Embryopathy in experimental diabetic gestation: assessment of PGE_2 level, gene expression of cyclooxygenases and apoptosis

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ABSTRACT

The causes of, and predisposing conditions for, increased congenital anomalies in embryos of experimental diabetic gestation are not fully identified. In the present study, some possible factors involved in diabetes-induced embryopathy are explored. The concentration of PGE_2, the gene expression of cyclooxygenases (COX-1 and COX-2) and level of apoptosis (measured by caspase-3 activity) are assessed during organogenesis in the embryos of streptozotocin-induced diabetic rats. The concentrations of PGE_2 in the embryos of diabetic rats were lower than controls, with the lowest values in malformed embryos and their associated membranes (yolk sacs). The pattern of change in PGE_2 was similar in the embryos of the control and diabetic groups, which showed a steady decline between days 9 and 11 of gestation. These changes in PGE_2 were accompanied by a small decrease in COX-1 expression in all embryos and associated membranes during the same gestational period. Expression of COX-2, which was below normal in diabetic embryos, decreased between days 9 and 11 of gestation in all groups. In the membranes of non-malformed embryos, COX-2 expression peaked on day 10 of gestation. It was found that there was little or no detectable COX-2 expression in the membranes of malformed embryos on day 9 of gestation and although its expression was detectable on the following days it was much lower than in the other groups. Caspase-3 activity increased substantially between days 9 and 11 of gestation. Embryos from the experimentally diabetic group showed higher activity than did controls, with the largest increases in the malformed embryos. It would appear that COX-2 expression and PGE_2 concentration (in both embryo and associated membranes) play a significant role in organ formation. The data presented here suggest that an unhealthy placenta may be instrumental in the development of malformed embryos.

KEY WORDS: Apoptosis. Cyclooxygenases. Diabetes mellitus. Fetal development. Prostaglandins. Teratogens.

Introduction

Despite numerous improvements in the management of diabetes mellitus, increased incidence of congenital malformations remains a major complication in infants of diabetic mothers. The cause of such phenomena and the predisposing factors that determine which embryos are at risk and why some embryos are spared are not clear.

A variety of potential metabolic disturbances, including maternal hyper- and hypoglycaemia, hyperketosis, nutritional deficiencies, oxidative stress and increased oxygen free-radical production, together with many other non-genetic and genetic factors have been implicated.[1] Diabetes-induced alteration of prostaglandin E_2 (PGE_2) and arachidonic acid metabolism have been identified as having teratological capacity.[2] Addition of PGE_2 or arachidonic acid to murine embryos exposed to a diabetic-like environment *in vivo* and *in vitro* diminished embryonic dysmorphogenesis.[3,4] During organogenesis, murine embryos were shown to have functional COX enzymes and active prostaglandin production. However, it has been suggested that COX-2, but not COX-1, plays a crucial role during the initial stages of pregnancy.[5]

A proposed hypothesis for the aetiology of diabetes-associated malformation is hyperglycaemia-induced apoptosis.[6] In experimental models of both pre- and post-implantation diabetes-induced anomalies, accelerated apoptosis has been detected in tissues destined to show evidence of malformation.[7] Maternal hyperglycaemia is proposed to trigger exaggerated apoptosis in the developing murine embryo, which could result in congenital malformation or fetal death.

Caspases are cysteine proteases involved in the initiator and effector phases of apoptosis.[8] Caspase-3 represents a common final pathway of the execution of apoptosis in highly divergent systems,[9] and its activity is used to assess the level of apoptotic activity.

