Pathogenesis and Prevention of Diabetes-Induced Embryopathy

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“The natural course of pregnancy in diabetes even in the insulin era can be summarised in the word destruction. Maternal survival can be assured, but if a non-intervention program is followed only one diabetic pregnancy in three terminates in a live, surviving infant”

Priscilla White 1959
Abstract

Pregnancy in women with spontaneous autoimmune-induced insulin-dependent diabetes mellitus (IDDM) is associated with a perinatal mortality rate (PNMR) 2-4 times higher than that for non-diabetic pregnancy. This is due to increased rates of (1) fetal early growth delay, (2) congenital malformation, (3) prematurity due to spontaneous pre-term labour, and (4) babies small for gestational age. The mechanisms underlying these phenomena have not been precisely defined but circumstantial evidence suggests that they may result primarily from embryonic damage in the earliest stages of pregnancy. Since practical and ethical considerations limit direct investigation of these areas in human subjects, this hypothesis has been examined directly using the BioBreeding/Edinburgh (BB/E) rat, which spontaneously develops autoimmune-induced IDDM, as a model for the human disorder.

Established IDDM in the BB/E rat was associated with severe disturbance in the development of the pre-implantation embryo, as indicated by (1) a five fold increase in the number of unfertilised oocytes, (2) a reduced percentage of expanded blastocysts, and (3) a -20 % inner cell mass (ICM) cellular deficit in embryos which reached the expanded blastocyst stage. Moreover, blastocysts removed from diabetic rats and cultured in vitro for 24 h showed no sign of 'catch-up' growth of the ICM, although under these conditions, the trophectoderm (TE) exhibited a 25 % cellular accretion.

Data obtained from pregnant Diabetes Prone (DP) BB/E rats during the pre-diabetic period (when the maternal metabolic profile was normal), showed that the full complement of IDDM susceptibility genes was not in itself, sufficient to disturb early
complement of IDDM susceptibility genes was not in itself, sufficient to disturb early embryo development and/or give rise to an ICM cell deficit. In addition, experiments involving the transfer of two-cell embryos showed that the oviductal/uterine environment of a Diabetic DP-BB/E recipient could induce not only an ICM deficit but also a TE cell accretion in embryos from Non-Diabetic Diabetes Resistant (DR) BB/E donor rats. However, transfer of two-cell embryos from Diabetic DP- into Non-Diabetic DR-BB/E mothers did not prevent the development of an ICM cellular deficit or a TE cell accretion,- implying earlier damage to the germ cells.

Greatly improved metabolic control was achieved in Diabetic DP-BB/E rats before and over the period of conception using sustained release insulin implants. This resulted in a reduction in the number of unfertilised oocytes, an increase in the percentage of expanded blastocysts but no change in ICM cellular deficit.

A sensitive and accurate quantitative RT-PCR method was developed and used to determine the ratio of bcl-2 (a cell death agonist gene) to bax (a cell death antagonist gene) expression in single blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats. Although the bcl-2 to bax ratio was not significantly increased in blastocysts from diabetic rats, these data demonstrate for the first time that bax and bcl-2 mRNA is present in the pre-implantation embryo.
Declaration

I hereby declare that this work is my own. Although part of a research group, the investigations described in this thesis represent the studies in which I have a major contribution. Acknowledgement of specific assays or procedures not performed by myself is clearly stated throughout the thesis, and unless otherwise stated, the investigations were performed primarily by myself. I have not submitted this thesis for any other degree, diploma or professional qualification.

Jane E. McCracken

Date: 4 September 1998
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Figure 3.15C: Ratios of standard pActMutPCR2.1 DNA/ovary cDNA band intensity plotted against pActMutPCR2.1 DNA concentration

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<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
<tr>
<td>BB/E</td>
<td>BioBreeding/Edinburgh</td>
</tr>
<tr>
<td>BG</td>
<td>blood glucose</td>
</tr>
<tr>
<td>BOHB</td>
<td>β-hydroxybutyrate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CBM</td>
<td>capillary basement membrane</td>
</tr>
<tr>
<td>CBV</td>
<td>coxsakie B virus</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freud's adjuvant</td>
</tr>
<tr>
<td>CIT</td>
<td>conventional insulin therapy</td>
</tr>
<tr>
<td>cM</td>
<td>centimorgan</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CRS</td>
<td>congenital rubella syndrome</td>
</tr>
<tr>
<td>Cy</td>
<td>cyclophosphamide</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotides</td>
</tr>
<tr>
<td>DP</td>
<td>Diabetes Prone</td>
</tr>
<tr>
<td>DR</td>
<td>Diabetes Resistant</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra-acetate</td>
</tr>
<tr>
<td>EGD</td>
<td>early growth delay</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EMC</td>
<td>encephalomyocarditis virus</td>
</tr>
<tr>
<td>fg</td>
<td>femptograms</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>GABA</td>
<td>gamma-amino butyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GBM</td>
<td>glomerular basement membrane</td>
</tr>
<tr>
<td>GTT</td>
<td>glucose tolerance test</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
</tr>
<tr>
<td>HPL</td>
<td>human placental lactogen</td>
</tr>
<tr>
<td>IAA</td>
<td>insulin autoantibody</td>
</tr>
<tr>
<td>ICA</td>
<td>islet cell antibody</td>
</tr>
<tr>
<td>ICSA</td>
<td>islet cell surface antibody</td>
</tr>
<tr>
<td>ICM</td>
<td>inner cell mass</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IGFR</td>
<td>insulin-like growth factor receptor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IUGR</td>
<td>intra-uterine growth retardation</td>
</tr>
<tr>
<td>kb</td>
<td>kilo basepairs</td>
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</tbody>
</table>
Abbreviations

kDa  kilo dalton
kg  kilogram
KRV  Kilham's rat virus
kV  kilo volts
LCMV  lymphocytic choriomeningitis virus
LIF  leukaemia inhibitory factor
M  molar
mA  milliamp
mg  milligram
min  minute
ml  millilitre
mm  millimetre
mmol/L or mM  millimolar
MODY  maturity onset diabetes in the young
NIDDM  non-insulin-dependent diabetes mellitus
NOD  non-obese diabetic mouse
NTD's  neural tube defects
pActPCR2.1  plasmid (PCR2.1) containing the native actin insert
pActMutPCR2.1  plasmid (PCR2.1) containing the mutated actin insert
pBaxPCR2.1  plasmid (PCR2.1) containing the native bax insert
pBaxMutPCR2.1  plasmid (PCR2.1) containing the mutated bax insert
pBcl-2PCR2.1  plasmid (PCR2.1) containing the native bcl-2 insert
pBcl-2MutPCR2.1  plasmid (PCR2.1) containing the mutated bcl-2 insert
PCD  programmed cell death
PCR  polymerase chain reaction
pg  picograms
pmoles  picomoles
PNMR  perinatal mortality rate
RDS  respiratory distress syndrome
RNA  ribonucleic acid
rNTP  ribonucleotides
RT-PCR  reverse transcriptase polymerase chain reaction
SD  standard deviation
SEM  standard error of the mean
sec  second
SRII  sustained release insulin implant
STZ  streptozotocin
TE  trophectoderm
TGFα or β  transforming growth factor alpha or beta
TNFα  tumour necrosis factor alpha
V  volts
W  watt
µg  microgram
µl  microlitre
µmol/L  micromolar
Experimental Aims

Pregnancy in women with insulin-dependent diabetes mellitus (IDDM) is associated with a high incidence of fetuses exhibiting early growth delay and congenital malformation, babies small for gestational age, and spontaneous pre-term labour. The mechanisms underlying the teratogenic effect of maternal IDDM have not been precisely defined. It is however, recognised that these complications tend to be associated with each other (in both diabetic and non-diabetic pregnancy) and are not only responsible for the excess perinatal morbidity and mortality still incurred by women with IDDM, but are also associated with long-term morbidity in children born to these mothers.

