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Pathogenesis of Diabetes-Induced Congenital Malformations

Ulf J. Eriksson, L. A. Håkan Borg, Jonas Cederberg, Hanna Nordstrand, C. Martin Simán, Christian Wentzel, and Parri Wentzel

Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden

ABSTRACT

The increased rate of fetal malformation in diabetic pregnancy represents both a clinical problem and a research challenge. In recent years, experimental and clinical studies have given insight into the teratological mechanisms and generated suggestions for improved future treatment regimens. The teratological role of disturbances in the metabolism of inositol, prostaglandins, and reactive oxygen species has been particularly highlighted, and the beneficial effect of dietary addition of inositol, arachidonic acid and antioxidants has been elucidated in experimental work. Changes in gene expression and induction of apoptosis in embryos exposed to a diabetic environment have been investigated and assigned roles in the teratogenic processes. The diabetic environment appears to simultaneously induce alterations in several interrelated teratological pathways.

The complex pathogenesis of diabetic embryopathy has started to unravel, and future research efforts will utilize both clinical intervention studies and experimental work that aim to characterize the human applicability and the cell biological components of the discovered teratological mechanisms.

Abbreviations

COX = cyclooxygenase, ECM = extracellular matrix, NAC = N-acetylcysteine, HbA_{1c} = glycosylated hemoglobin A, PGE_2 = prostaglandin E_2, PGH_2 = prostaglandin H_2, PI = phosphatidyl inositol, PIP = phosphatidyl inositol monophosphate, PIP_2 = phosphatidyl inositol diphosphate. ROS = reactive oxygen species, SOD = superoxide dismutase

BACKGROUND

The embryo-fetal dysmorphogenesis in diabetic pregnancy has been extensively investigated in experimental [26, 134] and clinical [168, 239] studies. The research focus has shifted during the last decades as a consequence of the successively improved clinical treatment of diabetic pregnancy [40, 42, 100]. Thus, in the early days of insulin therapy the offspring faced a significant risk of both intrauterine and perinatal mortality, as well as markedly increased risk for postnatal morbidity com-

pared with non-diabetic pregnancy [190]. At present, almost eighty years after the introduction of insulin therapy, when the rate of most previous complications has been reduced, the incidence of congenital malformation remains increased [130] and constitutes a major threat to the health of the offspring of the diabetic woman [23, 38, 45, 94, 96, 129, 221, 224]. The magnitude of the expected risk for congenital malformation varies from a doubling to 5–6 times that of non-diabetic gestation [3, 10, 14, 24, 31, 53, 84, 100, 102, 103, 113, 128, 157, 172, 220, 247, 252, 256], and congenital malformations are commonly regarded as one major cause of mortality and morbidity in the offspring of diabetic pregnancy [24, 34, 95, 102, 130].

The continued risk for fetal malformation justifies future intensified clinical and experimental research efforts. Clinical investigations have reported increased incidences of congenital malformation both in type-1 [128, 130, 155, 168], and type-2 [14, 95, 194, 221, 247] diabetic pregnancies. The offspring of women with gestational diabetes show increased rate of congenital malformations [126, 127, 129, 147, 180, 193, 221], however, in these studies there may be a proportion of pregnant women with undiagnosed type-1 or type-2 diabetes contributing to the high rate of fetal dysmorphogenesis. Also, clinical studies have indicated that the risk for congenital malformations is dependent on the blood glucose regulation during the periconception period and the first trimester [82, 138, 139, 150, 153, 211, 220], in particular during the first seven weeks of pregnancy [154].

Experimental studies have suggested that the major teratogen in diabetic pregnancy is hyperglycemia [35, 55, 158, 212], although other diabetes-related factors may also influence the fetal outcome, *e.g.* increased levels of ketone bodies [79, 109, 110, 140, 164, 181, 214, 225-227], triglycerides [59, 238], and branched chain amino acids [70, 238]. Several teratological pathways in the embryonic tissues have emerged from the research efforts, such as alterations in the metabolism of inositol [7, 101, 106, 107, 240, 258], arachidonic acid/prostaglandins [7, 58, 198] and reactive oxygen species [69-71, 97]. The embryonic formation of sorbitol [63, 67, 101, 106, 240], glycated proteins [72, 244, 270], and the maternal and fetal genotypes [25, 26, 64, 68, 182] are also expected to influence the complex teratological events in diabetic pregnancy. The cell biological details and interplay of these factors and pathways will be the subject for future research efforts in the field of diabetic embryopathy.

HYPERGLYCEMIA IS A MAJOR DIABETIC TERATOGEN

The metabolic alterations in the diabetic mother have profound effects on embryogenesis. Several reports have suggested that hyperglycemia acts as a primary teratogen [120, 239]. This is inferred from clinical demonstrations of a positive correlation between maternal HbA1c levels in early pregnancy and fetal malformation rate [42, 93, 116, 138, 143, 153, 169, 177, 192, 210, 211, 239, 277], and the decreased rate of fetal dysmorphogenesis achieved by intensified control of the maternal diabetic state during this time period [6, 82, 117, 211, 271]. Experimental results sup-

Anti-teratogenic intervention

The dysmorphogenesis found in rodent embryos exposed to high glucose concentrations in vitro can be diminished by addition of inositol [7, 101, 107], arachidonic acid [89, 199], or antioxidants [69, 70, 260, 261] to the culture medium. In addition, the offspring of diabetic rodents show less dysmorphogenesis if the mother is supplied with inositol [2, 206, 208], arachidonic acid [89], or antioxidants [71, 121, 228, 229, 232, 254] during pregnancy (*cf.* Figure 3). Furthermore, addition of the mitochondrial pyruvate uptake inhibitor α-cyano-hydroxycinnamic acid to embryos cultured in high concentration of glucose or pyruvate also diminishes dysmorphogenesis [70] and embryonic mitochondrial swelling [276].

port this notion of hyperglycemia as a teratogen, since high glucose levels [77, 238], or maternal diabetes in vivo [2, 16, 29, 33, 44, 47, 49, 56, 59–62, 65, 66, 71, 77, 83, 86, 87, 89, 111, 115, 123, 134, 136, 137, 145, 160, 179, 182, 184, 186, 195, 228, 229, 232, 233, 238, 240, 246, 251, 254, 274, 276, 281], as well as exposure to high glucose concentration [7, 35, 64, 69, 70, 72, 74, 89, 101, 106, 159, 161, 199, 212, 235, 249, 261], or diabetic serum [17, 92, 167, 178, 204, 213, 215, 237, 259, 260, 281, 283] in vitro cause embryonic maldevelopment.