In the present study, some of the proposed mechanisms involved in diabetes-induced embryopathy are explored in an attempt to clarify their possible role in the development of congenital anomalies. The results for oxidative stress and antioxidant defence have already been reported.[10]

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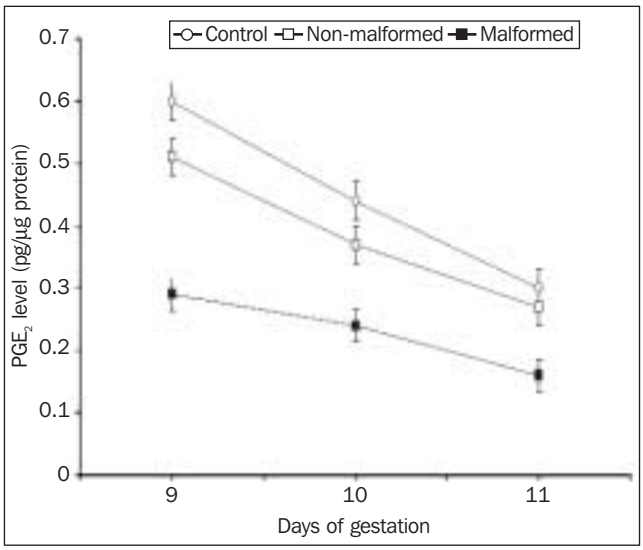

Fig. 1. Changes in PGE_2 level in rat embryos of diabetic and non-diabetic control groups during organogenesis. Each point represents the mean of six determinations (vertical bars represent SD).

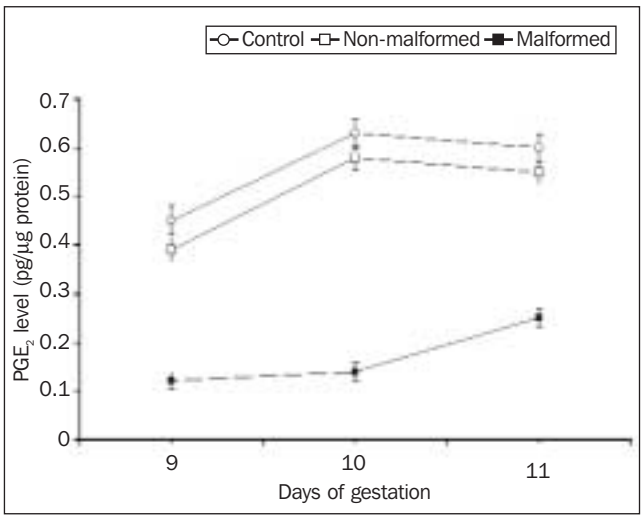

Fig. 2. Changes in PGE_2 level in the membranes of rat embryos of diabetic and non- diabetic control groups during organogenesis. Each point represents the mean of six determinations (vertical bars represent SD).

The additional factors considered here are COX gene expression, PGE_2 level and caspase-3 activity as a measure of apoptosis.

Materials and methods

Rats from a local Wistar-derived strain were used. Induction of chemical diabetes by streptozotocin (STZ) and mating of female diabetic rats have already been described.[10]

Pregnant diabetic and non-diabetic female rats were sacrificed on gestational days 9, 10 and 11. Embryos and their membranes were dissected from the uterine horns and examined for gross anatomical malformation,[10] and those with apparent anomalies were regarded as malformed.

In the present study, embryos and their membranes were snap-frozen in liquid nitrogen for subsequent PGE_2 analysis

