The status of diabetic embryopathy

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Diabetic embryopathy is a theoretical enigma and a clinical challenge. Both type 1 and type 2 diabetic pregnancy carry a significant risk for fetal maldevelopment, and the precise reasons for the diabetes-induced teratogenicity are not clearly identified. The experimental work in this field has revealed a partial, however complex, answer to the teratological question, and we will review some of the latest suggestions.

Introduction
Claes Hellerström (1), the senior and mentor of the authors of this text, strongly inspired the early work in the field of embryonic development (2–4) and maldevelopment (5), particularly in a diabetic environment (6–9).

Despite increased clinical efforts to improve glycemic control during diabetic pregnancy, the rate of congenital malformations remains increased in studies of diabetic gestation of type 1 (10–21) and type 2 diabetes (16–25). In two recent large meta-studies it was found that the malformation rate in type 1 diabetic pregnancy did not differ from that of type 2 diabetic pregnancy (26,27), and both rates were estimated to be around 5%–6%. The similar rates of malformation may relate to the higher age and concomitant adiposity in type 2 diabetic women, both of which may increase the malformation incidence in this group (28–30).

The cell biological reason for the teratogenic effect of the diabetic state is not known. However, both environmental factors (maternal diabetic state and intrauterine conditions) and genetic predisposition seem to be of importance in diabetic embryopathy, i.e. this is a case of environment–gene interaction. The congenital malformations are likely to be induced in early gestation (31–33), and the risk for giving birth to a child with a malformation is enhanced by increased maternal metabolic dysregulation (34–37).

Alterations of maternal metabolism
Several teratological factors in maternal serum have been suggested, often from clinical experience, and subsequently characterized in various experimental systems. The maternal teratogenic factors most often indicated are hyperglycemia and hyperketonemia.

Glucose
Increased glucose levels are the hallmark of the diabetic state, and there is ample clinical evidence that increased glucose/HbA1c levels correlate with increased risk for congenital malformation in the offspring (32–35,37–40). In experimental studies the analogous correlation between increased serum glucose levels and increased risk for fetal malformations has been demonstrated in diabetic rodents (8,41–76). In addition, injections of glucose to pregnant non-diabetic animals have also yielded teratological effects (77,78). Furthermore, in vitro culture of rodent embryos in increased glucose concentrations (48,57,64,79–95) as well as in diabetic serum (83,96–105) yields disturbed development (Figure 1).

The alleged teratological effect of hyperglycemia is likely to be correlated to the metabolism of glucose, since exposure to L-glucose in vitro has no negative developmental effect (79). Increased embryonic uptake of glucose by existing (106), often up-regulated (107,108), glucose transporters also seems to be necessary for the teratogenic effect. The increased influx of glucose would yield increased glycolytic flux, as well as increased mitochondrial activity of the citric acid cycle and enhanced oxidative phosphorylation. Several consequences of increased glucose metabolism have been suggested (109) where increased mitochondrial production of superoxide may possess the most pronounced teratogenic potential. However, also increased hexose monophosphate shunt activity, reactive oxygen species (ROS)-mediated inhibition of
glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and increased formation of reactive alpha-oxoaldehydes may play roles in the diabetes-induced embryonic dysmorphogenesis. In addition, increased formation of advanced glycosylation end-products (AGEs) and decreased intracellular availability of arachidonic acid (and its products, the prostaglandins) are suspected players in the teratogenic drama of diabetic pregnancy (Figure 2).

Of note, the pre-implantation embryo follows another route toward maldevelopment, since a diabetes-induced decrease of the inner cell mass (55,110) is likely to be induced by hypoglycemia, due to down-regulation of glucose transporters in the blastocyst, leading to decreased intraembryonic glucose levels (111–113) and enhanced apoptosis (110,114).

**Ketone bodies**

The ketone bodies are synthesized in increased amounts in the (maternal) liver in response to the diabetic state. The two major compounds, beta-hydroxybutyrate and acetoacetate, traverse the placenta (115) and are utilized as substrate (acetoacetyl-CoA) in the embryonic mitochondria. There is experimental evidence for a teratogenic role for diabetes-associated metabolites other than glucose. Thus, serum from diabetic rats with normalized glucose levels is still teratogenic in vitro cultures (103,104), and the direct exposure to increased ketone body levels in vitro yields disturbed development in cultured rodent embryos (116–123). The mechanism for ketone body teratogenesis should also include metabolism of the compounds (124).

**Combination effect**

Culture of rat embryos in a combination of sub-teratogenic levels of glucose and beta-hydroxybutyrate yields disturbed in vitro embryonic development, thereby supporting the notion of a synergistic relationship between diabetes-associated metabolites (90). Both of these putative teratogens serve as metabolic substrates in the embryo (and mother), and the most prominent teragen, glucose, has the most complex metabolism involving glycolysis. Furthermore, the metabolic pathways of the teratogens converge at the citric acid cycle and oxidative phosphorylation in the mitochondrion, which has led to the speculation that an overactivity of the
oxidative phosphorylation pathway may be at the core of the diabetic teratogenicity.

**Alterations of fetal metabolism**

Major teratogenic processes in embryonic tissues so far identified include alterations of signaling systems such as metabolism of arachidonic acid/prostaglandins (73,86,94,125–129).

