Embryopathy in experimental diabetic gestation: assessment of oxidative stress and antioxidant defence

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ABSTRACT

Maternal diabetes is associated with an increased rate of congenital fetal anomaly. In the present study, diabetes was induced by streptozotocin in female rats one week prior to conception and the embryos were examined during organogenesis. Experimental diabetes is associated with over-production of free radicals and disturbed antioxidant defence, particularly in malformed embryos. Oxidative stress is demonstrated by increased MDA accumulation and reduced glutathione levels. Despite large differences in the reduced/oxidised glutathione ratios during organogenesis in the control, diabetic non-malformed and malformed embryo groups, the half-cell redox potential was constant for each group during the experimental period. Calculated redox potentials indicated that although embryo cells from the control and diabetic mother groups were of the same chronological age, the stages of development were different. Increased oxidative stress in rat embryos was associated with increased glutathione peroxidases and glutathione-S-transferase activity. This may, in part, provide an explanation for the observed accumulation of oxidised glutathione in malformed embryos. Moreover, decreased levels of vitamin C and selenium were observed. Increased oxidative stress and perturbations in antioxidant defence contribute to the high incidence of congenital anomalies in experimental diabetic gestation.

KEY WORDS: Diabetes mellitus. Fetal development. Oxidative stress. Teratogens.

Introduction

Diabetes during pregnancy is a recognised medical problem, as maternal metabolic disturbances have a detrimental effect on embryonic development. Despite tight glycaemic control, the frequency of congenital fetal malformation and prenatal death in the offspring of diabetic mothers is substantially higher than is seen in non-diabetics. Furthermore, the incidence of major malformations is higher when diabetes is diagnosed during pregnancy.[1] The development of diabetic complications has been linked to reactive oxygen species (ROS), and different antioxidants appear to improve fetal outcome in *in vitro*[2–4] and *in vivo*[5–7] models of diabetic embryopathy.

As the dominant non-protein thiol in the mammalian cell, glutathione (GSH) is essential for maintaining the intracellular redox balance.[8] It plays a key role in the regulation of many transcription factors involved in differentiation, proliferation and/or apoptosis during development.[9] Shifting the intracellular redox status, via excessive or inappropriately timed GSH oxidation, could disrupt signal cascades and result in altered development.[8] High concentrations of thiols are associated with a reducing environment and increased proliferation, while apoptosis is initiated by an oxidising environment; thus, the redox status of the cell might determine whether it proliferates, differentiates or dies.[10]

This study aims to assess the impact of maternal experimental diabetic gestation on oxidative stress and the efficiency of antioxidant defence in malformed and non-malformed embryos and associated placentas. In addition, the same parameters were determined in the livers and placentas of pregnant rats.

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Materials and methods

A local Wistar-derived strain of rats was used. Diabetes was chemically induced by two successive daily intraperitoneal doses of 55 mg/kg streptozotocin (STZ). Diabetes was confirmed one week later by a blood glucose level above 20 mmol/L, as measured by a glucometer (Elite).

Diabetic females were mated with healthy non-diabetic males overnight. The presence of a sperm mucus plug in the vagina the following morning was taken to signify pregnancy (gestation day 0). Non-diabetic females were mated with healthy non-diabetic males overnight and used as the control group.

Pregnant diabetic and non-diabetic female rats were sacrificed on gestation days 9, 10, 11 and 15, and each uterus

Table 1. Parameters of oxidative stress in rat embryos of non-diabetic control and in experimental diabetic gestation.