Multifactorial analysis of maternal parameters in diabetic rat pregnancy has shown a correlation between maternal levels of glucose, ketone bodies (β-hydroxybutyrate), branched chain amino acids and adverse embryo outcome (malformation and resorption rates) [238]. Furthermore, *in vitro* studies indicate that diabetic serum is teratogenic to rodent embryos even when the glucose level is normal [17, 237, 259].

It has also been shown that diabetic pregnant women with a minimal increase in HbA1c levels still have an increased risk for offspring with congenital malformation, and that the risk is not related to the HbA_{1c} concentration [156, 239]. Furthermore, several studies have indicated that other metabolites than glucose (*e.g.* lactate, pyruvate, ketone bodies, several amino acids, glycerol and free fatty acids) may be altered in diabetic individuals, despite normoglycemia [4, 11, 20–22, 146, 174, 279].

The conclusion from these studies is that hyperglycemia may be a major teratogen in diabetic pregnancy and that alterations in several maternal and fetal metabolites (*i.e.* β-hydroxybutyrate and branched chain amino acids) are additional teratogens.

INOSITOL DEPLETION IS TERATOGENIC

In embryos subjected to high glucose concentration in vitro, the inositol levels decrease due to impaired uptake [258], yielding an embryonic deficiency of inositol [106, 235, 240]. Supplementation of inositol to high glucose cultured embryos [7, 101, 107], or dietary addition to diabetic pregnant rodents [2, 121, 206, 208], yields less embryonic maldevelopment. Adding the competitive inhibitor scyllo-inositol to

Anti-teratogenic intervention

Depletion of embryonic inositol by administration of scyllo-inositol to the culture medium of rat embryos yields developmental damage, which can be markedly diminished by addition of inositol [236] to the medium, but not by addition of PGE_2 [263].

the culture medium induces both inositol deficiency and embryonic dysmorphogenesis of similar type as the damage caused by high glucose alone [235, 236]. Both the inositol deficiency and the embryo maldevelopment elicited by scyllo-inositol exposure can be diminished by addition of inositol to the culture medium [235, 236]. These findings identify inositol deficiency as a likely component of diabetic teratogenesis [8].

The immediate effect of lowered inositol concentration would be decreased levels of the phosphoinositides (PI, PIP and PIP_2) and their products in the embryonic tissue [235]. A lack of PIP_2 would subsequently yield less IP_3 and diacylglycerol, both of which are stimulators of protein kinase C activity. A lowered protein kinase C activity would exert a number of effects, including lowered activity of phospholipase A_2, the key enzyme in the metabolism of triglycerides and phospholipids [133]. A decrease of phospholipase A_2 activity would subsequently diminish the availability of free arachidonic acid, and thereby diminish the production and metabolism of prostaglandins. This would constitute a link to other teratological pathways, as discussed below.

ARACHIDONIC ACID ALTERATIONS ARE TERATOGENIC

Disturbed metabolism of arachidonic acid and prostaglandins has been found in previous studies of experimental diabetic pregnancy. Addition of arachidonic acid to the culture medium was shown to block the embryonic dysmorphogenesis elicited by high glucose concentration [89], a finding that has been repeated [199], and expanded [261] in subsequent studies. Intraperitoneal injections of arachidonic acid to pregnant diabetic rats diminished the rate of neural tube damage [89], as did enriching the diet of the pregnant diabetic rats with arachidonic acid [206, 207], thereby indicating disturbance in the arachidonic acid cascade as a consequence of a diabetic environment [269]. Addition of PGE2 to the culture medium also blocks glucose-induced teratogenicity *in vitro* [7, 261], as well as maldevelopment of embryos cultured in diabetic serum [92]. Measurements of PGE_2 have indicated that this prostaglandin is decreased in embryos of diabetic rodents during the period of neural tube closure [197, 262], in high glucose cultured embryos [262], as well as in the yolk sac of embryos of diabetic women [223].

Previous studies have shown, however, that the uptake of arachidonic acid by embryonic yolk sacs is increased in a hyperglycemic environment [58]. This finding

