Embryonic Gene Alterations in rats Caused by Exposure to Diabetes and/or Obesity

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Abstract

There is ample evidence that both diabetes as well as obesity leads to various metabolic disturbances that leads to oxidative stress. Oxidative stress has been shown to be associated with congenital malformations of which neural tube defects and cardiac malformations are more common. The cellular and molecular mechanisms through which oxidative stress induces these defects during the developmental stage are not well known. Previous work in this field suggests that oxidative stress results in lipid peroxidation and altered expression of genes that have key roles in the developmental processes. The present study aimed to investigate gene alterations in embryos from pregnant diabetic or obese rats. Embryos and adipose tissue obtained from the locally bred diabetic and obese Sprague-Dawley inbred rat strain were subjected to Total RNA extraction and were quantified using Real time PCR for relative gene expressions analysis. The present study showed that maternal diabetes as well as obesity diminishes the antioxidative defense mechanisms by down regulating the gene expressions of the key reactive oxygen species scavenging enzymes copper zinc superoxide dismutase and manganese superoxide dismutase in day 10 rat embryos. There was also altered embryonic gene expression for several developmental genes due to maternal diabetes at gestational day 11 and 13 in rat embryos.
### Important terms and Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTD</td>
<td>Neural Tube Defect</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BMP 4</td>
<td>Bone morphogenetic factor 4</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Copper Zinc Superoxide dismutase</td>
</tr>
<tr>
<td>Edn 1</td>
<td>Endothelin 1</td>
</tr>
<tr>
<td>Ednra</td>
<td>Endothelin receptor type A</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose 6 phosphate dehydrogenase</td>
</tr>
<tr>
<td>GD</td>
<td>Gestational day</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial derived neurotrophic factor</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione Peroxidase</td>
</tr>
<tr>
<td>IL 6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>Inhba</td>
<td>Inhibin beta A</td>
</tr>
<tr>
<td>MD</td>
<td>Manifestly diabetic</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese Superoxide dismutase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>Msx2</td>
<td>msh homeobox 2</td>
</tr>
<tr>
<td>N</td>
<td>Normal (non diabetic, nonobese)</td>
</tr>
<tr>
<td>OB</td>
<td>Obese</td>
</tr>
<tr>
<td>q PCR</td>
<td>quantitative Polymerase chain reaction</td>
</tr>
<tr>
<td>Ret</td>
<td>Ret proto-oncogene</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>Tbx5</td>
<td>T-box 5</td>
</tr>
<tr>
<td>TNFa</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
</tbody>
</table>
# Table of Contents

- Introduction 1
- Material and Methods 5
- Results 10
- Discussion 19
- Conclusion 24
- References 25
- Acknowledgements 30
Introduction

The recent increase in prevalence of metabolic disorders such as diabetes and obesity poses a global threat as they are among the five leading risks for mortality resulting in 6 and 5% respectively of the total deaths each year (WHO, 2009), these diseases affect increasingly greater numbers of women in childbearing age worldwide. Metabolic alterations resulting from diabetes and obesity during pregnancy have detrimental effects on pregnancy outcomes.

Apart from increasing the risk of maternal and perinatal mortality both diabetes (Fine et al., 1999; Wentzel et al., 1999; Cederberg et al., 2003; Pavlinkova et al., 2009) and obesity (Shaw et al., 1996; Cedergren and Källen, 2003; Igosheva et al., 2010; Jungheim et al., 2010) have been shown to increase the risk of congenital malformations of which neural tube defects (NTDs) and cardiac malformations are more common.

Diabetes is a chronic disorder characterized by high blood glucose resulting either from lack of insulin or from insulin resistance. This disease affects 346 million people worldwide and the estimated death toll in 2004 from this disease was 3.4 million (WHO, 2011). There are two main types of diabetes namely Type 1 and Type 2 diabetes. Type 1 diabetes, which was previously called insulin-dependent or childhood-onset diabetes results from a lack of insulin due to beta cell destruction. Type 2 diabetes, known previously as non-insulin-dependent or maturity onset diabetes, results from the inability of the body to use insulin effectively due to insulin resistance.

The fact that maternal diabetes increases the risk for congenital malformations has been shown in many previous studies (Fine et al., 1999; Wentzel et al., 1999; Cederberg et al., 2003; Pavlinkova et al., 2009) and the incidence when compared to non-diabetic pregnancies has been found to be 2-5 times higher in diabetic pregnancies (Albert et al., 1996; Mironiuk et al., 1997; Aberg et al., 2001). Though there are some metabolic differences between Type 1 and Type 2 diabetes such as blood ketone levels, somatomedin binding proteins and
triglycerides, both types of diabetes result in the same kind of congenital malformations in pregnancy (Schaefer- Graf et al., 2000; Aberg et al., 2001; Farrell et al., 2002).

Hyperglycemia is a common feature in both Type 1 and Type 2 diabetes, thus suggesting elevated glucose as one of the causes of congenital malformations. This notion is supported by previous studies on rat embryos exposed to high glucose containing media in vitro which resulted in increased rate of malformations compared with embryos cultured in low glucose containing media (Eriksson et al., 1982; Eriksson and Borg, 1991). On the other hand normoglycemic serum from insulin treated diabetic rats still retained the teratogenic effects in whole embryo culture (Wentzel and Eriksson, 1996) thereby suggesting the presence of other factors than only high glucose as teratogenic agents in diabetic pregnancy.