**Arachidonic acid/prostaglandins**

Arachidonic acid is a polyunsaturated fatty acid present in the phospholipids of membranes of the cells. The metabolism of arachidonic acid and its products, the prostaglandins, is crucial for cellular life. In particular, arachidonic acid is involved in cellular signaling as a lipid second messenger. Disturbed metabolism of arachidonic acid and prostaglandins has been found in previous studies of experimental diabetic pregnancy. Intraperitoneal injections of arachidonic acid to pregnant diabetic rats diminished the rate of neural tube damage (NTD) (48) as did enriching the diet of pregnant diabetic rats with arachidonic acid (130–132). Addition of arachidonic acid to the culture medium was shown to block the embryonic dysmorphogenesis elicited by high glucose concentration (48,82,94). Addition of PGE2 to the culture medium also blocked glucose-induced teratogenicity in vitro (86,94) as well as maldevelopment of embryos cultured in diabetic serum (101). Measurements of PGE2 have indicated that this prostaglandin is decreased in embryos of diabetic rodents during neural tube closure (128,133) in high-glucose-cultured embryos (128) as well as in the yolk sac of embryos of diabetic women (134).

Previous studies have shown that the uptake of arachidonic acid by embryonic yolk sacs is increased in a hyperglycemic environment (126). This finding would preclude an uptake deficiency of arachidonic acid in the conceptus of diabetic pregnancy, a result supported by the demonstration of uptake deficiency of arachidonic acid in the conceptus of diabetic pregnancy, a result supported by the demonstration of uptake deficiency of arachidonic acid in the conceptus of diabetic pregnancy (132). Addition of arachidonic acid to the culture medium was shown to block the embryonic dysmorphogenesis elicited by high glucose concentration (48,82,94). Addition of PGE2 to the culture medium also blocked glucose-induced teratogenicity in vitro (86,94) as well as maldevelopment of embryos cultured in diabetic serum (101). Measurements of PGE2 have indicated that this prostaglandin is decreased in embryos of diabetic rodents during neural tube closure (128,133) in high-glucose-cultured embryos (128) as well as in the yolk sac of embryos of diabetic women (134).

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Diabetes-induced teratological processes

Several studies have suggested that diabetic embryopathy is associated with alterations of various signaling systems, which results in disturbed intracellular conditions, such as oxidative stress (62,88,90,138), nitrosative stress (139,140), endoplasmic reticulum (ER) stress (140–142), and hexosamine stress (143,144). In addition, enhanced embryonic apoptosis (77,78,93,114,145–149) has been regarded as a component of diabetic embryopathy (Figure 2).

**Oxidative stress**

Oxidative stress reflects an imbalance between production of reactive oxygen species (ROS) and an ability to detoxify the reactive intermediates or to repair the resulting damage. ROS can damage all components of the cell, including proteins, lipids, and DNA. Some ROS act as cellular messengers in redox signaling, and a state of oxidative stress can therefore disturb normal cellular signaling. ROS are produced through multiple mechanisms, e.g. by NADPH oxidase (NOX) enzymes, and in mitochondria, where about 1%–2% of electrons passing through the electron transport chain are incompletely reduced and give rise to the superoxide radical (\(O_2^\cdot\)). The ROS with the highest capacity to cause cellular damage is the hydroxyl radical, which, once formed, will alter DNA, RNA, proteins, lipids, or carbohydrates without being removed by any scavenging enzyme system.

The notion that diabetes is associated with oxidative stress has been suggested by several authors (150–154). For instance, increased lipid peroxidation and ROS generation were found in diabetic rats, measured as increased serum 8-epi-PGF2α levels (155) and increased electron spin clearance rate (156). Cyclic voltammetric studies have also indicated increased levels of lipid peroxidation in diabetic rats (157), and the isoprostane 8-epi-PGF2α is increased in embryos exposed to high glucose levels in vitro (128) and diabetes in vivo (158). Examination of litters of diabetic rats demonstrated lowered a-tocopherol (vitamin E) concentration in day-11 embryos and in the liver of day-20 fetuses (69).

The first evidence of an involvement of oxidative stress in the pathogenesis of diabetic embryopathy was the
demonstration that treatment of rodent embryos with antioxidative agents largely normalizes increased glucose-induced malformation rates in vitro (88, 90), observations that were repeated (91,123,159,160) and extended to in vivo studies (62,65,66,69,70,94,105,131,161–163). Furthermore, antioxidative treatment was found to normalize several markers of oxidative stress, such as serum 8-epi-PGF$_2$α levels (155), electron spin clearance rate (156), and the concentration of embryonic isoprostanes in vitro (128) and in vivo (158). Evidence for diabetes-induced oxidative stress has subsequently been found in several rat models of diabetic pregnancy (164).

Several different compounds with antioxidative properties have been shown to diminish embryonic maldevelopment resulting from exposure to high glucose levels in vitro or to a diabetic intrauterine environment in vivo. Thus, adding scavenging enzymes, e.g. superoxide dismutase (SOD) (88), catalase (88), or glutathione peroxidase (88), to the culture medium protects rat embryos from dysmorphogenesis induced by high glucose concentration in vitro. The antioxidant NAC blocks dysmorphogenesis in high-glucose-cultured rodent embryos (94,105,128,165–167) and neural crest cells (168,169), and addition of glutathione ester to high-glucose medium diminishes embryonic maldevelopment (91). Actually, teratogenic concentrations of beta-hydroxybutyrate or the branched chain amino-acid analog alpha-ketoisocaproic acid (KIC) can also be blocked by addition of SOD to the culture medium (90).

