Effects of excess glucose on mammalian post-implantation embryos

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ABSTRACT Studies on the effects of excess glucose on mammalian post-implantation embryos grown in culture are reviewed and selected examples of these studies are discussed to demonstrate the value, and the limitations, of the embryo-culture system. By use of culture techniques the precise concentrations of exogenous glucose causing morphological and biochemical changes are defined for a range of developmental stages in both rat and mouse embryos. The critical window of exposure is known and the morphological, cellular and biochemical changes associated with hyperglycemic culture are described. The effects of hyperglycemic serum are briefly compared with those of serum prepared from diabetic rats and the contribution of the high glucose concentrations to the embryopathic effects of the "diabetic" serum is assessed. The advantages and the limitations of the culture system are discussed.

KEY WORDS: *embryo culture, glucose transporters, hyperglycemia, maternal diabetes, organogenesis*

Introduction

The embryos of many mammals, including man and rodents, undergo interstitial implantation; passing through the uterine epithelium and into the uterine stroma (Finn, 1977). With the resultant decidualization the developing embryo becomes completely enveloped by maternal tissue. The bulk of the decidual tissue is supplied with a fine capillary network, however, in the implantation chamber, which immediately surrounds the conceptus, the maternal blood is extra-vascular. The precise origin, and the drainage, of the blood from the implantation chamber is unknown. Thus it is neither possible to observe embryonic development *in vivo* nor to assay the afferent and efferent blood supplies of the embryo for metabolite concentrations, etc. Prior to the development of successful methods for the culture of post-implantation mammalian embryos there were many excellent accounts of the anatomy of embryogenesis (e.g. Snell and Stevens, 1966; Theiler, 1972) but comparatively little information on mechanisms of normal or abnormal development.

The culture techniques developed by Denis New and his colleagues for the *in vitro* growth of post-implantation mammalian embryos allow frequent, or constant, monitoring of embryonic development and metabolism throughout organogenesis and provide a suitable system for the manipulation of development by, for example, the addition of a specific teratogen, such as excess glucose, to the culture medium (New, 1978; Shepard *et al.*, 1983; Cockroft, 1990).

Over the past twenty years the embryo culture techniques have been used in a range of studies of the effect of excess glucose on embryos undergoing organogenesis *in vitro*. The most common disease associated with excess glucose is diabetes mellitus. Many epidemiological studies have shown that the incidence of congenital malformations is approximately three to four times greater in infants of diabetic mothers than in the offspring of non-diabetic women (e.g. Freinkel, 1980; Eriksson, 1984; Becerra *et al.*, 1990). By examining the range of lesions observed, which include cardiac, skeletal and central nervous system abnormalities, Mills (1982) concluded that the initial damage occurred between the 6th-8th weeks of pregnancy, that is during organogenesis.

Although diabetes is characterized by high concentrations of glucose in the blood, it is also associated with a range of other metabolic perturbations including, for example, disrupted fatty acid metabolism and electrolyte imbalance. *In vivo* it is very difficult to isolate factors causing embryonic lesions because of the range of abnormal factors presenting simultaneously. The embryo-culture system, however, provides a unique opportunity for investigating effects of single factors associated with diabetes at precise stages of development. Morphological, cellular and intracellular changes in response to specifically defined environments can be monitored and, because embryos from a single mother can be exposed to a range of different treatments, the overall number of animals used can be comparatively low.

The following article concentrates on the effects of excess glucose in the culture medium and reviews selected examples of these investigations in order to demonstrate the value, and the limitations, of the culture system in such studies.