Obesity that is defined by WHO as excessive and abnormal accumulation of fat that results in an increased risk for disease and puts the health in jeopardy. Obesity has become a pandemic these days and has more than doubled since 1980 (WHO, 2011). Obesity is the fifth leading cause of mortality globally resulting in 5% of the deaths each year (WHO, 2009). WHO statistics from 2008 show that 1.5 billion adults (20 years and older) worldwide, were overweight and out of these 300 million women were obese (having body mass index (BMI) > 30 kg/m²) (WHO, 2011).

It has been shown in recent studies that maternal obesity also increases the risk for congenital malformations including neural tube defects and cardiac malformations in the fetus (Shaw et al., 1996; Cedergren and Källen, 2003; Igosheva et al., 2010; Jungheim et al., 2010). The cellular and molecular mechanisms through which obesity results in these congenital malformations are not clear yet.

Neural tube defects (NTDs) are the most common type of congenital malformations that affects 0.5-2/1000 established pregnancies globally (Mitchell, 2005). They are congenital malformations of the brain and spinal
cord resulting from failure or incomplete closure of the neural tube during the first 4-5 weeks and 6-10 days of development in humans (Blencowe et al., 2010) and animal models (Eriksson et al., 1989) respectively. Those having severe defect have 15-fold increased risk of death and 9 to 10% of the infants with severe NTDs die in their first year of life (Malcoe et al., 1999). Maternal diabetes and obesity confers increased risk for NTDs in humans as well as in animal models (Wentzel et al., 1999; Cederberg et al., 2003; Igosheva et al., 2010; Jungheim et al., 2010).

Oxidative stress has been shown to be associated with neural tube defects and there is ample evidence that both diabetes (Trocino et al., 1995; Wentzel et al., 1999) as well as obesity (Chang et al., 2003) leads to various metabolic disturbances. These metabolic changes result in oxidative stress that is either the result of increased oxidative metabolism and superoxide production (Yang et al., 1997), or the free radical scavenging system immaturity (Chang et al., 2003) or both (Wentzel, 2009). This fore mentioned notion of association of oxidative stress to NTDs is supported by the fact that antioxidant supplementation has been shown in animal models to prevent developmental defects including NTDs (Eriksson and Borg, 1991; Viana et al., 1996; Wentzel and Eriksson, 1998; Wentzel et al., 1999).

The molecular mechanisms through which oxidative stress induces these defects during the developmental stage is not well known but previous work in this field (Chang et al., 2003) suggests that oxidative stress results in lipid peroxidation and altered expression of genes that have key roles in the developmental processes. There is no single gene or group of genes that can be solely held responsible for oxidative stress induced embryonic dysmorphogenesis. In addition for NTDs has been suggested that Pax-3, which is essential for neural tube closure, is inhibited by oxidative stress resulting in increased neural tube defects (Phelan et al., 1997; Chang et al., 2003).

The notion that obesity is a chronic inflammatory state has gained popularity recently and previous studies have shown that obesity results in increased inflammation in animal models (Zhu et al., 2010; Shankar et al., 2011) as well
as in term placenta of obese women (Challier et al., 2008). Obesity has also been suggested previously to be a state of chronic oxidative stress (Vincent et al., 2007). There is little knowledge at the molecular level to suggest any definite relation between the oxidative stress and inflammation in obesity but some investigators have suggested that the cytokine tumor necrosis factor alpha (TNFa) which is secreted in increased amounts from the increased adipose tissue in obesity (Guilherme et al., 2008) may have a role. TNFa has been suggested to mediate several cellular processes at the molecular level to result in insulin resistance, which ultimately results in oxidative stress (Guilherme et al., 2008). As mentioned above oxidative stress increases the risks for congenital malformations including NTDs.

The present study aimed at investigating the genetic alterations in embryos from pregnant diabetic or obese rats, in particular the genes related to the defense against oxidative stress and if possible to the genes related to inflammation and skeletal and cardiac development. Superoxide dismutase (SOD), glutathione peroxidase (Gpx) and catalase (CAT) are reactive oxygen species (ROS) scavenging enzymes that play an important role in the defensive mechanisms against oxidants (Halliwell and Gutteridge, 1995; Cederberg et al., 2000). The mRNA levels of these ROS scavenging enzymes in embryos may to a large extent reflect the antioxidative capacity of embryos.

For this study, rats from the locally bred Sprague-Dawley inbred strain (L) that have an increased incidence for congenital malformations (Eriksson et al., 1982) were used as models for both diabetes and obesity. Diabetes was induced by a single injection of Streptozotocin (Sigma-Aldrich Stockholm, Sweden) while obesity was induced, by feeding the female rats with a high fat diet (Research Diets, Inc. New Brunswick, NJ08901 USA). Embryos and adipose tissue obtained from these rats were subjected to Total RNA extraction and after synthesis of cDNA were quantified using Real time qPCR for relative gene expression analysis.
Materials and Methods

Ethical Considerations

Uppsala University’s Ethical committee on Animal Experiments approved all the procedures and experiments involving animals.