In vivo studies of the pathogenesis of diabetic embryopathy have involved animals with chemically-induced diabetes and have largely focused on the period of organogenesis. Studies of the pre-implantation period are less common.

The aim of the study was to elucidate the mechanism of the teratogenic effect of IDDM by studying the interaction of maternal genetic background and metabolic profile on pre-implantation embryo development using the spontaneously diabetic, insulin-dependent BB rat as a model for human IDDM. In addition, the effect of the degree of diabetic metabolic control achieved before and at various points after conception on embryonic development was examined.
Chapter One

Introduction

1.1 Diabetes Mellitus - what is it?

Diabetes mellitus is a common clinical syndrome characterised by an elevated level of glucose in the blood (hyperglycaemia) and in the urine (glycosuria) due to an absolute or relative deficiency of insulin. There are two main categories of diabetes: primary and secondary diabetes. Primary diabetes can be further divided into two main clinical types: insulin-dependent diabetes mellitus (or Type 1 diabetes) and non-insulin-dependent diabetes mellitus (or Type 2 diabetes). At clinical presentation, insulin-dependent diabetes mellitus (IDDM) is characterised by a virtual disappearance of pancreatic \( \beta \) cells and hence an absolute deficiency of endogenous insulin. Without insulin replacement therapy patients die rapidly in ketoacidosis. In contrast, most patients with non-insulin-dependent diabetes mellitus (NIDDM) have only a moderate reduction in pancreatic \( \beta \) cell mass, a relative deficiency of endogenous insulin, insignificant ketonaemia, and mild symptoms. Most NIDDM patients can survive for long periods of time without insulin treatment.

Diabetes may also occur secondary to (i) another pathology, either in the pancreas (for example pancreatitis or neoplastic disease) or in other endocrine glands where there is excess production of hormonal antagonists of insulin (such as growth hormone, thyroid hormone, cortisol, catecholamines or glucagon), or (ii) pregnancy.
Diabetes occurring for the first time during pregnancy is known as Gestational Diabetes Mellitus (GDM). GDM is a controversial area and a common clinical problem. Several studies such as the one by Metzger et al (1), have emphasised the heterogeneity of GDM indicating that women with previously undetected NIDDM as well as women in the pre-diabetic period of both IDDM and NIDDM can be involved. The hormonal/metabolic adaptation to pregnancy results in increased resistance to the action of insulin. Maintenance of normoglycaemia requires a greater secretion of insulin and those with pre-diabetes may be unable to meet this demand. GDM is present in about 2% of all pregnancies and is typically a disorder of the second half of pregnancy. For this reason, the problems associated with untreated GDM mainly consist of babies large for gestational age (macrosomia) and late intra-uterine death.

Diabetes may also be found associated with genetic syndromes such as Down’s, Klinefelter’s or Turner’s syndrome.

Diabetes is a world-wide problem and the incidence of both types of primary diabetes is increasing. However, the prevalence of both varies considerably in different parts of the world. In Britain, the prevalence of diabetes is between 1 and 2%, with a 0.4% incidence rate for IDDM in Caucasians of European descent (2,3). In Europe and North America the ratio of NIDDM : IDDM is approximately 7:3, but it is estimated that almost 50% of cases of NIDDM remain undetected.
1.1.1 Metabolic Consequences of Insulin Deficiency

Insulin has profound effects on the metabolism of carbohydrate, protein, fat and electrolytes. These can be divided into anabolic and anticatabolic actions. The balance of these effects in the fasting and post-prandial states is controlled by (i) a variation in the relative circulating concentration of insulin (the only anabolic hormone) and several catabolic hormones - glucagon, growth hormone, cortisol, catecholamines and thyroid hormones, and (ii) the fact that insulin exerts its anticatabolic effects at a lower concentration than that required for its anabolic actions.

Diabetes mellitus is characterised by hyperglycaemia due to an absolute (IDDM) or relative (NIDDM) lack of insulin. In both types of diabetes this leads to decreased anabolism and increased catabolism. The metabolic consequences of untreated diabetes mellitus are shown in Figure 1.1. At first, hyperglycaemia is only evident after a meal but as the disease progresses and insulin deficiency becomes more severe, fasting hyperglycaemia develops. One of the first metabolic derangements caused by a lack of insulin is the inhibition of glucose utilisation in muscle and adipose tissue. Hepatic gluconeogenesis is also stimulated in the liver, releasing additional glucose into the blood. Glucose is excreted in the urine when the blood glucose level exceeds the reabsorptive capacity of the renal tubules (approximately 10 mmol/L). Glycosuria gradually becomes more continuous and is associated with increased water loss leading to polyuria and polydipsia, and eventually to dehydration. If hyperglycaemia develops very slowly over a period of months or years as in NIDDM, the renal threshold for glucose rises slowly and so glycosuria and the symptoms of diabetes
Figure 1.1: The metabolic consequences of untreated Diabetes Mellitus

- Decreased Anabolism
  - Fatigue
  - Hyperglycaemia
  - Vulvitis
  - Balanitis
  - Polyuria
  - Thirst
  - Polydipsia
  - Tachycardia
  - Hypotension

- Increased Catabolism
  - Increased secretion of glucagon, cortisol, catecholamines and growth hormone
  - Glycogenolysis
  - Gluconeogenesis
  - Lipolysis
  - Hyperketonaemia
  - Hypotension
  - Hypothermia
  - Peripheral vasodilation
  - Hyperventilation

- Salt and water depletion

- Death

From Baird JD (4)
may be slight for a prolonged period. This is the reason why so many cases remain undetected until a late stage. Insulin deficiency also results in reduced protein synthesis and increased protein breakdown, leading to a rise in circulating amino acids. One-third to one-half of this is converted to glucose via gluconeogenesis which further contributes to the hyperglycaemia.

The most profound effect of insulin deficiency is on fat metabolism - there is a decrease in triglyceride synthesis and an increase in triglyceride breakdown (lipolysis). Fatty acids are taken up by the liver and degraded through eight steps within the mitochondria of the liver cells, each stage yielding one molecule of acetyl co-enzyme A. Under normal circumstances, these molecules enter the Krebs cycle but in the absence of insulin, more acetyl co-enzyme A is formed than can enter the cycle. Instead, acetyl co-enzyme A is converted to aceto-acetic acid which is then reduced to form β-hydroxybutyric acid and some decarboxylated to form acetone. These ketone bodies are normally oxidised and utilised as metabolic fuel but when the rate of production exceeds that of removal by peripheral tissues, hyperketonaemia results. Ketone bodies are acids which dissociate almost completely at physiological pH, releasing hydrogen ions into the blood and body fluids. The fall in pH can be countered by buffers in the blood, in particular bicarbonate, but only to a certain extent and so the pH of the blood and other body fluids continues to fall resulting in acidosis. Elevation of blood β-hydroxybutyrate and acetoacetate concentration produces additional problems for the kidney, already troubled by osmotic diuresis caused by the hyperglycaemia. The ketone bodies are excreted in the urine but largely at the expense of sodium and potassium ion loss. This aggravates the dehydration and
further decreases the blood volume, leading to a decreased blood pressure and a diminished glomerular filtration rate.