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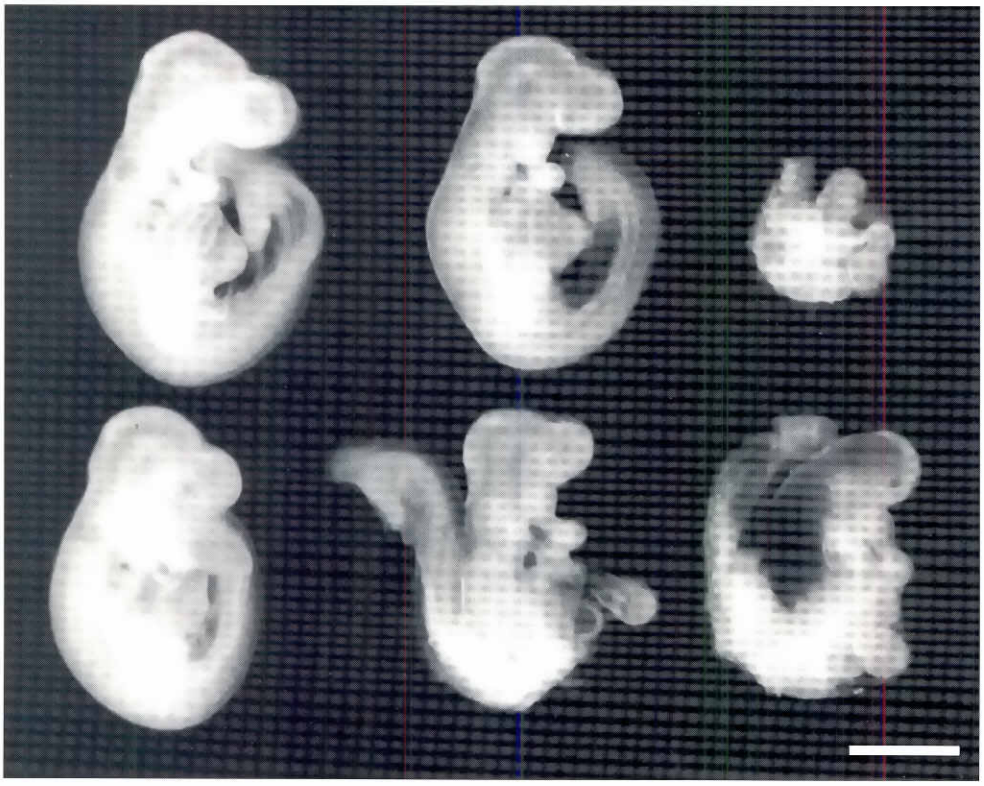

Fig. 1. 9.5d rat explants after 48 h in culture to show the gross morphological effects of excess glucose in the culture medium with or without adjustment of the osmolarity. *Embryos on the left were cultured in control medium, those in the middle in hyperglycemic medium with normal osmolarity and those on the right were exposed to excess glucose and high osmolarity. Reproduced with permission from Cockroft and Coppola (1977). Bar, 1 mm.*

Morphological effects of excess glucose

Gross morphological effects

The original study demonstrating the deleterious effects of excess glucose on embryonic development was that of Cockroft and Coppola (1977) in which embryos, explanted from CFHB rats at 9.5d (head-fold stage) were cultured in rat serum with added glucose. After 48 h of culture, at normal osmolarity (300-310 m osm/l), with glucose added to concentrations of up to 9 mg/ml (50 mM) the gross morphology of all the embryos appeared normal. However, with further increases in the glucose concentration morphological development was adversely affected. Growth and differentiation were retarded, and the incidence and severity of morphological abnormalities increased, in a dose-dependent manner. About 5% of embryos cultured with 12 mg/ml (66.7 mM) added glucose had major malformations compared to 50% of those embryos cultured with 15 mg/ml (83.3 mM) added glucose. In all the embryos with major malformations the neural tube had failed to close normally, the embryo had failed to rotate to the dorsally convex (fetal) position and the opposed edges of the anterior and posterior neural tubes had fused to one another giving the embryos a squirrel-like appearance. Excess osmolarity exacerbated the incidence of abnormalities in the presence of added glucose, but on its own was not dysmorphogenic (Fig. 1).

Reece *et al.* (1985) and Pinter *et al.* (1986b) also reported abnormal neural tube development in rat embryos exposed to excess glucose in culture. Embryos were explanted at 9.5d from CD strain rats and only those embryos with two somites were put into culture for 48 h. As observed by Cockroft and Coppola, embryos exposed to high glucose concentrations had retarded growth and differentiation and a high incidence of abnormalities. The severity of these effects was dose dependent, for example with 350 mg/dL (19.4 mM) added glucose 20% of the embryos had malformations, with 750 mg/dl (41.7 mM) added glucose 50% were abnormal and with 950 mg/dl (52.8 mM) added glucose 100% of the embryos were affected. In all the above studies, the dysmorphogenic effects of excess glucose were specific to d-glucose and were not induced by its non-metabolizable isomer, L-glucose.

Hence, by use of the embryo culture, the susceptibility of rat embryos to direct exposure to high glucose concentrations had been clearly demonstrated. However the susceptibility of the embryos in the two different laboratories (that of Cockroft and Coppola and that of Pinter and Reece) was markedly different. Cockroft and Coppola observed no malformed embryos until the added glucose concentrations were in excess of 50.0 mM, whereas Pinter and Reece reported malformed embryos following exposure to only 19.4 mM added glucose.

There were two obvious differences in the experimental protocols. In one study the embryos were explanted from CFHB rats and were put into culture at the headfold stage; in the other they were from CD rats and only embryos with two somites were put into culture. The developmental stage of embryos is known to affect their susceptibility to glucose (see below) or a wide range of other teratogens (Wilson, 1977). The strain of rat also has a marked effect on the embryonic response to a vast range of factors including maternal diabetes (Wilson, 1977; Eriksson, 1988).