Analogously, dietary supplementations with antioxidative compounds have been shown to diminish diabetic embryopathy in vivo. Thus, administration of butylated hydroxytoluene (BHT) (62), vitamin E (66,69,163), vitamin C (70,163), and folic acid (170) decreases the malformed rate in the offspring of diabetic rats and largely improves embryonic and fetal growth in vivo. Alpha-lipoid acid supplementation has been found to reduce the diabetes-induced high incidence of resorptions and malformations in offspring of diabetic rodents (171–173). Embryos exposed to a diabetic intrauterine milieu have demonstrated diminished malformation rates after maternal supplementation of NAC (174). Combined supplementation of antioxidative compounds, e.g. vitamin E and C (162), or folic acid and vitamin E (147), to pregnant diabetic rats also diminished diabetes-induced dysmorphogenesis. In a study of glucose-induced cardiac malformations in a mouse model, the administered antioxidants decreased all negative effects of hyperglycemia/oxidative stress, such as hampered migration and increased apoptosis of neural crest cells, and prevented outflow tract defects (175). In addition, pregnant diabetic mice, transgenic for the CuZnSOD gene (SOD1) have fewer malformed offspring than the diabetic wild-type mice (138,139,142,176), illustrating the anti-teratogenic capacity of increased ROS scavenging activity.

Embryonic neural tissue subjected to high glucose concentrations shows increased superoxide production, as measured in a Cartesian diver system (177). One effect of increased intracellular ROS production would be inhibition of the rate-limiting enzyme of glycolysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), since this enzyme has displayed sensitivity to ROS in several different conditions of oxidative stress (178). This sensitivity resides in the thiol group of cysteine residue 149 in the active site of the enzyme (179,180). Oxidation of the thiol group by NO or ROS leads to decreased enzyme activity (181), and blocking of this process by antioxidants protects the activity of the enzyme (182). Another mechanism for GAPDH inhibition also results from mitochondrial production of ROS, activating poly-(ADP-ribose) polymerase 1 (PARP 1) by damaging DNA. PARP 1, in turn, induces ADP-ribosylation of GAPDH, leading to its inactivation and an accumulation of metabolites earlier in the metabolism pathway. In line with these considerations, decreased GAPDH activity was found in rat embryos subjected to a diabetic environment both in vivo and in vitro (183). Furthermore, addition of the antioxidant NAC prevented the decrease in activity (183). In addition, fetuses and embryos of diabetic rodents display increased rates of DNA damage (60,75,184), another indication of enhanced ROS activity and damage in embryonic tissues. High-amplitude mitochondrial swelling was demonstrated in embryonic neuroectoderm of embryos exposed to a diabetic environment (185,186). This swelling decreased after antioxidative treatment of the mother (74), implicating an embryonic ROS imbalance, with conceivable consequences for the rate of apoptosis in susceptible cell lineages in the embryo (167,187). The mitochondrial plays an important role in the apoptotic machinery, and previous studies have suggested that an altered apoptotic rate may affect the maldevelopment of embryos subjected to a diabetic milieu (93,145).

Furthermore, diabetic transgenic mice, overexpressing thioredoxin-1, have a lower incidence of malformations and decreased oxidative stress markers than diabetic wild-type mice (188), demonstrating a parallel relationship between dysmorphogenesis and degree of oxidative stress. Also, it has been suggested that diabetes-induced oxidative stress in embryos may cause maldevelopment via altered TNF-alpha levels (189).

In a recent study of the activity of AMP-activated kinase (AMPK) in embryos of hyperglycemic mice, it was demonstrated that maternal hyperglycemia stimulated AMPK activity and that stimulation of AMPK with 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) increased the rate of NTD in the embryos (190). In addition, stimulation of AMPK by hyperglycemia, hypoxia, or antimycin A could be inhibited by antioxidants. The AMPK inhibitor compound C blocked the effects of hyperglycemia or antimycin A on NTD occurrence, suggesting that diabetes/glucose-induced stimulation of embryonic AMPK activity is a teratogenic consequence of oxidative stress in diabetic embryopathy (190). In support of that notion, it was reported in a subsequent study that sole stimulation of AMPK disrupts embryonic gene expression and causes neural tube defects (191).

The bulk of data implicates oxidative stress and ROS excess as an important component in the etiology of diabetic embryopathy. The data also suggest that long-term exposure to high glucose creates embryonic ROS excess either from increased ROS production (177) or from diminished antioxidant defense capacity (91,159,192). The ROS excess may be small, restricted to particular cell populations (193,194), and likely to vary with gestational time and nutritional status, making direct ROS determinations difficult.
Increasing ROS levels in embryos lead to malformations (195,196), suggesting that ROS excess may also play a role in the teratogenic process(es) of phenytoin medication (197,198), ethanol abuse (193,194,199), and, possibly, thalidomide administration (200). Therefore, ROS excess may constitute a common element in a number of teratogenic situations, including diabetic pregnancy (cf. Figure 2) (201).

**Hypoxic stress**

In early organogenesis, oxygen levels are likely to be very low in the embryonic environment, and excess glucose metabolism could accelerate the rate of O₂ consumption, thereby exacerbating the hypoxic state. Since hypoxia can increase mitochondrial superoxide production, excessive hypoxia may contribute to oxidative stress. In a study of O₂ availability in embryos of glucose-injected hyperglycemic mice, it was found that O₂ availability was reduced by 30% in embryos of hyperglycemic mice (202). When pregnant hyperglycemic mice were housed in 12% O₂, the NTD rate increased 8-fold in the offspring. Conversely, housing pregnant hyperglycemic mice in 30% O₂ significantly suppressed the effect of maternal diabetes to increase NTD (202). These observations suggest that maternal hyperglycemia depletes O₂ in the embryo and that this contributes to oxidative stress and the adverse effects of maternal hyperglycemia on embryo development (Figure 2).

