Upsala J Med Sci 102: 61–98, 1997

Congenital Malformations in Experimental Diabetic Pregnancy: Aetiology and Antioxidative Treatment

Minireview based on a doctoral thesis

Martin Simán

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

Abstract

Diabetes mellitus in pregnancy causes congenital malformations in the offspring. The aim of this work was to characterize biochemical and morphologic anomalies in the conceptus of an animal model of diabetic pregnancy. In addition, a preventive treatment against diabetes-induced dysmorphogenesis was developed.

Congenital cataract was often found in the offspring of diabetic rats. The fetal lenses had increased water accumulation, sorbitol concentration and aldose reductase activity compared to control lenses. The results suggest that the cataracts form via osmotic attraction of water due to sorbitol accumulation in the fetal lens.

Another set of malformations, with possible neural crest cell origin, occurred frequently in offspring of diabetic rats. These included low set ears, micrognathia, hypoplasia of the thymus, thyroid and parathyroid glands, as well as anomalies of the heart and great vessels. Furthermore, diabetes caused intrauterine death and resorptions more frequently in the late part of gestation.

When the pregnant diabetic rats were treated with the antioxidants butylated hydroxytoluene, vitamin E or vitamin C, the occurrence of gross malformations was reduced from approximately 25% to less than 8%, and late resorptions from 17% to 7%. This suggests that an abnormal handling of reactive oxygen species (ROS) is involved in diabetes-induced dysmorphogenesis *in vivo*. Indeed, an increased concentration of lipid peroxides, indicating damage caused by ROS, was found in fetuses of diabetes rats. In addition, embryos of diabetic rats had low concentrations of the antioxidant vitamin E compared to control embryos. These biochemical alterations were normalized by vitamin E treatment of the pregnant diabetic rats.

The antioxidants are likely to have prevented ROS injury in the embryos of the diabetic rats, in particular in the neural crest cells, thereby normalizing embryonic development. These results provide a rationale for developing new anti-teratogenic treatments for pregnant women with diabetes mellitus.

Introduction

Diabetes mellitus is a disease characterized by abnormal carbohydrate handling caused by reduced/absent insulin production (diabetes type 1, Insulin Dependent Diabetes Mellitus, IDDM) or by decreased sensitivity to insulin (diabetes type 2, Non-Insulin Diabetes Mellitus, NIDDM). The diabetic individual is prone to develop complications induced by the diabetic state, which may affect the morphology and function of the vascular system, kidney, peripheral nerves, skin, retina, lens, and - in the case of pregnancy - the developing conceptus. The risk for acquiring one of these complicating conditions is closely related to the glycaemic control prior to onset, suggesting that the aetiology may be similar for several of these complications. In particular diabetic embryopathy has been suggested to possess aetiologic characteristics in common with retinopathy and neuropathy. The present communication reviews some of the literature on pregnancy in diabetes and discusses some recent advances on the mechanisms behind and possibilities for preventive treatment of congenital malformations in an experimental model for diabetic pregnancy.

Clinical background; spontaneous abortions and congenital malformations in pregnancy complicated by type 1 diabetes

One of the greater challenges for obstetric medicine is given by IDDM, although the disease complicates less than 0.5% of all pregnancies (66). Before the discovery of insulin, the fetal mortality rate not only reached approximately 60% but diabetic pregnancy was also associated with high maternal mortality (36, 223). The introduction of insulin, however, improved the situation drastically by diminishing maternal and fetal morbidity and mortality (62). Several problems with IDDM and pregnancy remain, such as maternal infections, pre-eclampsia, nephropathy, retinopathy and neuropathy. The effects of IDDM on the conceptus include altered fetal growth, polyhydramnios, fetal and neonatal loss, and congenital malformations (66, 128).

During the period from World War II to the 1980s, the malformation rate is generally reported to be 2-3 times higher in diabetic pregnancy than in non-diabetic pregnancy (6, 77, 97, 114, 123, 124, 133, 135, 137, 143, 144). With intensified insulin treatment, the occurrence of congenital malformations has been reduced, thus indicating the importance of good metabolic control for normal embryonic development (85, 112, 147, 163, 174, 208). A high glycosylated haemoglobin (HbA_{1c}) concentration in maternal serum during early gestation is associated

with a high risk of congenital malformations and spontaneous abortions (77, 85, 133, 174, 208). There are some conflicting reports on the correlation of HbA_{1c} with malformations (135, 163) and spontaneous abortions (102), but these reports have only a few observations of patients in bad metabolic control. Taken together, the compiled data from all studies suggest the existence of a threshold for the degree of severity of the maternal diabetic state, a threshold above which spontaneous abortions and congenital malformations occur in greater frequency in diabetic compared to normal pregnancy (77). This threshold has been suggested to be reflected by an HbA_{1c} value of about 10% (77, 174).

The organ systems that most often develop abnormalities during fetal life due to diabetes in the mother are the skeletal (*e.g.* caudal regression syndrome), circulatory, neural, renal and gastrointestinal. In addition, two extremely rare anomalies, caudal regression syndrome and situs inversus occur at much higher relative frequency in diabetic pregnancy (6, 114, 130, 134, 169).

Table 1. *Occurrence of malformations in children of diabetic mothers. Percentage of all malformations in offspring of an IDDM population, and relative malformation frequency in offspring of diabetic mothers compared to the malformation rate in offspring of non-diabetic mothers. ND: not determined.*

Type of malformation:	% of all malformations Ref. (130)	Relative risk Ref. (114)
Caudal regression	5	252
Situs inversus	ND	84
Cardiac anomalies	31	4
CNS anomalies	31	2-3
UG anomalies	15	5
GI anomalies	3	3

However, it should be remembered that only 8-16% of babies with caudal regression have mothers with IDDM (66). In absolute numbers cardiac and CNS defects occur most commonly in IDDM pregnancies (4% and 1.9%, respectively) (66). The diabetes induced cardiac malformations are primarily found in the ventricular septum and the outflow tract of the heart (54). The CNS defects are mainly spina bifida, hydrocephalus and anencephalus. In addition to the major malformations of neural tissue, disturbed learning and impaired psychomotor performance have been reported in some infants of diabetic mothers, especially in children in whom a reduced body size was observed in the early part of pregnancy (106, 164).

The congenital malformations in diabetic pregnancy are likely to be induced before the seventh week of gestation (134). This conclusion is based on the assumption that the malformations are induced primarily during the period of organogenesis, *e.g.* gestational weeks 3-8 in human pregnancy (176) and is corroborated by experimental findings in diabetic rats. By systematically omitting insulin treatment during different time periods of pregnancy in rats, it was shown that the teratogenic period for skeletal malformations occurs between gestational day 6 and 10 in this species (39, 42). Interestingly, it was not possible to decrease the length of the teratogenic period.

Although the clinical appearance of dysmorphogenesis induced by diabetes is well defined, the cellular and embryonic mechanisms remain unclear. In addition, anti-teratogenic treatment of pregnant diabetic women, other than intensified metabolic control, has not yet been reported. Therefore, research in experimental models is needed to investigate teratogenic mechanisms and to evaluate the potential of new therapeutic regimens.

Experimental models for studies of diabetes-induced congenital malformations

In order to obtain information on the underlying mechanisms of complications in the offspring of women with diabetes, several experimental models have been used. These include pregnancy in animals with spontaneous or experimentally-induced diabetes, culture of whole embryos (preimplantatory or during organogenesis), and culture of embryonic tissue or cells in a diabetes-like environment.

The most important complications in human offspring of diabetic pregnancy are macrosomia, spontaneous abortion and congenital malformations. Rodents are most often used in animal models of diabetic pregnancy and the alterations in growth and development of the offspring show both similarities and dissimilarities with human embryo/fetal changes.

