Diabetes Publish Ahead of Print, published online August 26, 2008

Decreased cardiac glutathione peroxidase levels and enhanced mandibular apoptosis in malformed embryos of diabetic rats

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Received 9 January 2008 and accepted 14 August 2008.

Additional information for this article can be found in an online appendix at
http://diabetes.diabetesjournals.org

ABSTRACT

Objective: To characterize normal and malformed embryos within the same litters from control and diabetic rats for expression of genes related to metabolism of reactive oxygen species (ROS) or glucose, and developmental genes.

Research design and methods: Embryos from non-diabetic and streptozotocin-diabetic rats were collected on gestational day 11 and evaluated for gene expression (PCR) and distribution of activated Caspase-3 and glutathione peroxidase-1 (Gpx-1) by immunohistochemistry.

Results: Maternal diabetes (MD) caused growth retardation and increased malformation rate in the embryos compared to controls (N). We found decreased gene expression of Gpx-1 and increased expression of Vascular endothelial growth factor-A (Vegf-A) in malformed embryos of diabetic rats (MDm), compared with non-malformed littermates (MDn). Alterations of mRNA levels of other genes were similar in MDm and MDn embryos. Thus, expression of Copper Zinc superoxide dismutase (CuZnSOD), Manganese superoxide dismutase (MnSOD), and Sonic hedgehog homolog (Shh) were decreased, and Bone morphogenetic protein-4 (Bmp-4) was increased in the MD embryos compared to N embryos. In MDm embryos, we detected increased activated Caspase-3 immunostaining in the first visceral arch and cardiac area, and decreased Gpx-1 immunostaining in the cardiac tissue, both findings differed from the Caspase / Gpx-1 immunostaining of the MDn and N embryos.

Conclusions: Maternal diabetes causes growth retardation, congenital malformations and decreased general antioxidative gene expression in the embryo. In particular, enhanced apoptosis of the first visceral arch and heart, together with decreased cardiac Gpx-1 levels may compromise the mandible and heart to an increased risk of developing congenital malformation.

Abbreviations

AOE – antioxidative enzyme, AR – Aldose reductase, Bmp-4 – Bone morphogenetic protein-4, CuZnSOD – Copper Zinc superoxide dismutase, G6PDH – Glucose-6-phosphate dehydrogenase, GAPDH – Glyceraldehyde-3-phosphate dehydrogenase, Gpx-1 – Glutathione peroxidase-1, Gpx-2 – Glutathione peroxidase-2, IL-6 – Interleukin-6, MnSOD – Manganese superoxide dismutase, MD – manifestly diabetic rat / offspring of MD rat, MDm – malformed MD offspring, MDn – non-malformed MD offspring, N – normal rat / offspring of N rat, Nm – malformed N offspring, Nn – non-malformed N offspring, NAC – N-acetylcysteine, PARP – Poly (ADP-ribose) polymerase, PGE2 – Prostaglandin E2, p53 - tumor protein 53, Ret - Ret proto-oncogene, ROS – reactive oxygen species, Shh – Sonic hedgehog homolog, TNF-alpha – Tumor necrosis factor-alpha, Vegf-A - Vascular endothelial growth factor-A

The cellular and molecular mechanisms of diabetic embryopathy are not completely clarified. Previous experimental studies have suggested that the teratological impact of a diabetic environment partly depends on excess of reactive oxygen species (ROS) in the embryo (1) as a consequence of either increased free oxygen radical formation (2-4) or decreased capacity of ROS-scavenging enzymes (5-9), or both. Previous work has furthermore demonstrated that supplementation of antioxidative agents such as Copper Zinc superoxide dismutase (CuZnSOD) (1; 2), N-acetylcysteine (NAC) (10), vitamin E & C (8) and folic acid (11) *in vitro*, as well as BHT (12), vitamin E (13-19), vitamin C (18; 20), NAC (21) and folic acid (11) *in vivo* attenuate malformation rate and diminish markers of oxidative stress, *e.g.* by normalizing tissue levels of TBARS (15), isoprostane 8-iso-$PGF_{2\alpha}$ (22; 23) and carbonylated proteins (24).

The driving cellular force behind the diabetes-induced oxidative stress is likely to be associated with the enhanced glucose metabolism (25-27) in the embryonic/fetal cells exposed to the increased ambient levels of glucose. One putative primary source of the reactive radical compounds would be the mitochondria receiving a high influx of pyruvate and oxygen, and subsequently producing a large amount of ROS (mainly superoxide) (3) in the oxidative processes of the electron transport chain. The ensuing leakage of superoxide into other compartments of the mitochondria and the cytosol, and the further formation of hydrogen peroxide and hydroxyl radicals should yield mitochondrial alterations (28), as well as lipid peroxidation (22) and DNA damage (29) in the embryo. There are several observations in support of this notion. The structural alterations, mainly high-amplitude swelling of the embryonic mitochondria (28), are diminished by maternal antioxidative treatment (16), thereby supporting the notion of a ROS-related etiology of the structural changes. The enhanced lipid peroxidation, as evidenced by increased levels of the isoprostane 8-iso-$PGF_{2\alpha}$ (22; 23; 30), may induce several teratogenic pathways in addition to the developmental disturbance caused by peroxidation of structural lipids in mitochondrial and cellular membranes. For instance, it has been demonstrated that 8-iso-$PGF_{2\alpha}$, which is produced non-enzymatically by ROS-mediated oxidation of arachidonic acid in the offspring (30) has its own teratogenic potency (23). In addition, an excessive peroxidation of arachidonic acid may hamper prostaglandin biosynthesis by depleting precursor pools, and, in particular, yield decreased concentration of prostaglandin E2 (PGE2) (31), which could obstruct neural tube closure (22).