Cultured mouse embryos have also been shown to be damaged by excess glucose in their culture media. As with rat embryos, the most commonly occurring induced abnormality was failure of normal closure of the neural tube. However the gross morphological damage was primarily restricted to only the anterior neural tube and added glucose concentrations as low as 5 mg/ml (27.8 mM) caused such damage (Sadler, 1980).

Cellular effects

Histological studies of the dysmorphogenesis associated with culture under hyperglycemic conditions have concentrated on two specific tissues.

The neural tube

Reece *et al.* (1985) examined neural tubes from 9.5d rat explants after 48 h in culture with normal or high ambient glucose. In control embryos the neuroepithelium consisted of three distinct

layers, the marginal, mantle and ependymal layers. Cells were rounded or flask-shaped and were separated by wide inter-cellular spaces, contained few organelles, and the nuclei, nucleoli and cytoplasm were relatively electron dense. Mitotic figures were observed only in the ependymal layer and were in direct contact with the lumen of the neural tube.

Following culture in the presence of 750 mg/dL (41.7 mM) added glucose the neuroepithelium had two distinct appearances. In those regions of the tube which had closed normally the neuroepithelium resembled that of the control embryos. However in areas where the neural tube had failed to close, marked differences were observed; there was no clear division between the ependymal, mantle and marginal layers; cells were closely packed and contained mitochondria and structures resembling microtubules. Mitotic figures were present, but these were fewer in number than in the controls and not all were in contact with the lumen of the neural tube. Examination of *in vivo* controls revealed that the cellular appearance of areas of open neural tubes in those embryos exposed to excess glucose resembled that of neural tubes of 14.d embryos. It was thus suggested that excess glucose inhibits the normal cell division and proliferation typical of earlier stages of development and induces premature maturation of the neural tube.

Sadler (1980) in his examination of cultured mouse embryos observed less derangement of the neural tissues following exposure to high ambient glucose. Although the anterior neural tubes failed to close following culture with excess glucose, the neuroepithelium appeared healthy except for small quantities of pyknotic debris.

Marked differences were thus observed in the extent of cellular and tissue disruption in the neural tubes of rat versus mouse embryos following culture under hyperglycemic conditions. These differences may reflect the duration of the exposure to excess glucose; the mouse embryos were examined after 24 h of culture, whereas the rat embryos were cultured for 48 h, or they may be attributable to species differences.

Further studies on the morphological responses of the cells of the neural tube of 10.5d rat embryos to ambient glucose concentration have investigated glucose-dependent changes in microvilli of the neural plate prior to closure of the neural tube. It was observed that immediately after explantation or following exposure to glucose-free Hanks balanced saline solution (HBSS) the microvilli of the neural plate were relatively short but after exposure to HBSS with glucose the microvilli lengthened by about 10-fold. It was suggested that cells of the neural plate may modulate glucose uptake by changing the length of the microvilli and hence regulating the length of the diffusion barrier between the extra- and intracellular compartments (Shepard *et al.*, 1993; Shepard and Park, 1994).

TABLE 1

EFFECTS OF EXPOSURE TO HIGH GLUCOSE CONCENTRATIONS AT DIFFERENT STAGES OF RAT EMBRYO DEVELOPMENT

Experiment	*Equivalent age at start of exposure (days)	Duration of exposure (h)	Number of malformed embryos (total number)	
1	not exposed	0 h (control culture)	0	(18)
1	9.5	48	9	(18)
1	9.5	24	11	(18)
1	10.5	24	0	(18)
2	not exposed	0 h (control culture)	0	(12)
2	9.5	48	7	(12)
2	9.5	12	6	(12)
2	10.0	12	0	(12)

*All embryos were explanted at 9.5d at which stage some were put directly into hyperglycemic medium whilst others were cultured in control medium for 12 or 24 h prior to transfer to hyperglycemic medium. The "equivalent" age is the age at explantation plus the number of hours in culture under normal conditions prior to exposure to high added glucose (66.7 mM). Data from Cockroft (1984).