**Isoprostane formation**

Isoprostanes, e.g. 8-epi-PGF₂α, are prostaglandin-like compounds formed in situ from peroxidation of arachidonic acid by non-enzymatic, free radical-catalyzed reactions, and they therefore serve as indicators of lipid peroxidation (203–205). These non-classical eicosanoids possess potent biological activity as inflammatory mediators. Also, the formation of isoprostanes may, in itself, consume arachidonic acid, and therefore diminish the available pool of arachidonic acid in the embryo (see above). It has been shown that a diabetes-like environment increases the isoprostane levels in embryonic tissues (128). In addition, supplementation of 8-epi-PGF₂α to the culture medium caused malformations in whole-embryo culture, thereby illustrating the independent teratogenic activity of isoprostanes (206). Furthermore, adding SOD or NAC to the culture medium with isoprostane excess normalized almost all morphological and biochemical parameters, including the elevated tissue concentration of 8-epi-PGF₂α, thereby illustrating the teratogenic potential of diabetes/glucose-induced oxidative stress (cf. Figure 2) (206).

**AGE formation and RAGE activation**

The biochemical process of advanced glycosylation end-product (AGE) formation, which is accelerated in diabetes as a result of chronic hyperglycemia and increased oxidative stress, has been postulated to play a central role in the development of diabetic complications (207). AGES are known to accelerate oxidative damage to cells in a diabetic environment. Examples of AGE-modified sites are carboxymethyllysine (CML), carboxymethyllysine (CEL), and argpyrimidine. Under oxidative stress due to hyperglycemia in patients with diabetes, AGE formation is increased beyond normal levels.

RAGE, the receptor for AGE, is a transmembrane pattern recognition receptor. Except for AGES, RAGE has also other agonistic ligands: high mobility group protein B1 (HMGB1), S100/calgranulins, amyloid-beta, and MAC-1. The interaction between RAGE and its ligands is thought to result in pro-inflammatory gene activation. Ligand stimulation of RAGE initiates a signaling cascade resulting in activation of NFκB and activation of NAPDH-oxidase, thereby yielding increased intracellular oxidative stress. An increased accumulation of AGE has been found in the pathogenesis of several diabetic complications, such as cataract, retinopathy, atherosclerosis, nephropathy, and nephropathy. Inhibition or knockout of RAGE attenuates the detrimental effects of hyperglycemia in nephropathy and nephropathy (208).

It has also been suggested that AGE–RAGE activation is involved in diabetic embryopathy. Thus, the embryonic formation of glycated proteins (85,209,210) has been suggested to influence the teratological events in diabetic pregnancy. It has been shown in rodent embryos cultured in high glucose that the levels of the AGE precursor 3-DG increase in embryonic tissues, and the addition of 3-DG to the culture medium with physiologic concentrations of glucose induces malformations, an effect that is reversible with the addition of SOD (85).

In a recent study of diabetic embryopathy with RAGE knockout mice, it was found that maternal diabetes induced more fetal resorptions, malformations (facial skeleton, neural tube), and weight retardation in the wild-type fetuses than in the RAGE−/− fetuses, despite similar maternal hyperglycemia. In wild-type offspring, maternal diabetes increased fetal hepatic levels of 8-iso-PGF₂α and activated NFκB in the embryos, in contrast to unchanged 8-iso-PGF₂α levels and NFκB activity in diabetes-exposed RAGE−/− offspring. These findings suggest that RAGE activation and oxidative stress are associated phenomena in diabetic embryopathy (Figure 2).

**Nitrosative stress**

Nitrosamine overproduction, or ‘stress’, has been implicated in diabetic embryopathy, as a concomitant—and, in some studies, a ‘downstream’—event to oxidative stress. Thus, in a study of yolk sacs of CuZnSOD-(SOD1)-overexpressing embryos from normal and diabetic mice, it was found that the SOD1-transgenic embryos were largely protected from several of the negative effects of diabetes (139). Thus, diabetes-induced elevated markers of oxidative stress (4-hydroxynonenal and malondialdehyde reductions) were diminished in the SOD1-transgenic embryos compared to the wild-type embryos. Furthermore, hyperglycemia-increased iNOS expression and nitrosylated protein were also diminished, and caspase-3 and caspase-8 cleavages were blocked, in the SOD1-transgenic embryos. This finding suggests that oxidative stress induces iNOS expression, nitrosative stress, and apoptosis in diabetic embryopathy (139). In another
study, diabetic pregnant mice were fed via gavage an inhibi-
tor of nitric oxide (NO) synthase (NOS) 2-L-N6-(1-iminoethyl)lysine (L-NIL; 80 mg/kg), once a day from embryonic (E) day 7.5 to 9.5 during early stages of neurulation. The treatment significantly reduced the NTD rate in the embryos, compared with that in vehicle (normal saline)-treated diabetic animals (140). In addition to alleviation of nitrosative stress, ER stress was also ameliorated, as assessed by quantification of associ-
ated factors. Apoptosis was reduced, indicated by caspase-8 activation. These results show that nitrosative stress is import-
ant in diabetes-induced NTDs via exacerbating ER stress, lead-
ing to increased apoptosis (140). The combined observations suggest a role for nitrosative stress in diabetic embryopathy, and suggest a ‘cross-talk’ between oxidative stress, nitroso-
mine stress, and ER stress (see below and Figure 2).

**ER stress**

ER stress, also named unfolded protein response (UPR), is a cellular stress response related to the ER, which is induced by an accumulation of misfolded proteins in the ER lumen. The ER stress/UPR diminishes protein translation, degrades mis-
folded proteins, and produces chaperones involved in protein folding in order to restore normal ER function. If this is not achieved, or the disruption is prolonged, the ER stress/UPR shifts towards promoting apoptosis.

In a study of the effects of maternal diabetes on the develop-
ment of oocytes and early embryos by using time-lapse live cell imaging confocal microscopy, the ER displayed an increased percentage of homogeneous distribution patterns throughout the entire ooplasm during oocyte maturation and early embryo development. In addition, a higher frequency of large ER aggregations was detected in oocytes and two-cell embryos from diabetic mice. These results suggest that the diabetic condition adversely affects the ER distribution pattern during mouse oocyte maturation and early embryo develop-
ment (211).