When there is a relative insulin deficiency as in NIDDM, there may be sufficient circulating insulin to preserve its anticatabolic but not its anabolic effects. In these circumstances lipolysis is not markedly accelerated and the concentration of ketone bodies in the blood remains relatively normal despite severe hyperglycaemia. Patients with IDDM however, have an absolute deficiency of endogenous insulin and without insulin replacement therapy die rapidly in ketoacidosis.

1.1.2 Clinical Features of Diabetes

Table 1.1 shows the classical clinical features of the two main types of diabetes.

<table>
<thead>
<tr>
<th>Table 1.1</th>
<th>Clinical features of IDDM and NIDDM</th>
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<tbody>
<tr>
<td><strong>IDDM</strong></td>
<td><strong>NIDDM</strong></td>
</tr>
<tr>
<td>Age at onset</td>
<td>&lt; 40 years</td>
</tr>
<tr>
<td>Duration of symptoms</td>
<td>Weeks</td>
</tr>
<tr>
<td>Body weight</td>
<td>Normal or low</td>
</tr>
<tr>
<td>Ketonuria</td>
<td>Yes</td>
</tr>
<tr>
<td>Rapid death without treatment</td>
<td>Yes</td>
</tr>
<tr>
<td>Autoantibodies</td>
<td>Yes</td>
</tr>
<tr>
<td>Diabetic complications at diagnosis</td>
<td>No</td>
</tr>
<tr>
<td>Family history of diabetes</td>
<td>No</td>
</tr>
<tr>
<td>Other autoimmune disease</td>
<td>Yes</td>
</tr>
</tbody>
</table>
The difference between the two types is not distinct as overlap occurs particularly in the age of onset, duration of symptoms and family history. For example, Maturity Onset Diabetes in the Young (MODY) is an uncommon genetic syndrome with a dominant mode of inheritance which results in a NIDDM-like disorder in young adults (5), whilst some elderly patients can present with typical autoimmune IDDM. Since the age of onset of NIDDM is usually after the age of 40, most of the women with NIDDM are not of reproductive age. There are however some exceptions to this, including: (i) the Pima Indians from Arizona in the USA; (ii) ethnic groups in Britain such as the Asian Indians; and (iii) patients with MODY. These groups have high prevalence rates for NIDDM and disease often develops at a young age, and so women from these groups may have diabetes during their child-bearing years. In these NIDDM pregnancies, the problems mainly consist of macrosomia and late intrauterine death. These complications also occur in pregnant women with IDDM but they may experience additional severe fetal morbidity and mortality not encountered in NIDDM pregnancies.

1.1.3 Aetiology of Insulin-Dependent Diabetes Mellitus

Although the precise aetiology of IDDM is still uncertain, it is known that environmental factors interact with genetic susceptibility to determine which of those genetically predisposed actually develop clinical disease.

1.1.3.1 Genetic Factors

To date, 12 separate chromosome regions have been implicated in the development of IDDM. These include the major histocompatibility complex (MHC) region on
chromosome 6p21 (IDDM1) (6,3); the variable number tandem repeat (VNTR) (7) in the insulin gene region (8,9,10,11) on chromosome 11p15 (IDDM2). Linkages were also seen at chromosome 15q26 (IDDM3) (12), chromosome 11q (IDDM4), chromosome 6q (IDDM5) chromosome 18q (IDDM6) and the X chromosome (13). In addition, a disease locus on chromosome 2 (IDDM7) has been mapped (14).

Two recent studies have used dense microsatellite maps (approximately 300 markers at a spacing of 11 cM) and reconfirmed the major importance of IDDM1 but provided limited, if any, support for IDDM2 (15,16). Both studies identified a new susceptibility locus (IDDM4) in the vicinity of the FGF3 gene on the long arm of chromosome 11. Davies et al (16) found linkage with another locus on the long arm of chromosome 6, near the ESR marker. From these studies there seems no doubt that IDDM is a polygenic disorder in humans, with the strongest effect at IDDM1 and more minor effects seen at a number of other loci spread throughout the genome. The identities of IDDM1 and IDDM2 are known but the other mutations remain to be identified (17).

The MHC is responsible for encoding three HLA Class II molecules known as HLA-DP, -DQ, and -DR. It has been shown that susceptibility to IDDM is directly related to the amino acid at position 57 of the N-terminal B-1 domain of the HLA-DQ beta chain (18). Maximum susceptibility is associated with substitution of aspartic acid at position 57 with either alanine, valine or serine (this is seen in more than 80 % of IDDM patients), whereas almost complete resistance to the development of IDDM is conferred by the inheritance of two alleles with aspartic acid at position 57. The
HLA-DQ genes encode cell surface DQ molecules which present antigens to T cells. The occurrence of both positive and protective DQ associations suggests that different DQ molecular structures may influence disease susceptibility by their effects on antigen binding or presentation. Thus, DQ polymorphisms may determine the specificity and extent of an autoimmune response against pancreatic islet insulin-secreting cells.

A major problem which confounds the interpretation of the genetics of autoimmune diabetes is the problem of penetrance i.e. the degree to which a particular genotype is actually expressed in the form of a clinical disease. This is illustrated by the relative low concordance for IDDM between monogygotic twins (36 %) (19) which indicates a role for environmental factors in the precipitation of disease. The most important candidate environmental risk factors are early nutritional intake and early viral exposure (20).

1.1.3.2 Environmental Factors

(i) Dietary Factors

Dietary factors have been proposed as a possible explanation for the rising incidence of IDDM in Europe and North America. Indirect evidence for this has come from observations that (i) during periods of relative starvation, such as times of war, there is a decrease in the incidence of IDDM (21, 22); (ii) when the diet is altered and protein intake is increased (for example, in migrants from Western Samoa to Auckland, New Zealand), there is an increased incidence of IDDM (23); (iii) in Iceland, the unusually high incidence of IDDM in boys born in October, has been
linked to the high nitrosamine content of meat traditionally consumed at Christmas (24); and (iv) the mean incidence of childhood IDDM is correlated with the mean consumption of unfermented cow's milk (25).

Helgason and colleagues reported that there was circumstantial evidence of a causal relationship between IDDM in young Icelandic males and the consumption of smoked/cured mutton by their parents at about the time of conception (24). This was found to be linked to the high nitrosamine content of the smoked/cured mutton. This hypothesis was tested in CD1 mice fed Icelandic nitrosamine-rich smoked/cured mutton before mating, during pregnancy and to offspring from day 19 until 5 weeks of age (26). Although the parent mice did not develop diabetes, over 16 % of the male progeny and 4.2 % of the female progeny did. Upon histological examination, the islet β cells were found to be morphologically damaged, but lymphocytic infiltration was infrequent. When the mutton was fed to the parents for 10 days prior to fertilisation, and the weanlings fed standard mouse diet, there was first a fall and then a rise in plasma glucose in males at 3 weeks of age, and in females at 5-6 weeks of age. Therefore, the diabetogenic effect of nitrosamine appeared to be mediated via the parental germ cells rather than by a direct effect upon the fetal β cells.