The ROS-induced DNA damage (29) may directly disrupt development by altered expression of key genes. In addition, the cellular DNA repair processes may activate poly(ADP-ribose) polymerase (PARP), which may cause Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibition by poly(ADP-) ribosylation (32). The net result would be diminished embryonic GAPDH activity, which has been demonstrated in rat embryos subjected to diabetes in vivo and high glucose in vitro (33). Furthermore, the decreased glycolytic flux proximal to GAPDH (32) and the presence of increased ambient glucose levels will yield enhanced flux in the polyol (34; 35) and hexosamine pathways (36). An increased availability of proximal glycolytic intermediaries would increase DAG production and cause activation of several PKC isoforms (37; 38), as well as enhancing the flux in the AGE pathway (39). All of the consequences of inhibited GAPDH activity may thus

contribute to the teratogenic outcome of diabetic pregnancy. Evidently, there are multiple ways for a diabetes-induced state of oxidative stress in the embryo to disturb embryonic/fetal development, several of which enjoy considerable experimental support.

Another consequence of a state of oxidative stress would be enhanced apoptosis in the embryonic/fetal tissue (40), which has been described (26; 41-43), and suggested to be mediated by enhanced Jun-amino-terminal kinase 1 & 2 (JNK1 & 2) activity (19; 44). It has also been suggested that maternal diabetes induces an inflammatory state in the embryo, where pro-inflammatory cytokines, i.e. TNF-alpha (45; 46), act to down-regulate the principal ROS-scavenging enzymes via increased activity of MAP kinases (47). The exact relation between enhanced apoptosis and induction of malformations is still unclear, mainly since we do not fully comprehend the specific transmission of a general increase in programmed cell death into precisely restricted developmental damage to embryonic organs or organ system (42).

Based on earlier studies (7; 48; 49) and based on a question, which has been raised several times – *what genes are involved in diabetes-induced embryonic dysmorphogenesis?* (5; 8; 26; 42; 50) – we wanted to add to the teratological knowledge by identifying differences in gene expression between the malformed and non-malformed offspring within the same litters of diabetic animals.

Our working hypothesis was that genes of the malformed offspring with an expression pattern different from that in the non-malformed offspring within the same litter may be associated with the teratogenic process, either directly or indirectly. We thus decided to compare gene expression of embryos that were morphologically normal and those that were malformed in litters of normal and diabetic rats. By comparing gene expression and protein distribution in embryos of the same age and with the same exposure to an identical intrauterine milieu, we aimed to control for these possible confounding factors of the teratogenic process. From earlier work, we knew that the two tissues of the rat fetuses, which are particularly vulnerable to the diabetic state are the mandible and the heart (17) (Figure 1). We also knew that a susceptible period in diabetic rat pregnancy occurs during gestational days 6-10 (51), which corresponds to week 2-4 in human pregnancy. Bearing in mind that the size and relative immaturity of day-10 embryos makes them difficult to evaluate for developmental defects, we chose to interrupt the rat pregnancies on gestational day 11, in order to be able to clearly distinguish between malformed and non-malformed embryos. We decided to relate embryonic (mal)development to expression of the major oxidative defense genes (SODs, Gpx-1, Gpx-2, catalase), to key genes of glucose metabolism (Aldose reductase (AR), GAPDH), to developmental/teratological genes (Poly (ADP-ribose) polymerase (PARP), tumor protein 53 (p53), Bone morphogenetic protein-4 (Bmp-4), Ret proto-oncogene (Ret), Sonic hedgehog homolog (Shh), Vascular endothelial growth factor-A (Vegf-A), Tumor necrosis factor-alpha (TNF-alpha), Interleukin-6 (IL-6)), and to the tissue distribution of activated Caspase-3 (denoting apoptotic rate) and Gpx-1.

METHODS

Animals. Non-diabetic and diabetic rats from a local Sprague-Dawley strain (bred in the Animal Facility of the Biomedical Center of Uppsala University) with an increased

incidence of congenital malformations in diabetic pregnancy were used as embryo donors (12). The rats had free access to food (AB Analycen, Lidköping, Sweden) and tap water and were maintained at an ambient temperature of 22°C with 12h light/dark cycle.