In a study of oxidative and ER stress in SOD1-overexpress-
ing mice, it was found that maternal diabetes causes increased levels of ER stress markers, e.g. C/EBP-homologous protein (CHOP), calnexin, phosphorylated (p)-elf2alpha, p-
PERK, and p-IKE1alpha; triggered XB1 mRNA splicing; and enhanced ER chaperone gene expression in wild-type embry-
as, whereas all these changes were blocked in the embryos of diabetic transgenic mice. This supports the notion that diminishing diabetes-induced oxidative stress, e.g. by SOD1 overexpression, blocks ER stress in embryos (142).

A downstream effect of the ER stress would be an activa-
tion of C-Jun N-terminal kinase (JNK). The possible relation-
ship between JNK1/2 activation and ER stress in diabetic embryopathy was investigated in mice. Maternal diabetes increased ER stress markers and induced swollen/enlarged ER lumens in embryonic neuroepithelial cells during neurulation. Deletion of both jnk1 or jnk2 genes diminished hyperglycemia-increased ER stress markers and ER chaperone gene expression. In high-glucose cultured embryos, the addition of the ER chaperone 4-phenylbutyric acid (4-PBA) diminished ER stress markers and abolished the activation of JNK1/2 and its downstream transcription factors, caspase-3 and caspase-8, as well as Sox1 neural progenitor apoptosis. Consequently, 4-PBA blocked high-glucose-induced NTD in vivo. It was concluded, therefore, that hyperglycemia induces ER stress, which yields activation of the proapoptotic JNK1/2 pathway, which yields induced neural tube apoptosis, and thereby NTD (212).

Autophagy is an intracellular process to degrade dysfunc-
tional proteins and damaged cellular organelles, which, in addition, regulates embryonic cell proliferation, differentiation, and apoptosis. Furthermore, blockage of this process in embryos causes NTDs reminiscent of those observed in dia-
abetic pregnancies. In a study of NTD induction in diabetic mice with or without 2%-5% trehalose water, the role of autophagy was investigated. Maternal diabetes suppressed autophagy in neuroepithelial cells and altered autophagy-
related gene expression. Trehalose treatment reversed autophagy impairment and prevented NTDs in the embryos of diabetic pregnancies. The study demonstrates that mater-
nal diabetes suppresses autophagy in neuroepithelial cells of the developing neural tube, leading to NTD formation (213).

In a recent study of diabetes-induced cardiac malforma-
tions, it was found that the rate of atrio-ventricular septal defects (AVSDs) was increased concomitant with enhanced ER stress in embryonic hearts. Blocking of glucose-induced ER stress with 4-PBA in an endocardial cushion explants culture restored endocardial cell migration. The findings suggest that development of the endocardial cushions is susceptible to the insult of maternal hyperglycemia, and that diabetes-
induced ER stress in the developing heart mediates the nega-
tive effect on endocardial cell migration (214). The studies suggest that ER stress is involved in the teratogenesis of neural and cardiac malformations in embryos exposed to dia-
abetes or hyperglycemia, and that ER stress, nitrosative stress, and oxidative stress each enhance other (Figure 2).

**Hexosamine stress**

Increased ambient glucose concentrations yield increased uptake, phosphorylation, and metabolism of glucose, primarily by enhanced flux in the glycolytic pathway and, also, in the hexosamine biosynthetic pathway (109). This is a pathway that converts glucose to uridine diphosphate N-acetylglucos-
amine (UDP-GlcNac).

Under normoglycemic conditions, approximately 1%-3% of total glucose consumed by somatic cells are directed down the hexosamine pathway (215). UDP-GlcNac is the substrate for the majority of glycosylation in the cell, producing mucopolysaccharides (216). In this process UDP-
GlcNac is attached to serine or threonine residues of pro-
teins, thus becoming a post-translational modification (beta-
O-linked glycosylation), which regulates protein function in an analogous manner to phosphorylation (217). Altered beta-O-linked glycosylation has been associated with a num-
ber of disease states, including cancer, inflammatory condi-
tions, and neurodegenerative diseases (218). Notably, it is also implicated as a primary mechanism behind the
development of insulin resistance and pancreatic beta-cell destruction in type-2 diabetes (215,218).

It has been suggested that hexosamine stress may play a role in diabetic teratogenesis (143,144,219). Indeed, defect development has been demonstrated in pre-implantation mouse embryos, treated with glucose (27 mM) or glucosamine (0.2 mM), which was added to embryo culture media. Both treatments disturbed embryo development, increased apoptosis, and decreased cell number in the resulting blastocysts (219). Addition of benzyl-2-acetamido-2-deoxy-a-D-galactopyranoside (BADGP), an inhibitor of O-linked beta-N-acetylglucosaminyltransferase (OGT), the enzyme which adds O-GlcNAc to proteins, rescued all these phenotypes in the hyperglycemia treatment group, although only mild improvement was seen in the glucosamine group (219). This may reflect the relative potencies of each hexose in their capacity to stimulate the UDP-GlcNAc production (215). In another study, pregnant mice were injected with glucose to induce hyperglycemia, or glucosamine, to activate the hexosamine pathway directly. Both treatments increased the NTD rate in the embryos, decreased GSH levels, and increased oxidative stress, as indicated by increased 2,7-dichloro-dihydrofluorescein fluorescence. Glucose and glucosamine also inhibited expression of Pax-3; however, all these effects were prevented by GSH ethyl ester administration (143). These findings suggest a role for hexosamine stress in diabetic embryopathy (Figure 2).

**Apoptosis**

The notion that apoptosis may be a component of the teratogenic process of diabetic pregnancy has been studied thoroughly. Thus, there are several reports of increased rates of apoptosis in embryos exposed to a diabetic environment (77,78,146–149,167,187,220–222). In particular, there are findings indicating increased apoptotic rates already in pre-implantation embryos (93,114,145).