In the first trimester of human pregnancy, the embryo of a diabetic mother tends to be smaller than that of a non-diabetic mother, as demonstrated by early ultra-sound measurement (162). In rodent embryos before implantation, high ambient glucose concentration or a diabetic uterine environment were shown to selectively hamper the growth of the inner cell mass which is destined to form the embryo proper (159). The macrosomia observed at birth in human diabetic

pregnancy is caused by excessive growth in late gestation (156). In diabetic rodents macrosomia may be found only in mild maternal diabetes or if the animals are allowed to give birth spontaneously (1, 51, 117, 166, 191). It should, however, be noted that diabetic animals often give birth later than the controls (40). If the gestation is interrupted at a defined time point in pregnancy, decreased fetal weight is a common observation (24, 33, 38-42, 45, 47-49, 67, 69, 81, 171-173, 189, 216, 228, 230). The differences between humans and rodents may originate in the relatively shorter fetal period in rodents. Thus, the finding of hampered embryonic growth in diabetic rat pregnancy is difficult to interpret with respect to human gestation. The rodent models may mainly represent early human diabetic pregnancy and thus, embryonic size may still be a valid estimate of disturbed development.

Rodents do not abort embryos or fetuses dying *in utero*. Instead, the dead conceptus is resorbed by the surrounding tissue. Similar resorptive events may be encountered in human twin pregnancy when one of the fetuses die. Little is known about the mechanisms causing intrauterine death. In experimental non-diabetic pregnancy in mice, increased concentrations of macrophages and the growth factor TNF-α in the uterine wall have been associated with resorptions in early pregnancy (35), thus indicating that the "soil" rather than the "seed" is defective (141). Furthermore, in experimental diabetic pregnancy an increased TNF-α concentration has been found in the uterus (160) which may be involved in the abnormal early development of the conceptus. Intrauterine death in the late part of pregnancy, however, may be caused by organ malformations (21), possibly in the circulatory system.

Studies of congenital dysmorphogenesis in animal models of diabetic pregnancy have demonstrated gross malformations (*e.g.* omphalocele, exencephaly, micrognathia, cleft palate, subcutaneous edema), visceral malformations (vacuolar cataract, microphthalmia, coloboma, heart anomalies, dilatation of the renal pelvis and ureter) and skeletal malformations (fused ribs, reduced bone ossification) when the offspring were studied perinatally (33, 38, 47, 64, 67, 69, 140). In a study of NOD mice, an increased frequency of situs inversus with splenic abnormalities was found (140). Studies of embryos in early gestation mainly report disturbed closure of the neural tube (2, 74, 189, 216). As discussed above, the critical time point for malformations has been defined in experimental models to occur during organogenesis (39, 42). Hence, the animal models produce several malformations similar to those seen in children of diabetic mothers.

Another important approach in the search for mechanisms of disturbed development is the culture of whole embryos *in vitro* (146). By systematically testing different metabolites that are altered in diabetes, it has been shown that glucose, ketone bodies, branched chained amino acids, and somatomedin inhibitors can induce malformations in the embryos (29, 177, 178, 228). Furthermore, addition of arachidonic acid, prostaglandins, inositol, and antioxidants (4, 43, 44, 90, 165) have the potential to prevent abnormal development caused by diabetes-related substances (*i.e.* mainly high glucose concentration). Culture experiments with dispersed cells or isolated neuroepithelium have enabled the study of morphologic development (199, 200, 202, 204) and biochemical alterations (58, 229) of embryonic tissue in a diabetes-like environment.

Based on the animal and culture experiments, several hypotheses for the cause of diabetes-induced teratogenesis have been suggested. These include deficiency in myo-inositol (2, 4, 89-91, 171, 197, 198, 218), arachidonic acid (74, 165, 173), and prostaglandin (4, 76, 181, 219) concentration, excess production of sorbitol (46, 49) and 3-deoxyglucosone (52), abnormal handling of reactive oxygen species (ROS) (43, 45) and increased levels of somatomedin inhibitors (178). It is likely that several of these mechanisms act simultaneously, thus providing a multifactorial aetiology for the diabetes-induced dysmorphogenesis (20). The following summary will concentrate on two of the proposed mechanisms for diabetes-induced complications, *i.e.* enhanced flux in the sorbitol pathway, which appears to have adverse effects on fetal lens development, and an excess of ROS and its potentially harmful effects on neural crest cells (NCC).

Aetiology of diabetes-induced embryonic dysmorphogenesis

The polyol pathway in lenticular complications

Increased concentration of sorbitol has been found in several tissues affected by increased glucose concentration *e.g.* nerves, kidneys, embryos and lenses (27, 49, 65). Two enzymes are involved in the polyol pathway; aldose reductase (AR) and sorbitol dehydrogenase (SDH). In the first reaction, AR reduces glucose to sorbitol

$$\text{I.}\quad \text{Glucose} + \text{NADPH} \xrightarrow{\text{AR}} \text{Sorbitol} + \text{NADP}^+$$

and in the second reaction sorbitol is catalysed to fructose by SDH.

$$\text{II.}\quad \text{Sorbitol} + \text{NAD}^+ \xrightleftharpoons{\text{SDH}} \text{Fructose} + \text{NADH}$$

Under normal conditions only small quantities of glucose are metabolized in the polyol pathway due to rapid phosphorylation of intracellular glucose (86). In diabetes-induced hyperglycaemia, however, a large proportion of the increased intracellular glucose remaines unphosphorylated due to saturation of the phosphorylating enzymes, and is therefore shunted through the polyol pathway. This pathway may metabolize up to one-third of the available glucose, which yields an accumulation of sorbitol in the tissue (27). Similarly, other hexoses may be metabolized by AR. For example, AR catalyses the conversion of galactose to dulcitol, but, in contrast to sorbitol, dulcitol cannot be further metabolized by SDH and hence, accumulates more rapidly in the tissue than sorbitol. Galactose has been used frequently to study effects of the AR enzyme.

Sorbitol does not readily pass through the plasma membrane of the cell (107). Thus, under diabetic conditions which induce increased sorbitol content within the cells, there may be cellular swelling due to an increased intracellular osmotic pressure. Based on this, it has been suggested that the tissue damage and reduced function in organs showing diabetes-induced complications should be secondary to the intracellular osmotic changes (28, 65, 101, 213). This view is supported by studies of cataract induction where the lens is exposed to high glucose concentration *in vitro*. These cataracts may be prevented by the addition of osmotically active compounds to the culture medium (108).

The claims that sorbitol accumulation and AR activity are involved in the osmotic disturbance of the lens are supported by two lines of evidence. First, treatment of diabetic rats with AR inhibitors prevents development of cataracts (9, 107). However, several AR inhibitors have other effects than AR inhibition on the metabolism and have therefore been critisized (226). It has, however, been argued that the non-specificity of AR inhibitors is not relevant *in vivo* (196). Secondly, the AR activity in lenses and the required time for cataract development in different species are inversely correlated. Mice have very low AR activity and do not develop hyperglycaemia-induced cataracts (214). Diabetic rats, on the other hand, which have an AR activity ten-fold greater that in mice, develop cataract within three weeks after induction of diabetes, and degus (Octodon), which have lenticular AR activity three times that in rats, develop vacuoles in the lens in only ten days (100). However, monkeys do not follow this pattern as they, despite having lower AR activity than mice, develop diabetic cataract albeit after a long time of disease (years). The conclusive experiment regarding the role of AR in diabetes-induced cataract formation has recently been reported in a mouse strain transgenic for AR. These mice, in contrast to normal mice, form lenticular vacuoles after induction of diabetes (120). The results

clearly show the permissive nature of high AR activity in cataract formation in diabetes. Thus, in adult cataract formation, AR appears to be of major importance, although other mechanisms cannot be excluded (105, 121, 193, 194, 210).

It has been argued that sorbitol itself does not have the osmotic power to cause rupture of lens fibres (27). Instead, other mechanisms, such as damage by free oxygen radicals, have been suggested to cause disturbances in the osmotic balancing systems, for instance decreased activity of the Na/K-ATPase (194). This has been supported by the finding of antioxidants preventing diabetes-induced cataract in adult animals (105, 121, 193, 210). Preliminary investigation of fetal lenses in rats treated with antioxidants at doses effective for prevention of other congenital malformations (see below) appear to have some effect against congenital cataract formation in diabetic pregnancy (Kent Berg and Glenn Dormer, personal communication). Further, the concept of AR in diabetes-induced complication is a matter of controversy. It has been shown by Crabbe *et al.* (31) that glucose can be converted to sorbitol without any protein present, and suggested that AR is not a reductase for aldoses. In response, Harrison *et al.* (87) point out that the reactions referred to cannot occur under physiological conditions. It would appear, from the compiled arguments, that the AR enzyme may be involved in diabetes-induced complications, although the relative importance of the polyol pathway remaines to be elucidated.