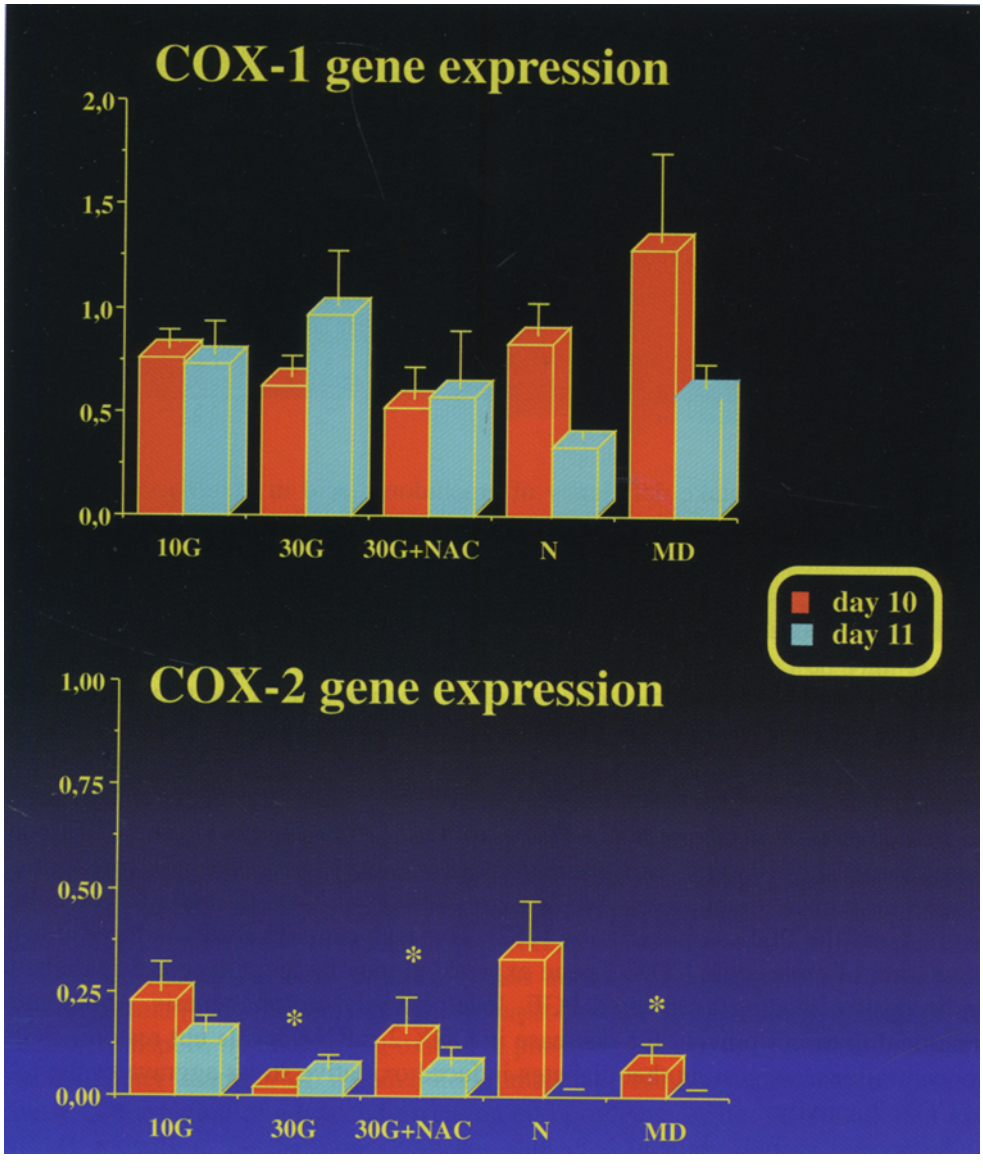


Figure 1. Expression of COX-1 and COX-2 in embryos sub-jected to 10 mmol/l, 30 mmol/l glucose in vitro (10G, 30G) for 24 or 48 hours, corre-sponding to gestational days 10 and 11, respectively. To the latter concentration was also added 0.5 mM NAC (30G + NAC). Expression was also estimated in day 10 and day 11 embryos from normal (N) and manifest-ly diabetic (MD) rats. Significance: * = $p < 0.05$ (30G vs. 10G, 30G+NAC vs. 10G, MD *vs.* N) [262].

Anti-teratogenic intervention

Culture of rat embryos with COX inhibitors, indomethacin and acetylsalicylic acid, resulted in malformations similar to those caused by high glucose culture, a maldevelopment that was blocked by supplementation of arachidonic acid or PGE_2 [261]. Addition of SOD or NAC diminished the COX inhibitor-induced dysmorphogenesis, analogous to the effect of the antioxidants on glucose-induced embryonic maldevelopment [261]. This result, together with the finding of diminished glucose-induced embryopathy by addition of arachidonic acid [89, 199, 261] and PGE_2, [7, 261] suggest a cross-talk between teratogenic effects caused by a decreased prostaglandin synthesis and ROS excess in embryos subjected to a diabetic environment, as well as between inositol and prostaglandins [7] (cf. Figure 3).

would preclude an uptake deficiency of arachidonic acid in the conceptus of diabetic pregnancy, a result supported by the demonstration of unchanged concentration of arachidonic acid in membranes of high glucose cultured embryos *in vitro* [200]. Recently, however, measurements in day-12 embryos indicate a decreased arachidonic acid concentration in offspring from diabetic rats [121]. From these data, it may be concluded that decreased availability of arachidonic acid and the prostaglandin products of arachidonic acid, is a component of the teratogenicity of diabetic pregnancy [269].

In a recent study, we found a downregulation of the gene expression of COX-2, the inducible form of the COX enzyme, as well as a GSH-dependent enhancement of the conversion of the precursor PGH_2 to PGE_2 [262] (Figure 1). The PGE_2 concentration of day 10 embryos and membranes was decreased after exposure to high glucose *in vitro* or diabetes *in vivo*. *In vitro* addition of NAC to high glucose cultures restored the PGE_2 concentration [262]. Hyperglycemia/diabetes-induced downregulation of embryonic COX-2 gene expression may be an early event in diabetic embryopathy, leading to lowered PGE_2 levels and dysmorphogenesis. Antioxidant treatment does not prevent the decrease in COX-2 mRNA levels but restores PGE_2 concentrations, suggesting that diabetes-induced oxidative stress aggravates the loss of COX-2 activity.

ROS EXCESS IS A COMPONENT OF DIABETIC TERATOGENESIS

The notion that diabetes is associated with oxidative stress has been suggested by several authors [9, 88, 176, 222, 264]. Increased lipid peroxidation and ROS generation were found in diabetic rats, measured as increased serum F_2-isoprostane levels [183], and increased electron spin clearance rate [219]. Both of these indica-

