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Effects of Glucose on Rat Embryos in Culture

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Summary. To elucidate the role of hyperglycaemia in causing fetal malformation, rat embryos have been grown in vitro from the head-fold stage for 48 h in the presence of excess glucose during different parts of the culture period. Culture of rat embryos for 10 or 21 h in 55 mmol/l exogenous glucose produced abnormalities observable after 48 h in culture. When embryos cultured for 10 or 21 h in excess glucose were observed by scanning electron microscopy at the end of the glucose treatment, abnormalities could be observed which may indicate how later malformations are formed. Thus it is possible that a relatively brief hyperglycaemic episode at a critical stage of embryogenesis may endanger the fetus of a diabetic mother.

Key words: Mammalian embryo culture, diabetes, glucose, hyperglycaemia.

Since Pederson et al. [1] observed that the incidence of congenital abnormalities in diabetic women was approximately three times that in a control group, considerable clinical evidence has accumulated which suggests that diabetic mothers are more likely to give birth to malformed infants than non-diabetic mothers. These malformations include defects of the heart and skeleton and abnormalities of the central nervous system such as anencephaly and spina bifida [2–3]. Abnormalities are also found in the offspring of laboratory rodents with natural diabetes [4], or with diabetes which has been induced by injection with alloxan or streptozotocin [5–7]. In addition, insulin replacement in alloxan- or streptozotocin-induced diabetic rodents prevents the increased incidence of abnormal embryos [8, 9], and thus fetal abnormality appears to be due to some aspect of the diabetic state.

Drug-induced diabetes in rodents may provide a useful model for the study of diabetes-associated abnormal development in man. However, with in vivo experiments it is difficult to separate the teratogenic effects of the diabetic state from any direct effects of the diabetogenic agents and to distinguish which of the metabolic disturbances associated with diabetes are responsible for fetal malformation. The culture of rodent embryos in vitro allows the effects of individual teratogens to be assessed. For this reason in vitro culture of mammalian embryos has been employed to analyse the mechanisms of diabetes-associated abnormal development. Deuchar [10] cultured rat embryos in serum from normal and diabetic rats from the late neurula stage for 24 h and found that diabetic serum had a beneficial effect on embryo growth compared with normal rat serum. However, Cockroft and Coppola [11] tested more directly the teratogenicity of glucose on rat embryos by culturing embryos from the head-fold stage in normal serum to which glucose had been added. Their results showed that 83 mmol/l glucose produced 'squirrel-like' abnormalities in which fusion of the neural folds was abnormal. Mouse embryos are apparently more susceptible to glucose, since embryos grown in serum which contained 22–33 mmol/l glucose showed abnormalities [12].

In the present study we have used the advantages of embryo culture in vitro to determine the effects of exposure to glucose for shorter periods within the 48 h culture. In addition, we have tried to gain information about the more immediate effects of glucose on the rat embryo by examining embryos which have been cultured in excess glucose at earlier developmental stages. So that the morphological abnormalities could be better assessed, embryos were examined by scanning electron microscopy.

Materials and Methods

Rat Embryo Culture

Embryos at the head-fold stage were obtained from random-bred Wistar rats at 9.5 days of gestation (timed from midnight preceding