Diabetes was induced with a single injection of 40 mg/kg streptozotocin (SZ) (Sigma-Aldrich Chemie Gmbh, Stenheim, Germany) into a tail vein (12). Within one week after SZ injection, the blood glucose concentration was measured in a Free Style Mini Glucose Meter (Thera Sense Inc, Alameda, CA, USA). Rats with a glucose concentration exceeding 20 mM were considered to be manifestly diabetic (MD), non-SZ-injected female rats were denoted non-diabetic controls (N). After establishing the diabetic status, the MD and N rats were mated overnight with non-diabetic male rats. A positive vaginal smear (containing sperm) the following morning designated gestational day 0. On gestational day 11 the MD and N rats were killed by cervical dislocation after light ether anesthesia. This gestational day was chosen since it corresponds to an early stage of embryogenesis where malformations of the heart, neural tube and general rotation pattern can be identified, and, consequently, their severity be assessed. The embryos were dissected out and evaluated with regard to presence of malformations (cardiac hypertrophy, neural tube defects, somatic malrotation), crown rump length and somite number. Viability of the embryos was confirmed by the presence of heartbeat. Embryos were prepared for immunostaining (4 N and 4 MD litters) and gene expression determination (4 N and 5 MD litters).

Preparation of total RNA. Total RNA from each embryo was isolated with RNeasy mini kit (QiaGEN GmbH Hilden, Germany) according to the manufacturer's instructions. Briefly, each embryo was lysed in 350 µl buffer. The samples were DNase treated (RNase-free DNase; QIAGEN GmbH, Hilden, Germany) and incubated at room temperature for 25 min. The isolated total RNA was washed twice with 50 µl RNase-free water and the accumulated flow-through was collected, yielding the total RNA sample. Lastly, 1 µl of an RNase inhibitor (RNA Guard; Amersham, Pharmacia Biotech, Uppsala, Sweden) was added to each sample.

Preparation of cDNA. One µg of total RNA was used for cDNA preparation. For cDNA synthesis, we used first-strand beads (Ready To Go, Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's instructions. The resulting cDNA was diluted threefold with water treated with RNAse-free water.

Analysis of mRNA levels. 4 litters with 32 embryos yielded the mRNA samples of the N group, whereas 5 litters with 35 embryos produced the mRNA samples of the MD group. One microliter of the cDNA purified from each embryos with 10 ng of converted total RNA was amplified and measured in duplicates with Real Time PCR using the MiniOpticon System (Bio-Rad Laboratories, Inc, Hercules, CA) with SYBR green used to detect the PCR product. Specific primers for CuZnSOD, MnSOD, Gpx-1, Gpx-2, Catalase, AR, GAPDH, p53, PARP, Shh, Ret, Bmp-4, Vegf-A, TNF-alpha and IL-6 were constructed with the aid of the “primer3” free software (http://primer3.sourceforge.net/) and subsequently purchased from TIB Molbiol (Berlin, Germany) (Table 1). We have previously assessed the stability of expression of various housekeeping genes and found the Glucose-6-phosphate dehydrogenase (G6PDH) gene to be constant in day-10 and day-11 embryos exposed to high glucose *in vitro* or diabetes *in vivo* (data not shown);

therefore, we chose the G6PDH gene as a reference in the real-time PCR protocol.

Controls were included in each run of the Real Time PCR assay; for each primer pair one sample with no cDNA (with only RNAse free water) was included. To exclude the possibility of remaining DNA fragments in the samples, 10 ng of the total RNA of each sample was amplified in the LightCycler. We found no PCR product in the water or the total RNA samples. Furthermore, we excluded the avian myoblastosis virus-RT enzyme in the cDNA preparation and found no amplified PCR product.

Results were analyzed for each sample with relative quantification comparing the difference between sample and reference crossing point (Cp) values. The relative abundance of mRNA/G6PDH in each sample was determined using the following equation:

$$2^{-(Cp,sample-Cp,G6PDH)}$$

to yield the ratio sample/G6PDH.

Immunohistochemistry of activated (cleaved) Caspase-3 and Gpx-1. 35 embryos from 4 litters and 28 embryos from 4 litters were used for immunohistochemistry in the N and MD groups, respectively. Embryos from different rats were fixed in 4% paraformaldehyde for 24 h and stored in 70% ethanol before they were embedded in paraffin and sectioned in 5-µm-thick sections. The slides were deparaffinized and rehydrated in accordance with standard procedures. Primary antibody, Cleaved Caspase-3, asp 175, 1:50 (1.2 µg), or Gpx-1 1:25 (2.5 µg) (Cell Signaling Technology, Inc. Beverly, MA, USA) were applied. The slides were incubated with secondary antibody (Dako EnVision+ Peroxidase, goat-anti-rabbit, DAKO A/S, Glostrup, Denmark). Thereafter the slides were incubated with chromogenic substrate solution Sigma Fast™ 3,3'-Diaminobenzidine tablet sets (Sigma-Aldrich Chemie, Stenheim, Germany). Lastly, the slides were counterstained (Mayer Haematoxylin, Histolab, Gothenburg, Sweden) and mounted with cover slips. The slides were viewed and photographed in a Leitz DMRB Leica fluorescence microscope (Leica AB, Stockholm, Sweden).