In a study of early post-implantation embryos, the expression of Bcl-2 mRNA was decreased and the number of deoxynucleotidyl transferase-mediated nick end labeling (TUNEL)-positive cells increased in embryos of diabetic rats compared to control embryos. These results suggest that a Bax-regulated mitochondrial cytochrome c-mediated caspase-3 activation pathway might be involved in the diabetic embryopathy (146). In another early study, it was reported that combined supplementation of folic acid and vitamin E to pregnant diabetic rats diminished diabetes-induced dysmorphogenesis and normalized apoptotic-associated protein levels (147). In another study of rodent embryos subjected to high glucose in vitro or diabetes in vivo, disturbed development was found, concomitant with increased activation of caspase-3 and other markers of apoptosis. Supplementation of NAC or an apoptosis inhibitor diminished both the dysmorphogenesis and apoptosis (167). Exposure to a diabetic milieu during organogenesis thus increases dysmorphogenesis and apoptosis in embryos.

In mice transgenic for the thioredoxin-1 gene with overproduction of the antioxidant thioredoxin-1, the incidence of diabetes-induced malformation is markedly lower in diabetic transgenic mice compared with diabetic wild-type mice. Furthermore, the diabetes-induced increased markers for oxidative stress, apoptosis-promoting proteins, and cleaved caspase-3 production are all diminished in the offspring of diabetic transgenic mice compared with the offspring of diabetic wild-type mice (188).

A new apoptotic pathway was recently suggested to be activated by a diabetic/hyperglycemic environment, involving the gene products of the ASK1-FoxO3a-TRADD-caspase-8 gene (149). Blocking components of this pathway diminished the NTD rate in diabetic mice. Thus, hyperglycemia-induced apoptosis and the development of NTD was reduced with genetic blockage of either FoxO3a or Casp8, or by inhibition of ASK1 by thioredoxin. In addition, examination of human neural tissues affected by neural tube defects revealed increased activation or abundance of the genes in the cascade. The conclusion was that activation of the ASK1-FoxO3a-TRADD-caspase-8 pathway participates in the development of NTDs, which could be prevented by inhibiting intermediates in this cascade (149). There is, clearly, strong experimental evidence for a role of apoptosis in diabetic embryopathy (Figure 2).

**Genetics and epigenetics of diabetic dysmorphogenesis**

Several lines of evidence support the notion that the metabolic alterations in the embryonic tissues are followed by changes in genetics (gene expression) and epigenetics (regulation of gene expression). Also there should exist permissive conditions in the mother and offspring that pave the way for the diabetes-induced genetic/epigenetic changes that ultimately lead to embryonic maldevelopment, i.e. genetic predisposition in mother and child.

**Genetic predisposition**

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 (223,224). Although predisposing genetic conditions for diabetes are clearly present in offspring of diabetic parents (225,226), as the offspring of a diabetic father has higher risk of developing the disease than the offspring of a diabetic mother (227–231), it has been established that diabetic men do not have an increased risk of fathering malformed offspring (232,233). This indicates that the genes predisposing for diabetes do not induce congenital malformations. In contrast, maternal diabetes has been suggested to be associated with Down’s syndrome (234–236) and has also been suggested to predispose for optic nerve hypoplasia in female offspring (237). A genetic element may be present in the etiology of diabetic embryopathy (238), a notion supported by experimental data (58,84,138,239–242).

The contribution of the fetal genome and maternal (diabetic) environment was evaluated in a rat model where the
outcome of diabetic pregnancy in two outbred substrains of Sprague-Dawley rats (with low incidence, H, and high incidence, U, of skeletal malformations in the offspring), and F1 hybrids between them was studied (84). The fetuses of diabetic H mothers had no skeletal malformations, regardless of embryo type (H/H or H/U). When the diabetic mother was U or from the hybrid strain (H/U) and the offspring of the mixed H/U type, increased skeletal malformation (3%-5%) rates resulted. When the embryos contained a major U genome, either U/U or U(H/U), further increased skeletal malformations (17%-19%) were found. These findings indicate that both the maternal and fetal genomes are involved in the etiology of diabetes-induced (skeletal) malformations in rodent diabetic pregnancy (84). When pre-implantation embryos were transferred from diabetic NOD mice to non-diabetic ICR mice and allowed to develop until gestation day 13, as were embryos from the reverse transfers, as well as from ICR-to-ICR transfers, we found 8/58 (14%), 18/45 (40%), and 0/73 (0%) malformed embryos in the NOD-ICR, ICR-NOD, and ICR-ICR transfers, respectively. The result thus suggests that both the embryo genotype and the maternal environment are of importance for diabetic embryopathy (58). Moreover, in a recent study one-cell mouse zygotes and blastocysts were transferred from diabetic or control mice into non-diabetic pseudopregnant female recipients, and were evaluated at embryonic day 14 (243). The offspring from the diabetic rats had higher rates of malformations than the controls, and the conclusion was that exposure to maternal diabetes during oogenesis, fertilization, and the first 24 hours is enough to program permanently the fetus to develop significant morphological changes (243). In order to characterize the relative importance of the maternal and paternal genome in relation to the teratogenicity of the maternal (intrauterine) milieu, rats from two different strains were cross-mated. Thus, male rats from a malformation-prone (L) and a malformation-resistant (W) strain were mated with diabetic females from the other strain to produce F1 offspring: LW (L male x W female) and WL (W male x L female), which would be genetically identical with exception of imprinted genes and mitochondrial types. However, the malformation rate was 0% in the LW and 9% in the WL offspring (244), demonstrating both a teratologic ‘dilution’ effect, as well as the importance of the maternal environment. Based on metabolic data from the two types of pregnancy (indicating a more disturbed metabolic state in the diabetic L rats), the study suggested that the fetal genome controls the embryonic dysmorphogenesis in diabetic pregnancy by instigating a threshold level for the teratological insult and that the maternal genome controls the teratogenic insult by (dys)regulating the maternal metabolism (244). Furthermore, when the F1 crosses were mated in a subsequent study, the malformation rate of the F2 combinations WL x WL_diabetic and LW x LW_diabetic was around 5% (245), a further dilution of the teratogenic induction; however, the malformed WL x WL offspring had only agnathia/micrognathia, whereas the malformed LW x LW offspring had 60% agnathia/micrognathia and 40% cleft lip and palate. Thus, despite identical autosomal genotypes, the diabetic WL and LW female rats gave birth to offspring with markedly different malformation patterns. This study suggested a teratological mechanism in diabetic pregnancy influenced by maternal metabolism and parental strain epigenetics (245).