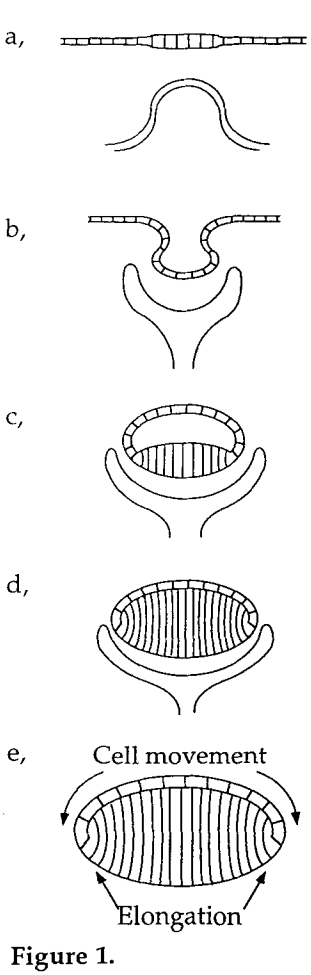


Figure 1.
Lens development

Lens development

The lens in rat fetuses begins to form at gestational day 11, by the formation of a lens placode in the surface ectoderm after induction of the optic cup from the neural tube (Fig. 1a). The lens placode invaginates to form a lens vesicle (Fig. 1b). As the lens vesicle breaks away from the ectoderm, cells on the posterior lens surface will elongate to form primary lens fibres (Fig. 1c). By gestational day 14 these fibres have obliterated the cavity of the lens vesicle (Fig. 1d). The cells on the anterior surface of the lens vesicle on the other hand, are stimulated to proliferation by the fluid in the eye chamber (94). Subsequently, the cells in the

anterior epithelium will move toward the equator of the lens (Fig. 1e), and as they reach the equator the lens cells begin to elongate and undergo morphological and biochemical differentiation characteristic for lens fibres. New fibres are added in onion-like layers superficially throughout life, but the lens growth is most active during early life (71).

The physiological function of AR in lenses has not been clarified. During the development of the lens, high quantities of AR mRNA are expressed in the lens and optic vesicle. Later, AR mRNA is found in the anterior epithelium and in the equatorial region (15). It has been suggested that AR is of importance for the elongation process of lens fibres, by causing increased osmotic pressure via sorbitol formation thereby expanding the cell volume. As the lens continues to grow throughout life, this mechanism may be valid in adulthood as well. Another possible function for AR is the counter-regulation of osmotic changes in the environment, as seen in the kidneys (16). This, however, is an unlikely event in the lens since the regulation of AR expression is slow in lenses (15). Although the physiologic function of AR remains to be elucidated, the polyol pathway appears to be of central importance in cataract formation, both in adult and fetal lenses exposed to a diabetic environment.

Reactive oxygen species (ROS) as a cause of congenital malformations

The possible involvement of ROS in the aetiology of diabetic complications has been discussed for more than two decades (5, 155). The arguments in favour are mainly based on the protective effects antioxidative of treatment (99, 115), the finding of reduced endogenous antioxidative capacity in diabetic patients (212), and increased tissue damage in diabetic individuals (usually measured as lipid peroxidation) presumably caused by enhanced free oxygen radical activity (180).

In 1991, Eriksson and Borg presented the first evidence of ROS-involvement in glucose-induced malformations *in vitro* by showing that antioxidants added to the culture medium normalized the development of embryos exposed to high glucose concentrations (43). Later, this finding was repeated in embryos exposed to other diabetes-associated teratogens (pyruvate, β-hydroxybutyrate, α-ketoisocaproate) (44) and to serum from diabetic rats (220). Furthermore, addition of an extra CuZnSOD-gene in a transgenic mouse strain resulted in fewer malformations and improved growth both *in vitro* (45) and *in vivo* (81) of embryos subjected to a diabetic environment.

In subsequent studies, attention was given to the mitochondrion as a possible source of ROS (44). By addition of α-cyano-4-hydroxycinnamic acid (CHC), an

inhibitor of pyruvate uptake to the mitochondrion, malformations caused by high glucose and high pyruvate concentrations were prevented. However, CHC was not effective against dysmorphogenesis induced by high levels of β-hydroxybutyrate and α-ketoisocaproate, both of which do not enter the mitochondrion via the pyruvate uptake mechanism. Thus, these results suggest that metabolism of oxidative substrates in the mitochondrion is the source of ROS. Studies of the mitochondria in a diabetic environment during organogenesis have shown high amplitude swelling of this organelle in neuroepithelial cells and in blood cells, both *in vitro* and *in vivo* (228). Treatment with the antioxidants BHT and vitamin E *in vivo* and CHC *in vitro* normalized the mitochondrial morphology (230). Examination of embryonic neural tube tissue, using the Cartesian Diver method, suggested a leakage of superoxide from the mitochondrion when exposed to high glucose concentrations. This would be secondary to a Crabtree effect, *i.e.* increased glycolytic activity and lowered oxygen uptake leading to a state-four respiration condition of the mitochondria with increased leakage of ROS (229). Hence, there are ample findings in favour of an important role of the mitochondria in diabetes-induced teratogenesis.

Increased ROS activity has also been shown in embryos cultured in high glucose concentrations (211) and in dispersed embryonic cells from 12-day-old embryos exposed to β-hydroxybutyrate (58). Further, damage to DNA, which may originate from increased ROS activity, has been found in rat embryos transgenic with the lacZ-gene, both *in vivo* (119) and *in vitro* (118). However, a study of mitochondrial DNA did not reveal any increased mtDNA damage in diabetic rat embryos (231). Thus, there is experimental evidence in favour of both increased ROS production and ROS damage in diabetic pregnancy.

The rat strain used by us, the Sprague-Dawley U-substrain with a high susceptibility to diabetes-induced malformations, has been found to have a different isoenzyme of catalase, a major scavenger of hydrogen peroxide (48). The activity of catalase in embryos of this strain was lower than in the ancestral (and malformation-resistant) H-strain of rats, and the difference in activity was further exacerbated by maternal diabetes (24). This suggests that decreased antioxidative capacity may cause increased susceptibility to the teratogenic activity of a diabetic environment. In other studies, maternal diabetes also increased the activity and amount of superoxide dismutase in embryos (57). Thus, an altered antioxidative capacity is induced by a diabetic milieu. However, arguments have been raised against the possibility of ROS excess in the aetiology of diabetic embryopathy by pointing out the low oxygen tension (55, 138) and low mitochondrial activity (125) of the early conceptus. The low mitochondrial oxidation, and consequently

low ROS production, may be balanced by an extremely low ROS scavenging capacity in the embryo in early embryo (37). Furthermore, different populations of embryonic cells may have different mitochondrial activity and susceptibility to ROS damage. Altogether, the available data strongly indicate abnormalities in the ROS production and/or the antioxidant defence capacity in embryos of diabetic pregnancy, abnormalities likely to be of importance in the induction of embryonic dysmorphogenesis.

Lipid peroxidation

Increased ROS levels may cause damage to all types of biological molecules, including DNA, proteins, lipids, lipoproteins, carbohydrates, and connective tissue macromolecules (32). Damage to lipids is of particular interest, since it may induce a chain reaction resulting in excessive peroxide formation, a process denoted as lipid peroxidation (79).