Statistical and ethical considerations. The Uppsala Regional Ethical Committee on Animal Experiments approved the research protocol including all experimental procedures involving animals. The comparisons between different experimental groups were based on individual embryos. Differences between means were evaluated by Student's two-tailed t-test, as well as by ANOVA with Fisher's PLSD as post hoc test. The rates of normal embryos and embryos with malformations were compared with χ^2-statistics. A value of $p < 0.05$ was considered to denote a statistically significant difference between groups. The calculations were performed with the aid of the Macintosh version of the statistical program Statview.

RESULTS

Maternal diabetes increased malformation and resorption rates in embryos compared with embryos of normal pregnancy (Figure 1). We also found decreased crown rump length as well as diminished somite number in embryos from diabetic rats compared with embryos from non-diabetic rats (Table 2).

The expression patterns of the antioxidative enzymes (AOEs) are shown in Figure 2. We found decreased gene expression of CuZnSOD, MnSOD and Gpx-1 in MD embryos (both MDn and MDm) compared with N embryos, In addition, Gpx-1 mRNA levels were more decreased in MDm embryos than in the MDn embryos, Gpx-2 and catalase expression did not differ between the groups (Figure 2). No significant

differences were found with regard to gene expression of AR, GAPDH, p53 and PARP (Figure 3).

Shh was decreased, Ret was unaltered and Bmp-4 mRNA levels were increased in MD embryos compared to N embryos (Figure 4). The Vegf-A mRNA levels were increased in both the MDn and MDm embryos compared with N embryos, and, in addition, the MDm embryos had higher mRNA levels than the MDn embryos (Figure 4). The TNF-alpha mRNA levels were decreased in the MD embryos (Figure 4), whereas we could not demonstrate IL-6 mRNA in the embryos (data not shown).

We detected increased activated Caspase-3 immunostaining in the first visceral arch and cardiac area in the MDm embryos compared with the MDn and N embryos (Figure 5). The immunostaining of Gpx-1 displayed a general accumulation of positive cells in the cardiac tissue of all embryos, however, the MDn embryos had less staining than the N embryos, and the MDm embryos had less staining than the MDn embryos (Figure 6).

DISCUSSION

One important finding in the present study was that maternal diabetes diminishes the antioxidative defense gene expression in the embryos, as evidenced by decreased expression of CuZnSOD, MnSOD, and Gpx-1 in MDn and MDm offspring, which may be responsible for a decrease in AOE activity (52). This observation is in concert with previous reports of diminished AOE activity in embryonic tissues exposed to diabetes (7-9; 48), however, not with all (2; 7; 9), which may be related to differences in mode and time of exposure to the diabetic milieu. In addition, in the present study we detected decreased Gpx-1 mRNA levels in MDm embryos and a corresponding reduction of Gpx-1 protein in the cardiac tissues of the MDm embryos, both findings differing from MDn embryos of the same litters, as well as from Gpx-1 estimations in N embryos. In a similar rat model, Gpx activity was found decreased in embryos with neural tube defects compared to non-malformed embryos from same litters of diabetic rats (7).

The specific decrease in Gpx-1 expression/occurrence in MDm embryos may suggest an association between this isoform of glutathione peroxidase and the teratogenicity of diabetes, tentatively with the processes behind cardiac malformations. Such an association has not been suggested before, to our knowledge, and the localization of Gpx-1 protein in the cardiac wall on day 11 may suggest the occurrence of a protective antioxidative mechanism. The net effect of the Gpx-1-deficiency – further enhanced by the decreased levels of the other AOEs - would be enhanced cellular damage, as suggested from mice with a targeted Gpx-1 mutation, displaying mitochondrial dysfunction (53) and apoptosis (54), changes in line with a teratological disturbance of normal development. Evidently, however, the association between malformation and reduced Gpx-1 expression may also be the opposite, the embryonic maldevelopment may actually induce the alteration in Gpx-1 mRNA levels, and, putatively, decreased activity of the enzyme (7).

In previous work, we have only been able to secure suggestive evidence of enhanced regional apoptotic activity in embryos subjected to a diabetic environment. We thus regard the results of the immunohistochemistry efforts of the present study with great interest. We thus found increased Caspase-3 immunostaining in the first visceral arch and in cardiac tissue of the MDm embryos compared with MDn and N embryos, indicative of enhanced apoptosis in

the two regions corresponding to the two major types of diabetes-induced malformations in this rat model, micrognathia and cardiac out-flow defects (cf. Figure 1).

We found only one malformed N embryo, which displayed gene expression patterns similar to the MDm embryos, further supporting the view of generalized changes of the gene clusters paving the way for specific morphogenetic alterations. In particular were the SODs, Gpx-1 and Shh genes decreased, and Vegf increased in the Nm embryo thus indicating a coupling to oxidative stress and neural crest cell development. However, the small sample size (n = 1) precludes any far-reaching conclusions.

The effect on AOE gene expression was strikingly similar in the CuZnSOD, MnSOD and Gpx-1, which were all decreased. The finding of these diabetes-related genes suggests - in concert with previous studies (4; 5; 7; 9) - that maternal diabetes causes a general down-regulation of the principal AOEs in rat embryos prone to develop congenital malformations, and allow the speculation that this putative weakening of the antioxidative defense may be involved in the teratogenic process. The physiological reasons for the diminished ROS scavenging gene expression in the diabetes-exposed embryos are not clear, and may therefore presently be subject to speculation only. It is noteworthy, that in the present study we found decreased TNF-alpha mRNA levels and could not detect IL-6 in the embryos, therefore a "generalized inflammation" concept causing the diminished AOE gene expression does not seem to be likely, at least not at this embryonic stage (45; 46).