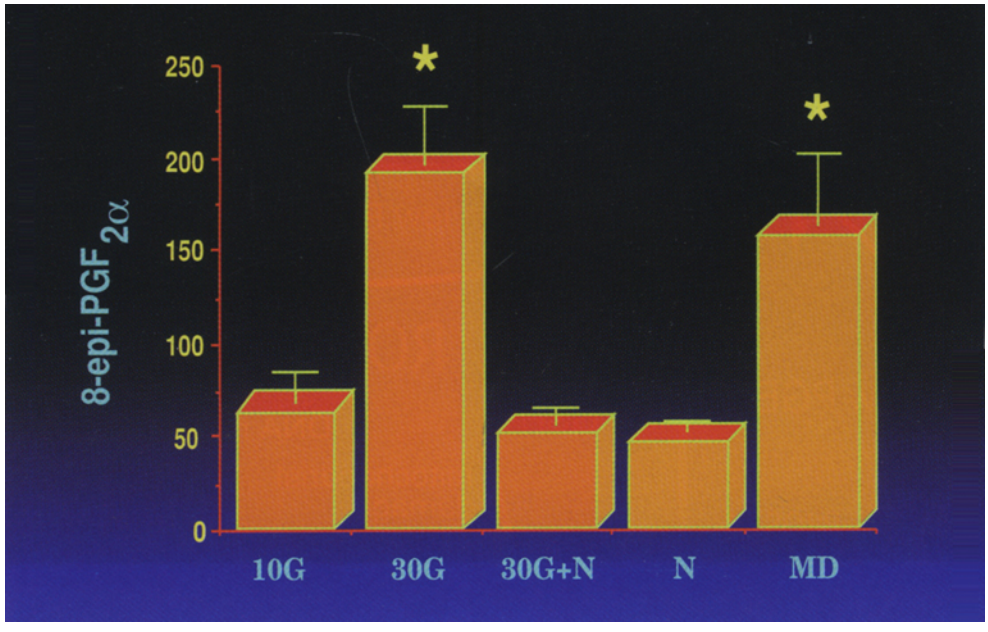


Figure 2. F2-isoprostane-concentration in day 9 embryos exposed to 10 mM or 30 mM glucose (10G, 30G) *in vitro* for 48 hours (30G embryos with added NAC are denoted 30G+N), and in day 11 embryos from normal (N) and manifestly diabetic (MD) rats. Significance: * = $p < 0.05$ (30G vs. 10G and MD vs. N) [262].

tors of oxidative stress were normalized by vitamin E treatment of the diabetic rats [183, 219]. Cyclic voltammetric studies have indicated increased levels of lipid peroxidation in diabetic rats [54], and it was recently demonstrated that mitochondria of vascular endothelial cells produce excess amount of superoxide in response to hyperglycemia [173]. Diminishing this overproduction of ROS via inhibition of the electron transport, by uncoupling oxidative phosphorylation, or by addition of SOD, blocked other markers of intracellular imbalance, such as activation of protein kinase C, formation of advanced glycation end products, sorbitol accumulation and NF-kB activation. Furthermore, embryos subjected to high glucose concentration show evidence of increased superoxide production, as measured in a Cartesian Diver system [275].

In a recent study we found an increased F_2-isoprostane (8-epi-$PGF_{2\alpha}$) concentration in embryonic tissue exposed to hyperglycemia *in vitro* and diabetes *in vivo* (Figure 2). This finding suggests an embryonic increase in lipid peroxidation rate that can be blocked by antioxidative treatment utilizing NAC.

Adding scavenging enzymes, *e.g.* SOD, catalase or glutathione peroxidase, to the culture medium protects rat embryos from dysmorphogenesis induced by high glucose concentration *in vitro* [69]. Teratogenic concentrations of β-hydroxybutyrate or the branched chain amino acid analogue α-ketoisocaproic acid can be blocked by

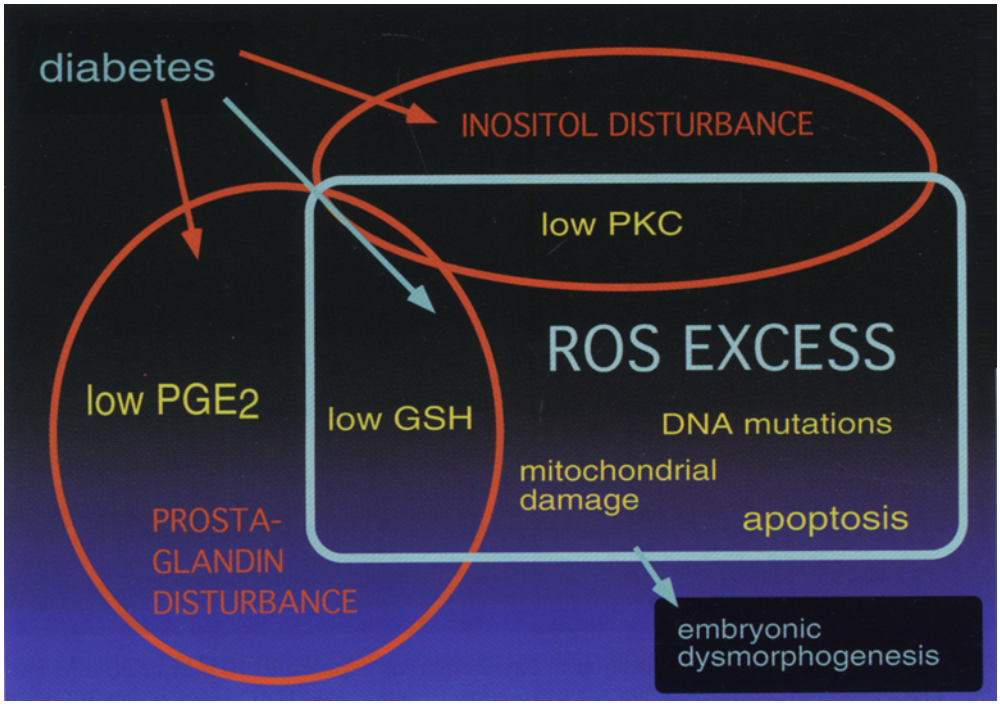


Figure 3. Tentative scheme of diabetic teratogenesis, showing three major changes – inositol disturbance [106], prostaglandin disturbance [89], and ROS excess [69], the latter change executing several of the effects on the embryo, DNA mutation [137], mitochondrial alterations [274], apoptosis [195], and, lastly, embryonic dysmorphogenesis.

addition of SOD to the culture medium [70], and addition of SOD or NAC diminishes the dysmorphogenesis caused by diabetic serum [260]. In a study of the early development of cranial neural crest cells, it was shown that high glucose inhibited, and NAC normalized, the migration and proliferation of these cells, and that control cells of non-neural crest origin were not affected by either treatment [241]. Examination of litters of diabetic rats demonstrated lowered _-tocopherol (vitamin E) concentration in day-11 embryos and in the liver of day-20 fetuses [228].