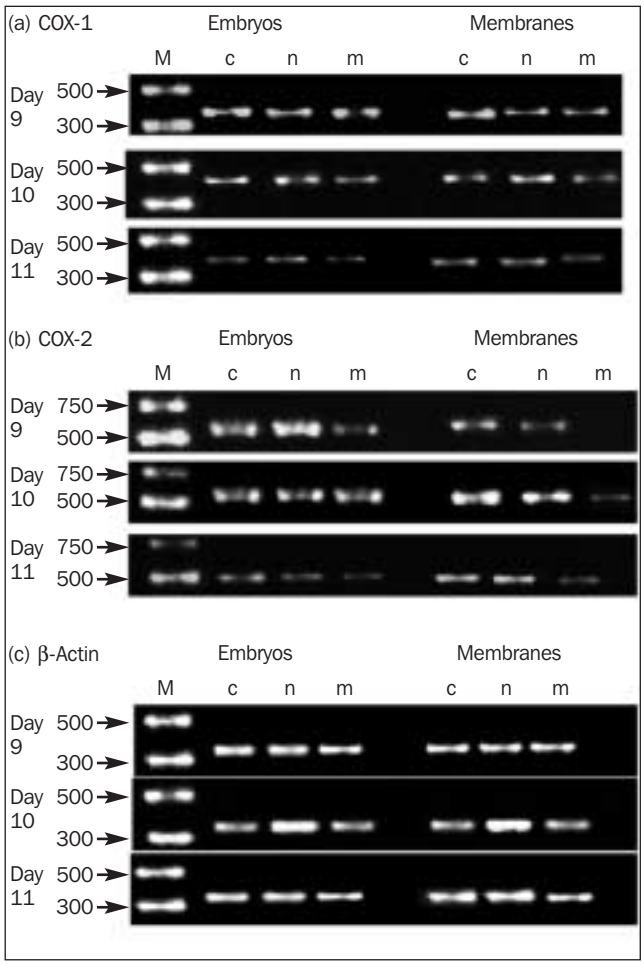

Fig. 3. Expression of genes encoding a) COX-1, b) COX-2 and c) β-actin in the different categories of embryos and their membranes. Control: c; non-malformed: n; and malformed: m. Marker DNA: M.

by a competitive enzyme immunoassay (EIA) kit (Assay Designs).[11] Gene expressions of both COX-1 and COX-2 were determined by reverse transcription–polymerase chain reaction (RT-PCR).[12,15] Messenger RNA (mRNA) expression of the genes encoding these two COX isoforms was semi-quantified by running the reaction products of PCR on agarose and comparing their relative band densities to the β-actin band used as the internal standard.[12]

Caspase-3 activity was determined in embryos only by the method described by Thronberry.[13] Protein was assayed by the method of Lowry *et al*.[14] All determinations were carried out on individual samples.

Data obtained were assessed statistically by one-way ANOVA for repeated measurements. Results are presented as the mean and standard deviation (SD).

Results

Data on the changes in PGE_2 concentration in the embryos and their membranes are presented in Figures 1 and 2, respectively. PGE_2 levels in the homogenates of the embryos from the diabetic group were lower than in controls, with the lowest values found in the malformed embryos. Between days 9 and 11 of gestation, PGE_2 levels in non-