The metabolic state of the severely streptozotocin-diabetic non-insulin treated rat has been characterized previously by us (10; 25). In general, the diabetic state yields blood glucose levels in the range of 20-25 mM, increased concentrations of ketone bodies (10) and increased levels of branched chain amino acids (25). The particular characteristics of the diabetic state, which may be primarily affecting gene expression, are not clear, but increased glucose levels should be of importance, since in vitro exposure of embryos to high glucose levels promotes gene expression changes analogous to those found in vivo. Furthermore, existence of a functioning glucose transporter gene has been shown to be of fundamental importance for diabetes-induced embryonic (mal)development (27), illustrating the importance of high intracellular levels of glucose in the diabetic embryopathy. Regarding the genes controlling glucose metabolism others and we have previously identified an association between increased sorbitol accumulation and dysmorphogenesis in the embryo (34; 35). However, attempting to diminish such accumulation by using AR inhibitors did not prevent the maldevelopment in the offspring (34; 35).

Another set of reasons for the interest in the AR and GAPDH genes are the result of a recent linkage study of skeletal malformation of diabetic rats, where we found linkage to the chromosomal regions harboring these two genes (data not published). In the present study, however, we found no significant changes of mRNA levels of AR and GAPDH in the MD embryos, as noted previously with regard to the GAPDH expression (33), a difference we are unable to explain. The previously demonstrated diabetes-induced decrease in GAPDH enzyme activity (33) may thus be a ROS-mediated alteration of the GAPDH enzyme itself (either directly or via DNA-damage-induced PARP activity) (32), thereby causing a block in the glycolytic pathway with a number of metabolic effects with teratological importance, such as DAG-mediated activation of (several isoforms of)

PKC (37; 38), increased hexosamine flux (36), and enhanced production of glycosylation precursors/products (39).

Maternal diabetes did not alter embryonic p53 (as noted previously (43)) and PARP expression in the present study, despite a putative PARP activity increase due to the presence of DNA damage in the MD embryos (29). The increased Bmp-4 levels of the embryos of diabetic rats suggested specific affection of the precursors of the skeletal system, which backs earlier findings of delayed and defect chondrogenesis and osteogenesis in the offspring (55).

With regard to the diabetes-induced malformations of this rat model (Figure 1), suggested to have a neural crest cell-derived origin (17), the two neural crest-associated genes Shh and Ret were expected to be affected. Whereas the Ret gene was unaltered by maternal diabetes, we found diabetes-induced decrease in the Shh mRNA levels in the MD embryos. Despite the absence of a significant difference in Shh mRNA levels between the MDn and MDm embryos, the decreased Shh expression in the MD offspring is of particular interest in relation to the findings of embryonic craniofacial (56) and aortic (57) anomalies resulting from direct inhibition of Shh, as well as the findings of an early role for Shh to secure the neural crest cell survival in the early development of the lower jaw (58). Taken together, these results support a diabetes-induced disturbance in the neural crest cell development (17; 59) of the embryos of the present model, and, putatively also in human diabetic pregnancy. However, since isolated neural stem cells exposed to glucose in vitro show increased gene expression of both Shh and Bmp-4 (50), the exact relationship(s) between gene expression changes in the surrounding tissues and the neural crest cells themselves caused by exposure to a diabetic environment will have be subject to further studies.

We found markedly increased Vegf-A expression in the MD embryos, and further increased expression in the malformed embryos compared to the non-malformed MD embryos, This is of particular interest with regard to the coupling between Vegf alterations and hampered embryonic development (60), although an *increased* Vegf expression has not been previously reported in association with embryonic dysmorphogenesis. We suggest that the enhanced embryonic Vegf-A levels in the present study may be interpreted as a compensatory change in response to a diabetes-induced general developmental delay.

The demonstrated gene expression changes in malformed (and non-malformed) embryos of diabetic rats will evidently demand future work efforts, in order to clarify the association to embryonic maldevelopment – cause, effect, or no relation at all – efforts that to some extent are ongoing.

One major, and slightly surprising, observation was that there was no clear *individual* pattern of gene expression in the embryos for any of the genes studied (cf. Appendix). Thus, when examining individual gene expression values there was no evident relation to uterine position, growth, or morphological status – with the possible exceptions of Gpx-1 and Vegf-A. The Gpx-1 mRNA was markedly decreased, and the Vegf-A mRNA were markedly increased in malformed compared to non-malformed embryos from the same litter of diabetic rats. Further efforts in the search for the etiology of diabetes-induced congenital malformations may therefore benefit from considering a putative impact of a decrease in embryonic Gpx-1 expression, and, possibly, function, as well as considering the importance of

enhanced Vegf-A expression for embryonic development.