High-amplitude mitochondrial swelling was demonstrated in embryonic neuroectoderm of embryos exposed to a diabetic environment [274], a swelling diminished by antioxidative treatment of the mother [276], implicating an embryonic ROS imbalance, with conceivable consequences for the rate of apoptosis in susceptible cell lineages in the embryo [80]. In addition, fetuses and embryos of diabetic rodents display increased rates of DNA damage [136, 137], another indication of enhanced ROS activity in the embryonic tissues.

Direct evidence of ROS excess in embryonic tissue has been difficult to demonstrate, however. In neuroepithelial cells mitochondrial swelling results from exposure to a diabetic state *in vivo* or hyperglycemia *in vitro* [108, 274]. This high-amplitu-

Anti-teratogenic intervention

Supplementation of antioxidants has been shown to be beneficial for the development of embryos in a diabetes-like environment *in vivo* and *in vitro*. Dietary addition of butylated hydroxytoluene [71], vitamin E [206, 228, 230, 233, 254, 276], vitamin C [229], combinations of vitamins E and C [27], glutathione ester [217], lipoic acid [268] diminish perturbed embryonic development *in vivo*, whereas addition of SOD [69, 70, 72], catalase [69], glutathione peroxidase [69], NAC [141, 241, 260-262], glutathione ester [249] diminish embryonic dysmorphogenesis *in vitro* (*cf.* Figure 3).

Anti-teratogenic intervention

In summary, despite difficulties in demonstrating ROS in embryos acutely exposed to high glucose, the combined data support the notion that the teratogenic process in diabetic pregnancy does involve excess radicals at a late stage, and, by blocking this excess by antioxidants the diabetes-induced dysmorphogenesis can be substantially diminished (*cf.* Figure 3).

de swelling can be diminished by antioxidant supplementation [276]. Also, in the neuroectodermal cells indirect evidence of superoxide production in response to hyperglycemia was found [275]. This suggests that long-term exposure to high glucose creates an embryonic ROS excess either from increased ROS production [275], or from diminished antioxidant defense capacity [112, 151, 249]. ROS excess may be relatively small, restricted to particular cell populations [30, 41], and likely to vary with gestational time and nutritional status, making direct ROS determinations difficult. Nevertheless, a cyclic voltametry measurement of oxidation potential in pre-implantation rodent embryos cultured in diabetic serum indicated the presence of ROS excess also at this stage [179].

Increasing ROS in embryos leads to malformations [5, 114], suggesting to several authors that ROS excess may also have a role in the teratogenic process(es) of phenytoin medication [266, 267], ethanol abuse [30, 41, 125], and, recently, thalidomide administration [189]. Therefore, ROS excess may constitute a common element in a number of teratogenic situations, including diabetic pregnancy [73].

NEURAL CREST CELLS ARE TARGETS IN DIABETIC TERATOGENESIS

Cardiac anomalies are the most common malformations in offspring of diabetic mothers [1, 3, 10, 90, 122, 144, 148, 152, 203, 278], but the rate of skeletal malformations is also increased in diabetic pregnancy [75, 122, 203]. The infants frequent-

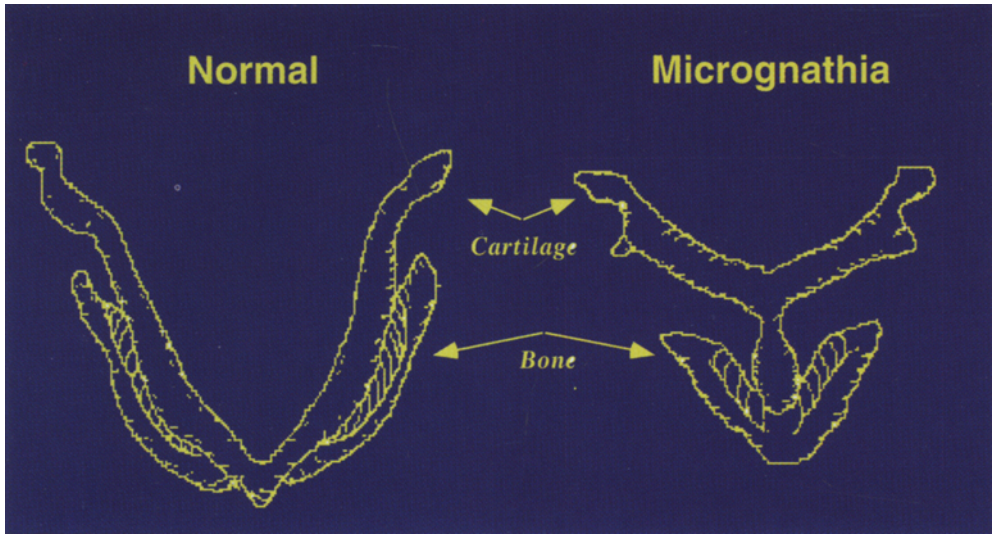


Figure 4. Reconstruction of the mandible of a rat fetus from a normal (left) and a diabetic (right) rat mother, the latter exhibiting micrognathia [230].