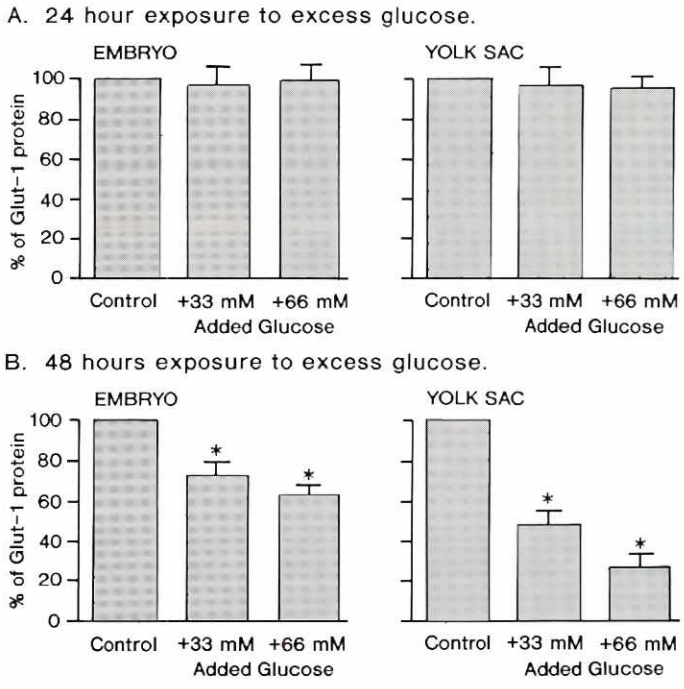

Fig. 2. Summary of the expression of GLUT 1 protein in the embryo and yolk sac after exposure of 9.5d rat explants to excess glucose in culture for 24 or 48 h. *Adapted from Takao et al. (1993).*

Visceral yolk sac

Pinter *et al.* (1986b) also examined the visceral yolk sacs of 9.5d rat explants following 48 h of culture under normal or hyperglycemic conditions. The visceral yolk sacs of control embryos appeared thin and translucent and had well developed capillary networks containing circulating embryonic blood. By contrast, yolk sacs of explants exposed to 750 mg/dL (41.7 mM) glucose concentration were smaller, thicker and more opaque. Furthermore, the capillary networks were poorly developed. Ultrastructural studies of the visceral endoderm cells suggested that the absorptive function of these cells was severely disrupted by excess glucose; the microvillous surface was reduced; there were fewer vesicles on the apical surface of the cells; fewer mitochondria and a sparser distribution of endoplasmic reticulum were observed compared to the yolk sacs of control embryos.

(Similar structural damage to that described above has also been observed in the yolk sacs of rat embryos cultured in serum prepared from rats with streptozotocin-induced diabetes [Rashbass

TABLE 2

TISSUE METABOLITES IN 9.5d EXPLANTS AFTER 24 h OF CULTURE IN NORMAL OR HIGH GLUCOSE CONCENTRATIONS

Culture conditions	Metabolites (n mol/mg wet weight)±S.E.			
	Glycogen	Glucose	Glucose 6-phosphate	Fructose 6-phosphate
Control glucose	1.35 (7) ±0.159	1.86 (11) ±0.175	0.03 (10) ±0.001	0.056 (10) ±0.003
+ 50 mM glucose	*5.17 (7) ±0.362	*22.7 (11) ±0.820	*0.06 (10) ±0.003	*0.123 (10) ±0.003

*Significantly different from the values for control embryos $p<0.001$ (Student's *t* test). Each sample was prepared from the homogenate of 6 explants from one culture bottle. The number of samples assayed is shown in parentheses. Data from Ellington, Passoneau and Freinkel (unpublished).

and Ellington, 1988] and also in the yolk sacs of embryos from pregnant rats maintained on a high sucrose diet or with streptozotocin-induced diabetes [Zusman and Ornoy, 1984]).

Since the visceral yolk sac is known to be the prime route for the uptake of nutrients during organogenesis (Padykula *et al.*, 1966; Payne and Deuchar, 1972; Freeman *et al.*, 1981). Pinter *et al.* suggested that excess glucose in the culture medium may have a primary effect on the visceral yolk sac with the resultant embryopathy caused by lack of nourishment. However, results from other embryo-culture experiments in which yolk sac function has been compromised indicate that yolk sac damage is not the prime cause of neural tube abnormalities. For example, New and Brent (1972) and Freeman *et al.* (1982) cultured rat embryos in the presence of yolk sac antibody, which severely compromised yolk sac function, and observed a dose-related retardation of growth and differentiation, but no neural tube defects or other consistent morphological abnormalities. Similarly, lack of glucose in the culture medium for even brief periods results in small, poorly differentiated embryos, but not in open neural tubes (Ellington, 1987a; Akazawa *et al.*, 1989).

Developmental stages susceptible to damage by excess glucose

Cockroft (1984) used embryo-culture techniques to investigate the sensitivity to excess glucose of rat embryos of different ages. In an elegant series of experiments, he initially explanted embryos at 9.5d and cultured them for 48 h in medium with either normal glucose or 15 mg/ml (83 mM) added glucose. By varying the duration of the exposure to the excess glucose from 48 to 12 h and the age of the embryos at the start of the exposure, it was determined that the maximum sensitivity of 9.5d explants to excess glucose occurred between 9.5-10.0d (Table 1).