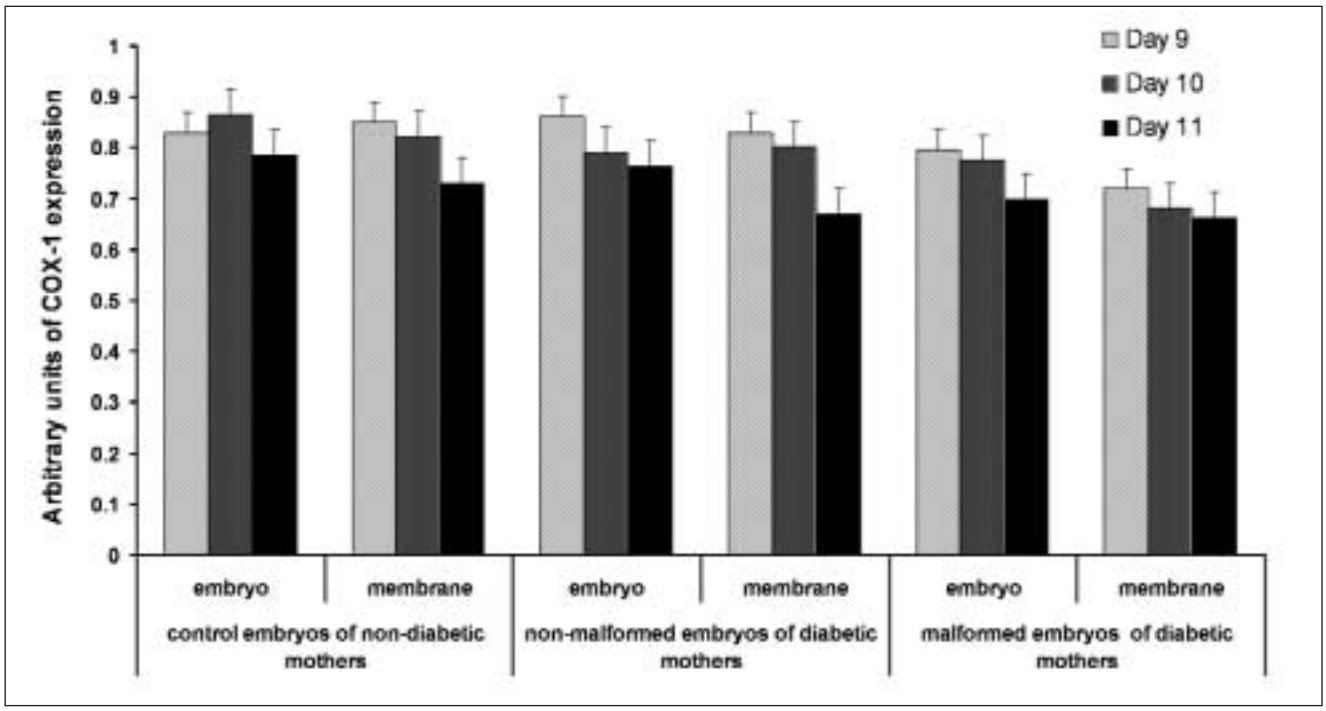

Fig. 4. Semi-quantitative estimation of COX-1 gene expression in the rat embryos and their membranes in diabetic and non-diabetic control groups during organogenesis. Data represent the mean of five determinations (bars represent SD).