In a previous study, it could be demonstrated that a specific variant of the catalase enzyme is present in rats that are malformation-prone (Cs-1a), whereas another variant of the catalase protein was present in rats that do not develop malformations in response to maternal diabetes (Cs-1b) (241). Thus, embryonic catalase activity was lower in embryos from normal U rats than in embryos from normal H rats, and maternal diabetes augments this difference (242). The catalase cDNA and the promotor region of the catalase gene in the U and H rat were sequenced (246) and yielded one nucleotide mutation in the 5′-UTR region of the U rat cDNA and a heterozygocity in the U rat gene promoter. Therefore, the decreased catalase mRNA levels may result from different regulation of transcription (promotor), and the difference in the electrophoreotic mobility in zymograms (241) may be a result of post-translational modifications of the catalase protein.

Using an inbred Sprague-Dawley strain (L) with about 20% skeletal malformations when the mother is diabetic, and inbred Wistar Furth rats (no diabetes-inducible skeletal malformations), a global gene linkage analysis of the skeletal malformations was performed with micro-satellites, a study which yielded strong coupling of the malformations to seven regions on chromosomes 4, 10, 14, 18, and 19, and a weaker coupling to 14 other loci in the genome. Altogether we found loci on 16 chromosomes. Searching for candidate genes within a distance of 10 cM from each micro-satellite yielded 18 genes that had been implicated in previous studies of diabetic embryopathy. These genes were involved in embryonic development/morphogenesis (Map1b, Shh, Tgfb3, Vegfa, Dvl2, Nf1, Gsk3b, Gap43, Tgfb3, Gdf1, Csfr1) (247–253), regulation of DNA/RNA metabolism (En2, Brc3, Tp53) (166,247,254–256), regulation of apoptosis (Noi3, Bak1) (247), and cellular metabolism (Folr1, Akr1b1) (170,254,257).

**Gene expression**

Altered gene expression in the offspring has been demonstrated in several studies (247,250,252–254,258–262) and appears to be an integral component of the diabetic embryopathy (263). Pax3 gene expression was found to be reduced in embryos of diabetic mice (77,78), and this transcription factor may regulate the gene expression of the licensing factor cdc-46 (264) and a gene, Dep-1 (265), as well as p53 (255), all of which may be of importance for a correct neural tube closure. Null mutation of the Pax3 gene yields the Splotch mouse displaying neural tube defects (77,266). It has also been shown that the decreased Pax3 expression in embryos of diabetic mice could be normalized by treatment of the mother with antioxidants (202), thereby demonstrating a coupling between ROS excess and a teratologically important change in gene expression. In a later study, neural crest cells (NCCs) of Pax3-deficient embryos displayed impaired migration and increased apoptosis. Suppression of p53, either by null mutation of the p53 gene, or administration of a p53 inhibitor, pifithrin-alpha,
prevented the defective NCC migration and apoptosis in Pax3-deficient embryos, and also restored proper development of cardiac outflow tracts. Pax3 thus appears required for cardiac outflow tract septation because it blocks p53 expression during NCC migration (256).

The embryonic expression of genes controlling the defense against oxidative stress is sensitive to maternal diabetes. Thus, in a study of pregnant diabetic rats, the embryos demonstrated decreased expression of CuZnSOD and MnSOD (267). In addition, the expression of Gpx-1 and Gpx-2 was decreased compared with embryos from normal rats, enzymes that function in the detoxification of hydrogen peroxide, an important antioxidant system. The decrease in gene expression of Gpx-1 was further enhanced in the malformed embryos. The immunostaining of Gpx-1 displayed a general accumulation of positive cells in the cardiac tissue of all embryos. However, the non-malformed embryos had less staining than embryos from normal rats, and malformed embryos from diabetic rats had almost no staining at all (267).

In a study of cardiac malformations in diabetic mouse pregnancy demonstrating dilated heart tube, smaller ventricles, conotruncal stenosis, and abnormal heart looping, ventricular septal defects were observed and actors in the TGF-β signaling that regulate heart development were down-regulated by maternal diabetes. It was concluded that the TGF-β signaling is involved in cardiac malformations in diabetic embryopathy (250). Also, in a study of global gene expression in a transgenic mouse model of cugal dysgenesis, and in a pharmacological model using in situ hybridization and quantitative real-time PCR, altered expression of several molecules that control developmental processes and embryonic growth was observed. The most pronounced finding was that of altered Wnt signaling, which suggests that impaired signaling in this pathway may be involved in diabetic embryopathy (252). A genome-wide investigation of gene expression in embryos from normal and diabetic mice followed by quantitative RT-PCR yielded several genes with altered expression. Sequence motifs in the promoters of diabetes-affected genes suggest potential binding of transcription factors that are involved in responses to oxidative stress and/or to hypoxia, and, furthermore, around 30% of the diabetes-affected genes encoded transcription factors and chromatin-modifying proteins or components of signaling pathways that affect transcription (258). In a genome-wide expression profiling in the developing heart of embryos from diabetic and control mice it was found that a total of 878 genes exhibited more than 1.5-fold changes in expression level in the hearts of experimental embryos compared with their respective controls. Several genes involved in a number of molecular signaling pathways such as apoptosis, proliferation, migration, and differentiation in the developing heart were differentially expressed in embryos of diabetic pregnancy (262).