Animal Model

Rats, from the locally bred Sprague-Dawley inbred strain (L) (bred in the animal facility of Biomedical Center of Uppsala University), that have an increased incidence for congenital malformations, were used as models for both Diabetes and Obesity. Manifestly diabetic, obese and control were denoted as (MD), (OB) and (N) respectively.

Diabetes was induced by injecting the rats with Streptozotocin (Sigma-Aldrich Stockholm, Sweden) with a single dose of 40 mg/kg in the tail vein. The diabetic state was confirmed within one week with the help of glucose analyzer (Free style mini; Abbot Laboratories, Chicago, IL). Rats with blood glucose values over 20 mmol/l were labeled as manifestly diabetic (MD) while those Labeled as N (control) were not injected at all.

Obesity was induced, by feeding three weeks female L rats, either with high fat diet (60%) (From Research Diets, Inc. New Brunswick NJ 08901 USA) or regular diet (5%) respectively. The female rats were mated with normal diet fed L males at 12-14 weeks of age, and the starting of pregnancy was confirmed by a positive vaginal smear at gestational day 0.

The pregnancy was interrupted either on gestational day 10, 11 or 13 for mRNA evaluation or gestational day 20 for evaluation of fetuses with regard to size, malformation, resorptions and collection of visceral adipose tissue from rat-mother for mRNA evaluation.
All the animals were maintained in an environment having an ambient room temperature of 22 °C and 12:12-h light and dark cycle and had free access to food and water.

**Experimental Material**

Whole Embryos were used as experimental material and were obtained by mating the rats overnight and then confirming the pregnancy with a positive vaginal plug or presence of sperms in vaginal smear and designating the day as gestational day 0 (GD0). The pregnancies were interrupted at GD10 for obese and their controls and GD10, GD11 and GD13 for diabetic and controls by euthanizing the rats, after mild ether anesthesia, by cervical dislocation and the uterine horns were dissected out and the embryos were removed and dissected free from their membranes. Visceral adipose tissue was taken at gestational day 20 from obese and controls for mRNA evaluation by euthanizing the rats, after mild ether anesthesia, by cervical dislocation. The number of samples used in the study for each group of the experimental material is shown in Table 1.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>GD 10 Whole Embryos</th>
<th>GD 11 Whole Embryos</th>
<th>GD 13 Whole Embryos</th>
<th>Visceral adipose tissue from GD 20 pregnant rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (Control)</td>
<td>24</td>
<td>46</td>
<td>36</td>
<td>15</td>
</tr>
<tr>
<td>MD (Manifestly Diabetic)</td>
<td>6</td>
<td>41</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>OB (Obese)</td>
<td>32</td>
<td></td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>

**Total RNA Preparation**

Total RNA from each Embryo was isolated with Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Dr. Hercules, CA 94547 USA) according to the manufacturer’s instructions. Briefly 350 ul of lysis
buffer was added to each day 10 and 11 embryo samples while doubling the amount of lysis buffer for day 13 embryos. An equal amount of 70 % ethanol was added and the mixture was then shifted to the spin column, washed with 700 ul of low stringency wash solution and treated with DNase 1 and incubated at room temperature for 20 minutes and washed again with 700 ul high and then low stringency wash solution before finally eluting it.

While total RNA from freshly frozen (in liquid nitrogen) samples of visceral adipose tissue (from gestational day 20 rats) was isolated using Aurum™ Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Dr. Hercules, CA 94547 USA) following the manufacturer’s instructions for the spin protocol. Briefly 1 ml of PureZol was added to each sample and immediately homogenized and then centrifuged for 10 minutes. After centrifugation 0.2 ml chloroform was added to the supernatant and after incubating at room temperature for 5 minutes centrifuged again to phase out the mixture from which the upper aqueous phase was taken out carefully and an equal amount of 70 % ethanol was added. The mixture was then shifted to the spin column washed with 700 ul of low stringency wash solution and treated with DNase 1 and incubated at room temperature for 20 minutes and washed again with 700 ul high and then low stringency was solution before finally eluting it.

cDNA Synthesis

1 ug of the total RNA was used to synthesize cDNA with iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc, 2000 Alfred Nobel Dr. Hercules, CA 94547 USA) according to the manufacturer’s instructions. The reaction mixture contained 4 ul of 5x iScript reaction mix, 1 ul iScript reverse transcriptase and RNA template required and nuclease-free water to have 20 ul of volume per reaction. Complete reaction mix was then incubated for 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C. The cDNA was then diluted with RNase Free water (provided within the iScript™ cDNA Synthesis kit) to obtain a final volume of 100 ul.
mRNA Analysis

The cDNA was then amplified using iCycler™ Thermal cycler (Bio-Rad Laboratories, Inc) mRNA expression analysis and was measured with MY iQ™ Single color Real-Time PCR Detection System (Bio-Rad Laboratories, Inc). All the primers used were obtained from TIB Molbiol (Berlin, Germany) (Table 2). 1 ul of the final cDNA was amplified in a total volume of 10 ul for each sample containing 5 ul of iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc), 3 ul of RNase Free water and 0.5 ul of each primer (sense and antisense having 10 uM Conc: each).