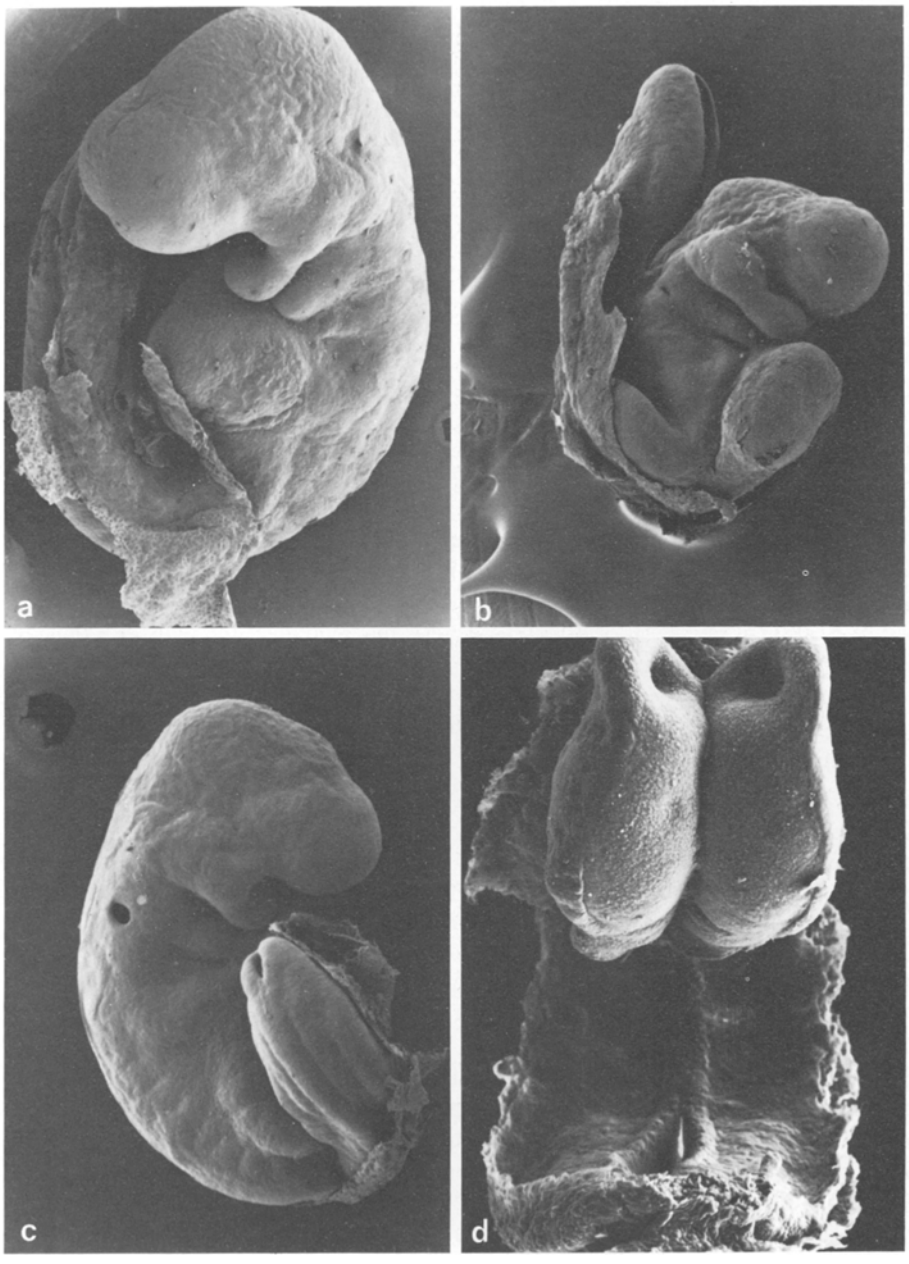

Fig. 1 a–d. Scanning electron micrographs of rat embryos explanted at 9.5 days and cultured in vitro. **a** Control embryo after 48 h culture is fully rotated and shows well developed brain vesicles (× 30). **b** After 48 h culture, the first 21 h being in 55 mmol/l exogenous glucose, the embryo has formed a 'squirrel-like' abnormality where the anterior and posterior regions of the neural tube have fused. In addition the posterior neuropore is open and the brain vesicles are not fully formed (× 34). **c** After 48 h culture, the last 27 h being in 55 mmol/l exogenous glucose, the embryo shows minor abnormalities and development is delayed; the optic pit and posterior neuropore are open and rotation is abnormal (× 33). **d** After 21 h culture, the embryo is dorsally concave and the neural folds are raised and closed in the cervical region, and the cephalic region is well developed. The optic sulci have formed (× 109)

the morning on which vaginal plugs were observed [13]). Embryos were explanted in Hank's balanced saline with sodium bicarbonate (Flow Laboratories, Irvine, UK) and cultured at 38 °C in rotating glass bottles according to the method of New et al. [14]. Each bottle contained five embryos in 6 ml of culture medium. The culture medium was pooled rat serum obtained from blood centrifuged immediately after withdrawal with added streptomycin (100 µg/ml) and penicillin (100 IU/ml, final concentrations) respectively. The serum was stored at − 20 °C and heat-inactivated at 56 °C for 30 min immediately before use. Glucose (300 mmol/l, 1·1 ml, corrected to serum osmolarity of 300 mOsm) was added to 5 ml serum to give a final concentration of 55 mmol/l exogenous glucose. Control cultures received 1.1 ml Hank's saline.

Initially the culture bottles were equilibrated with $O_2/CO_2/N_2$ (5:5:90). After 24 and 45 h the cultures were re-equilibrated with gas ratios (20:5:75) and (40:5:55) respectively [15].