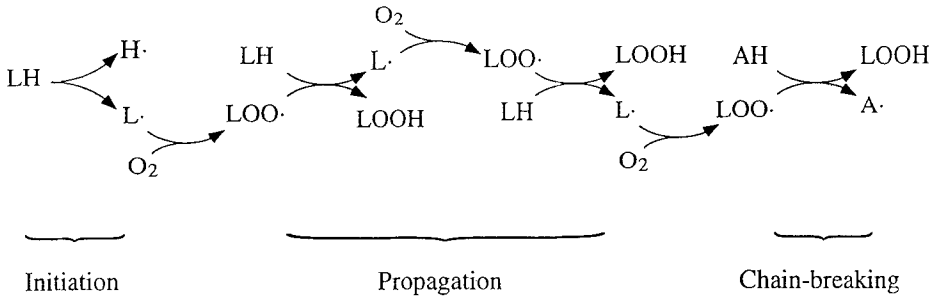


Figure 2. *Sequence of reactions in lipid peroxidation.*

Lipid peroxidation (Fig. 2) is initiated by the extraction of a hydrogen atom from a polyunsaturated fatty acid (L), leaving the fatty acid with an unpaired electron as a lipid radical (L·). The lipid radical will then react with molecular oxygen to form a peroxyl radical (LOO·). The peroxyl radical may extract a hydrogen atom from an adjacent lipid, thereby forming a lipid peroxide (LOOH) and a new lipid radical (L·). Thus, a chain reaction is initiated which will continue until the radical is neutralised by reaction with another radical or is transferred away from the membrane. This may occur by reaction with another peroxyl radical, a membrane-bound protein, or an chain-breaking antioxidant (AH), such as BHT or vitamin E (see below).

A commonly used method for detection of lipid peroxides is the thiobarbituric acid (TBA) assay, in which a degradation product of lipid peroxides, malonyl-dialdehyde (MDA), is detected after reaction with TBA. The TBA method has

been criticized for not being specific for lipid peroxides, since MDA is produced *in vivo* from other sources than lipid peroxides and TBA may also yield fluorescent products with other substances than MDA. Thus, results from this assay should be interpreted with caution (79). Several other methods are used for estimation of lipid peroxidation, such as measurement of conjugated dienes, chemiluminescence with microperoxidase (227), and ferrous oxidation in xylenol orange (FOX) (153). Most of these methods render similar interpretational problems as the TBA assay, or are not suitable for measurement in tissues (79). Thus, due to their simplicity, TBA-based methods are the most frequently used assays for estimation of lipid peroxidation.

Antioxidants

Butylated hydroxytoluene (BHT)

The compound butylated hydroxytoluene (BHT, Fig. 3) is a chain-breaking antioxidant (148) that is commonly used as a preservative in lipid-containing products, such as stains and cosmetics, and as additive to various foods. BHT is primarily distributed in the hydrophobic phase of cellular membranous structures (104).

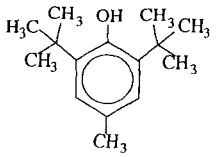


Figure 3. *BHT, redrawn from ref.* (13)

The compound has been demonstrated to have several therapeutical effects. BHT administered to animals prevents sugar-induced cataracts (3, 121), blocks cholesterol-induced atherosclerosis (10), and diminishes tumourigenesis in rats that have been exposed to carcinogenic compounds (131, 206, 222). On the other hand, experimental studies have also shown that BHT can promote cancerogenesis when the compound is given together with a tumourigenic agent (127, 161, 225). BHT is also hepatotoxic in rats pre-treated with phenobarbital (168). The putative teratogenicity of BHT has also been discussed (59, 195, 217). However, it was recently shown in a multigenerational study that BHT does not have any adverse effects on either reproductive or neurobehavioural parameters when mice were given a diet containing 0.4% BHT (205).

The toxicological effects of BHT may be exerted via an bioactivation of BHT to form BHT quinone methide (BHT-QM), a reaction catalysed by the enzyme complex cytochrome P-450 (13, 80). In contrast to BHT, BHT-QM may act as an prooxidant, and hence, be harmful to the cell rather than protective. Based on the toxicological evidence, WHO recommends a daily BHT intake of no more than 0.3 $mg*kg^{-1}$ for humans (221).

Vitamin E

α-Tocopherol is a naturally occurring vitamin (Fig. 4). It was initially recognized to be associated with reproduction and fertility in rats (53). Currently, however, the primary action of vitamin E is regarded to be a lipid soluble chain-breaking antioxidant (96), with the particular function of preventing lipid peroxidation in membranes. In contrast to BHT, α-tocopherol is located in the perimetry of membranes (104). This location gives the molecule the possibility of scavenging radicals in the membrane, and then exporting the radical to the water phase via ascorbic acid (157). Absorption of vitamin E from the intestine is facilitated by simultaneous consumption of fats (126, 190), and the vitamin is transported within lipoproteins in the blood (209). Although all forms of vitamin E are absorbed from the intestine, only α-tocopherol is incorporated into VLDL particles by a tocopherol transfer protein in the liver (233).

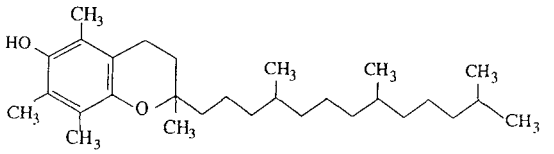


Figure 4. *α-Tocopherol, redrawn from ref.* (126)

In experimental diabetes, vitamin E has been shown to prevent complications, such as cataract development (210) and neuropathy (30). The low toxicity of vitamin E makes it attractive for therapeutical use. In studies of vitamin E toxicity, adverse effects were rarely observed with dosages up to 2000 mg vitamin $E*day^{-1}$ in human subjects. At higher dosages, side effects and intolerance were observed. However, the only side effect that repeatedly occurs is a coagulation defect in individuals with previous vitamin K deficiency (103). Teratological studies of vitamin E at very high dosage have not revealed any negative effects on outcome of pregnancy or reproductive capacity (93, 113, 129). However, preliminary reports with 2 $g*kg^{-1}$ of vitamin E to diabetic rats indicate an increased frequency of intrauterine death in the early part of the pregnancy

(17). Hence, vitamin E is traditionally regarded as a safe pharmacological agent, although new data stresses caution with use at very high doses.

Vitamin C

Ascorbic acid (vitamin C, Fig. 5) is a naturally occurring water soluble antioxidant, and its concentrations in tissues and plasma are decreased in both animals and humans with diabetes (26, 132, 192, 232, 235, 236). One of the vital roles for vitamin C is to act as an antioxidant to protect cellular components from ROS-induced damage (7, 149). In addition, ascorbic acid plays an important role in many biochemical processes, such as collagen synthesis (56), cholesterol synthesis (72, 88) and iron absorption (183).

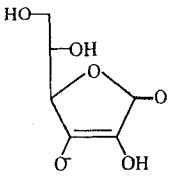


Figure 5. *Ascorbic acid, redrawn from ref. (7).*

Ascorbic acid is synthesized from D-glucose in most species, with the exception of primates and guinea pigs (150). The transport over biological membranes of vitamin C is facilitated by glucose transporters, especially GLUT-1 (73, 139, 215), and hence, hyperglycaemia has been shown to reduce placental transport of ascorbic acid to the fetus (95, 152). As ascorbic acid is a water soluble antioxidant, it has the potency to scavenge the superoxide and hydroxyl radicals (8), the ROS which are produced under physiological conditions (63). In addition, ascorbic acid may function as a chain-breaking antioxidant in the lipid soluble phase by an interaction with lipid soluble antioxidants such as vitamin E and coenzyme Q (8, 157). Ascorbate is the first antioxidant to be consumed in plasma exposed to oxidative stress (60, 61) and is regarded as the most important defence against oxygen radicals in the aqueous phase (149). However, in the presence of transition metals, autooxidation of ascorbate occurs *in vitro*, resulting in formation of superoxide (78). The significance of the prooxidant activity *in vivo* is unclear, mainly due to low concentrations of free metals under physiological conditions (136) and high concentrations of urate in plasma which inhibits ascorbate auto oxidation (182).

Results and Discussion

The results discussed in this review originates mainly from six different publications (50, 184-188). The rationale for the performed experiments was based on three previous observations in rats, which were further investigated.

1, The high frequency of congenital cataracts in lenses of offspring of diabetic rats (33, 68, 70). We show that fetal lenses of diabetic rats have accumulated high amounts of water and sorbitol compared to the controls, thereby indicating that an osmotic mechanism is causing the cataracts (188).