In additional experiments, embryos were explanted at 9.0d or 8 days+20 h and were exposed to excess glucose — 12 mg/ml (66.7 mM) for 8 hour periods. A high proportion of the explants exposed to high glucose concentrations for the first or the second 8 hour period developed malformations, whereas those exposed later were less affected or unaffected. A dose-response experiment also revealed that the younger embryos (9d explants) were sensitive to added glucose concentrations as low as 6-9 mg/ml (33.3-50 mM). Embryo culture experiments were similarly used to demonstrate that the earlier stages of development of mouse embryos were also more sensitive to excess glucose than later stages (Sadler, 1980). Hence, by use of embryo-culture techniques, a very precise window of susceptibility to excess glucose, or any other teratogen, can be determined.

Molecular mechanisms of glucose uptake

Ellington (1987b) used embryo-culture techniques to study glucose uptake by 9.5d rat explants and concluded that, at physiological glucose concentrations, glucose uptake was largely carrier mediated. Glucose transporters fall into two main classes, facilitative (uniports) and concentrative (symports). Studies of uptake of deoxy-D-glucose by isolated rat visceral yolk sacs cultured with or without specific transport inhibitors demonstrated that hexose transports in the yolk sac are facilitative (Koszalka *et al.*, 1988). So far, seven distinct facilitative glucose transporters, GLUT 1-7, have been identified, each with tissue specific distributions and distinct kinetic properties (Mueckler, 1994). In rodent post-implantation embryos, GLUT 1, 2 and 3 are expressed with specific spatial and temporal distributions. No evidence has been found for the presence of GLUT 4, the insulin-dependent transporter either in mouse embryos from pre-implantation to 7.5d or in 10.5 or 12.5d rat embryos (Hogan *et al.*, 1991; Smith and Gridley, 1992; Takao *et al.*, 1993).

In preliminary experiments Dorman and Ellington (unpublished) used embryo culture techniques to look at the effects of specific blockers for GLUT transporters (phloretin and cytochalasin B) and for the glucose/sodium concentrative transporter (phlorizin). Each of the blockers severely disrupted normal development indicating that not only facilitative but also concentrative glucose transporters may be functioning in post-implantation rat embryos.

Glucose uptake and metabolism at high glucose concentrations

Molecular mechanisms

Takao *et al.* (1993) used embryo culture methods to assess the effects of high ambient glucose concentrations on the expression of the glucose transporter genes and protein. 9.5d rat explants were cultured in normal serum (diluted 3:1 with saline to obtain a final glucose concentration of 6.6 mM) or in serum with the isosmotic addition of 33.3 or 66.6 mM glucose for 24 or 48 h. Expression of GLUT 1 protein was unaffected by 24 h exposure to excess glucose following explantation at 9.5d. However, in explants subjected to 48 h of exposure to high ambient glucose, there was significant downregulation of GLUT 1 protein expression in both the yolk sac and embryo (Fig. 2). The lack of downregulation of GLUT 1 transporter protein during the first 24 h may explain the vulnerability of 9.5d explants to excess glucose during earlier stages of culture.

Glycolytic flux

During early organogenesis embryos grow extremely rapidly, for example rat embryos increase their protein content by about 40 fold between 10.5-12.5d (New, 1973). During this stage of development embryos derive almost all their energy from glycolysis (Tanimura and Shepard, 1970). With such rapid growth, and the dependence on glucose as an energy source, embryos are vulner-

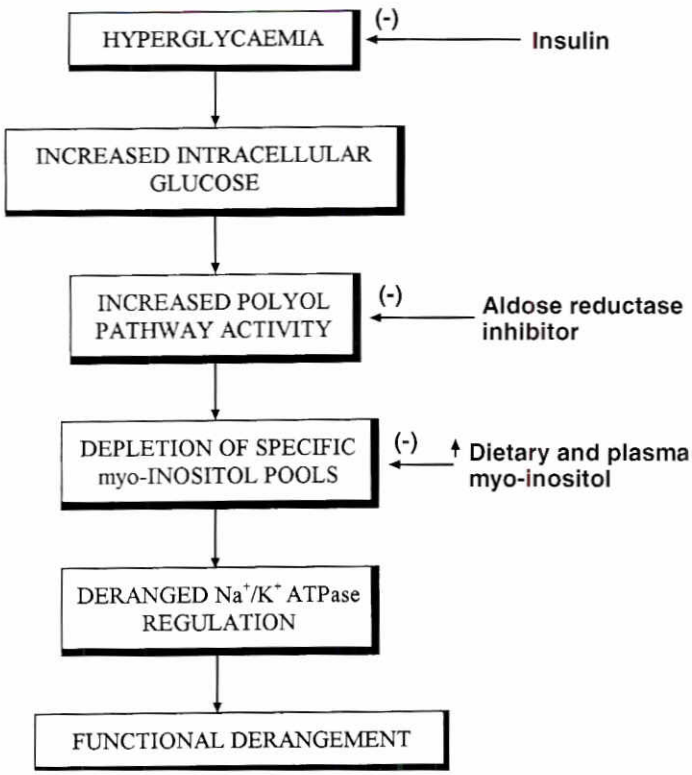

Fig. 3. Summary of the metabolic derangement suggested by Winegrad to be a common cause of diabetic damage. *Adapted from Winegrad (1987).*