In 1984 Borch-Johnsen and colleagues (27) suggested that the incidence of IDDM was inversely proportional to the percentage of children breast-fed for less than 3 months. Subsequently it was shown that prolonged exclusive breast-feeding is partially protective against the risk of subsequent diabetes imposed by being a first degree relative of an IDDM patient (28). The reasons for this may be a delay in the
introduction of a putative diabetogen into the weaning diet, or some protective moiety in the human milk. As cow’s milk is the predominant weaning food in high diabetes incidence countries, it’s involvement as a diabetogen has been studied. Other investigators have shown that patients with IDDM have increased levels of IgA class antibodies towards cow’s milk proteins such as β-lactoglobulin (29, 30) and bovine serum albumin (BSA) (31). Analysis of the BSA molecule has identified a 17 amino acid sequence known as ABBOS which differs markedly from the equivalent sequence in man and other species and that antibodies to this region could cross-react with a β cell specific 69 kDa surface protein (P69) (32). P69 is not presented on the β cell membrane and it is thought that it does not enter the repertoire of self-antigens tolerated by the immune system. However, P69 can be induced on the β cell membrane by the inflammatory cytokine gamma interferon which is secreted in response to infection. It has been postulated that individuals with genetic susceptibility to IDDM are capable of generating antibodies against the ABBOS sequence and these antibodies are then able to cross-react with P69 on the β cells, but only when P69 is expressed on the surface of the β cells, for example during an infection (33).

These associations between early nutritional status and risk for IDDM must still be regarded as speculative. It is unlikely that the introduction of cow’s milk proteins is the only trigger of autoimmunity against the β cell. More studies are needed including large prospective intervention studies in high-risk groups, before advice to the general population regarding a change in eating habits could be used for primary prevention.
(ii) **Viral Infections**

Infections with many unrelated viruses have been associated with the pathogenesis of IDDM. Viral infections may induce diabetes either by direct damage to the pancreatic islets or by the induction of an autoimmune reaction. It is also possible that both mechanisms may operate together. The complete nucleotide sequence of the diabetogenic Coxsakie B virus (CBV4) has been compared to that of non-diabetogenic strains and 25 nucleotide sequence differences were identified affecting the amino acid changes of the viral coat proteins (34). Three recent papers suggest that molecular mimicry exists between the protein P2-C of CBV4 and the β cell enzyme glutamic acid decarboxylase (GAD). The T cell mediated immune-response aimed at fighting the Coxsakie virus infection may also destroy the β cell (35,36,37).

Encephalomyocarditis (EMC) virus has two variants, a diabetogenic strain (strain D) and a non-diabetogenic strain (strain B). There is a 14 nucleotide sequence difference between the two strains, and it appears that only two amino acid changes may be responsible for the diabetogenicity of EMC virus (38). The effects of EMC on the pancreatic islets are similar to those of CBV, namely damage to the β cells and a reduction in the production of insulin (39).

Rubella is the only known maternal infection which can induce diabetes in the offspring. About one third of the long-term survivors of congenital rubella syndrome (CRS) develop diabetes 5-20 years after the initial infection (40). Although diabetes is the most common of the delayed manifestations of CRS, autoimmune thyroid
disease (41), Addison’s disease (41) and growth hormone deficiency (42) have also been reported.

In the majority of cases, IDDM autoimmunity develops months, if not years before its clinical onset and so a recent viral infection cannot be responsible for initiating β cell autoimmunity. However, an infection may stimulate memory T cells reactive against self antigens which have already been primed by an ongoing autoimmune process.

Although these studies point to a viral involvement in the pathogenesis of IDDM, additional factors must also be required since only a small fraction of patients infected with these viruses actually develop diabetes. Moreover, the introduction of mumps, measles and polio vaccines decreased the incidence of these diseases but did not change the incidence or age of onset of IDDM.

1.1.4 Pathogenesis of Insulin-Dependent Diabetes Mellitus

The pathogenesis of IDDM is associated with a series of chronic autoimmune reactions against the pancreatic β cells. By the time a clinical diagnosis is made more than 90% of the β cell mass has been lost. However, there is an array of autoantibodies against islet cell antigens which are detectable months, sometimes years before the clinical onset of hyperglycaemia and insulin deficiency.

Islet cell antibodies (ICA) have been detected in 80 - 90% of new-onset IDDM patients. The presence of ICA in genetically predisposed individuals before the onset of clinical disease supports the hypothesis that the autoimmune β cell destruction is a
chronic and progressive process. The first autoantigen to be characterised was insulin (43). Insulin auto-antibodies (IAA) (defined as antibodies present in individuals not yet treated with insulin) occur in 40-50 % of IDDM patients and appear to inversely correlate with the patient’s age at onset of disease (44).

Glutamic acid decarboxylase (GAD) catalyses the synthesis of the inhibitor neurotransmitter γ-amino butyric acid (GABA) from glutamate. Autoantibodies against the 65 kDa isoform of GAD (GAD65) appear to be the best markers for IDDM. IDDM antibodies immunoprecipitate mainly GAD65 in 75 - 80 % of new onset IDDM patients compared with 1 -2 % of healthy individuals (45,46). GAD65 autoantibodies are also found in another autoimmune disorder, the stiff-man syndrome although two different epitopes are involved (47).

Another autoantigen was isolated when a human islet λgt11 expression library was screened with sera from ICA positive first degree relatives of IDDM patients (32) and named ICA 69. The number of potential IDDM autoantigens has increased as molecular cloning techniques rapidly advance. However, the role of ICA in the destruction of the β cells has not been clarified. An immune reaction to many of the autoantigens may be secondary to the massive β cell damage associated with the release of these intracellular molecules.

The sequence of events which leads to the destruction of the pancreatic β cell begins with hyperexpression of Class I MHC antigens within the islets and on the vascular endothelium, followed by insulitis. The invading infiltrate contains macrophages,
helper and cytotoxic and suppressor T lymphocytes, natural killer cells and B lymphocytes. The intense expression of Class I MHC antigens now extends to all pancreatic cells - resulting in the specific destruction of the β cells, leaving the other islet cells intact. Finally, an end-stage islet with no residual insulin is left as the infiltrate regresses. The sequence of events leading to the destruction of the pancreatic β cell in the human has been shown to be identical to that described in the BB/E rat (48, 49, 50).

1.2 Pregnancy in women with Insulin-Dependent Diabetes Mellitus

1.2.1 A Historical Overview

Before the discovery of insulin, few cases of pregnancy complicated by IDDM were reported since most female patients of reproductive age died within one to two years of disease onset. The first true case of diabetes in pregnancy was described in 1824 by Heinrich Gottlieb Bennewitz of Berlin (51). The patient, Fredrica Pape was a 22 year old who after several successful normal pregnancies was admitted to the Berlin Infirmary at 36 weeks gestation with classic symptoms and signs of gestational diabetes. She suffered an intrapartum fetal death and delivered a 12 lb. still-born infant.

In the limited number of pregnancies reported in the pre-insulin era, both the perinatal and maternal mortality rates were approximately 50 %, with half of the maternal deaths occurring during pregnancy and the remainder during the following two years
In 1915 Elliott Joslin presented a paper to the Boston Medical and Surgical Society in which he reported that almost every case of diabetes mellitus ended in death from ketoacidosis and that this fatal outcome was accelerated by pregnancy. It was emphasised by diabetologists and obstetricians alike that pregnancy should be terminated if complicated by diabetes as the risks to mother and fetus were too high.

With the discovery of insulin in 1921 by Banting and Best, life expectancy was dramatically improved for all diabetic patients. In eight short months insulin had been isolated and clinical trials begun. The first injection of insulin was administered on January 11 1922 to Leonard Thompson, a 13 year old boy. He weighed only 64 lb and had been kept alive on only 450 calories per day. Insulin treatment improved the fertility of women with diabetes allowing women with more severe diabetes to become pregnant. Between 1923 and 1927 the maternal survival rate continued to improve but the perinatal mortality rate (PNMR) remained unchanged.