We conclude that maternal diabetes causes growth retardation, congenital malformations and decreased general antioxidative gene expression in the embryo. In particular, enhanced apoptosis of the first visceral arch and heart, together with decreased cardiac Gpx-1 levels may compromise the mandible and heart to an increased risk of developing congenital malformation.

ACKNOWLEDGEMENTS

The authors are indebted to Ing-Marie Mörsare for valuable technical support. Financial support was obtained from The Ernfors Family Fund, The Swedish Diabetes Association, The Novo Nordisk Foundation, and The Swedish Research Council Grant No. 12X-7475.

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TABLE 1. Primers used in the study.

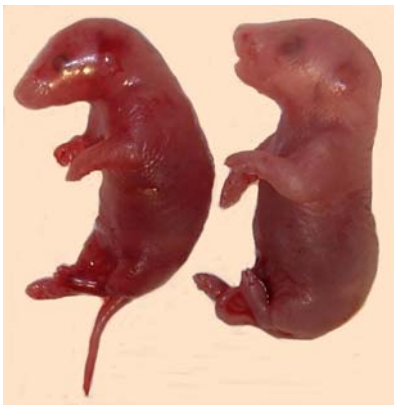
gene	forward primer	reverse primer
G6PDH	gTCATgCAgAACCACCTCCT	ACATACTggCCAAggACCAC
CuZnSOD	AAgCggTgAACCAgTTgTg	CCAggTCTCCAACATgCC
MnSOD	ggTggAgAACCCAAAggAgA	AgCAgTggAATAAggCCTgT
Gpx-1	TgAgAAgTgCgAggTgAATg	AACACCgTCTggACCTACCA
Gpx-2	TgCCCTACCCTTATgACgAC	ggAgATTCCTAggCTgAgCA
Cat	TTATgTTACCTCACAgCCTggT	gTgTTgTgTgTTCTgTgTgTgTAg
AR	CgCCAggATCTCTTCATTgT	CTCCATAgCCgTCCAAgTgT
GAPDH	ggCATTgCTCTCAATgACAA	TgTgAgggAgATgCTCAgTg
PARP	CTggACAACCTCCTggACAT	CgCgTgAgTgTTCTTCACAT
p 53	CCCTgAAgACTggATAACTgTCAT	CTCAgTTCCAggTTCCTgTg
Shh	TTAAATgCCTTggCCATCTC	TTTCACAgAgCAgTggATgC
Ret	CTggAgCCAACAAggAgAAg	CCACATCTgCATCAAACACC
Bmp-4	CgTTACCTCAAgggAgTggA	AgTCCACgTAgAgCgAATgg
Vegf-A	gCCCTgAgTCAAgAggACAg	CAggCTCCTgATTCTTCCAg
TNF-α	ggCATggATCTCAAAgACAACC	gAggCTgACTTTCTCCTggTAT
IL-6	TgATggATgCTTCCAAACTg	gAgCATTggAAgTTggggTA

TABLE 2. Crown-rump length and somite number in embryos of control (N) and manifestly diabetic (MD) rats. The N and MD offspring is also subdivided into non-malformed (N/MD normal) and malformed (N/MD malformed). Significances: *) = $p < 0.05$ vs. N, #) = $p < 0.05$ vs. N/MD normal (Student's t-test).

	n	Crown-rump length (mm)	Somites
N normal	66	3.85 ± 0.03	29.8 ± 0.2
N malformed	1	1.50	17.0
MD normal	36	2.95 ± 0.07*	25.7 ± 0.3*
MD malformed	27	2.20 ± 0.11* ∫	15.8 ± 1.2* ∫

FIGURE LEGENDS

Figure 1. A) Fetuses displaying a) micrognathia (left fetus) and normal morphology (right fetus) B) Outcome of pregnancy in the control (N) and manifestly diabetic (MD) groups, distributed as normal (□), malformed (■), and resorbed (▨) embryos on gestational day 11. Significances: *) = $p < 0.05$ vs. N (chi-square statistics).



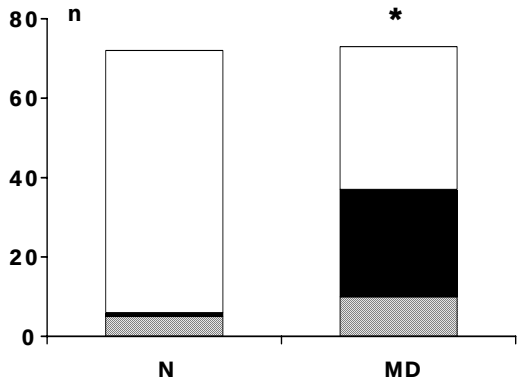


Figure 2. Gene expression of CuZnSOD, MnSOD, Gpx-1, Gpx-2 and catalase on gestational day 11 in the control (N) and manifestly diabetic (MD) groups containing 29 and 32 embryos. Non-malformed and malformed N and MD offspring are denoted N n (28 embryos), MD n (20 embryos) and N m (1 embryo), MD m (12 embryos), respectively.

Mean + SEM. Significances: *) = $p < 0.05$ vs. N, #) = $p < 0.05$ vs. MD n (ANOVA).