able to any factor disrupting the normal supply, or metabolism, of glucose — "fuel-mediated teratogenesis" (Freinkel *et al.*, 1984; Freinkel, 1988). Accordingly, Ellington, Passoneau and Freinkel (unpublished) assayed metabolite accumulation in 9.5d explants after 24 h of culture in either normal or excess glucose (50 mM). There were significant increases in tissue glucose, glycogen, glucose 6-phosphate and fructose 6-phosphate following exposure to excess glucose (Table 2). Hence there is strong evidence that embryos cultured in the presence of excess glucose are more likely to develop morphological abnormalities than embryos cultured in control medium and that the excess ambient glucose results in excess intracellular glucose in those embryos. Further studies into the causes of the dysmorphogenic action of excess glucose on embryos were based on evidence accumulated in the study of diabetes.

There have been numerous studies investigating the causal factors of tissue damage in patients (or experimental animal models) with long term diabetes. In a wide range of tissues the same biochemical abnormalities have been observed; that of high intracellular sorbitol and low intra-cellular myo-inositol; Winegrad (1987) proposed that much of the observed diabetic damage could be attributed to a common cause, that of elevated sorbitol and depleted intracellular myo-inositol (Fig. 3).

The elevated blood glucose associated with diabetes is thought to lead to increased intracellular glucose with, probably, saturation of the hexokinase enzyme, increased activity of the (low affinity) aldose reductase enzyme and hence increased sorbitol synthesis. The sorbitol molecule is relatively impermeable in the cell membrane and remains in the cell causing osmotic swelling. (Such elevated intracellular sorbitol has been observed in a range of tissues from diabetic patients including the lens of the eyes where the osmotic swelling is associated with cataract formation) (Tomlinson *et al.*, 1993).

Abnormal levels of myo-inositol have also been observed in diabetic tissue. Myo-inositol depletion is associated with two factors. Myo-inositol is taken up against its concentration gradient by carrier mediated transport; this uptake is competitively inhibited by glucose and hence high extra-cellular glucose reduces the uptake of myo-inositol into cells. Also, through a less well understood mechanism, high intracellular sorbitol causes depletion of myo-inositol (Olgemöller *et al.*, 1993).

Low intra-cellular myo-inositol affects two separate pathways. It impairs sodium-potassium ATPase activity in the cell and also reduces the synthesis of arachidonic acid and prostaglandins (Greene *et al.*, 1988). Hence further studies of the effects of excess glucose on embryonic development initially concentrated on sorbitol and myo-inositol and their associated pathways.

Intracellular sorbitol

In vivo studies have shown that pregnancies in rats (Sprague-Dawley substrain) made diabetic by streptozotocin injection resulted in between 10-20% of offspring with skeletal malformations. The mean blood glucose of these diabetic rats was 30 mM±7 (n=17) and the sorbitol levels in 11.5d embryos were between 3-5 times those found in the embryos of control rats. A daily dose of statil (an aldose reductase inhibitor) administered to the diabetic mothers from 0d until the termination of pregnancy significantly reduced the levels of sorbitol in embryos, but had little effect on the incidence of malformations. It was therefore concluded that sorbitol accumulation was not a major factor in the dysmorphogenesis observed in diabetic pregnancies (Eriksson *et al.*, 1986).

TABLE 3

SORBITOL AND MYO-INOSITOL CONTENT OF 9.5d EXPLANTS AFTER 48 h OF CULTURE IN NORMAL OR HIGH GLUCOSE CONCENTRATIONS

Culture conditions	Content per intact concepts			
	Protein (μg)	DNA (μg)	Sorbitol (ng)	Myo-inositol (ng)
Controls (9)	315±23	28.7±1.9	144±32	481±31
+ Added glucose (mM)				
16.7	307±23	29.1±2.3	***565±28	**327±21
66.7	*226±23	*21.2±2.9	***880±14	***122±11
+ Added glucose (mM) + Sorbinil (10 μM)				
16.7	264±15	25.0±1.0	†233±89	320±28
66.7	209±15	19.4±2.4	†275±102	150±15

Embryos cultured with added glucose alone were compared with control embryos. Embryos cultured with added glucose and Sorbinil were compared with those cultured with just added glucose. Significantly different from the values for control embryos: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Significantly different from the values for embryos cultured in high glucose without Sorbinil: † $p<0.05$. Data from Hod *et al.* (1986).