In the first set of experiments embryos were cultured for 48 h but exposed to excess glucose from 0 to 21 h or 21 to 48 h. At the end of the 48-h culture period, the yolk-sac diameter and crown-rump lengths of embryos were estimated, and the embryos scored for heart beat, yolk-sac circulation and somite number, and any abnormalities were noted. The embryos were then fixed for scanning electron microscopy. As a sub-set of this experiment, embryos were incubated in excess glucose from 0 to 21 h and then examined by scanning electron microscopy. In the second set of experiments, embryos were cultured for 48 h but exposed to excess glucose from 0 to 10 h or 10 to 48 h. At the end of the 48-h culture period embryos were scored as above and fixed for scanning electron microscopy. As a sub-set of this experiment, embryos were incubated in excess glucose for 0 to 10 h and then examined by scanning electron microscopy.

Scanning Electron Microscopy

Embryos were fixed overnight in 2% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.3 [16]; they were washed in changes of 0.1 mol/l cacodylate buffer (pH 7.3) and dehydrated in a graded acetone series. The absolute acetone was replaced with liquid CO_2, and the embryos

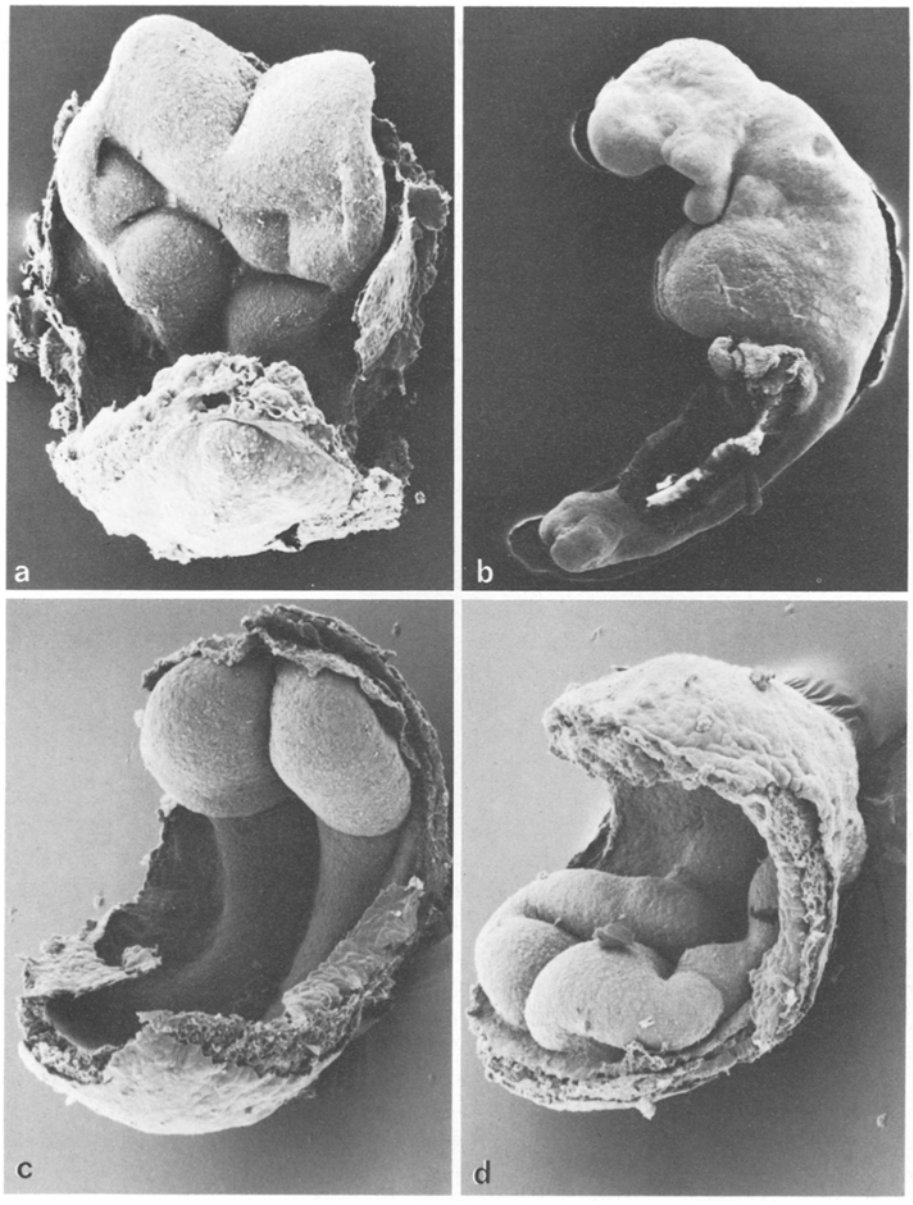

Figure 2 a–d. Scanning electron micrograph of rat embryos explanted at 9.5 days and cultured in vitro in exogenous glucose (55 mmol/l). **a** After 21 h culture the embryo is more dorsally concave than control embryos so that the tail and cephalic region are more closely apposed. The neural folds are poorly developed in the cephalic region and are abnormally grooved (× 121). **b** After 48 h culture, the last 38 h being in 55 mmol/l exogenous glucose, the embryo has failed to rotate and is delayed in development; the optic pit and posterior neuropore are open and the brain vesicles are retarded (× 44). **c** After 10 h culture, the embryo is dorsally concave and the neural folds are beginning to rise (× 118). **d** After 10 h culture, the embryo is dorsally concave and the neural folds show abnormal grooves (× 133)

were dried using the critical point method. The dried specimens were mounted on stubs. Finally the embryos were coated with a gold-palladium mixture and observed and photographed using a Phillips 501B scanning electron microscope (Pye Unicam, Cambridge, UK).

Results

By the end of the 48-h culture period control embryos had reached the 21–23 somite stage. The neural folds had fused and the major brain vesicles formed (Fig. 1 a). The heart was beating and a yolk-sac circulation had been established. Preliminary experiments in which embryos were exposed to 55 mmol/l excess glucose throughout the culture period confirmed that the major abnormality following this treatment was a 'squirrel-like' embryo in which the cephalic region of the neural folds was fused with the caudal region of the neural folds, rather than with the contralateral cephalic fold [11]. A number of other abnormalities were observed including microcephaly, failure of rotation, irregular somites and crooked neural tube.