Table 2. Showing the used Primers along with their Sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'→ 3'</th>
<th>Tm °C</th>
<th>Product size (bp)</th>
<th>Annealing temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PDH</td>
<td>GTC ATG CAG AAC CAC CTC CT</td>
<td>56.8</td>
<td>152</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>ACA TAC TGG CCA AGG ACC AC</td>
<td>56.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Msx2</td>
<td>CCT CGG TCA AGT CGG AAA AT</td>
<td>57.6</td>
<td>192</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>ACT TCT GTC GGC GGA ACT TG</td>
<td>57.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edn1</td>
<td>ACT TCT GCC ACC TGG ACA TC</td>
<td>56.6</td>
<td>203</td>
<td>58</td>
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<tr>
<td></td>
<td>GGC TCG GAG TTC TTT GTC TG</td>
<td>56.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>AAG CGG TGA AGT ATG TGT G</td>
<td>57.2</td>
<td>182</td>
<td>58</td>
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<tr>
<td></td>
<td>CCA GGT CTC CAA CAT GCC</td>
<td>56.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnSOD</td>
<td>GGT GGA GAA CCC AAA GGA GA</td>
<td>57.5</td>
<td>212</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>AGC AGT GGA ATA AGG CCT GT</td>
<td>56.1</td>
<td></td>
<td></td>
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<tr>
<td>Ednra</td>
<td>GCC CTT TTG ACC ACA ATG AT</td>
<td>55.9</td>
<td>225</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>CTT CTT GGT TCC AGA TA</td>
<td>55.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>TTA TGT TAC CTC ACA GCC TGG T</td>
<td>55.4</td>
<td>156</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>GTG TTT TGT GTC TGT GGT TAG</td>
<td>54.9</td>
<td></td>
<td></td>
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<td>GPx-1</td>
<td>TGA GAA GTG CGA GGT GAA TG</td>
<td>55.5</td>
<td>187</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>AAC ACC GTG ACC TAC CA</td>
<td>56.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tbx5</td>
<td>GCA TGG AAG GAA TCA AGG TG</td>
<td>56.0</td>
<td>245</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>CAT AGC AGG CTC GGC TTT AC</td>
<td>56.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GDNF</td>
<td>CCC GAA GAT TAT CCT GAC CA</td>
<td>55.0</td>
<td>242</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>TAG CCC AAA CCC AAG TCA GT</td>
<td>56.8</td>
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<td></td>
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<tr>
<td>SHH</td>
<td>TTA AAT GCC TGG GCC ATC T</td>
<td>55.6</td>
<td>243</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>TTT CAC AGA GCA GTG GAT GC</td>
<td>55.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ret</td>
<td>CTG GAG CCA ACA AGG AGA AG</td>
<td>56.7</td>
<td>183</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>CCA CAT CTG CAT CAA ACA CC</td>
<td>55.6</td>
<td></td>
<td></td>
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<tr>
<td>BMP-4</td>
<td>CGT CAC ACC CAA AAG GAG AAG</td>
<td>56.6</td>
<td>177</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>AGT CCA CGT AGG GCG AAT GG</td>
<td>56.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhba</td>
<td>GAT CAT CAC CTT TGC CAA GT</td>
<td>55.7</td>
<td>245</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>GTT CAC TCC TCT CCC CCT TC</td>
<td>56.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFa</td>
<td>GAC CCT CAC ACT CAG ATC ATC TTC T</td>
<td>55.6</td>
<td>64</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>TGC TAC GAC GTG GGC TAC G</td>
<td>56.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>TGA TGG ATG CTT CCA AAC TG</td>
<td>54.8</td>
<td>230</td>
<td>58</td>
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<tr>
<td></td>
<td>GAG CAT TGG AAG TTG GGG TA</td>
<td>56.0</td>
<td></td>
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</tr>
</tbody>
</table>

Real-Time PCR running conditions
The Real-Time PCR running conditions were set as denaturation and activation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 58°C for 10 sec and elongation at 72°C for 15 sec while each run finished with melting point analysis as shown in Table 3. mRNA levels for each sample were assessed individually and were run in duplicates. The stability of expression of various housekeeping genes was previously assessed and the glucose-6-phosphate dehydrogenase (G6PDH) gene found to be constant in day-10 and day-11 embryos (data not shown); therefore, G6PDH gene was chosen 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 being present in the samples, 10 ng of the total RNA of each sample was amplified in the MyIQ Optical Thermal Cycler. No PCR product was found in the water or the total RNA samples. Furthermore, the avian myoblastosis virus-RT enzyme was excluded in the cDNA preparation and no amplified PCR product was found.

Results for each sample were then analyzed and the relative quantification calculated as the difference between the sample and housekeeping gene crossing point (CP) values yielding \(^{\text{CP}}\) value. The \(^{\text{CP}}\) value thus obtained was transformed with the help of the following formula (2\(^{-\text{CP}}\)).

Table 3. Showing the Real-Time PCR running conditions.