One of the most important pioneers in the treatment of diabetic pregnancy was Dr. Priscilla White of the Joslin Clinic in Boston, USA. In 1928 she made several important observations that revolutionised the treatment of diabetic pregnancy, she concluded that (i) good control of diabetes was essential to fetal welfare and that the high maternal blood glucose concentration was probably responsible for excessive fetal growth, and (ii) "close and persistent supervision" of the patient by both the diabetologist and obstetrician was the most important part of the treatment programme. In 1932 she described the first three successful cases of diabetes
complicating pregnancy which had been treated with insulin - all of the mothers survived and delivered healthy live infants (56).

Over the 77 years since the discovery of insulin the PNMR has continued to decline and the maternal mortality rate is now almost the same as that found in the non-diabetic population. This improvement has resulted from the ability to achieve better regulation of the maternal diabetes than was formerly possible following the introduction of home glucose monitoring and more intensive insulin therapy. However, even in “centres of excellence”, the PNMR for diabetic pregnancy remains approximately four times that for non-diabetic women (4-8 % vs 1-2 %) (57) and 15-20 % of the infants born to mothers with IDDM experience neonatal morbidity severe enough to require a stay of more than 10 days duration in intensive care (58, 59). Thus today pregnancy complicated by maternal diabetes remains an important clinical problem.

1.2.2 Classification of Diabetes in Pregnancy

Women whose pregnancies are complicated by diabetes can be broadly divided into those who are known to have diabetes before pregnancy and those with diabetes diagnosed for the first time during their pregnancy (known as gestational diabetes). The White classification was designed by Dr. Priscilla White (56) to predict outcome of pregnancy on the basis of severity of maternal diabetes. She recognised that long duration of diabetes, particularly if complicated by vascular disease, had a progressively adverse effect on maternal and fetal outcome. The most recently revised White classes are shown in Table 1.2 (60).
Table 1.2  The White classification of Diabetic Pregnancy

<table>
<thead>
<tr>
<th>White Class</th>
<th>Maternal Metabolic Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational Diabetes A</td>
<td>Abnormal glucose tolerance test (GTT) - euglycaemia maintained by diet / insulin Diet alone, any duration or onset age</td>
</tr>
<tr>
<td>B</td>
<td>Adult onset (age 20 years or older), short duration (&lt; 10 years)</td>
</tr>
<tr>
<td>C</td>
<td>Relatively young onset (age 1-19 years) or relatively long duration (10-19 years)</td>
</tr>
<tr>
<td>D</td>
<td>Very young onset (&lt; 10 years of age), very long duration (20 years or more) or evidence of background retinopathy</td>
</tr>
<tr>
<td>F</td>
<td>Renal Disease</td>
</tr>
<tr>
<td>R</td>
<td>Proliferative Retinopathy or vitreous haemorrhage</td>
</tr>
<tr>
<td>RF</td>
<td>Renal Disease and Proliferative Retinopathy</td>
</tr>
<tr>
<td>H</td>
<td>Atheriosclerotic heart disease</td>
</tr>
<tr>
<td>T</td>
<td>Pregnancy after renal transplant</td>
</tr>
</tbody>
</table>
Although this system has been used extensively for the last 50 years there is now general agreement that a new system of classification needs to be introduced as it is too complicated and some patients do not fit into any one of the classifications. A system which is simple, addresses both the maternal and fetal issues and correlates with fetal outcome is required. It must also take into account the type of diabetes and the presence of maternal complications.

1.2.3 The Effect of Pregnancy on Maternal IDDM

1.2.3.1 Ketoacidosis

Ketoacidosis now only affects about 1% of IDDM pregnancies but it remains one of the most serious complications. It may occur particularly in association with infections, hyperemesis gravidarum, treatment with β-sympathomimetic agents (used to suppress uterine contractions), corticosteroid use and insulin pump failure (61). Since pregnancy is marked by a relative insulin resistance, enhanced lipolysis and ketogenesis, diabetic ketoacidosis may occur with minimal hyperglycaemia. Ketoacidosis results in a considerable loss of fluid and electrolytes, particularly potassium. Treatment involves the administration of unmodified insulin by intramuscular or intra-venous injection, fluid and potassium replacement and the administration of antibiotics if an infection is present. If ketoacidosis does occur during pregnancy the fetal loss can be as high as 20%. It is essential that the ketoacidosis is correctly rapidly in order to save the life of the fetus and the mother.

1.2.3.2 Pre-eclampsia

Diabetic pregnancies complicated with pre-eclampsia cause much concern because of
poor perinatal outcome. In one study, pre-eclampsia complicated 13.6% of IDDM pregnancies and 5% of non-diabetic pregnancies (62). The proportion of pregnancies complicated with pre-eclampsia rises with increasing severity and duration of diabetes according to the White classification. The perinatal mortality rate is 60 per 1000 livebirths for pre-eclamptic diabetic women, compared with 3.3 per 1000 livebirths for normotensive diabetic women. The occurrence of pre-eclampsia does not seem to be related to glycaemic control during pregnancy (62). Pre-eclampsia is difficult to diagnose in diabetic women with nephropathy and hypertension: it has been estimated that 30% of diabetic women with nephropathy also have pre-eclampsia.

1.2.3.3 Hypoglycaemia

Hypoglycaemia is the most common untoward event in insulin-treated diabetes. The first trimester is a period of glycaemic instability and hypoglycaemic reactions may be more frequent and more severe. Therefore patients and physicians should not strive for normoglycaemia at the expense of more frequent episodes of hypoglycaemia. Hypoglycaemia may not only be detrimental to fetal development and contribute to the progression of maternal retinopathy, but may also lead to serious injury and impairment of cognitive function.

1.2.3.4 Vascular Disease

Disease of the small blood vessels (microangiopathy) is specific to diabetes and affects all the small blood vessels in the body, but its effect is most destructive in three particularly sensitive areas: the retina (causing ‘diabetic retinopathy’ which may result
in blindness); the nerves (causing 'diabetic neuropathy' resulting in difficulty in walking, chronic ulceration of the feet, bladder and bowel dysfunction); and the renal glomerulus (causing 'diabetic nephropathy' and resulting in renal failure).

(i) Diabetic Retinopathy
Studies have shown that pregnancy can worsen proliferative retinopathy when compared with progression of the disease over a 40 week period in non-pregnant diabetic women (63). However, data from Denmark (64, 65) show that in women with well-controlled IDDM, background retinopathy progresses to proliferative retinopathy only occasionally and that most cases regress spontaneously after delivery.

(ii) Diabetic Nephropathy
Overt diabetic nephropathy is present in 5 % IDDM pregnancies and a first trimester finding of >300 mg / 24h proteinuria is associated with an adverse pregnancy outcome. Creatinine clearance rate is used as a diagnostic indicator of kidney function for pre-pregnancy counselling. A creatinine clearance rate of less than 50 ml/min before pregnancy has been associated with high rates of pregnancy-induced hypertension and fetal wasting (66). Fetal death is more common in hypertensive mothers than in pregnancies in which blood pressure is normal, however, recent developments in obstetric management and techniques have improved the outcome quite considerably. Pre-pregnancy counselling must emphasise that (i) poor perinatal outcome is predicted by proteinuria greater than 3 g/24 hours, a serum creatinine >130 mmol/L or a mean arterial pressure >107 mmHg, and (ii) even though
pregnancy may be successful, maternal long-term morbidity and mortality are high and may compromise a diabetic mother's ability to raise her child.