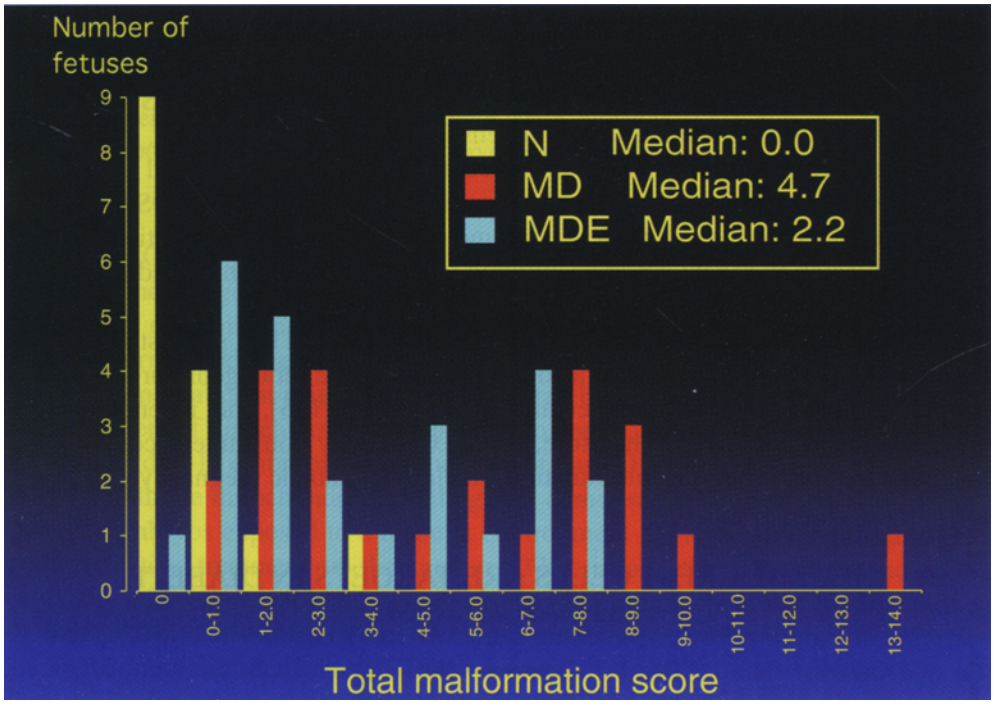


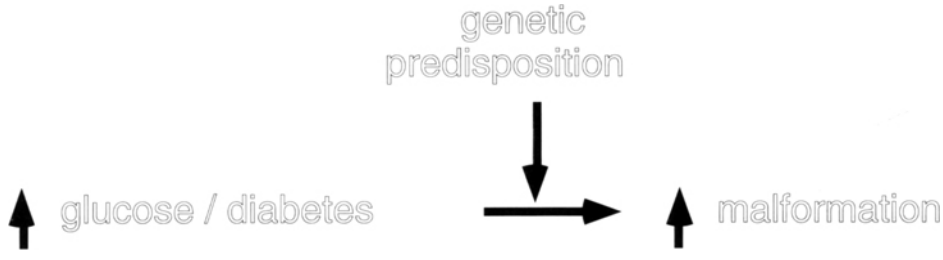
Figure 5. Distribution of total malformation score of fetuses of normal (N), diabetic (MD), and vitamin E-treated diabetic (MDE) rats. "0" denotes fetuses with a score of exactly zero. "0–1.0" and "1–2.0" denote $0 < \text{score} \leq 1$, and $1 < \text{score} \leq 2$, respectively [230].

ly show developmental changes related to diGeorge anomaly, a complex syndrome that has been detected in infants of diabetic mothers [52, 76, 91, 131, 175, 265].

DiGeorge anomaly affects the cranial and cardiac [132] neural crest cell derivatives – *i.e.* the cells forming the facial bones, including the mandible, the thyroid and parathyroid glands, the thymus, the cardiac outflow tract, and the great vessels close to the heart [13, 99, 124, 165]. The most common genetic defect associated with diGeorge anomaly is a microdeletion on chromosome 22 (22q11) [132, 163], but when the syndrome has been detected in infants of diabetic mothers this chromosomal lesion is most often absent [52, 76, 91, 131, 175, 265]. The maternal diabetic state, therefore, is able to induce a generalized defect in cranial and cardiac neural crest cell development in some, presumably genetically predisposed, individuals among the offspring.

Recently we found malformations resembling the diGeorge anomaly in fetuses of diabetic rats, which showed low set external ears and severely malformed Meckel's cartilage (*i.e.* micrognathia, cf. Figure 4) [230]. The fetuses of the diabetic rats also displayed small thyroid and thymus and absence of parathyroid glands. Cardiac anomalies were frequently observed; rightward displacement of the aorta, double outlet right ventricle, and persistent truncus arteriosus combined with ventricular septal defects. The malformations in the outflow tract included abnormalities of the great arteries; right-sided aortic arch/descending aorta, and double aortic arches. These defects tended to occur together within individual fetuses. Maternal dietary treatment with vitamin E markedly reduced the severity of the malformations (Figure 5) [230].

GENETIC PREDISPOSITION INFLUENCES THE OUTCOME OF PREGNANCY



Despite similar teratological exposure, the effect of any teratogen, including maternal diabetes/hyperglycemia, varies between individuals. In addition to stochastic conditions, genetic predisposition determines the effect of each teratogen on a particular individual [119, 166].

Although predisposing genetic conditions for diabetes are clearly present in offspring of diabetic parents [46, 245] as the offspring of a diabetic father has higher risk of developing the disease than the offspring of a diabetic mother [39, 149, 201, 250, 257] – it has been established that diabetic men do not have an increased risk of fathering malformed offspring [32, 36]. This indicates that the genes predi-