<table>
<thead>
<tr>
<th>Cycle 1</th>
<th>Cycle 2 (40 X)</th>
<th>Cycle 3</th>
<th>Cycle 4 (51 X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>Step 1</td>
<td>Step 2</td>
<td>Step 3</td>
</tr>
<tr>
<td>95°C</td>
<td>95°C</td>
<td>58°C</td>
<td>72°C</td>
</tr>
<tr>
<td>180 sec</td>
<td>10 sec</td>
<td>10 sec</td>
<td>15 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cycle 1</th>
<th>Cycle 2 (40 X)</th>
<th>Cycle 3</th>
<th>Cycle 4 (51 X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>180 sec</td>
<td>10 sec</td>
<td>15 sec</td>
<td>10 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical Calculations

All statistical calculations including mean and standard deviations were obtained using the non-transformed values. Student’s t-test was applied to evaluate the data. ANOVA with Fisher’s protected least significant difference
test was applied as post hoc test (means of normal vs obese and/or diabetic embryos). P < 0.05 was taken to designate a significant difference.

**Results**

**Gene Expression in day 10 Embryos.**

The gene expressions of the principal ROS scavenging enzymes CuZnSOD, MnSOD and Gpx-1 in day 10 embryos from obese as well as diabetic rats are shown in Figures 1, 2 and 3 respectively. The mRNA level of CuZnSOD was significantly decreased by maternal diabetes as well as obesity in day 10 embryos in comparison to controls as shown in Figure 1. MnSOD mRNA levels were significantly decreased by maternal diabetes as well as obesity in day 10 embryos in comparison to control embryos. In addition MnSOD mRNA levels in day 10 embryos from diabetic rats decreased further, when compared with mRNA levels in embryos from obese rats, Figure 2. The Gpx-1 mRNA levels increased in day 10 embryos from obese rats in comparison to embryos from control rats. There were no significant differences in embryonic Gpx-1 mRNA levels due to maternal diabetes. In addition there was a significant decrease in Gpx-1 mRNA levels in day 10 embryos from diabetic mothers compared to embryos from obese rats, as shown in Figure 3.

![CuZnSOD Gene Expression Embryo E10](image)

*Figure 1. Gene Expression of CuZnSOD in day 10 embryos from N (open bar), OB (grey bar) and MD (mesh bar) rats. Transformed means ± SE (ANOVA and Student’s t-test). Significance: * p < 0.05 vs N; # p < 0.05 vs OB. G6PDH, glucose-6-phosphate dehydrogenase; CuZnSOD, copper zinc superoxide dismutase; N, control; OB, obese; MD; manifestly diabetic.*
Gene expression in day 11 and 13 embryos

The gene expressions of the principal ROS scavenging enzymes CuZnSOD, MnSOD, Gpx-1 and Catalase in day 11 and 13 embryos from diabetic rats are shown in Figures 4, 5, 6 and 7 respectively. There were no significant differences in mRNA levels of CuZnSOD in day 11 and 13 embryos due to maternal diabetes compared to embryos from control rats as shown in Figure 4. The same pattern was found when we compared MnSOD mRNA levels of day 11 and 13 embryos from diabetic and control rats as shown in Figure 5.
results for mRNA levels of Gpx-1 and Catalase also failed to show any significant differences in day 11 and 13 embryos due to maternal diabetes when compared with their respective controls as shown in Figure 6 and 7 respectively.

![CuZnSOD Gene Expression in Embryos E11 & 13](image1)

Figure 4. Gene Expression of CuZnSOD in days 11 and 13 embryos from N (open bar) and MD (grey bar) rats. Transformed means ± SE (Student's t-test). Significance: * p < 0.05 vs N; ** p < 0.005 vs N. G6PDH, glucose-6-phosphate dehydrogenase; CuZnSOD, copper zinc superoxide dismutase; N, control; MD, manifestly diabetic.

![MnSOD Gene Expression in Embryos E11 & 13](image2)

Figure 5. Gene Expression of MnSOD in days 11 and 13 embryos from N (open bars) and MD (grey bars) rats. Transformed means ± SE (Student's t-test). Significance: * p < 0.05 and ** p < 0.005 vs N respectively. G6PDH, glucose-6-phosphate dehydrogenase; MnSOD, manganese superoxide dismutase; N, control; MD, manifestly diabetic.
Figure 6. Gene Expression of Gpx-1 in day 11 and 13 embryos from N (open bars) and MD (grey bars) rats. Transformed means ± SE (Student’s t-test). Significance: * p < 0.05 vs N; ** p < 0.005 vs N. G6PDH, glucose-6-phosphate dehydrogenase; Gpx-1, glutathione peroxidase-1; N, control; MD, manifestly diabetic.

Figure 7. Gene Expression of Catalase in day 11 and 13 embryos from N (open bars) and MD (grey bars) rats. Transformed means ± SE (Student’s t-test). Significance: * p < 0.05 vs N; ** p < 0.005 vs N. G6PDH, glucose-6-phosphate dehydrogenase; N, control; MD, manifestly diabetic.

Furthermore, the gene expressions of some of the developmental genes were studied in day 11 and 13 embryos from diabetic and control rats, the results are shown in Figures 8, 9, 10 and 11. Maternal diabetes decreased mRNA levels of Shh significantly in day 11 embryos but did not affect day 13 embryos when compared with their respective control embryos as shown in Figure 8. Ret mRNA levels were significantly increased by maternal diabetes in both day 11 and 13 embryos in comparison to embryos from control rats as shown in Figure 9. GDNF mRNA levels failed to show any significant difference in day 11 embryos but showed a significant decrease in day 13 embryos due to maternal diabetes in comparison to controls as shown in Figure 10. There were no
significant differences in mRNA levels of *BMP-4* in both day 11 and 13 embryos as shown in Figure 11.