(iii) **Diabetic Neuropathy**

There have been few studies in which the effects of pregnancy on the development of progression of diabetic neuropathy has been examined. Autonomic neuropathy may be troublesome in pregnancy with worsening postural hypotension, diminished counter-regulatory hormonal responses (notably catecholamines, growth hormone, cortisol and glucagon) to hypoglycaemia, and deterioration of gastroparesis. Diabetic gastroparesis contributes to nausea and vomiting, nutritional problems and difficulty with glucose control (67).

1.2.4 **Maternal Metabolic Adaptations to Pregnancy**

1.2.4.1 **Non-Diabetic Pregnancy**

Metabolic adaptations to pregnancy are directed towards (i) ensuring appropriate growth and development of the fetus, (ii) equipping the fetus with a store of energy to see it through the immediate neonatal period, and (iii) establishing a maternal bank of energy to ensure survival of the fetus, the neonate and the infant, should the supply of dietary energy be inadequate during and after pregnancy (68).

During the first half of pregnancy there is an increased secretion of insulin in response to glucose, minimal increase in total insulin resistance, and a rise in the number of insulin receptors on adipocytes. This results in increased triglyceride synthesis and secretion by the liver, which is then stored as fat.
In late pregnancy however, the plasma concentration of insulin remains raised but the number of insulin receptors returns to normal levels and so there is an increase in insulin resistance (69, 70). This insulin resistance parallels the growth of the fetoplacental unit (71) and there is a progressive rise in the ratio of plasma immunoreactive insulin to plasma glucose, reaching a peak at 32 weeks gestation with a gradual decline thereafter to non-pregnant levels post-partum (68). The result of this insulin resistance is a reduction in fasting blood glucose levels (72) which appears to be due to (i) a reduction in maternal gluconeogenesis and (ii) an increase in glucose use by the growing fetus. The maternal metabolism copes with the outflow of glucose to the fetus by switching to fat metabolism (73).

During pregnancy the ovary, placenta, anterior pituitary, pancreas and the fetal adrenal cortex are all involved in bringing about hormonal changes which have a profound effect on carbohydrate, fat and amino acid metabolism. The concentration of human placental lactogen (HPL) rises steadily during the first and second trimesters, reaching a peak in the last four weeks of pregnancy. Increased HPL causes the mobilisation of lipids as free fatty acids which serve as a maternal energy source thereby making glucose and amino acids available to the fetus (74). The concentration of free cortisol also progressively increase throughout pregnancy. Cortisol stimulates endogenous glucose production, glycogen storage and decreases glucose utilisation thereby reducing the effectiveness of insulin (75).

Thus, the biphasic metabolism of carbohydrate, fat and protein is used to spread the energy cost and protein requirement of pregnancy over the whole of the gestation
period. This is programmed by the placental steroid hormone balance which is
determined by the growth of the placenta. These placental hormones are responsible
for (i) conserving energy in early pregnancy, described as 'facilitated anabolism' by
Freinkel (71), and (ii) redirecting available energy to the fetus in late pregnancy,
'accentuated catabolism' (71).

1.2.4.2 Diabetic Pregnancy

The various derangements in metabolic fuels such as carbohydrates, amino acids,
lipids and ketones, characteristic of IDDM, have the potential to adversely affect the
development of the fetus. Poorly controlled maternal IDDM during the first
trimester, in particular during the first six weeks of pregnancy, has been associated
with a spectrum of developmental abnormalities ranging from spontaneous abortion to
congenital malformations and growth delay. In contrast, poor metabolic control
during the latter two thirds of gestation has mainly been associated with accelerated
fetal growth and a risk of large-for-gestational age babies.

The effect of increasing resistance to the action of insulin during pregnancy is
clinically expressed as (i) a substantial and progressive rise in the dose of insulin
required to maintain glycaemic control in women with established IDDM, and (ii) the
development of impaired glucose tolerance or frank gestational diabetes in some
apparently normal women with a (hitherto occult) deficit in pancreatic β-cell secretory
reserve (76).
In normal pregnancy the maternal blood glucose is maintained within a narrow range (3-6 mmol/L) and the concentration of glucose in the fetal circulation is half the maternal level. This means that stimulation of the fetal β-cells is usually minimal. Any deterioration in regulation of the maternal metabolism (as occurs in diabetes) results in the premature over-stimulation of the fetal β-cells with major consequences for fetal well-being (see Figure 1.2). Therefore the aim of intensive treatment in IDDM pregnancy is to maintain maternal normoglycaemia. This goal is not often achieved in practice because the insulin delivery systems currently available for treating diabetic patients are not glucose-responsive.

1.2.5 The Placenta in IDDM Pregnancy

Studies of placentae from women with IDDM have shown focal syncytial necrosis, dilation of syncytial endoplasmic reticulum, a normal or increased number of syncytial pinocytotic vesicles, an increased number of syncytial secretory droplets, focal basement membrane thickening and evidence of cytotrophoblast proliferation (77). The placentae of these women also have decreased utero-placental blood flow (78), and it has been speculated that the diameter of the uterine vessels supplying the placenta is slightly reduced, thereby restricting the access of blood to the fetal-placental unit (80). As described above, the histological structure of placentae of IDDM pregnancy shows numerous changes, including increased villous branching and an increased fetal-maternal exchange area (80, 81), in addition to a significant increase in the density of microvilli on the free surface of the villous syncytiotrophoblast (82).
However, there is no correlation between the degree and extent of the abnormalities noted in placentae from women with IDDM and the severity or duration of the maternal diabetic state (77). The authors were unable to show that the placental changes were any less marked in patients with well-controlled diabetes than in placentae from women with poor metabolic control (77). The mechanism controlling villous vascularisation and the cause of the abnormalities is still unknown, but it is thought that some component(s) of the diabetic state must play a pathogenic role.

In the rat with streptozotocin (STZ)-induced diabetes, similar morphological changes in placental histology have been reported. In particular, distorted placental morphology (83) and a decreased placental blood flow (84, 85, 86). In addition, changes in the capacity to transfer nutrients from mother to fetus have been demonstrated in the chorioallantoic placenta of rats with STZ-induced diabetes (87, 88). However, the mechanisms underlying the damaging effects of IDDM on the development and function of the placenta remain obscure.

1.2.6 Causes of Fetal and Neonatal Mortality and Morbidity in Established IDDM Pregnancy

1.2.6.1 Early Pregnancy

(i) Early spontaneous abortion

Spontaneous abortion is defined as death of the embryo or fetus before 20 weeks gestation. It has been shown that women with poorly controlled IDDM in the preconception period and early pregnancy have increased rates of spontaneous abortion (89, 90). Midovnik and colleagues (91) showed that women who later had
spontaneous abortion had significantly elevated HbA1c levels at 8 weeks gestation but no elevation of either glycosylated albumin (reflecting mean blood glucose levels in the preceding 2-4 weeks) or glycosylated total protein. They concluded that spontaneous abortion was related to poor glycaemic control around the time of conception rather than control immediately before the actual abortion.

(ii) Early Growth Delay

Studies from Copenhagen have examined fetal growth in IDDM pregnancy using ultrasound scanning to measure crown-rump length accurately and have reported a high incidence of small fetuses in the first trimester of pregnancy which was associated with an increased risk of subsequent fetal malformation (92) and spontaneous abortion (93). Fog-Pedersen et al (94) have presented data supporting their view that EGD results from the metabolic disturbance associated with IDDM, particularly if metabolic control was poor around the time of conception, and that EGD is associated with poorer psychomotor development post-natally (95).

EGD in fetuses from IDDM pregnancies has been confirmed in three other centres (96, 97, 98). However, one centre has reported that the phenomenon of EGD disappears when the time of conception is determined more accurately (99).

