal hyperinsulinism or maternal hyperglycaemia are key factors in causing developmental abnormalities associated with maternal diabetes, is a problem for future investigation. It is planned in the next phase of the present work to test the effects of insulin and of serum-borne factors from diabetic females on rat embryos and their organs grown in vitro. One conclusion to be drawn from the results presented here is that even mildly diabetic animals can produce foetuses with larger numbers of skeletal abnormalities than controls (cf. Table 3). It is therefore possible that even well controlled diabetes, or undetected pre-diabetic stages, may offer serious risks to the foetus during pregnancy.

I wish to thank the British Diabetic Association for a research grant, and the Department of Biological Sciences, Exeter University, for facilities for this work. Mrs Carol Jeynes has rendered skilled technical assistance, for which I am also grateful.

REFERENCES


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(Received 20 January 1977, revised 19 April 1977)