Figure 8. Gene Expression of *Shh* in day 11 and 13 embryos from N (open bars) and MD (grey bars) rats. Transformed means ± SE (Student’s *t*-test). Significance: * p < 0.05, ** p < 0.005 and *** p < 0.0005 vs N respectively. G6PDH, glucose-6-phosphate dehydrogenase; Shh, sonic hedgehog homolog; N, control; MD, manifestly diabetic.

Figure 9. Gene Expression of *Ret* in day 11 and 13 embryos from N (open bars) and MD (grey bars) rats. Transformed means ± SE (Student’s *t*-test). Significance: * p < 0.05, ** p < 0.005 and *** p < 0.0005 vs N respectively. G6PDH, glucose-6-phosphate dehydrogenase; Ret, ret proto-oncogene; N, control; MD, manifestly diabetic.
Furthermore, embryonic mRNA levels of some other developmental genes were also evaluated, as shown in Figures 12-16. mRNA levels of Tbx 5 and Msx 2 had no significant differences in day 11 embryos but showed a significant increase in day 13 embryos in comparison to controls as shown in Figures 12 and 13 respectively. Inhb A had no difference in gene expression in day 11 embryos but showed a significant decrease in day 13 embryos due to maternal diabetes in comparison to control embryos as shown in Figure 14. An increased embryonic Edn 1 mRNA levels were found in day 11 embryos due to maternal diabetes compared with embryos from control rats, whereas no difference was found between the groups on day 13, shown in Figure 15.
Maternal diabetes decreased the embryonic *Ednra* gene expression on day 11 but did not affect the gene expression on day 13 as shown in Figures 16.

**Figure 12.** Gene Expression of *Tbx 5* in day 11 and 13 embryos from N (open bars) and MD (grey bars) rats. Transformed means ± SE (Student's *t*-test). Significance: * p < 0.05, ** p < 0.005 and *** p < 0.0005 vs N respectively. G6PDH, glucose-6-phosphate dehydrogenase; Tbx 5, t box 5; N, control; MD, manifestly diabetic.

**Figure 13.** Gene Expression of *Msx 2* in day 11 and 13 embryos from N (open bars) and MD (grey bars) rats. Transformed means ± SE (Student's *t*-test). Significance: * p < 0.05, ** p < 0.005 and *** p < 0.0005 vs N respectively. G6PDH, glucose-6-phosphate dehydrogenase; Msx 2, msh homeobox 2; N, control; MD, manifestly diabetic.
Figure 14. Gene Expression of *InhbA* in day 11 and 13 embryos from N (open bars) and MD (grey bars) rats. Transformed means ± SE (Student's *t*-test). Significance: *p* < 0.05, **p** < 0.005 and ***p*** < 0.0005 vs N respectively. G6PDH, glucose-6-phosphate dehydrogenase; *InhbA*, inhibin A; N, control; MD, manifestly diabetic.

Figure 15. Gene Expression of *Edn 1* in day 11 and 13 embryos from N (open bars) and MD (grey bars) rats. Transformed means ± SE (Student's *t*-test). Significance: *p* < 0.05, **p** < 0.005 and ***p*** < 0.0005 vs N respectively. G6PDH, glucose-6-phosphate dehydrogenase; *Edn 1*, endothelin 1; N, control; MD, manifestly diabetic.

Figure 16. Gene Expression of *Ednra* in day 11 and 13 embryos from N (open bars) and MD (grey bars) rats. Transformed means ± SE (Student's *t*-test). Significance: *p* < 0.05, **p** < 0.005 and ***p*** < 0.0005 vs N respectively. G6PDH, glucose-6-phosphate dehydrogenase; *Ednra*, endothelin receptor type A; N, control; MD, manifestly diabetic.
Gene expression in Adipose tissue

The gene expressions of the ROS scavenging enzymes CuZnSOD, MnSOD, Gpx-1 and that of the cytokines TNFα in adipose tissue from day 20 pregnant obese and control rats are shown in Figures 17, 18, 19 and 20 respectively. There was a significant increase in the expression levels of CuZnSOD due to maternal obesity in adipose tissue when compared to controls as is shown in Figure 17. In contrast, the mRNA levels of MnSOD and Gpx-1 in adipose tissue did not differ between control and obese rats as shown in Figures 18 and 19 respectively. Likewise, the cytokine TNFα in adipose tissue yielded no significant differences due to maternal obesity in comparison to controls as shown in Figure 20. In addition, IL-6 mRNA levels in adipose tissue were not detectable (data not shown).

Figure 17. Gene Expression of CuZnSOD in visceral adipose tissue from day 20 gestational N (open bar) and OB (grey bar) rats. Transformed means ± SE (Student’s t-test). Significance: * p < 0.05 vs N. G6PDH, glucose-6-phosphate dehydrogenase; CuZnSOD, copper zinc superoxide dismutase; N, control; OB, obese.

Figure 18. Gene Expression of MnSOD in visceral adipose tissue from day 20 gestational N (open bar) and OB (grey bar) rats. Transformed means ± SE (Student’s
t-test). Significance: * p < 0.05 vs N. G6PDH, glucose-6-phosphate dehydrogenase; MnSOD, manganese superoxide dismutase; N, control; OB, obese.