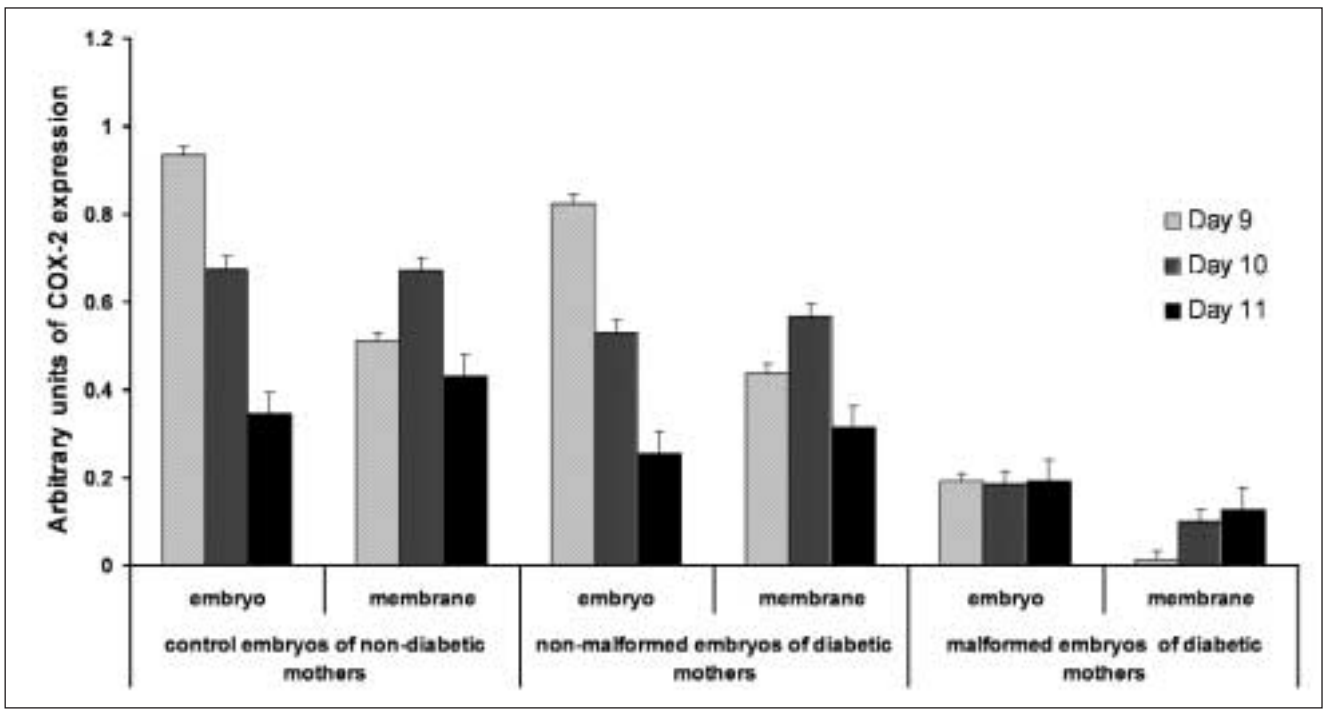

Fig. 5. Semi-quantitative estimation of COX-2 gene expression in the rat embryos and their membranes in diabetic and non-diabetic control groups during organogenesis. Data represent the mean of five determinations (bars represent SD).

malformed embryos were only 10–15% below those of the control group, while the decrease was 45–50% in the malformed embryos. In the membrane samples there was a sharp rise in the control and non-malformed embryo groups, followed by a slight decline on day 11 (Fig. 2). The membranes of malformed embryos had the lowest PGE_2 concentrations, averaging 75% below the control group on days 9 and 10 and approximately 60% on day 11.

Examples of COX-1 and COX-2 expression are presented in Figure 3. There was a tendency for a small decrease in COX-1 expression in all embryos and membranes from day 9 to 11 of gestation (Fig. 4). Estimates of COX-1 expression were similar in embryos and membranes of the control and diabetic groups. The only noted exception was the relative decrease in expression in the membranes of the malformed embryos.

Table 1. Changes in caspase-3 activity (pmol/h per μg protein) in rat embryos of diabetic and non-diabetic control mothers during the period of organogenesis.

Gestation*	Embryos of control mothers	Embryos of diabetic mothers	
		Non-malformed	Malformed
Day 9	3.37±0.52 (6)	5.01±0.072† (6)	5.34±0.41† (5)
Day 10	10.19±0.70 (6)	14.17±1.50† (5)	23.21±0.80†‡ (5)
Day 11	13.10±0.48 (6)	22.19±2.13† (5)	26.74±1.52†‡ (5)

Data presented as the mean ± SD.

*Day of mating was considered day 0 of gestation.

†Significantly different from embryos of non-diabetic control mothers ($P<0.05$).

‡Significantly different from non-malformed embryos of diabetic mothers ($P<0.05$).

Data on COX-2 expression, presented in Figure 5, emphasise the difference between this isoform and COX-1. The tendency for COX-2 expression to decrease between days 9 and 11 is apparent. In addition, the results indicate that COX-2 expression in embryos of experimental diabetic gestation was below normal, especially in malformed embryos. In contrast, COX-2 gene expression in the membranes of control and malformed groups (Fig. 5) shows a tendency to peak on day 10, followed by a fall on day 11. There was little or no COX-2 expression in the membranes of malformed embryos on day 9, and, although expression was detectable on days 10 and 11, it was much lower than in the membranes of the control or non-malformed groups (Figs. 3 and 5).

Caspase-3 activity increased substantially in all categories of embryo between days 9 and 11 (Table 1). Embryos in the experimentally diabetic group showed higher activity than in the control group, with the highest levels seen in the malformed embryos.