Gestation (days)	TBARS (nmol MDA/mg protein)	Glutathione tGSH	Glutathione GSH	Glutathione GSSG	Half-cell redox potential (mV)
		(nmol/mg protein)			
Embryos of non-diabetic control mothers					
9	0.033±0.004	8.34±1.38	8.15±1.37	0.09±0.005	-235.2±3.81
10	0.046±0.004	13.22±1.37	12.76±1.34	0.23±0.03	-235.6±2.13
11	0.062±0.003	16.87±1.74	15.97±1.67	0.45±0.05	-232.7±2.11
15 (liver)	0.537±0.033	19.39±0.51	18.63±0.47	0.38±0.02	-238.7±0.43
15 (placenta)	0.219±0.048	14.26±0.22	13.33±0.24	0.46±0.03	-227.7±1.19
Non-malformed embryos of diabetic mothers					
9	0.060±0.007[a]	4.60±1.04[a]	3.92±0.95[a]	0.34±0.05[a]	-199.8±4.71[a]
10	0.088±0.007[a]	9.8±0.58[a]	8.38±0.39[a]	0.71±0.19[a]	-210.7±3.66[a]
11	0.134±0.026[a]	12.97±0.41[a]	10.84±0.26[a]	1.06±.11[a]	-211.8±1.09[a]
15 (liver)	1.968±0.110[a]	17.90±0.79[a]	13.47±0.54[a]	2.21±0.31[a]	-208.1±2.19[a]
15 (placenta)	1.237±0.303[a]	11.86±0.55[a]	9.02±0.68[a]	1.42±0.09[a]	-203.2±2.65[a]
Malformed embryos of diabetic mothers					
9	0.069±0.007[a,b]	3.48±0.45[a]	2.82±0.40[a]	0.33±0.05[a]	-192.2±3.87[a,b]
10	0.092±0.004[a]	9.22±0.38[a]	6.83±0.37[a,b]	1.20±0.04[a,b]	-197.6±0.88[a,b]
11	0.150±0.019[a]	11.25±1.34[a]	8.22±1.09[a,b]	1.52±0.17[a,b]	-199.9±2.65[a,b]
15 (liver)	2.958±0.092[a,b]	16.78±0.47[a,b]	11.63±0.30[a,b]	2.58 ± 0.21[a,b]	-193.3±1.32[a]
15 (placenta)	1.74±0.286[a,b]	11.18±0.38[a,b]	6.39±0.36[a,b]	2.39±0.14[a,b]	-187.8±1.85[a]

Data presented as the mean ± SD
[a]Significantly different from embryos of non-diabetic control mothers ($P<0.05$).
[b]Significantly different from non-malformed embryos of diabetic mothers ($P<0.05$).

was exposed. The number of resorptions was noted and the embryos and membranes were dissected from the uterine horns and examined for gross anatomical malformations.

Each embryo and membrane was separated by gentle dissection and rinsed carefully in phosphate-buffered saline (PBS). Overall growth was quantified by direct measurement of crown-rump length. The embryos were examined for body rotation, closure of neural tube and appearance of specific organs. Embryos with apparent anomalies were regarded as malformed.

Each embryo, 15-day fetal liver and placenta and maternal liver was homogenised separately in PBS. Parameters of oxidative stress included thiobarbituric acid reactive substances (TBARS), representing lipid peroxidation end-products and quantified as malondialdehyde (MDA),[11] and total and oxidised glutathione,[12] which were used to calculate the half-cell redox potential by the Nernst equation.[13] For antioxidant defence, glutathione peroxidase (total [tGPx], selenium-dependent [sGPx] and non-selenium dependent [nsGPx]),[14] glutathione reductase[15] and glutathione-S-transferase[16] activities were measured. Protein was assayed using the method described by Lowry *et al*.[17] Also determined were vitamin C,[18] vitamin E[19] and selenium[20] levels.

Results

Experimental STZ-induced diabetes in female rats resulted in increased resorptions and congenital embryonal anomalies. While no gross anatomical malformations were detected in the embryos of the non-diabetic controls, maternal diabetes was associated with malformation in approximately 41% of viable embryos. Growth retardation was the most common (52%), followed by malrotation (32.5%), short mandible (15.0%), maldevelopment of head (12.5%), absence of tail (10%) and open neural tube (7.5%).