**Figure 19.** Gene Expression of Gpx-1 in visceral adipose tissue from day 20 gestational N (open bar) and OB (grey bar) rats. Transformed means ± SE (Student's t-test). Significance: * p < 0.05 vs N. G6PDH, glucose-6-phosphate dehydrogenase; Gpx-1, glutathione peroxidase-1; N, control; OB, obese.

**Figure 20.** Gene Expression of TNF α in visceral adipose tissue from day 20 gestational N (open bar) and OB (grey bar) rats. Transformed means ± SE (Student's t-test). Significance: * p < 0.05 vs N. G6PDH, glucose-6-phosphate dehydrogenase; TNF α, tumor necrosis factor α; N, control; OB, obese.

**Discussion**

Maternal diabetes and obesity are associated with increased risk of congenital malformations and growth disturbances in the offspring. Developmental defects due to maternal diabetes have been shown in many studies to be prevented with the supplemental administration of antioxidants in animal models (Eriksson and Borg, 1991; Viana et al., 1996; Wentzel and Eriksson, 1998; Wentzel et al., 1999). In one of such studies antioxidative
supplementation with vitamin E and C resulted in decreased oxygen radicals and prevented developmental defects (Cederberg et al., 2001).

In addition exposure to ethanol (Chen and Sulkin, 1996) as well as enzymatically produced oxidative stress by ROS (Jenkinson et al., 1986) has been shown to produce the same damaging effects on embryonic development. These and other findings suggest that oxidants cause growth disturbances and dysmorphogenesis in the offspring.

There is ample evidence that both diabetes (Trocino et al., 1995; Wentzel et al., 1999) and obesity (Chang et al., 2003) lead to various metabolic disturbances resulting in increased oxidants that are either the result of increased oxidative metabolism and superoxide production (Yang et al., 1997), or the free radical scavenging system immaturity (Chang et al., 2003) or both (Wentzel, 2009). Investigators have also suggested that diabetes induced oxidative stress alter the expression of genes (such as pax-3) with key roles in embryonic development and ultimately resulting in maldevelopment (Chang et al., 2003). Maternal obesity also results in genetic alterations in rat embryos and renders them sensitive to oxidative stress (Shankar et al., 2011). The alterations in gene expression patterns of the antioxidative defense enzymes may be involved in the oxidative stress related embryopathy (Wentzel and Eriksson, 2002; Loeken, 2004) since 97 % of the genes that are altered have potential binding sites for transcription factors involved in oxidative stress defense mechanisms (Pavlinkova et al., 2009).

Superoxide dismutase (SOD), Glutathione peroxidase (Gpx) and catalase (CAT) are ROS scavenging enzymes that play an important role in the defensive mechanisms against oxidants (Halliwell and Gutteridge, 1995; Cederberg et al., 2000). The mRNA levels of these ROS scavenging enzymes in embryos may to a large extent reflect the antioxidative capacity of embryos. The present study showed that maternal diabetes as well as obesity diminishes the antioxidative defense mechanism by down regulating the gene expressions of the key ROS scavenging enzymes CuZnSOD and MnSOD in day 10 rat embryos.
These results are in line with previous studies (Cederberg and Eriksson, 1997; Zaken et al., 2001). A pattern of gene expression similar to the present study results for ROS scavenging enzymes has also been shown in day 10 embryonic neural crest cells (NCCs), from diabetic rats (Wentzel and Eriksson, 2011). This cell population is derived from the neuroectoderm. Abnormal NCCs are associated with heart and neural tube defects, which are most common malformations due to pre gestational diabetes. When compared with day 10 embryos from obese rats, embryonic gene expression of MnSOD and GPx-1 was found to decrease even further in day 10 embryos from diabetic rats.

In addition transgenic rodent models over expressing CuZnSOD (Hagay et al., 1995) and malformation resistant strains over expressing MnSOD (Cederberg et al., 2000) have been shown to confer protection against developmental defects in a diabetic setting. Day 10 embryos fall in the critical period of gestation (i.e. day 6 to 10) where teratogenesis can easily occur (Eriksson et al., 1989). Since the dependence of the cell’s antioxidative status on the equilibrium of the different antioxidants is well known (Forberg et al., 1995). A down regulation of the ROS scavenging enzymes which are an important part of the antioxidative systems should render the embryos more susceptible to damage from oxidative stress.

Though the present study failed to show any differences in the gene expressions of the ROS scavenging enzymes in day 11 and 13 rat embryos exposed to maternal diabetes, whereas other investigators have shown altered gene expressions of the ROS scavenging enzymes (Forsberg et al., 1995; Zaken et al., 2000). There is also evidence that there are different patterns of gene expression on different developmental stages (Cederberg et al., 2000) and that the comparison between antioxidative capacities becomes difficult with increasing developmental stage of the embryo because each organ acquire a different antioxidant capacity (Zangen et al., 2006). The cellular and molecular processes through which oxidants disturb the normal embryonic development by altering expression of genes are not known with clarity yet.
Earlier studies have shown that diabetes cause mitochondrial swelling which indicate diabetes induced oxidative stress (Eriksson and Borg, 1991; Yang et al., 1998) and thereby resulting in altered gene expression. Guilherme et al. (2008) suggested that there is an association between mitochondrial dysfunction and metabolic disorders, and there is also evidence that maternal diabetes results in swollen embryonic mitochondria and that this swelling is prevented by antioxidant treatment (Yang et al., 1998). Maternal obesity has also been shown to increase mitochondrial production of ROS and it is suggested by Igosheva et al. (2010) that altered mitochondrial function is the likely mechanism through which obesity results in developmental failure in both oocytes and zygotes.