Discussion

Experimental diabetic gestation may prove to be of value in understanding the aetiology of diabetic embryopathy. Maternal diabetes results in exposure of the developing embryo to excessive oxidative stress and an oxidising environment.[10] This is coupled, as can be seen in the present study, with inhibition of COX-2 but not COX-1, decreased PGE_2 level and exaggerated apoptosis. These abnormalities were especially evident in malformed embryos.

In culture, high glucose levels and COX-2 inhibitors were reported to cause similar types of embryonic damage. Addition of arachidonic acid (a COX substrate and PG precursor), PGE_2 or antioxidants prevented developmental abnormality. This led to the suggestion that the teratogenic pathways related to excess reactive oxygen species (ROS) or to deficiency in arachidonic acid or COX are probably linked.[15]

Although excess ROS and lipid peroxidation products are reported to be associated with increased COX-2 gene expression,[16] data obtained in the present study showed the opposite in embryos and their yolk sacs exposed to a diabetic environment. Excessive oxidative stress[10] was associated with lower PGE_2 level and COX-2 gene expression; findings supported by other published data.[15,17] Explanations of this phenomenon were offered either through lowered activity of nuclear transcription factor-kappa B (NF-κB), an endogenous stimulator of COX-2 expression, or increased p53 regulator protein, an inhibitor of COX-2.[15,18,19] However, it should be noted that despite similar expression of COX-1 in all categories of embryos in the present study, there were differences in PGE_2 level that paralleled COX-2 expression. Apparently, both PGE_2 and COX-2 were similarly affected by the prevailing experimental diabetic gestation conditions.

Many metabolic and biochemical factors prevalent in the embryo of diabetic mothers favour depressed COX-2 expression and lowered PGE_2 biosynthesis. These include the regulatory role of selenium in the enzymatic oxidation of arachidonic acid.[20] Lower selenium levels[10] would result in low PGE_2, and glutathione (GSH) is known to be required for the conversion of PGG_2 to PGH_2 and then on to PGE_2.[15] Low GSH concentration observed in rat embryos as a result of maternal diabetes, particularly accompanying cases of malformation,[10] would thus lead to diminished GSH-dependent synthesis of PGE_2. The resulting low level of PGE_2 could lead to congenital anomalies in susceptible embryos.[15]

Both COX-2 and PGE_2 have been linked to angiogenesis,[21] which is known to play a pivotal role in shaping the body during organ formation.[22] Decreased PGE_2 level and COX-2 expression in embryos and their associated yolk sacs in the present study may result in disturbed angiogenesis.

The possibility that embryopathies induced by hyperglycaemia are yolk sac-dependent has been raised by some investigators.[23,24] During organ formation, the yolk sac provides protection, transport of nutrient and is the origin of blood cells and vessels.[25] Disturbances in COX-2 expression and PGE_2 metabolism and availability could, therefore, lead to metabolic alterations and/or abnormalities in the developing embryo.

Apoptosis plays a vital role in normal organ development in the embryo, with a delicate cell balance between proliferation and differentiation signals. The biochemical environment prevalent in experimental diabetic gestation, represented by increased oxidative stress and a shift in redox potential towards a more oxidative state,[10] favours apoptosis and induction of the caspase cascade.[26] Maternal hyperglycaemia may disturb the expression of regulatory genes in embryonic development and cell-cycle progression, resulting in premature death of the progenitor cell with consequent defective morphogenesis.[7] Moreover, PGs are reported to induce synthesis of Bcl-2 protein, which is an anti-apoptotic factor.[27] Low PGE_2 levels observed in malformed embryos may therefore be insufficient to induce Bcl-2 and antagonise or inhibit the accelerated apoptotic process.

From the available data, factors are emerging that probably play a significant role in diabetes-induced congenital anomalies. In the diabetic environment, embryos are exposed to excessive oxidative stress with relatively diminished capacity for antioxidant defence.[10] This is coupled with disturbances in the metabolic expression of factors that control organogenesis and embryonic

development. These factors are probably interrelated and might work in a cascade fashion. However, the role of an unhealthy placenta cannot be ignored and should be clarified. □

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