(iii) Congenital Malformation

IDDM pregnancy is associated with an increased risk of fetal maldevelopment, the mechanism of which has not been precisely defined. Congenital malformations are now the most common cause of neonatal death. Most studies have demonstrated a 3-
4 fold increase in major congenital malformations in infants of diabetic mothers (100, 101, 71, 102). No organ system is exempt and almost any congenital malformation can occur. Cardiac anomalies affect approximately 4% of IDDM pregnancies and include ventricular septal defects, complex lesions of the great vessels, and coarctation of the aorta (103). Many infants of IDDM mothers have a thickened interventricular septum and enlarged left or right ventricular wall which can lead to left ventricular outflow obstruction and cardiac failure. Most infants are asymptomatic but some develop congestive cardiac failure in the neonatal period.

IDDM pregnancy also carries an especially high risk for neural tube defects: 19.5 per 1000 pregnancies compared to 2 per 1000 for normal pregnancies (104). A rarer complication (0.5% of IDDM pregnancies) is the caudal regression syndrome (sacral agenesis, phocomelic embryopathy) which is a continuum of malformations ranging from agenesis of the lumbosacral spine to sirenomelia with fusion of the lower extremities. This syndrome although very rare, is the type of malformation most closely linked to IDDM pregnancy.

1.2.6.2 Late Pregnancy

(i) Intra-Uterine Growth Retardation

Approximately 5-10% of infants born to IDDM mothers are small, undernourished and growth retarded rather than macrosomic. Intra-uterine growth retardation (IUGR) is generally accepted as birth weight below the 10th centile in accordance with the gestational age for infants in the same community (105). Predisposing factors for IUGR include poor maternal renal function, poor metabolic control during
organogenesis, pre-eclampsia and chronic hypertension (106). Babies born with IUGR may be susceptible to immediate medical problems such as hypoglycaemia. Other recognisable features of IUGR include asymmetrical organ growth, polycythaemia, impaired gluconeogenesis, depleted glycogen and fat stores, delay of skeletal growth and a higher incidence of asphyxia (107).

(ii) Macrosomia

It is well established that fetal macrosomia in diabetic pregnancy is associated with high perinatal morbidity and mortality rates. The term 'macrosomia' refers to a neonatal birth weight of more than 4500 g. Macrosomia occurs in 25-42 % of pregnancies in diabetic women compared with 8-14 % in control populations (108). These fetuses are at a higher risk for unexplained death in utero, birth trauma, hypertrophic cardiomyopathy, vascular thrombosis and neonatal hypoglycaemia (108). In a study of 21 macrosomic infants Fee and Weil (109) found hyperplasia and hypertrophy of most tissues - resulting in an overall increase in body weight of 141 % compared with controls. They found an increase in heart (174 %), liver (179 %), lung (127 %), adrenal glands (158 %), pancreas (110 %) and spleen (127 %), with the brain and kidney unaffected.

Despite improvements in glycaemic control in IDDM pregnancy, rates of macrosomia still vary between 20 and 50 % (110, 111) and may signal the importance of other factors predisposing to fetal macrosomia such as maternal weight, maternal weight gain during pregnancy, parity and genetic factors (112).
(iii) Intra-Uterine Death

Late intra-uterine death or unexplained stillbirth has been a very important contributor to high perinatal mortality in diabetic pregnancy. Between 1946 and 1952 the rate of unexplained stillbirth was reported to be 27 %, falling to 15 % between 1953 and 1955 (113). In the 1960's an attempt was made to reduce the incidence of sudden unexplained stillbirth by adopting a policy of premature delivery by elective caesarean section at 36 weeks gestation. This reduced the number of stillbirths but increased the number of neonatal deaths due to respiratory distress syndrome as these babies are often more immature than their actual gestational age. Unexplained stillbirth has become less common in recent years, since the introduction of stricter diabetic control during pregnancy and more sophisticated fetal monitoring. However, it still occurs at a rate four times that for non-diabetic patients and can happen with alarming swiftness despite a previously normal fetal cardiotocograph. Factors known to be associated with sudden fetal death include a pregnancy at more than 36 weeks gestation, a mother with long-term diabetes and vascular disease, poor diabetic control, pre-eclampsia, ketoacidosis, polyhydrominos and a macrosomic infant (114, 115).

1.2.6.3 Neonatal Period

(i) Respiratory Distress Syndrome

Respiratory distress syndrome (RDS) is defined as an acute restrictive pulmonary disease which develops shortly after birth in susceptible premature infants. It is principally due to pulmonary surfactant deficiency and often leads to progressive ventilatory failure (116). An increased risk of RDS in infants born to IDDM mothers was initially reported by Gellis and Hsia (117). Before the introduction of neonatal
intensive care in the 1950's and 1960's RDS was the most common cause of neonatal death. Since then, however, the emphasis on improved maternal metabolic control and prolongation of the pregnancy beyond 38 weeks gestation, RDS has become much less common (118). In addition to prematurity, other risk factors for RDS include fetal sex - RDS develops more frequently in males (119); and mode of delivery - higher incidence of RDS in babies born by caesarean section; (120).

(ii) Hypoglycaemia

Neonatal hypoglycemia is a very important complication because of its frequency and potential to cause long term mental handicap particularly if hypoglycaemia is prolonged (121). It may be associated with reduced myocardial cointractivity so that congestive heart failure develops. Once the umbilical cord has been cut the glucose source for the infant is withdrawn and the excess insulin circulating in the baby's blood quickly rids the plasma of the remaining glucose and so blood glucose levels may drop precipitously during the first hour or two of life. It is essential therefore, that the blood glucose of these babies is monitored frequently and they are fed high-carbohydrate, high-calorie feeds in an effort to prevent hypoglycaemia.

(iii) Hypocalcaemia and Hypomagnesaemia

Hypocalcaemia (serum calcium less than 1.7 mmol/l) occurs in approximately 25-50 % of all infants born to IDDM women and its incidence appears to be directly related to the severity of maternal diabetes. Hypomagnesaemia can occur in 70-80 % of these babies and seems to be unrelated to maternal diabetes control.
Infants of IDDM mothers often exhibit transient functional hypoparathyroidism at birth, leading in some, to neonatal hypocalcaemia (122). This functional hypoparathyroidism may be due to a state of magnesium deficiency, which in itself is secondary to maternal hypomagnesaemia created by the increase in urinary magnesium excretion that exists in IDDM (123) and low maternal and fetal levels of magnesium may lead to delayed development of fetal parathyroid gland function which is a condition frequently found in hypocalcaemic infants.

(iv) Jaundice

Pathological hyperbilirubinaemia (serum bilirubin above 200 μmol/L) is frequent in infants of IDDM mothers. Its incidence is related to macrosomia with 70 % of all macrosomic infants affected compared with 47 % of those not macrosomic. The actual cause of bilirubinaemia is unclear, but polycythaemic babies are more likely to have a serum bilirubin above 170 μmol/L than non-polycythaemic babies. The increased bruising and trauma at birth commonly associated with macrosomic infants contributes to increased bilirubin production and therefore to jaundice. Hyperbilirubinaemia remains a common problem and strict control of diabetes during pregnancy may be the best way to reduce its incidence.