2, The occurrence of micrognathia in offspring of diabetic rats (47). It has been speculated that this malformation may be caused by a maldevelopment of the cranial neural crest cells which are involved in the formation of the mandible (204). We demonstrate that offspring of diabetic rats display several malformations of possible neural crest cell origin, in addition to micrognathia. In particular, heart abnormalities in the offspring of the diabetic rats are similar to analogous malformations in children of diabetic mothers (187).

3, The protective effects of antioxidants against malformations in embryos cultured *in vitro* in high concentrations of glucose, β-hydroxybutyrate and α-ketoioscaproic acid (43, 44), as well as diabetic serum (220). In several reports, we confirm these findings in an *in vivo*-model for diabetes-induced malformations with three different antioxidants (50, 184-186). Some adverse effects of BHT, one of the antioxidants used, are also discussed (184).

Congenital cataract

Fetal lenses of normal and diabetic rats were examined histologically from gestational day 16 to 22 (Fig. 8). All lenses showed severe vacuolisation and swollen fibres centrally. In addition, an eosinophilic amorphic substance was often found close to the anterior epithelium. Most of the lens cortex appeared to be normal. This is in contrast to the cataract in adult diabetic rats, in which the vacuoles first appear in the cortical region of the lens. Furthermore, the vacuoles were present as early as gestational day 16, one day earlier than previously reported by others (68, 70).

The water content of normal fetal lenses decreased throughout the lens development. The lenses of fetuses of diabetic rats, however, successively accumulated water until gestational day 22. The activity of the first enzyme in the sorbitol

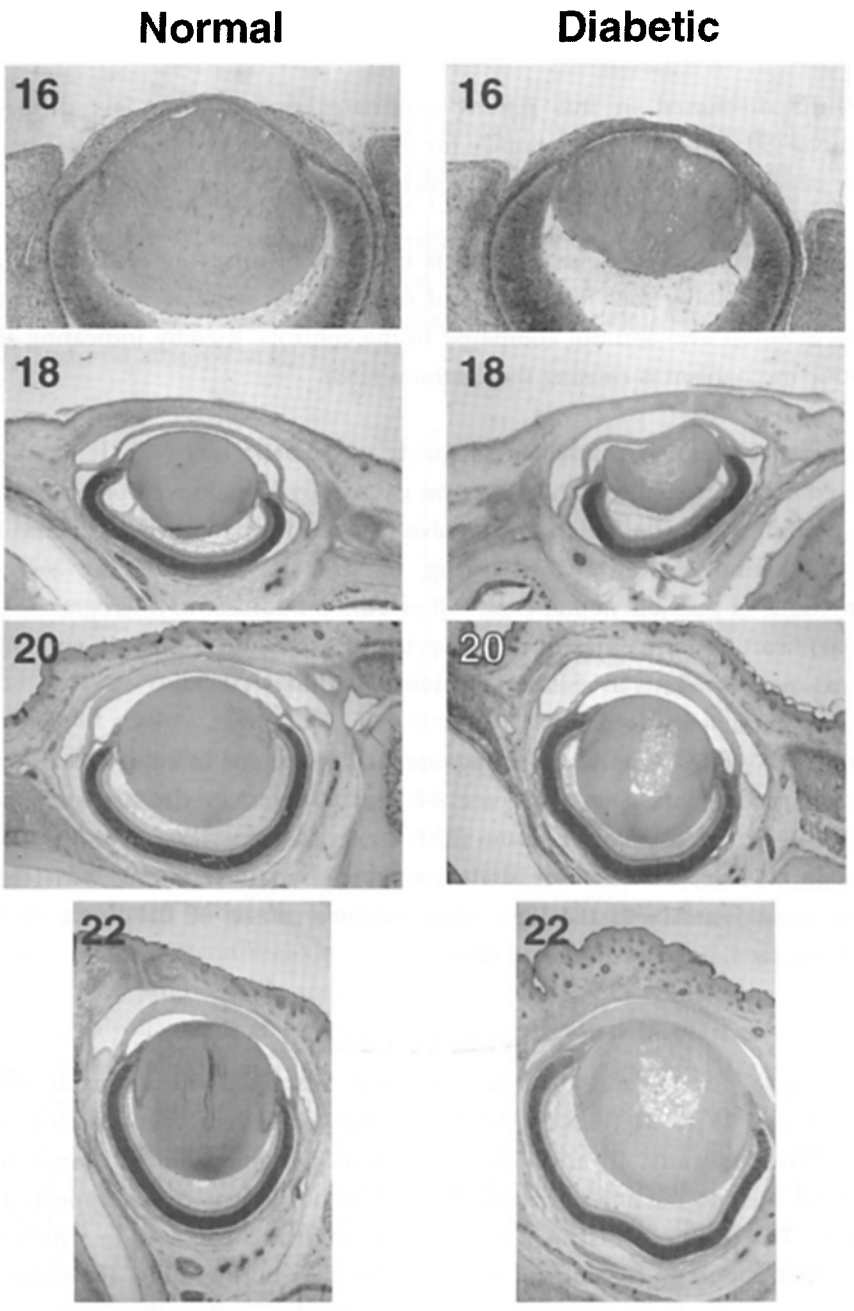


Figure 8. *Sections through fetal lenses from normal and diabtic rats on gestational day 16, 18, 20 and 22.*

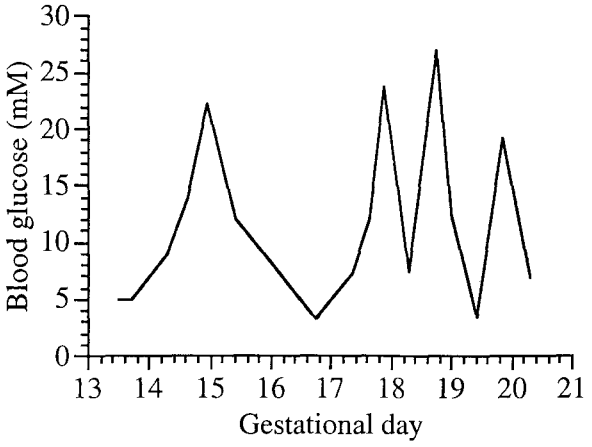
pathway, aldose reductase (AR), decreased towards the end of pregnancy, and the fetal lenses from diabetic rats had higher activity than the normal fetal lenses at all time points except day 20. The sorbitol concentration in fetal lenses lenses from diabetic rats was approximately ten times higher than the controls at all time points. Thus, the accumulation of water in fetal lenses from diabetic rats may be attributed to the increased sorbitol concentration (188). However, other osmotically active substances, such as fructose (145), may also be of significance for the development of congenital cataract.

In one single experiment, a pregnant rat was infused with high concentrations of glucose, in order to induce hyperglycaemia during the last gestational week. A catheter was placed in the jugular vein and glucose was continuously infused with a syringe pump from gestational day 13 to 20, when the pregnancy was interrupted and the eyes of the offspring were analysed histologically. Serum glucose of the rat was monitored throughout the infusion period (Fig. 9a).

As demonstrated in Fig. 9a, the experimental system yielded peaks of high glucose concentration during the infusion period. Histologic examination of the fetal lenses showed a distinct zone of vacuoles (Fig. 9b). This experiment further illustrates the importance of hyperglycaemia, even under short periods, for the induction of cataract. Of interest is the location of the vacuoles within the lens, in an intermediate region between the central and peripheral parts. These vacuoles were present in an area of the lens where the fibres have been involved in the terminal differentiation process around gestational day 15, *i.e.* the time point when the lens was first exposed to high a glucose concentration. Swollen lens fibres, but no vacuoles, were found in the central lens region.