Several different genes and pathways have been demonstrated to be affected in diabetic pregnancy; however, there is not, as yet, any universal gene identified to be responsible for enhanced (or decreased) susceptibility to diabetic embryopathy (Figure 2).

Epigenetics

There are considerable indications that epigenetic processes play a role in diabetic embryopathy (259,268–274). In a seminal study, it was found that transient hyperglycemia induced long-lasting activating epigenetic changes in the promoter of the NFκB subunit p65 in aortic endothelial cells both in vitro and in non-diabetic mice, resulting in increased p65 gene expression. Both the epigenetic changes and the gene expression changes persisted for at least 6 days of subsequent normoglycemia. Furthermore, the hyperglycemia-induced epigenetic changes and increased p65 expression were prevented by reducing mitochondrial superoxide production or superoxide-induced alpha-oxaldehydes (268).

Analysis of gene expression data from two sets of embryos of diabetic mice suggested that maternal diabetes may increase the overall variability of gene expression levels in embryos. The suggestion was that altered gene expression and increased variability of gene expression together constitute the molecular correlates for the incomplete phenotype penetrance in diabetic pregnancy. Based on this model, it was suggested that maternal diabetes reduces the precision of gene regulation in exposed individuals. Loss of precision in embryonic gene regulation may include changes to the epigenome via deregulated expression of chromatin-modifying factors (259,269).

In a study of maternal diabetes effect on DNA methylation of imprinted genes in oocytes, it was found in SZ-diabetic and NOD mice that the methylation pattern of Peg3 different methylation regions (DMR) was altered, and in the SZ mice demethylation was observed on day 35 after SZ injection. The expression level of DNA methyltransferases (DNMTs) was also decreased in diabetic oocytes. These results indicate that maternal diabetes has adverse effects on DNA methylation of the maternally imprinted gene Peg3 in oocytes, but also that methylation in the oocytes of the offspring is normal (271). Also, the expression was increased and the methylation level of H19 was decreased, whereas the expression and methylation levels of Peg3 were completely opposite in placentas of diabetic mice. When embryos of normal females were transferred to normal/diabetic pseudopregnant females, the methylation and expression of Peg3 in placentas were also clearly altered in the normal-to-diabetic group compared to the normal-to-normal group. However, when the embryos of diabetic females were transferred to normal pseudopregnant female mice, the methylation and expression of Peg3 and H19 in placentas were similar in the two groups. The data suggest that the effects of maternal diabetes on imprinted genes may primarily be caused by the adverse uterus environment (272).

In a study neural stem cells (NSCs) were exposed to high glucose/hyperglycemia, and alterations were found in chromatin reorganization, global histone H3 status, and global DNA methylation, as well as increased expression of Dcx and Pafah1b1 and decreased expression of four microRNAs targeting these genes. This study suggested that hyperglycemia alters the epigenetic mechanisms in NSCs, resulting in altered expression of developmental genes (273). In a genome-wide survey of histone acetylation in neurulation stage embryos
from diabetic mouse pregnancies, it was found that exposure to maternal diabetes and, independently, exposure to high-fat diet were associated with increases and decreases of H3 and H4 histone acetylation, respectively, in the embryo. These data suggest that epigenetic changes in response to diet and metabolic conditions may contribute to increased risk for NTD in diabetic and obese pregnancies (270).

In a study of embryos of pregnant hyperglycemic mice and mouse embryonic stem cells (ESC), the methylation of a Pax3 CpG island was decreased in embryos and ESC. Use of shRNA in ESC demonstrated that DNA methyltransferase 3b (Dnmt3b) was responsible for methylation and silencing of Pax3 prior to differentiation and by oxidative stress. These results indicate that hyperglycemia-induced oxidative stress stimulates Dnmt3b activity, thereby inhibiting chromatin modifications necessary for induction of Pax3 expression, and, thus, providing a molecular mechanism for defects caused by Pax3 insufficiency in diabetic pregnancy (274).

Epigenetic changes in the embryo caused by maternal diabetes are likely to be transferring the teratogenic input of the diabetic environment. Identifying these changes is important both for the increased knowledge generated, and also for the possible anti-teratological treatment that may emerge from the identified mechanisms (Figure 2).

Conclusions and future directions

Diabetic embryopathy has a complex etiology and pathogenesis. The studies of etiologic factors in the pathogenesis of congenital malformations have revealed a scenario in which the diabetic state simultaneously induces alterations in a series of teratogenically capable pathways. These pathways are intertwined, and several of them result in an imbalance of the ROS metabolism, yielding ROS excess in teratogenically sensitive cell populations, an imbalance ultimately causing the congenital malformations. Blocking the ROS excess may therefore be one valid way to diminish the disturbed development caused by the diabetic environment.

Upstream and downstream of the oxidative stress, however, several conditions of cellular stress are present, such as nitrosative, ER, hypoxic, and hexosamine stress, as well as a possible teratogenic involvement of AGES. In this area of metabolites and pathways, there may be possibilities of finding molecules to use for intervention therapy.

There is also a growing understanding of the diabetes-induced alterations in genetic and epigenetic systems, which will, again, increase our knowledge, and inspire to develop new ways to block diabetic embryopathy in the future.

Disclosure statement

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