Embryos of experimentally diabetic mothers in general, and malformed ones in particular, were lighter and of shorter crown-rump length, compared with controls. In contrast, placental weights were higher. Parameters of oxidative stress in rat embryos of non-diabetic controls and in experimental diabetic gestation are shown in Table 1. Although the rat embryos were of the same chronological age, redox potentials were indicative of different stages of development in different categories of embryos.

Increased production of ROS during early development necessitates an antioxidant defence system. Changes in the activities of glutathione peroxidases with time in developing embryos of control and experimentally diabetic rats are shown in Table 2. By day 11, maternal diabetes resulted in increased cellular GPx isoenzyme activities, reaching four-fold and seven-fold the control activity in non-malformed and malformed embryos, respectively. The relative contribution of nsGPx to the total activity of glutathione peroxidase was approximately 10% in control embryos, 25% in non-malformed embryos and 30–40% in malformed embryos of diabetic mothers. A similar increase in glutathione peroxidases was detected in the liver and placenta of 15-day embryos. The contribution of nsGPx isoenzyme in the placenta of malformed embryos was 34%, which was double that found in the other two embryo categories.

Table 2. Changes in glutathione peroxidase activities with time in developing embryos of control and experimentally diabetic rats.

Gestation (days)	tGPx	SGPx	NsGPx
		(mU/mg protein)	
Embryos of non-diabetic control mothers			
9	22.63±0.27	20.09±0.65	2.55±0.67
10	45.24±0.49	40.28±0.12	4.96±0.53
11	60.21±0.36	54.65±0.75	5.56±1.00
15 (liver)	58.78±0.70	51.12±0.34	7.66±0.75
15 (placenta)	49.09±0.52	41.70±0.81	7.39±0.57
Non-malformed embryos of diabetic mothers			
9	35.36±0.73[a]	27.18±2.14[a]	8.18±2.03[a]
10	72.87±0.51[a]	54.72±0.73[a]	18.15±0.67[a]
11	101.76±0.46[a]	79.43±0.60[a]	22.33±0.53[a]
15 (liver)	95.18±0.92[a]	77.76±0.94[a]	17.42±0.64[a]
15 (placenta)	60.62±0.80[a]	44.91±0.76[a]	15.78±0.57[a]
Malformed embryos of diabetic mothers			
9	35.33±0.27[a]	24.28±2.35[a,b]	11.05±2.45[a,b]
10	73.62±0.72[a]	45.26±0.29[a,b]	28.36±0.89[a,b]
11	99.44±1.33[a,b]	61.18±0.81[a,b]	38.26±1.24[a,b]
15 (liver)	95.74±1.11[a]	57.32±0.99[a,b]	38.43±1.31[a,b]
15 (placenta)	58.32±1.02[a,b]	38.40±0.52[a,b]	19.92±0.64[a,b]

Data presented as the mean ± SD
[a]Significantly different from embryos of non-diabetic control mothers ($P<0.05$).
[b]Significantly different from non-malformed embryos of diabetic mothers ($P<0.05$).

Table 3. Changes in glutathione-S-transferase activities (U/mg protein) with time in developing embryos of control and experimentally diabetic rats.

Gestation (days)	Embryos of controls	Embryos of diabetic mothers	
		Non-malformed	Malformed
9	0.81±0.04	1.21±0.10[a]	1.22±0.10[a]
10	2.09±0.35	3.85±0.44[a]	4.09±0.53[a]
11	3.30±0.36	4.95±0.20[a]	5.08±0.26[a]
15 (liver)	5.34±0.39	9.04±0.34[a]	10.05±0.38[a]
15 (placenta)	7.63±0.50	10.98±0.51[a]	11.59±0.62[a]

Data presented as the mean ± SD
[a]Significantly different from embryos of non-diabetic control mothers ($P<0.05$).
[b]Significantly different from non-malformed embryos of diabetic mothers ($P<0.05$).

Table 4. Changes in glutathione reductase activity (mU/mg protein) with time in developing embryos of control and experimentally diabetic rats.