In the first set of experiments embryos were cultured in 55 mmol/l excess glucose from 0 to 21 h, or 21 to 48 h and scored after 48 h of culture. The results show that rat embryos are more susceptible to excess glucose during the first 21 h of culture, from the head-fold stage to the 7–8 somite stage, than during the last 27 h of culture, from the 7–8 somite stage to the 23 somite stage (Table 1). Some of the embryos had formed the 'squirrel-like' abnormality (Fig. 1 b); some embryos showed more minor abnormalities (Fig. 1 c). Comparison of yolk-sac diameters showed no significant difference between the control and treated groups, and so glucose

Table 1. Effect on rat embryos of exposure to excess glucose (55 mmol/l) for first 21 h or last 27 h of 48 h culture in vitro

Rat embryos	Abnormalities		Somite no.	Yolk sac diameter (mm)	Crown-rump length (mm)
	'Squirrel'	Other			
Control ($n=30$)	1	3	23.8±0.6	3.47±0.11	2.85±0.07
Glucose 0–21 h culture ($n=33$)	14	5	24.7±0.3	3.19±0.10	2.85±0.11
Glucose 21–48 h culture ($n=28$)	3	7	23.8±0.8	3.19±0.21	2.82±0.08

Results expressed as mean±SEM

Table 2. Effect on rat embryos of exposure to excess glucose (55 mmol/l) for first 10 h or last 38 h of 48 h culture in vitro

Rat embryos	Abnormalities		Somite no.	Yolk sac diameter (mm)	Crown-rump length (mm)
	'Squirrel'	Other			
Control ($n=27$)	1	4	20.6±0.3	3.46±0.07	2.81±0.07
Glucose 0–10 h culture ($n=25$)	3	1	20.9±0.3	3.38±0.07	2.81±0.06
Glucose 10–38 h culture ($n=26$)	4	10	20.7±0.3	3.22±0.08	2.80±0.08

Results expressed as mean±SEM

appeared not to have a major effect on yolk-sac expansion. Because of the abnormal morphology of some embryos in the treated group, not all were scored for somite number; because of abnormal rotation some embryos were not scored for crown-rump length. Those that were scored showed no significant difference from the control group.

As a sub-set to this experiment embryos were exposed to 55 mmol/l excess glucose from the head-fold stage for 21 h and then scored and examined by scanning electron microscopy. Embryos cultured in normal serum for 21 h served as controls. Light microscopical observations showed that control embryos after 21 h were at the 7–8 somite stage. The embryos had not yet undergone rotation, and were still dorsally concave. Scanning electron microscopy of control embryos showed that the neural tube had closed in the mid-region, and that the cephalic folds were apposing each other. The optic sulci had formed (Fig. 1 d). Higher power observations of the neural tube revealed that the cells in the neural folds appeared rounded, whereas those on the outside of the neural folds were more flattened. This observation suggests that cells within the neural folds were under lateral compression as the folds closed and that cells on the outside of the neural folds were stretched. Scanning electron microscopy showed that of the 27 glucose-treated embryos 12 were abnormal, and revealed a variety of abnormalities. Some embryos were more dorsally concave than controls with the caudal region closely apposed to the cephalic region. In all adversely affected embryos the neural folds were abnormally grooved and the fusion of the folds appeared delayed since the cephalic folds were flatter than those of normal embryos, which were raised (Fig. 2 a). Such abnormalities might represent the precursor of the characteristic 'squirrel-like' embryo seen after 48 h culture.