TABLE 4

SUMMARY OF THE DEVELOPMENT OF 9.5d EXPLANTS AFTER 48 H OF CULTURE IN CONTROL, HYPERGLYCEMIC OR "DIABETIC" SERUM

	Serum		
	Control	Hyperglycemic	Diabetic
Initial serum glucose concentration (mM±SE)	8.5±0.75	29.7±0.85	32.0±1.1
Osmolarity of serum (mOsmol)±SE	276.5±0.7	295.5±0.7	294.8±1.1
No. of bottles	6	6	8
No. of embryos	24	24	32
Somite No. ±SE	25.8±0.4	25.8±0.2	24.5±0.2*‡
Protein content (μg)±SE	153±6	159±4	137±5†
% (No.) abnormal embryos	4 (1)	4 (1)	31 (10)

The values for the initial glucose concentrations are the results of assays on two separate batches of pooled sera for each of the experimental conditions. Significantly different from the values for explants cultured in hyperglycemic serum: †$p<0.05$; *$p<0.01$. Significantly different from the values for explants cultured in control serum. **$p<0.05$; ‡$p<0.01$ (Scheffé-test). Data from Rashbass and Ellington (1988).

Embryo-culture techniques, used to assess the effects of excess glucose in isolation to all the other factors in diabetic blood, also demonstrated elevated sorbitol in embryos exposed to excess glucose. The addition of the aldose reductase inhibitors (ARI), Statil or sorbinil, to hyperglycemic culture medium partly prevented the rise in the sorbitol content of the embryos, although it was still higher than control embryos, but had no effect on the incidence of malformations.

Although significant differences in the sorbitol content of embryos cultured in normal medium and in medium with 16.7 mM added glucose were noted, morphological abnormalities and growth retardation were only reported amongst those embryos exposed to 66.7 mM added glucose (Hod *et al.*, 1986). (66.7 mM was also the lowest concentration of added glucose at which Cockroft observed dysmorphogenesis).

Myo-inositol

Both *in vivo* and *in vitro* experiments have indicated that the increased sorbitol synthesis associated with exposure to excess glucose is not a cause of the observed embryonic malformations. In a later series of experiments Baker *et al.* (1990) used mouse embryo-culture to investigate the effects of the decreased intracellular myo-inositol pools. A range of concentrations of myo-inositol (2, 4 or 9 mM) were added to culture medium containing 44 mM glucose. Presomite mouse embryos were cultured for 48 h then examined for the incidence of closed neural tubes. Only 35% of the embryos cultured in the hyperglycemic medium had closed neural tubes compared to 77% of the control embryos. The addition of 4 mM myo-inositol to the hyperglycemic culture medium partially but, not completely, protected embryos against the dysmorphogenic effects of hyperglycemia as evidenced by the fact that 55% of the embryos cultured in the hyperglycemic medium with added myo-inositol had closed neural tubes. The protective effect of added myo-inositol was blocked by indomethacin, an inhibitor of arachidonic acid metabolism, suggesting that arachidonate or another factor in this metabolic pathway was contributing to the protective effects against hyperglycemia. In fact, both arachidonic acid and prostaglandin supplementation of hyperglycemic culture medium completely protected cultured mouse embryos from the effects of the excess glucose, thereby supporting this hypothesis (Goldman *et al.*, 1985; Pinter *et al.*, 1986a).

Free oxygen radicals

More recently it has been suggested that the complications of diabetes may arise from an increased production of free oxygen radicals. 9.5d rat embryos cultured in medium containing 50 mM glucose for 48 h displayed retarded growth and severe malformations. However, the addition of either citiolone (an inducer of oxygen scavenging enzymes) or oxygen scavenging enzymes such as superoxide dismutase, to the hyperglycemic medium protected embryos against both the growth-retarding and embryopathic effects of excess glucose. These results indicate that an increased production of free oxygen radicals by embryos cultured with excess glucose may contribute to the dysmorphogenic effects of hyperglycemia (Eriksson and Borg, 1991).

DNA metabolism

Derangement of normal DNA metabolism has also been implicated in malfunction of tissues from diabetics. Eriksson and Borg (1988) cultured 9.5d rat explants for 48 h in medium containing excess glucose and at the end of the culture assessed DNA repair activity (by measurement of hydroxyurea resistant thymidine incorporation) in embryos and yolk sacs. There was no evidence of alteration of DNA repair in response to hyperglycemic damage even at glucose concentrations as high as 67 mM. It was therefore concluded that there was no general increase in DNA repair following exposure to excess glucose, but the possibility of local repair in specific tissues was not eliminated.