Based on these observations with diabetic rats together with the glucose infusion experiment, the following hypothesis may be proposed: Cells in the lens become susceptible to vacuole formation if they are exposed to high glucose concentrations during their differentiation to fibres. If the high glucose concentration continues or is re-encountered, fibres which have been "primed" will swell and eventually form vacuoles, while other fibres will be less affected. This would explain why the vacuoles of congenital cataracts are located in the central lens area, while vacuoles in adult lenses are found in the cortical region. In addition, lenses from the glucose infusion experiment displayed vacuoles in a region which may correlate to the period of the first encounter of high glucose concentration. Furthermore, vacuoles induced by galactose-feeding are located closer to the lens surface and develop faster than diabetes-induced vacuoles (14). This indicates that the time required for vacuolar formation is of importance for

a,



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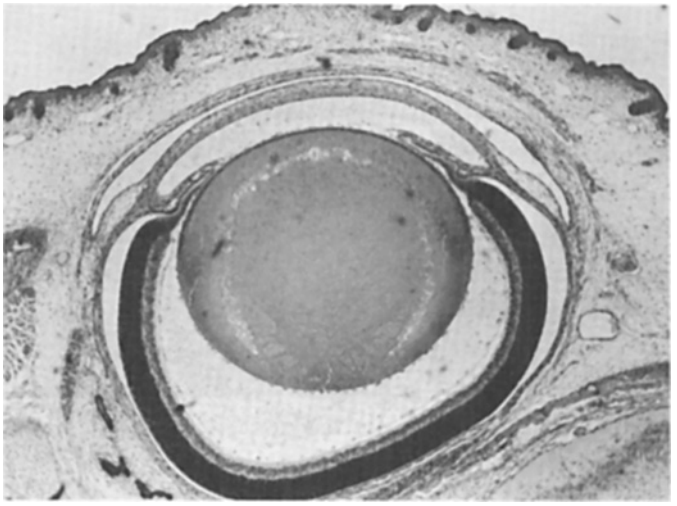

Figure 9. *a, Blood glucose in a rat during glucose infusion. b, Section through a fetal lens on gestational day 20 after glucose infusion for 7 days.*

their positioning in the lens. It may be speculated that the induction of susceptibility to vacuole formation is mediated by an increased expression of AR, which would cause excess sorbitol accumulation after exposure to high glucose concentrations. Thus, the model suggested provides a possible explanation for the selective positioning of vacuoles within the lens.

Preventive treatment with antioxidants

Morphology

Apart from congenital cataract, a high frequency of gross malformations are seen in offspring of diabetic rats. An underdevelopment of the mandible, micrognathia, is the most common external malformation at the end of gestation in U rats of the Sprague-Dawley strain (41). If embryos are examined by the end of organogenesis, at gestational day 11, neural tube defects are the most common abnormality. As stated in the Introduction, antioxidants can restore embryonic development in a diabetes-like environment *in vitro* both in cell culture (204) and whole embryo culture (43, 44, 211, 220). Mainly on the basis of these experiments, the hypotheses of involvement of ROS in the aetiology of diabetes-induced malformations was formulated. The primary aim of the reports discussed here (50, 185, 186) was to test whether these observations could be repeated in experiments *in vivo*.

In the treatment experiments, three different antioxidants were tested, butylated hydroxytoluene (BHT), α-tocopherol (vitamin E) and ascorbate (vitamin C). BHT was chosen in the first experiment because it has been shown to reduce complications in diabetic animals, and the dose of 1% in the diet was based on these reports (22, 121). Vitamin E was given by gavage in doses comparable to doses in a previous study (216), and as food supplementation (2%) in a molar dose similar to BHT. Vitamin C was also given in the diet at similar molar concentration as vitamin E and BHT, and in two higher doses (*i.e.* 0.9%, 1.8%, and 4%).

Pregnancy outcome on gestational day 20

In untreated diabetic rats, the mean proportion of malformations in the offspring at gestational day 20 was in the range of 19% to 27% (50, 185, 186). The malformation rate was reduced to 2%, 5% and 8% with the highest administered dose of BHT, vitamin E and vitamin C, respectively (Fig 10). Thus, the results clearly show that dietary supplementation of antioxidants substantially reduces the occurrence of gross malformations in the offspring of diabetic rats *in vivo*.

The effects of antioxidant treatment on fetal and placental weight is more difficult to interpret. In general, diabetes decreased fetal weight and increased placental weight. Both of the two lipid soluble antioxidants, BHT and vitamin E partially normalized the fetal weight, wheras no clear effect was seen with vitamin C, in concert with previously reported results (167, 170, 189, 216). The increased placental weight was somewhat reduced by antioxidant treatment of the diabetic pregnant rats, although the numerical difference never reached

statistical significance. Thus it appears that antioxidant therapy exerts a partially normalizing effect on the growth of the conceptus. However, the results do not provide evidence for excess ROS being the major cause of altered enbryo-fetal growth in diabetic pregnancy.

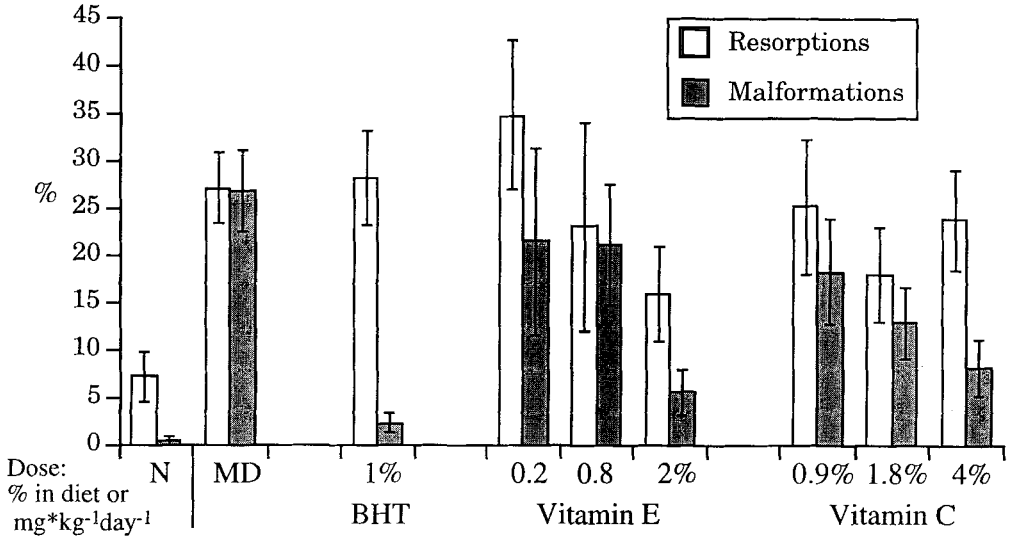

Figure 10: *Pregnancy outcome at gestational day 20. Antioxidants were administered by diet supplementation or by daily gavage (vitamin E only). Data adopted from ref 50, 185 and 186.*

Pregnancy outcome on gestational day 11

At gestational day 11, by the end of organogenesis, diabetes also caused altered embryonic development. Morphologic abnormalities consisted mainly of malrotation of the embryo and non-closure of the neural tube. This occurred with a frequency of 24% compared to 2% in the controls. With vitamin E supplementation in the diet, the occurrence of these abnormalities was reduced to 7%. The embryonic size, measured as somite number and crown-rump length was reduced in offspring of diabetic rats compared to controls. Again, vitamin E treatment partially restored the size. Thus, it appears that antioxidant treatment has the capacity to prevent malformations that are seen in both early and late pregnancy, and partly restores growth at both time points. However, although the number of malformations are similar at gestational day 11 and 20, it is not possible to ascertain that the malformations found on gestational day 11 are the same malformations as those found on pregnancy day 20 in a later developmental stage. Defects observed at gestational day 11 are mainly anomalies in the neural tube, and when the offspring is studied at day 20, damage to the neural crest cells (NCC) is a likely cause of the malformations (see below). However, since the neural tube and the NCC are adjacent during early embryo

development and originate from the same precursor cells, they may share similar biochemical alterations in diabetes.