Gestation (days)	Embryos of controls	Embryos of diabetic mothers	
		Non-malformed	Malformed
9	5.12±0.36	4.40±0.81[a]	2.81±0.33[a,b]
10	6.70±0.71	5.53±0.43[a]	3.58±0.44[a,b]
11	7.75±0.67	6.69±0.46[a]	3.88±0.33[a,b]
15 (liver)	9.63±0.75	9.08±0.59	8.00±0.73
15 (placenta)	1.74±0.43	1.36±0.26	1.19±0.29

Data presented as the mean ± SD
[a]Significantly different from embryos of non-diabetic control mothers ($P<0.05$).
[b]Significantly different from non-malformed embryos of diabetic mothers ($P<0.05$).

There was also a substantial increase in GST activity with gestation (Table 3). Higher values were observed in the embryos of the experimental diabetic rats, regardless of the malformation status.

A gradual increase in GR activity was noted in embryos between gestation days 9 and 11. However, GR activity in embryos of the experimental diabetic rats was lower than that in controls (Table 4), especially in malformed embryos, where activity was reduced by almost 50%.

Streptozotocin-induced diabetes was associated with disturbance of low molecular weight antioxidants. This resulted in a decrease in vitamin E and vitamin C levels (Table 5). Gradual accumulation of ascorbate in embryos was demonstrated between gestation days 9 and 11, and in fetal liver and placenta on day 15.

Experimental diabetic gestation also caused a decrease in selenium accumulation in fetal tissue, which was more apparent in malformed embryos (Table 5). The gap between malformed and control embryos steadily widened as gestation progressed. The concentration in malformed embryos was almost half that of controls by gestation day 11. Parameters of oxidative stress in livers of control and experimentally diabetic pregnant female rats on day 15 of gestation is shown in Table 6. In addition to a much higher

Table 5. Changes in the levels of vitamin E, vitamin C and selenium with time in developing embryos of control and experimentally diabetic rats.

Gestation (days)	Embryos of controls	Embryos of diabetic mothers	
		Non-malformed	Malformed
Vitamin E (μg/g tissue)			
9	2.18±0.11	1.91±0.08[a]	1.91±0.06[a]
10	2.94±0.29	2.34±0.38[a]	1.99±0.41[a,b]
11	2.90±0.12	2.42±0.22[a]	2.39±0.31[a]
15 (liver)	1.56±0.26	1.29±0.13[a]	1.16±0.13[a]
15 (placenta)	5.95±0.57	4.69±0.49[a]	3.73±0.65[a,b]
Vitamin C (μg/g tissue)			
9	29.39±3.08	20.40±2.90[a]	12.32±1.01[a,b]
10	55.56±4.46	50.42±7.37	25.87±8.91[a,b]
11	61.57±7.40	58.01±3.99	32.35±4.79[a,b]
15 (liver)	97.32±7.30	89.19±6.04	64.29±8.20[a,b]
15 (placenta)	61.40±5.01	50.28±3.74[a]	25.75±2.934.30[a,b]
Selenium (ng/g tissue)			
9	97.40±7.37	86.52±6.78[a]	69.36±5.78[a,b]
10	121.00±4.22	101.30±6.04[a]	68.44±3.60[a,b]
11	152.13±6.31	134.70±7.30[a]	79.32±6.21[a,b]
15 (liver)	259.45±3.91	224.90±7.56[a]	189.12±10.81[a,b]
15 (placenta)	351.26±5.56	323.00±2.80[a]	276.41±5.99[a,b]

Data presented as the mean ± SD
[a]Significantly different from embryos of non-diabetic control mothers ($P<0.05$).
[b]Significantly different from non-malformed embryos of diabetic mothers ($P<0.05$).

blood glucose level (388±32.2 mg/dL *vs*. 88±11.7 mg/dL in the control group), other markers were indicative of increased ROS production and oxidative stress.

Activity of all GPx isoforms in diabetic liver was higher than that in the control group (Table 7). However, the concentrations of low molecular weight antioxidants (vitamin E, vitamin C and selenium) were lower in diabetic livers.