Together, these and other similar experiments, clearly demonstrate that excess glucose in embryo culture medium causes abnormal morphological development, ultrastructural changes in the embryo and yolk sac, changes in glycolytic flux and sorbitol synthesis, depletion of tissue myo-inositol and production of free oxygen radicals. Hence we are beginning to gain a detailed description of the disruptive effects of excess glucose, but the

TABLE 5

SUMMARY OF THE INCIDENCE OF MALFORMATIONS AMONGST 8.5d MOUSE EXPLANTS AFTER 24 H IN CULTURE IN HYPERGLYCEMIC OR NORMOGLYCEMIC "DIABETIC" SERUM

Serum	Embryos (number)	Malformed embryos (%)
Control 23	0	
"Diabetic" (≈24 mM)	87	36
"Diabetic" (≈7.4 mM)		
(1) -	60	16
(2) + glucose (25 mM)	70	17
(3) + 3-OHB	73	29
(4) + glucose + 3-OHB	65	23

Data from Buchanan *et al.* (1994).

majority of these changes are observed only after culture of embryos in medium containing very high glucose concentrations. The glucose concentrations used were usually 50 mM or higher. Such extreme glucose concentrations are only occasionally observed even in severely uncontrolled diabetes (Fulop *et al.*, 1975).

Relevance of hyperglycemic studies to diabetes?

To evaluate the contribution of hyperglycemia to the dysmorphogenic effects of diabetes, Rashbass and Ellington (1988) cultured 9.5d rat explants either in serum prepared from rats with streptozotocin induced diabetes or in control serum with glucose added until its concentration approximated that of the diabetic serum (i.e. about 30 mM). After 48 h of culture embryos cultured in the "diabetic" serum had retarded growth and development compared to embryos cultured in control serum or in serum with added glucose. Thirty one percent of the embryos cultured in the "diabetic" serum had malformations compared to only four percent in each of the other two culture media (Table 4).

Similarly, Sadler *et al.* (1988) observed that mouse embryos of the same age cultured in "diabetic" serum developed a higher incidence of malformations than those cultured in control serum with glucose added to match the concentration in the "diabetic" serum. It was therefore concluded that hyperglycemia alone was not the teratogenic factor in diabetic serum. In fact additional studies of a number of other factors including ketone bodies, somatomedin inhibitors, insulin etc. suggest that the diabetic embryopathy is probably multifactorial in origin (reviewed in Freinkel *et al.*, 1986 and Sadler *et al.*, 1988).

Conclusions

The experiments described in this article scan a twenty year period during which embryo-culture techniques have been used to provide a fascinating insight into the effects of hyperglycemia in the developing post-implantation embryo *in vitro*. The precise concentrations of exogenous glucose causing morphological and biochemical changes have been defined for a range of developmental stages in both rat and mouse embryos. The critical window of exposure is known and the morphological, cellular and biochemical changes have been described. The effect of hyperglycemic serum has been compared with that of "diabetic" serum and attempts have been made to assess the contribution of hyperglycemia itself to the embryopathic effects of diabetic serum.

The value of the culture system is clearly demonstrated by these experiments. For example, by examination and selection of explants at the start of the culture the developmental stages used can be precisely defined and variation in developmental stages can be reduced. The concentration of the substance under investigation immediately surrounding the conceptus is known and any effects of maternal metabolism are eliminated. The periods of exposure to the test substance can be precisely defined and can be followed by further development *in vitro* to allow for the manifestation of any effects which were not immediately obvious. Thus, we can get extremely precise results from clearly defined experimental conditions.

However, conditions in culture are not the same as *in vivo*. *In vivo* the conceptus is static within the implantation chamber which itself is enveloped by the maternal decidua. Very little is known either of conditions in the implantation chamber or of the metabolic role of the enveloping decidual cells, although Christie (1966, 1967) has demonstrated the presence of glycogen and glycolytic enzymes in the decidual cells. It is possible that the decidual cells function, in a manner similar to the liver of the adult, to regulate the supply of glucose to the rapidly growing embryo. In general, the reported incidence of malformed rat and mouse embryos is much lower amongst embryos developing *in vivo* in diabetic mothers than amongst embryos cultured in "diabetic" serum. Thus there appears to be some protection proffered *in vivo* which is not active *in vitro*. Hence whilst the embryo culture experiments can yield extremely interesting results, the extrapolation of these results from *in vitro* rat or mouse studies to human *in vivo* situations should be undertaken only with extreme caution!

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