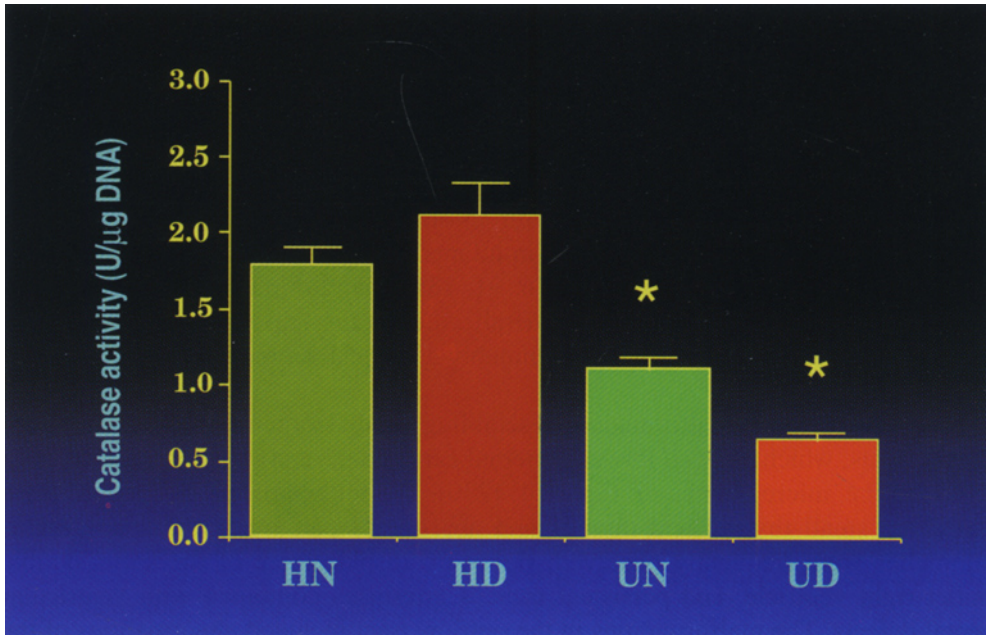


Figure 6. Catalase activity in embryos of normal (N) or diabetic (D) rat mothers from a malformation-resistant (H) and malformation-prone (U) strain. Significance's: * = $p < 0.05$ (UN *vs.* HN, UD *vs.* UN) [25].

sposing to diabetes are not inducing congenital malformations. In contrast, maternal diabetes has recently been suggested to be associated with Down's syndrome [170, 191, 234], and has also been suggested to predispose for optic nerve hypoplasia in female offspring [171]. A genetic element may be present in the etiology of diabetic embryopathy [253], a notion supported by experimental data [25, 57, 64, 68, 97, 182, 273].

It has been suggested that the absence of a specific malformation pattern for diabetic embryopathy signals the presence of several teratological factors and mechanisms in diabetic pregnancy [122]. Likewise, the number of different teratogenic agents identified would indicate that diabetic embryopathy is of complex etiology [17, 216, 283].

We have found lowered catalase activity in embryos of a malformation-prone rat strain, a decrease which is more pronounced when the rat mother is diabetic (Figure 6) [25]. This finding suggests that a genetic predisposition toward increased rate of embryonic dysmorphogenesis [64] may result from diminished capacity to scavenge endogenously formed ROS, a capacity that can vary between rat strains [97, 218] – and, tentatively, also among human pregnant diabetic women and their offspring. In fact, when we studied the catalase gene further in the two rat strains we found differences in the nucleotide sequences of the promoter and the mRNA. Thus, we uncovered a heterozygosity in the promoter region of the malformation-resistant

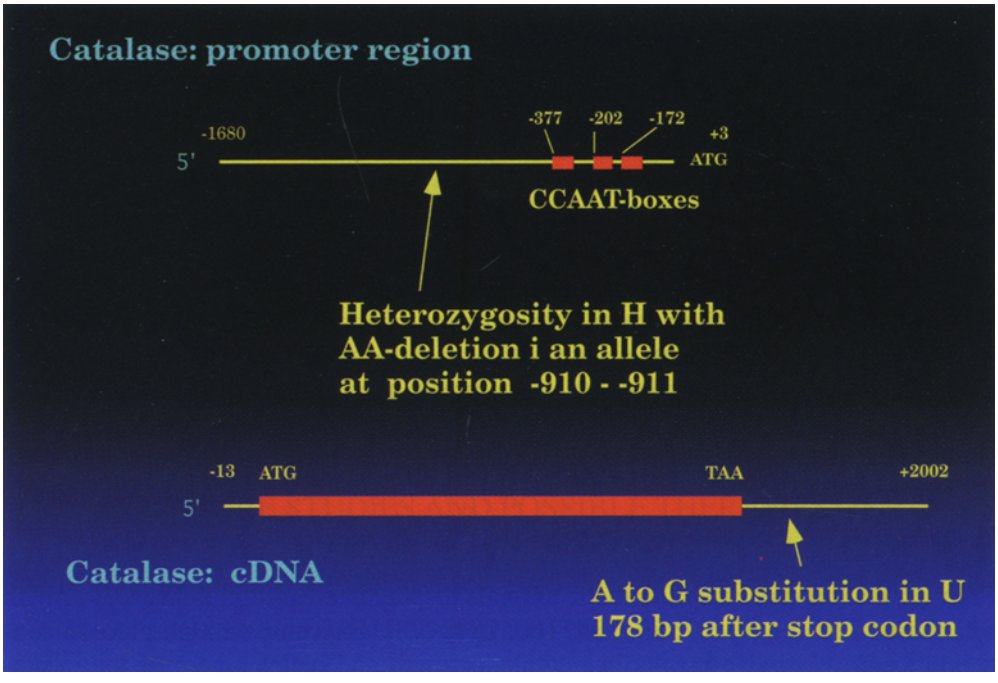


Figure 7. Structure of catalase promoter and cDNA. Comparison between the nucleotide sequences in a malformation-resistant (H) and malformation-prone (U) rat strain [26].

strain, and a base substitution in the 3'UTR region of the mRNA of the malformation-prone strain, respectively (Figure 7) [26]. The full significance of these structural changes is not yet clear, but, speculatively, the heterozygosity may affect the binding of transcription factors and the base substitution in the 3'UTR region could alter mRNA stability.

ALTERATIONS IN GENE EXPRESSION CAUSED BY A DIABETIC ENVIRONMENT

High glucose *in vitro* and diabetes *in vivo* alter the expression of several genes. It was previously shown that induction of a diabetes-like condition increased expression of the ECM genes B1-laminin and fibronectin in day-11 and day-12 rat embryos [18]. These genes are also altered in placentae of diabetic rats, fibronectin is overexpressed and laminin is suppressed [81], and similar changes have been demonstrated in human term placenta in diabetic pregnancy (K. Røge, personal communication). The functional importance of the ECM alterations is not clear at present, but may affect placental transfer in diabetic pregnancy [81].

The expression of genes controlling the defense against oxidative stress also shows diabetes-induced alterations in expression. Thus, MnSOD gene expression is altered in rat embryos exposed to a diabetes-like environment [78], which may rela-

te to the increased total SOD activity in these embryos [69]. In embryos from a malformation-prone rat strain, MnSOD and catalase expression did not increase in response to maternal diabetes, in contrast to the embryos of a malformation-resistant rat strain, in which the expression of these enzymes did increase [26].