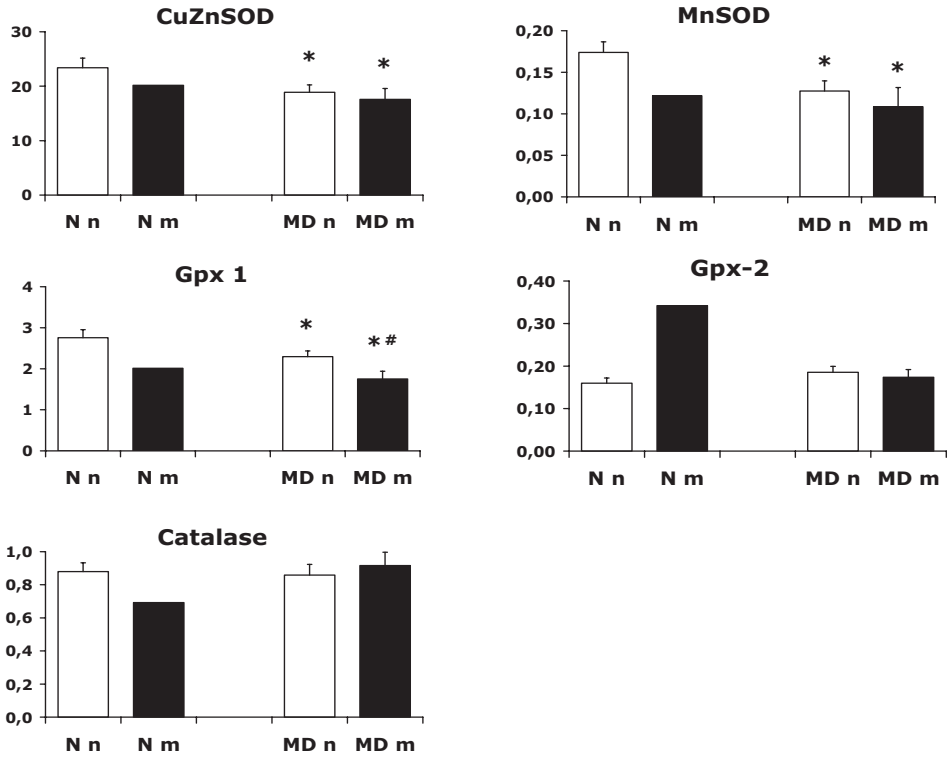


Figure 3. Gene expression of AR, GAPDH, p53 and PARP on gestational day 11 in the control (N) and manifestly diabetic (MD) groups containing 29 and 32 embryos. Non-malformed and malformed N and MD offspring are denoted N n (28 embryos), MD n (20 embryos) and N m (1 embryo), MD m (12 embryos), respectively.. Mean + SEM.

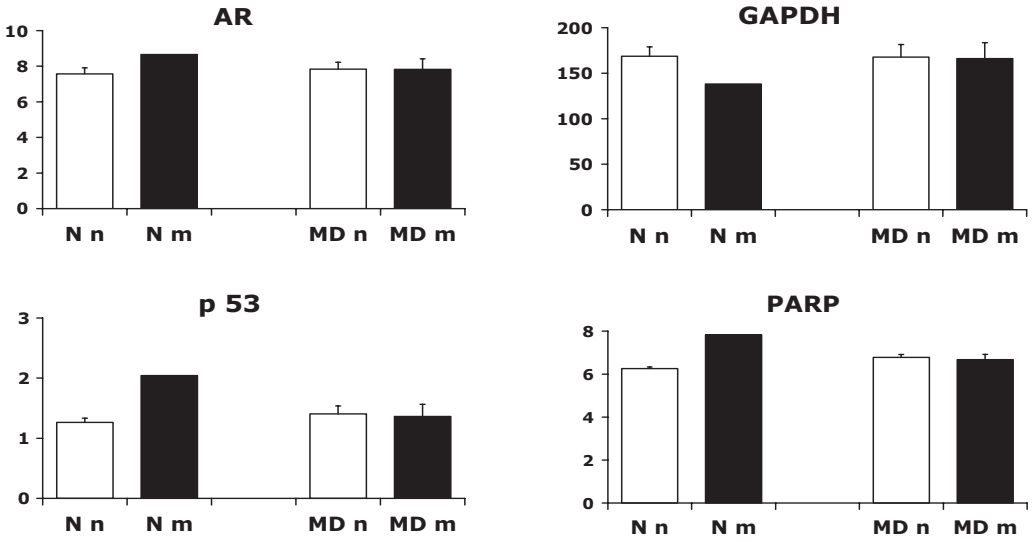


Figure 4. Gene expression of Shh, Ret, Bmp-4, Vegf-A and TNF-alpha on gestational day 11 in the control (N) and manifestly diabetic (MD) groups containing 29 and 32 embryos. Non-malformed

and malformed N and MD offspring are denoted N n (28 embryos), MD n (20 embryos) and N m (1 embryo), MD m (12 embryos), respectively.

Mean + SEM. Significances: *) = p < 0.05 vs. N, #) = p < 0.05 vs. MD n (ANOVA).