Discussion

Experimental diabetic gestation combines the stress of pregnancy and diabetes, which could lead to an increased incidence of congenital anomalies. This is reflected in the present study by an increased number of fetal deaths and consequent resorptions, as well as an increased frequency of malformations. However, not all embryos of diabetic mothers develop major malformations, even in pregnancies characterised by poor metabolic control.

The period of rat embryonic organogenesis between gestational days 9 and 11, which corresponds to post-conceptional weeks 3 to 5 in humans,[21] is characterised by a gradual increase in oxidative stress markers (e.g., elevation of TBARS) and depletion of GSH. Although the proportion of ROS released in the embryos is not known, it could be very high during organogenesis, a period when the embryo changes its energy metabolism from largely anaerobic (up to day 10) to aerobic glucose utilisation.[21] Furthermore, developing embryonic mitochondria possibly produce large amounts of ROS in response to an increased level of oxidative substrates.[22]

The results of the present study clearly indicate a definite over-production of free radicals and excessive exposure of embryos to oxidative stress in experimental diabetic gestation. In the population studied, the index of oxidative stress was higher in malformed embryos and this suggests its probable implication in diabetes-induced malformations. The results also indicate that increased free radical production in early diabetic gestation is coupled with disturbances to scavengers, especially the glutathione system.

Production of ROS during early developmental stages[23] necessitates the existence of antioxidant defence mechanisms, which are delicately balanced in the embryo. The effects of disturbing such a balance are difficult to predict and should be considered and interpreted carefully. Depletion of GSH in rat embryos *in utero* is reported to increase the incidence of malformations significantly,[24] as observed in the present study. Such depletion might result from either depressed synthesis or increased depletion by conjugation with electrophilic toxicants and removal from the embryonic biosphere.

Studies lend support to these probable causes. The activity of γ-glutamylcysteinyl synthetase (γ-GCS), which controls the rate-limiting step in GSH biosynthesis, and the expression of its messenger RNA (mRNA) are inhibited in embryos cultured under hyperglycaemic conditions.[25] However, the observed induction of GST probably represents an adaptive response to help eliminate accumulated toxic carbonyl-, peroxide- and epoxide-containing metabolites produced by the action of ROS on different cellular macromolecules.[26] Elimination of these S-conjugates is crucial to normal cellular function, as their intracellular accumulation causes cytotoxicity.[27]

Table 6. Parameters of oxidative stress in the liver of control and experimentally diabetic pregnant female rats on day 15 of gestation.

	Controls	Diabetic mothers
TBARS (nmol MDA/mg protein)	0.21±0.02	0.66±0.14[a]
Total GSH (nmol/mg protein)	45.00±2.10	38.99±0.65[a]
Reduced GSH (nmol/mg protein)	43.45±1.96	34.70±0.77[a]
Oxidised GSSG (nmol/mg protein)	0.78±0.10	2.15±0.31[a]
Redox potential (mV)	–251.5±1.2	–232.7±2.1[a]

Data presented as the mean ± SD
[a]Significantly different from embryos of non-diabetic control mothers ($P<0.05$). [b]Significantly different from non-malformed embryosof diabetic mothers ($P<0.05$).

Table 7. Parameters of antioxidant defence in the liver of control and experimentally diabetic pregnant female rats on day 15 of gestation.

	Controls	Diabetic mothers
TGPx (mU/mg protein)	83.96±2.98	108.88±5.33[a]
sGPx (mU/mg protein)	75.10±1.82	90.35±3.65[a]
nsGPx (mU/mg protein)	8.86±1.85	18.72±2.18[a]
GR (mU/mg protein)	15.26±1.57	8.15±0.69[a]
GST (mU/mg protein)	11.03±0.94	17.37±1.43[a]
Vitamin E (μg/g tissue)	30.82±1.15	26.12±1.62[a]
Vitamin C (μg/g tissue)	202.16±11.32	118.41±23.86[a]
Selenium (ng/g tissue)	395.51±15.13	346.24±15.80[a]

Data presented as the mean ± SD
[a]Significantly different from embryos of non-diabetic control mothers ($P<0.05$). [b]Significantly different from non-malformed embryos of diabetic mothers ($P<0.05$).