In the second set of experiments embryos were cultured in 55 mmol/l excess glucose from 0 to 10 h or 10 to 48 h and scored after 48 h in culture. The results suggest that the embryos are more susceptible to excess glucose during the last 38 h of culture, between the 3–4 somite stage and the 21 somite stage, than during the first 10 h of culture, from the head-fold stage to the 2–3 somite stage, judged by the occurrence of general abnormalities (Table 2). Some embryos had formed the 'squirrel-like' abnormality, whereas other embryos showed different abnormalities such as failure to rotate and open neuropore (Fig. 2 b). The incidence of 'squirrel-like' abnormal embryos was similar in both treated groups, although the total proportion of 'squirrel-like' embryos was less than the total proportion of such embryos under conditions of exposure for the first 21 h. Comparison of yolk-sac diameters showed no significant difference between embryos treated with glucose for the first 10 h of culture and controls, but embryos treated with glucose for the last 38 h of the culture showed less yolk sac expansion than controls ($p<0.05$). Somite number and crown-rump lengths of the scorable embryos were not different between control and treated groups.

As a sub-set of this experiment embryos were exposed to 55 mmol/l excess glucose for 10 h from the head-fold stage and then scored and examined by scanning electron microscopy. Embryos cultured in normal serum for 10 h served as controls. The control embryos had 2–4 somites and were at the neural groove stage, with the cephalic folds becoming prominent (Fig. 2 c). Scanning electron microscopy showed that of the 22 embryos exposed to excess glucose, 18 were abnormal. A common abnormality was that the neural folds were assymetric, the neural groove being crooked (Fig. 2 d).

Discussion

Glucose is teratogenic to both mouse [17] and rat [11] embryos. The present results confirm this observation for rat embryos, and demonstrate that the embryos may develop abnormally when exposed to glucose for relatively brief periods. In addition, scanning electron microscopy showed that abnormalities of the neural tissue are present after only 10 h exposure to glucose. Exposure to elevated levels of glucose may be one of the factors responsible for fetal abnormalities associated with maternal diabetes in women. The susceptibility of human early embryos to raised levels of glucose is not known but diabetic women might achieve teratogenic

levels of blood glucose during peaks of hyperglycaemia which may occur without symptoms in early pregnancy. In addition, a change in the level of blood glucose at a critical stage of embryonic development might be more deleterious to the embryo than a constant elevated level [18]. Thus it is possible that a relatively short, undetected episode of hyperglycaemia resulting from diabetes or even a disturbed glucose balance during non-diabetic pregnancy at a critical stage of embryogenesis might be sufficient to produce fetal malformation.

One of the major types of abnormality was a defect in the neural tube. Successful neurulation involves a morphogenetic event, the raising and subsequent apposition of the neural folds, followed by a cellular adhesive event, the fusion of neural folds to produce the neural tube. Observations of embryos after 21 h in culture suggest that the elevation of the neural folds was delayed and that the embryos had flexed abnormally such that the caudal and cephalic regions were apposed. The 'squirrel-like' abnormality characteristic of rat embryos exposed to excess glucose might be a result of the fusion of the anterior and posterior regions of either fold. In addition, spina bifida abnormalities could be a result of a failure of the apposition of the neural folds rather than a failure of their fusion; what appears to be a failure of cell adhesion might be due to a failure or delay of a preceding morphogenetic movement. However, any explanation of the cellular mechanisms of neural tube abnormalities must encompass the observation that other agents can cause neural defects; sodium salicylate can cause 'squirrel-like' abnormalities in rat embryos [19], and open posterior neuropore can be caused by exposure of embryos to homogenates of placental and decidual tissues [20], or by raised oxygen tension [21]. In addition, hyperglycaemia may not be the only teratogenic component of diabetes. There are other potentially teratogenic disturbances, such as hypoinsulinaemia, ketosis and lactic acidosis, and it is possible that the increased occurrence of fetal abnormality associated with maternal diabetes is due not to hyperglycaemia alone, but to the synergistic action of such factors with hyperglycaemia.

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