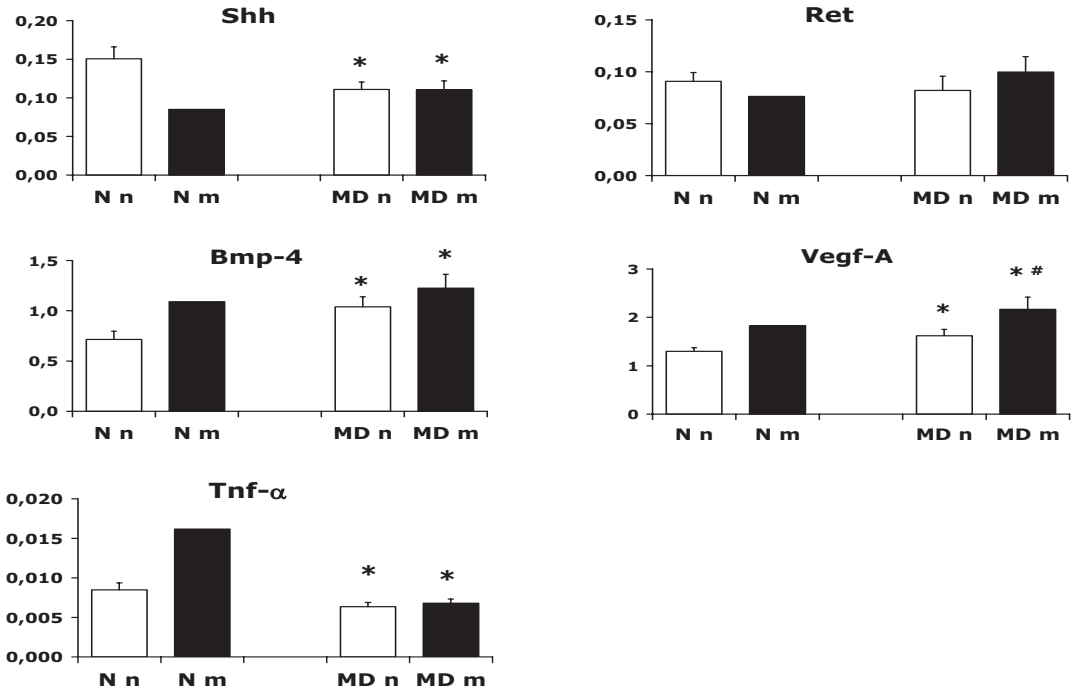


Figure 5. Distribution of activated Caspase-3 protein in A) non-malformed N embryo, B) non-malformed MD embryo, and C) malformed MD embryo. The first visceral arch (circle) and cardiac area (square) are marked, note differences in Caspase-3 staining between the embryos. Space bar = 400 µm.

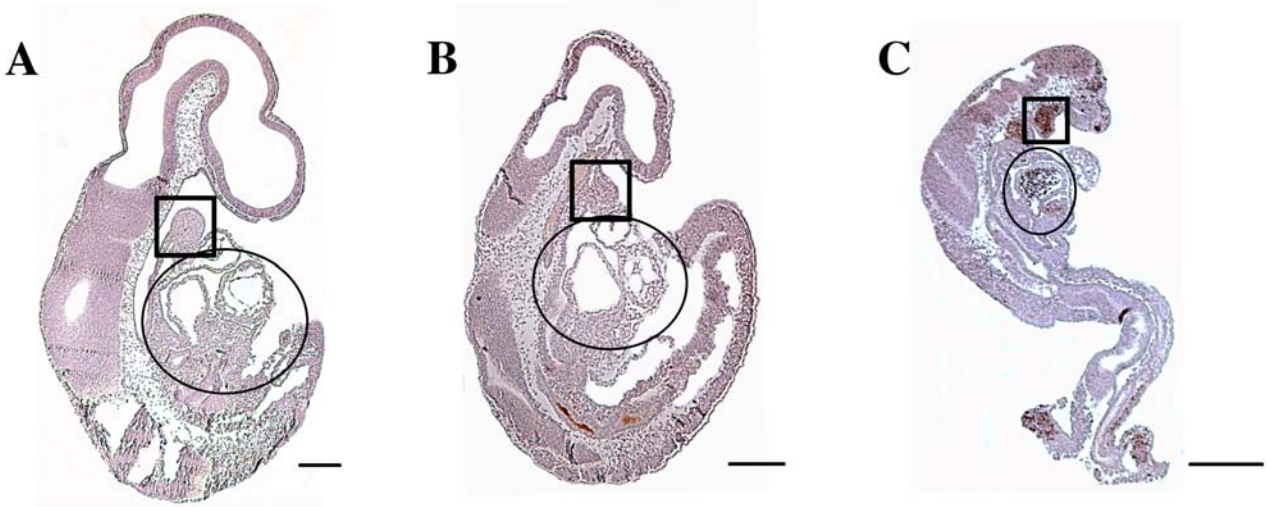


Figure 6. Distribution of Gpx-1 protein in whole embryo (A-C) and first visceral arch (D-F). Left column shows a N embryo (A+D), middle column a non-malformed MD embryo (B + E) and left column depicts a malformed MD embryo (C + F). Note differences in staining of cardiac area.
Space