Changes in redox potential of the oxidised/reduced glutathione couple appear to correlate with the biological status of the cell. The half-cell redox potential is estimated to be approximately –240 mV for the proliferation phase, –200 mV for differentiation and –170 mV for apoptosis. These estimates are considered to provide a rationale and understanding of the cellular mechanisms associated with cell growth and development, signalling and reductive or oxidative stress.[13]

The calculated redox potentials in the present study are nearly constant for each category of embryo throughout the gestational days investigated. The values obtained for the control embryos are almost identical to the proposed estimates for the redox potential during the proliferation phase. The experimental diabetic gestation embryos represent a lesser reductive state with values equivalent to the differentiation phase, while the redox potential for the malformed embryos was lowest on gestational day 9 but approached the values for non-malformed embryos on subsequent days. These results suggest that although cells from control embryos and those from embryos of diabetic mothers were of the same gestational age, they were probably at different stages of development.

The antioxidant capacity of rat embryo *in vivo* and *in vitro* is reported to increase with gestation.[28,29] In general, this is in accord with the observations reported here. The activities of all GPx isoenzymes and GST, but not GR, were higher in embryos affected by maternal diabetes. The lower GR activity in embryos from diabetic mothers provides a plausible explanation for the observed accumulation of GSSG in these embryos, particularly those with malformations, which showed the highest GSSG levels and the lowest GR levels.

Oxidative stress is not only coupled with modulations in the glutathione system and related enzymes but also with disturbances in other low molecular weight antioxidants. In murine gestation, the fetus relies on maternal blood for its supply of selenium.[30] There is a significant decrease in selenium concentration in maternal blood,[31] with corresponding increases in placental and fetal levels, which become more pronounced as gestation proceeds.[30]

In the present study, there was a gradual accumulation of selenium with gestational age in embryonic tissue. However, the lower levels of hepatic selenium in diabetic mothers, compared to those in the controls, were reflected in their embryos, particularly the malformed ones. In early murine life, disruption of the gene expression of selenocysteine tRNA proves lethal, suggesting an essential function for selenoproteins in embryonic development.[32] This may be a contributing factor to the increase in fetal deaths, resorptions and malformations associated with experimental diabetic gestation.

In the present study, differences in vitamin E level were minimal among the different categories of embryos, which was not what had been anticipated. Nevertheless, vitamin C levels showed substantial decreases only in malformed embryos from the experimental diabetic gestation group. This suggests a critical role for vitamin C in diabetes-induced embryopathy, and is supported by findings that the addition of vitamin C to embryo cultures normalises embryonic antioxidant defence and reduces the damage caused by the diabetic environment.[33,34]

These experiments on pregnant rats confirm the reported increases in oxidative stress in experimental diabetic gestation. Despite agreement with published data on elevated MDA and lowered GSH concentrations, the increased GPx activity contradicts reports of diminished enzyme activity.[35] However, in hyperglycaemic states, over-production of antioxidant enzymes, including GPx, is thought to constitute a response to glucose-induced oxidative stress.[36]

The changes in enzyme activity were coupled with reduction in low molecular weight antioxidants in the experimental diabetic gestation group, compared to the non-diabetic controls. Both groups of animals were housed under the same conditions, and consumed similar quantities of the same diet. Hyperglycaemia and a lack of insulin are known to depress ascorbate clearance from plasma into tissues, creating a condition known as tissue scurvy.[37] This may, at least in part, explain the decreased levels of vitamin C in samples of maternal liver and placenta under diabetic conditions.

In conclusion, the data presented here suggest that increased oxidative stress and the perturbation in antioxidant defence are contributing factors to the high incidence of congenital anomalies in experimental diabetic gestation. □

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