An intracellular redox state is maintained by normal functioning mitochondria, which replenish the antioxidant system as well as produce ROS (Dumollard et al., 2009). For maintaining a healthy cellular environment the antioxidants should be in sufficient amount to counter the effects of oxidants. The main substrates for mitochondrial oxidation, carbohydrates and fatty acids, are readily available in diabetes and obesity and are known to increase mitochondrial respiration (Iossa et al., 2002). Glucose and fatty acid oxidation increases mitochondrial overproduction of ROS and may surpass the cellular antioxidative capacity resulting in the activation of the damaging pathways such as advanced glycation end-products (AGEs), protein kinase C (PKC), hexosamine and polyol pathway (Brownlee, 2001 and 2005; Wentzel, 2009).

Embryos with compromised activity of the mitochondria may fail to control the balance between substrate supply and demand resulting in ROS production in excessive and developmentally damaging amounts (Leese et al., 2008) and this yields in DNA damage (Lee et al., 1999) which results in altered expression of some of the key developmental genes. In the present work several of the developmental genes, such as Shh (sonic hedgehog), Ret (ret proto-oncogene), GDNF (glial cell derived neurotrophic factor), Tbx-5 (T-Box 5), Msx-2 (msh homeobox 2), InhbA (inhibin beta A), Edn 1 (endothelin 1) and Ednra (endothelin receptor type A), were evaluated. There was altered embryonic gene expression at gestational day 11 and 13 due to maternal diabetes. All
these fore mentioned genes play important roles in the developmental processes and alteration in their expression can result in maldevelopment as has been shown in previous studies (Chang et al., 2003).

*Shh* encodes a protein which regulates the expression of genes that have important roles in growth, differentiation and survival of the cell in various tissues through the SHH signaling pathway (Riobo and Manning, 2007). The present study showed that *Shh* mRNA level was decreased by maternal diabetes in day 11 embryos. A decrease in expression levels of *Shh* would result in diminished activity of the SHH pathway and can result in damage. In addition the inhibition of SHH pathway by cyclopamine in neurons exposed to oxidative stress induced by H$_2$O$_2$ (hydrogen peroxide) treatment has been shown to result in more pronounced neuronal damage (Dai et al., 2010).

*GDNF* and *Ret* play an important role in the ret proto-oncogene signaling system and mediate various cellular processes such as differentiation, survival, motility and proliferation (Runeberg-Ross and Sarma, 2007). *Edn 1* and *Ednra* play key roles in the endothelin pathway and are involved in the development of cephalic and cardiac neural crest-derived craniofacial and cardiac structures (Kedzierski and Yanagisawa, 2001). *Inhba* belongs to the transforming growth factor-beta superfamily and plays an important role in growth and development (Bernard et al., 2001). Any alteration in the expression of the above mentioned genes would be expected to have an adverse effect on growth and development of the embryo as they all are equally important for normal embryonic development.

The notion that obesity is a chronic inflammatory state has gained popularity recently and previous studies have shown that obesity results in increased inflammation in sheep midgestational placenta (Zhu et al., 2010; Shankar et al., 2011) as well as in term placenta of obese women (Challier et al., 2008). Obesity has also been suggested previously to be a state of chronic oxidative stress (Vincent et al., 2007). There is little knowledge at the molecular level to suggest any definite relation between the oxidative stress and inflammation in obesity but some investigators have suggested that the cytokine TNFa secreted...
in increased amounts from the adipose tissue (Guilherme et al., 2008) may have a role.

TNFa has been suggested to mediate several cellular processes at the molecular level leading to insulin resistance, which ultimately results in oxidative stress (Guilherme et al., 2008). All this prompted for measuring the mRNA levels of the ROS Scavenging enzymes and the inflammatory cytokines TNFa and IL-6 in visceral adipose tissue from day 20 obese and control pregnant rats in order to study if they were altered. CuZnSOD mRNA level was up regulated in adipose tissue in contrast to the down regulation that was observed in the day 10 embryos from high fat diet rats. Although the mRNA levels of TNFa showed a tendency to increase in adipose tissue from obese rats compared to control rats IL6 gene expressions were not detectable.

Conclusion

Pregestational diabetes and obesity are associated with increased risk of fetal maldevelopment. The cellular and molecular mechanisms behind these are not known yet but it is likely that genetic predisposition, nutrition and environmental factors are involved. In both diabetes and obesity disturbed metabolic state and oxidative stress have been suggested as a major contributor to the developmental defects. It has been shown that antioxidant supplementation diminishes or abolishes diabetes and obesity induced malformations and growth disturbances in the rodent offspring (Eriksson and Borg, 1991; Viana et al., 1996; Cederberg et al., 2001). Furthermore, it has been shown that antioxidant supplementation improved the clinical outcome of various conditions in humans such as Crohn’s disease (Emerit et al., 1991) and in burned patients (Thomson et al., 1990).
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