The Pax-3 gene expression was recently found to be reduced in embryos of diabetic mice [195], and this transcription factor may regulate the gene expression of the licensing factor cdc-46 [105], and a gene, Dep-1 [19], both of which may be of importance for a correct neural tube closure. Null mutation of the Pax-3 gene yields the *Splotch* mouse displaying neural tube defects [37, 195]. It has also been shown that the decreased Pax-3 expression in embryos of diabetic mice could be normalized by treatment of the mother with NAC [141], thereby demonstrating a coupling between ROS excess and a teratologically important change in gene expression.

DIABETIC PREIMPLANTATION TERATOLOGY

Preimplantation embryos of diabetic animals display decreased viability and growth retardation [12, 49, 50, 57, 83, 135, 160, 184, 273]. Exposure to high glucose concentration *in vitro* also results in inhibited growth and development of the preimplantation embryo [43, 51, 280, 282]. The cells in the inner cell mass, *i.e.* the embryo proper, are particularly sensitive to the hyperglycemic or diabetic environment and display decreased cell number in response to exposure to a diabetes-like milieu [135, 184]. Recently, a paradoxical reaction to hyperglycemia was demonstrated in preimplantation embryos [162]. In embryos of diabetic mice, 48 hours and 96 hours old, a pronounced intracellular hypoglycemia was found despite maternal hyperglycemia [161]. The decreased intracellular glucose concentration was associated with a decreased glucose transport, and decreased levels of glucose transporters (GLUT-1, GLUT-2, GLUT-3), both at the protein and mRNA levels [161]. Furthermore, this decrease in intracellular glucose concentration leads to lower cell number in the embryoblast, either by increased apoptotic rate [159, 188], or by diminished proliferation of these cells [184]. The decrease in cell number may be induced by increased levels of the cytokine TNF-α which is overexpressed and excessively secreted by uterine cells in diabetic pregnancy [187]. After binding to receptors in the embryo [185], TNF-α may diminish proliferation of the inner cell mass [272] by increasing the production of other proteins, such as TGF-α1 [196], and the death promoting protein Bax [159] in the embryo. The interpretation of these data is that a preimplantation hyperglycemia causes either direct apoptosis [118], possibly related to ROS generation [142, 205], or predisposes the embryo for malformations later in the embryogenesis [162], as has been suggested for other teratogens [85, 202].

In term placenta of the rat the transport capacity of glucose is reduced after exposure to hyperglycemia or diabetes *in vivo* [243], and that human term placental trophoblast down regulate their GLUT-1 glucose transport system (mRNA and pro-

tein) after exposure to 25 mmol/l glucose *in vitro* for 96 hours [98]. Thus, the glucose-induced down regulation of glucose transporters in the preimplantation embryonic cells has a parallel situation in cells from older offspring. On the other hand, the glucose concentration in day-10 and day-11 embryos of normal and diabetic rats closely mirrors the maternal glucose level, suggesting no upper limit for glucose uptake by the embryonic tissue [240]. This is also supported by the finding of unchanged levels of GLUT-1, the major constitutive glucose transporter, in postimplantation embryos exposed to hyperglycemia *in vitro* or maternal diabetes, compared to control embryos [242, 248], indicating no down-regulation of the transport capability in these embryos by a high ambient glucose concentration. The uptake of glucose by the postimplantation embryo, therefore, appears to be proportional to the extracellular glucose level, and, therefore, is unlikely to control an apoptosis-inducing pathway via intracellular hypoglycemia.

FUTURE STUDIES AND POSSIBLE THERAPIES OF DIABETIC PREGNANCIES

Clinical studies, aiming at characterizing the maternal and fetal oxidative state in relation to fetal outcome should be executed. The future development in the field of diabetic pregnancy will need clinical implementation of the experimental findings; in particular investigations should be performed of the possible beneficial effect of dietary addition of antioxidants [71, 209, 255]. Of specific interest are two recent studies in pregnant women [15, 28]. One study demonstrated a marked decrease in the rate of pre-eclampsia in women given antioxidants (vitamin E+C) daily from gestational week 16–22 and onwards [28]. In the other report, women with periconceptional multivitamin intake had less risk for giving birth to infants with congenital heart malformations, particularly affecting the outflow tract and ventricular septum [15].

Experimental elucidation of the mechanisms involved, aiming to clarify the nature of the cross-talk between different teratological pathways should be conducted in the future. We are currently performing a linkage study in order to identify the predisposing genes in a malformation-prone rat strain. We will also specifically characterize the gene expression of several candidate genes, such as Pax-3, phospholipase A_2, and protein kinase C in normal and malformed offspring of diabetic rodents, as well as in specific target tissues, such as the cranial neural crest cells.

CONCLUDING REMARKS

The available clinical and experimental data indicate that there is more than one alteration in the embryonic environment capable of inducing a teratogenic development. One – perhaps the most important – alteration is the increase in glucose concentration [35], which seems to have a number of direct metabolic consequences in the embryo [104]. However, there are other changes with teratogenic importance, such as increased levels of ketone bodies [109] and branched chain amino acids

[70], to which the mechanism of action remains to be elucidated. Also, the study of etiologic and pathogenic factors in the pathogenesis of congenital malformations has revealed a complex process in which the diabetic state simultaneously induces alterations in a series of teratogenically capable pathways [8, 48, 89, 231]. These pathways are intertwined, and several of them seem to result in an imbalance of the ROS metabolism [173], yielding a ROS excess in teratogenically sensitive cell populations, an imbalance ultimately causing the congenital malformations [69]. This notion is important in developing therapies to counter the embryopathy. Blocking the ROS excess may be a valid way to diminish the disturbed development caused by the diabetic environment.

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Correspondance to:
Ulf J. Eriksson, MD, PhD,
Department of Medical Cell Biology
Biomedical Center, P.O. Box 571, SE-751 23 Uppsala, Sweden
e-mail:ulf.eriksson@medcellbiol.uu.se
http://www.bmc.uu.se/medcell/research/safari/uer/engindex.html