Resorptions

In addition to malformations, diabetic pregnancy in rodents is also complicated with a high frequency of intrauterine death and subsequent resorptions. The cause of intrauterine death has not been studied in experimental diabetes. It is assumed that severely abnormal embryos/fetuses will die because of their malformations. This is supported by the findings of malformations in early pregnancy but only resorptions at term in offspring exposed to excess mannose *in utero* (21). However, studies of pregnancy outcome in a non-diabetic resorption prone mouse strain have indicated that early resorptions are caused by a immunologic activation in the uterus rather than embryonic abnormalities (cf. Introduction). At gestational day 20 in the present work, the mean proportion of resorptions ranged from 23% to 29% (50, 185, 186). With BHT treatment, no protective effect was found on the resorption rate (Fig 10). With vitamin E supplementation, a trend toward fewer resorptions was noted at gestational day 20 (Fig 10). At gestational day 11, on the other hand, diabetes did not cause any major increase in resorption frequency, and no clear effect of vitamin E treatment was found, thus indicating a possible difference in aetiology of resorptions in early and late pregnancy. In the study with vitamin C treatment (185), special attention was given to resorptions, and each resorption was classified as early or late depending on size (diameter more or less than 4 mm, cf. Materials & methods). Using this approach, it was confirmed that diabetes does not increase the number of early resorptions compared to controls, and vitamin C supplementation did not effect these frequencies. The rate of late resorptions however, was clearly increased in the diabetic litters, whereas they rarely occurred among the control litters. With vitamin C treatment the rate of late resorptions was successively reduced to levels indistinguishable from the controls, in parallel with a substantial reduction of congential malformations in the offspring. The late resorption and malformations may therefore have similar aetiology, which suggests that the former represent lethal malformations. The occurrence of early resorptions on the other hand, may be a strain-specific characteristic of the U rat pregnancy with little influence from the diabetic state.

The morphologic results with BHT, vitamin E and vitamin C treatment are in agreement with other reports on antioxidants and diabetes-induced malformations (122, 179, 189, 216). In these studies, embryo outcome was only assessed in early gestation. The results presented here show that antioxidants can prevent the diabetes-induced malformations and intrauterine death that are

observed in late gestation. Furthermore, intrauterine death leading to resorption of the conceptus is most likely caused by at least two different mechanisms in early and late gestation, respectively.

Detailed morphologic evaluation

Although it is clear that antioxidants can prevent diabetes-induced malformations, little is known about the morphological processes that are disturbed by diabetes. Several clinical and experimental findings indicate that neural crest cells (NCC) may be involved in the abnormal development (54, 75, 154, 199, 200, 202, 204, 224). In order to investigate this relationship further, a histological examination of diabetic fetuses was performed on gestational day 16, with special emphasis on fetal organs with NCC-dependent development (187).

Mandible

Examination of the mandible in fetuses with micrognathia revealed both an abnormal Meckel's cartilage and a markedly altered mandibular bone. Instead of the normal horseshoe shape of Meckel's cartilage ranging from the developing malleus by the middle ears connecting at the tip of the mandible (Fig. 11a), a "bridge" of cartilage was found between the ears (Fig. 11b). From this bridge a rod of cartilage sometimes projected forward. Intramembranously formed mandibular bone was found adjacent to Meckel's cartilage, and the bone deposition appeared to be dependent on the position of Meckel's cartilage. Hence, the mandibular bone in micrognathic fetuses was more centrally positioned.

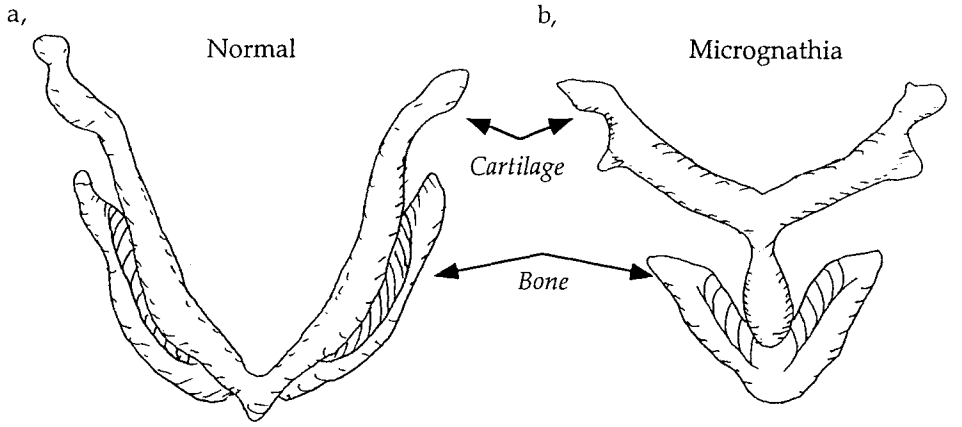

Figure 11. *Appearance of Meckel's cartilage and the mandibular bone in fetuses on non-diabetic (a) and diabetic (b) rats.*

Meckel's cartilage and the mandibular bone are formed from two populations of NCC (83). The cells that will become bone are induced to form osteoblasts by the surface ectoderm in the mandible (82), *i.e.* after their migration from the cranial neural crest region to the first visceral arch (71). Cells forming Meckel's cartilage, on the other hand, receive their differentiation induction before migration when they are still situated in the neuroectoderm (84). Combining this with the observation that diabetes induces the malformations before gestational day 10 in rats (39, 42), yields that the damage to the NCC occurs before migration. Assuming that bone abnormalities are secondary to cartilage defects, the diabete-induced damage should be specific to the NCC-population that will form cartilage. It also appears that the NCC induction of the pre-chondrocytes may be the primary target for the diabetes-induced developmental damage resulting in micrognathia.

Parathyroid, thyroid and thymus

The rodent parathyroid glands, which are situated antero-laterally on each thyroid gland, were easily identified in control fetuses. In fetuses of diabetic rats, on the other hand, the parathyroids were often missing. The thyroid itself often lacked the isthmus and was sometimes small or rudimentary. The thymus was reduced in volume and often displayed several lobules (c.f ref. 187). Similar defects of these glands can be induced by ablation of the cranial neural crest (11) and knock-out of the endothelin-1 gene, involved in morphogenesis of the neural crest (116). Thus, based on the morphologic appearances, the defects may originate from NCC developmental abnormalities.

Heart

Several types of heart malformations were noted in fetuses of diabetic rats, mainly consisting of subaortic ventricular septal defects (VSD) and double outlet right ventricle (DORV). These defects may have been caused by a rightward displacement of the aorta which was a very common finding in this study. The position of the aorta is determined by wedging of the heart tube and twisting of the conotruncal septum (151). Since the conotruncal septum is of NCC origin (109), heart defects are seen when this cell population is manipulated, for instance by ablation (110) or treatment with retinoic acid (18). Defects caused by these manipulations share several similarities with the findings in fetuses of diabetic rats. It should be noted, though, that other embryonic manipulations, such as physical prevention of embryonic bending also results in DORV and VSD (142). Therefore, it is not possible to state conclusively that the heart defects in the present study are of NCC origin. However, since the heart defects covariate with

malformations in the head-neck region, it seems reasonable that NCC are involved in the diabetes-induced heart malformations.

Great vessels

The great arteries were often abnormal in fetuses of diabetic rats, and persistence of retro-oesophagal vessels and right-sided aortic arch were consistent findings. These defects are likely to originate from an abnormal persistence of the antelateral dorsal aorta, which normally would degenerate. Interestingly, although this part of the aorta does not contain NCC, neural crest ablation can still cause several malformations in this vessel (111), some of which are similar to those found in offspring of diabetic rats. Blood flow from the heart is apparently of importance for the formation of the great vessels (12, 92) and hence, vessel malformations may be secondary to heart defects. Interestingly, the vessel defects found in diabetic fetuses covariated strongly with heart malformations but with less power than with other head/neck defects.

Analysis of associations between the defects described here showed that the malformations existed together within fetuses. This corroborates the view that they share aetiological characteristics. Based on the morphological observations, it seems likely that NCC are involved in these malformations. Also, treatment with vitamin E reduced the severity of the malformations, indicating that they are developmentally related.

Biochemical evaluation

In order to asertain that a supplemented compound had been absorbed by the animals, and to characterize the metabolism and protective effects of the anti-oxidants, the administered substances (50, 184-186) and TBARS (185, 186) were measured in the rats and their offspring.

BHT was analysed in maternal liver at gestational day 20 (50), thus confirming that BHT was absorbed from the diet by the rats. Interestingly, the BHT-treated diabetic rats had a three times higher BHT concentration in their livers than the BHT-treated controls. This may be explained by the higher food consumption in diabetic rats (201), and by the lipophilic properties of BHT. During the pretreatment period, most of the BHT accumulates in adipose tissue (207). As the animals become diabetic, the fat stores are gradually consumed and the stored BHT may have been released back to the circulation for redistribution in the animal. Thus, it is possible that fetuses of diabetic rats were exposed to a higher BHT-concentration than the controls during pregnancy.

Several antioxidants can interact by oxidizing/reducing each other, and therefore it was expected that BHT would keep α-tocopherol reduced and hence increased the tissue concentration of this vitamin. Much to our surprise, the opposite was found in adult liver. BHT caused a decrease in hepatic vitamin E concentration to less than 1/3 of the level in the untreated rats, both in diabetic and control animals. Based on these results, an experiment was performed in which healthy female rats were treated with BHT for four weeks at two dose levels, with or without vitamin E supplementation (184). It was found that BHT increased the liver weight in relation to the given BHT dose, but liver weight was not affected by vitamin E, in agreement with previous findings (19, 98). Furthermore, BHT decreased α-tocopherol concentration in the liver dose-dependently, an effect not changed by vitamin E supplementation. However, this interaction was not seen in abdominal adipose tissue. The BHT concentration in liver and adipose tissue was not affected by vitamin E supplementation. Studies of BHT metabolism have shown that during the process of conjugation BHT is metabolized to toxic compounds, one of which is BHT-quinone methide (BHT-QM) (13, 80). BHT-QM has oxidative activity and is formed by a bioactivation of BHT catalysed by the enzyme complex cytochrome P450. Hence, BHT-QM is only formed in tissue with cytochrome P450 activity. Thus, it is likely that there are prooxidative effects of bioactivated BHT that cause a consumption of α-tocopherol in the hepatic tissue, whereas this does not occur in adipose tissue, since it lacks cytochrome P450 activity. The toxic property of BHT would render this compound unsuitable for dietry treatment of diabetic pregnancy in the doses required for prevention of dysmorphogenesis.

The concentration of α-tocopherol, ascorbic acid and TBARS were analysed in both maternal tissues and in the offspring (185, 186). Since TBARS estimates oxidative damage in membranes, and α-tocopherol protects from oxidative damage in membranes, an inverse relationship was expected between these substances. This was not obvious in individual organs, but by comparing different organs it was clearly shown that decreasing α-tocopherol concentration correlated with increasing TBARS-concentrations (Fig. 12).

The finding of the lowest concentration of α-tocopherol and highest concentration of TBARS in fetal tissue were in agreement with previous reports (234). In contrast, maternal liver had high α-tocopherol concentration and low levels of TBARS (Fig. 12). For ascorbic acid, on the other hand, no clear correlations with tissue concentrations of either α-tocopherol or TBARS were found. Also, very high concentrations of ascorbic acid were found in fetal brain. It

has been suggested that ascorbic acid may be involved in brain development (237).

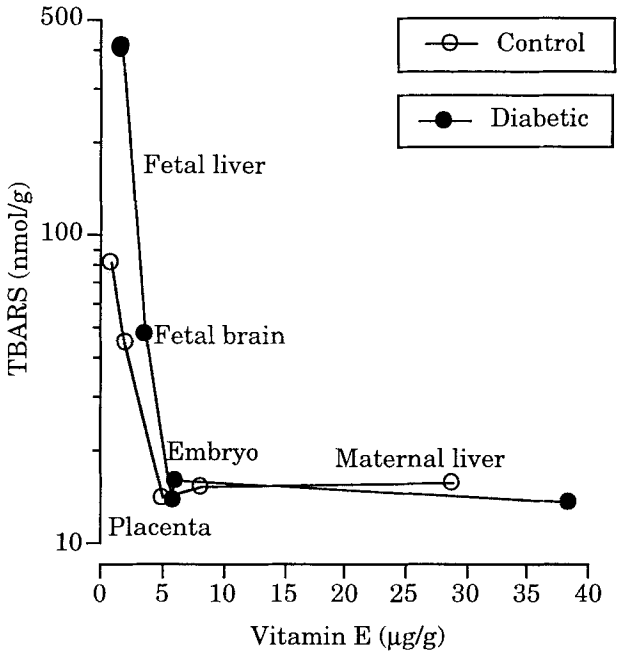

Figure 12. *Correlation between vitamin E and TBARS in different tissues.*

Diabetes caused an increase in α-tocopherol in maternal serum and a decrease in embryonic tissue. The differences are small, but all available reports estimating these parameters show the same trend (170, 189, 216). The increase of serum α-tocopherol could, at least partly, be explained by an increase in serum lipid concentration, since the vitamin is transported in this serum fraction (209). The decrease of α-tocopherol concentration in day-11 embryos may either be explained by diminished uptake or increased consumption of the vitamin. The latter effect would be in concert with an increased oxidative activity in the embryos. On the other hand, ascorbic acid concentration in fetal and maternal tissue, was not affected by diabetes. By contrast, non-pregnant streptozotocin diabetic rats have decreased concentrations of ascorbic acid (232, 235, 236).

Diet supplementation with vitamin E resulted in increased α-tocopherol concentration in all tissues examined. However, most of the administered vitamin appeared in the maternal liver (186). Supplementation with vitamin C resulted in increased α-tocopherol concentration in the placenta, but not in any of the other organs. This may have been due to a regeneration of oxidized

α-tocopherol in the placenta (8, 157), thereby decreasing the consumption of the vitamin.

Lipid peroxidation, estimated by TBARS, tended to be increased in maternal serum and was markedly increased in fetal liver in diabetic rats compared to controls. Supplementation with vitamin E to diabetic animals completely normalized TBARS in fetal liver. Supplementation with vitamin C decreased TBARS in serum but, had less effect on TBARS in fetal liver. This difference between vitamin E and C effects may simply be that ascorbic acid reduces α-tocopherol which, in turn, diminishes the TBARS concentration. Therefore, the low α-tocopherol concentration in fetal liver of diabetic rats without vitamin E supplementation eliminates the mechanism of action for ascorbic acid (cf. Fig 12).

The biochemical analyses showed that administered antioxidants are readily absorbed by the pregnant rats and transferred to the offspring. Furthermore, maternal diabetes caused oxidative damage, the degree of which was dependent on the capacity of the antioxidative defence. Supplementation of exogenous antioxidants partly prevented oxidative damage to the tissue.

Conclusions and perspectives

The data presented, in addition to the results previously reported in the literature, indicate that diabetes may cause disturbed embryonic/fetal development by at least two different mechanisms, *i.e.* over-activity of the sorbitol pathway and excess ROS production. Both are accompanied by different malformations, such as congenital cataract and defects in tissues of NCC origin, respectively.

The evidence for involvement of ROS in NCC-derived malformations are based on the observations that diabetes can cause lipid peroxidation (185, 186), that antioxidants can normalize the increased lipid peroxidation (185, 186), and that antioxidants can prevent diabetes-induced malformations (50, 185-187) Thus, it is likely that prevention of malformations in the embryo by antioxidants is mediated by scavenging ROS-induced developmental damage. Several different antioxidants have similar effects on malformations, both *in vitro* and *in vivo* (43-45, 122, 167, 179, 189, 211, 216, 220), implicating that the protective action has indeed been mediated through the antioxidative capacity of the compounds and not by some other unspecific mode of action. However, it was not possible to detect any increased lipid peroxidation in embryos in early gestation (186),

uniform antiteratogenic effects of antioxidants in several rats strains, it seems reasonable to assume that the animals used may serve as a good model for embryonic dysmorphogenesis induced by diabetes in humans. Furthermore, since abnormal ROS metabolism is frequently reported in patients with diabetes (23, 25, 203), this too appears to share similarities with rats. Therefore, antioxidants should be effective in preventing some of the malformations seen in children of diabetic mothers. It should, however, be kept in mind that there may be other malformations with different aetiology which are not prevented by antioxidants. One example of this is the limited effects of antioxidants in correcting the embryonic/fetal size in animal experiments. Although the importance of strict metabolic control during diabetic pregnancy must not be neglected, the future treatment regimen may include dietry antioxidative supplement as an adjunct to intensive insulin therapy.

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Address for reprints:
Martin Simán, Doctor of Medical Science
Department of Medical Cell Biology
Uppsala University
Biomedicum, P.O. Box 571
S-751 23 UPPSALA, SWEDEN
E-mail: Martin.Siman@medcellbiol.uu.se