1.2.7 The Metabolic Basis of Fetal and Neonatal Mortality and Morbidity in Established IDDM Pregnancy

Although the precise mechanism(s) of early embryopathy remains uncertain, clinical and experimental evidence suggests that poor maternal diabetic control during the period of organogenesis (from 2-10 weeks gestation) may result in an increased rate
of congenital malformation (71). While the risk of anomalous development can be reduced by establishing meticulous metabolic control prior to the period of organogenesis (124, 125), most women with IDDM continue to incur an increased risk of malformations in their offspring because of the inherent difficulties in achieving good enough metabolic regulation with the unresponsive insulin-delivery systems currently in use. Miller and associates (126) showed that when the HbA1c value at the 14th week of pregnancy was 6.9% there were no greater risk of congenital malformations, if the HbA1c was 7.0-8.5% (indicating fair diabetes control) the incidence of malformation was 5.1%, but when the HbA1c was greater than 8.6% (indicating poor control) the incidence of malformation increased to 22.4%. This result was later confirmed by Ylinen et al (127) and Reid et al (128). However, Hanson and colleagues (90) reported that the individual HbA1c value cannot be used to accurately predict fetal malformation since in their study more than 50% women who had HbA1c values of +8 SD or more above the control mean value had infants without malformations.

The use of HbA1c to assess glycaemic control in pregnancy is limited for the following reasons: (i) episodes of hypo- and hyperglycaemia can result in a mean value which seems quite acceptable, (ii) the influx of new young red blood cells into the circulation is increased in pregnancy leading to a misleadingly low HbA1c value, (iii) increased maternal blood volume in pregnancy which can also lead to a misleadingly low HbA1c value. The value of HbA1c is primarily in the pre-pregnancy period and in the very earliest stage of pregnancy. Otherwise, accurate and frequent
blood glucose measurements are mandatory during pregnancy to assess metabolic control.

Most of the evidence for the link between diabetes and early embryopathy has come from animal studies. Using whole rat embryos cultured in high glucose concentration during day 9.5-11.5 of gestation Sadler et al (129) showed that these embryos developed growth retardation, anomalies of fusion of neural folds, microencephaly, malformations of the eyes, vasculature and heart. Freinkel (130) later showed that this did not happen when other hexoses such as fructose, inositol, sorbitol or galactose were substituted - indicating the specificity of glucose.

Freinkel et al (131) showed that ketones and glucose together can be synergistic in their teratogenic effects. If used together in smaller doses they could cause a greater malformation rate than if used singly in larger doses. They termed this effect 'fuel-mediated teratogenesis'. These studies suggest that malformations in IDDM pregnancy result from the combined impact of several metabolic abnormalities.

Factors other than glucose reported to cause malformations in vitro include ketones (132, 133, 134, 131) and serum fractions that inhibit somatomedin activity (131, 135, 136). In vitro studies of embryogenesis revealed that deficiency of myoinositol (137, 138), abnormalities of arachidonic acid and prostaglandin metabolism (139, 140, 141) and over-production of oxygen radicals (142) may mediate the embryotoxic effects of hyperglycaemia. These animals studies and others, indicate that the aetiology of diabetic embryopathy is complex and involves more than just hyperglycaemia.
In the non-diabetic population spontaneous abortion is multifactorial whereby up to 60% are due to chromosomal abnormalities (143) and the remainder to maternal vascular, immunological and idiopathic factors. It is not known whether an increase in spontaneous abortion rates in diabetic women with poor glycaemic control is due directly to an increase in blood glucose or if some other pathology is responsible. Most authors speculate however, that the increased risk of spontaneous abortion is due to an increased rate of malformation incompatible with embryonic or fetal life.

Evidence has accumulated in the last 20 years to support the original Jorgen Pedersen hypothesis (101) that many of the pathophysiological events found in the infant of the IDDM mother may be attributed to fetal hyperinsulinaemia secondary to inadequate maternal metabolic control. The Pedersen hypothesis states that maternal hyperglycaemia results in increased delivery of glucose to the fetus, which experiences premature maturation of pancreatic insulin secretion. The resulting fetal hyperinsulinaemia causes overgrowth of the fetus, particularly in insulin-sensitive tissues such as the liver, subcutaneous fat, striated and cardiac muscle. This hypothesis was modified (71) to include the synergistic effect of other fuels such as amino acids and lipids. However, the impact of maternal hyperglycaemia on the developing fetus is not simply restricted to fetal overgrowth as shown in Figure 1.2.

Insulin and insulin-like growth factors are the main regulating hormones affecting fetal growth. Insulin is present in the pancreatic islets at 8-10 weeks gestation but is not secreted into the circulation until 20 weeks when the insulin response to glucose becomes evident (144). Approximately 80% of the maternal glucose level will be
Figure 1.2: The impact of maternal hyperglycaemia on the fetus

Maternal hyperglycaemia
  ↓
Fetal hyperglycaemia
  ↓
Fetal hyperinsulinaemia
  ↓
Fetal substrate uptake ↑

Neonatal Hypoglycaemia

Fetal lung surfactant ↓

Macrosomia

Fetal O₂ extraction ↑

Hypoxaemia

Erythropoeisis ↑
  ↓
Polycythaemia
  ↓
Tissue Fe ↓

Intra-Uterine Growth Retardation
Intra-Uterine Death

Respiratory Distress Syndrome

Placental size ↑
and Blood flow/function ↓
found in the fetus and the endogenous glucose level in the fetal circulation will regulate the glucose responsiveness of the fetal β cells (145).

The precise cause of intra-uterine death is unknown but sustained fetal hyperglycaemia in the sheep is associated with stimulation of fetal oxygen consumption, increased fetal uptake of substrates namely glucose and lactate, and placental lactate production and can result in fetal hypoxaemia resulting in metabolic acidosis and fetal death (146). Chronic fetal hypoxaemia stimulates extramedullary haematopoeisis resulting in fetal polycythaemia and an increase in platelet aggregation which may predispose the fetus to intravascular thrombosis (147). The additional haemoglobin synthesised requires elemental iron, but the placental transport of iron is relatively limited owing to vascular disease affecting placental blood flow (78) this results in fetal iron deficiency. Alterations in iron metabolism may contribute to some of the neonatal morbidity in infants born to IDDM mothers, since tissue iron deficiency is known to adversely affect the development of the brain, liver, gastrointestinal tract, heart and skeletal muscle. Intra-uterine growth retardation may also result from fetal hypoxaemia caused by reduced uterine blood flow which may be caused by pre-eclampsia, poor metabolic control, vascular disease or ketoacidosis.

There is evidence that maternal hyperglycaemia and fetal hyperinsulinaemia are involved in the process of delaying fetal lung maturation. High glucose and insulin can inhibit fetal lung maturation directly in a dose dependent manner (148, 149). Type II pneumocytes in the lung have insulin receptors which play a role in their uptake of glucose and it has been suggested that hyperglycaemia/ hyperinsulinaemia...
may lead to down regulation of glucose receptors in the fetal lung resulting in reduced glucose availability for the synthesis of surface-active phospholipids (150). After birth, the down-regulated receptors limit glucose uptake and phosphatidylcholine synthesis which contributes to the development of RDS.

Fetal hyperinsulinaemia may also result in neonatal hypoglycaemia during the first few hours of life. Infants of IDDM women are unable to respond to the hypoglycaemia by releasing glucagon from the α cells of the pancreas to mobilise glycogen (151). They are also relatively glucagon resistant, requiring ten times the usual amount of glucagon to cause glycogen to be released and broken down into glucose.

1.2.8 Long-term Morbidity in Infants of Diabetic Mothers

1.2.8.1 Neuropsychological Development

The relationship between maternal ketonuria and impaired intellectual development of the offspring was first described by Churchill in 1969 (121). It has been reported that moderate hypoglycaemia in the neonatal period can have serious neuro-developmental consequences (152, 153). They report that the number of days on which moderate hypoglycaemia occurred was strongly related to a reduction in mental and motor scores at 18 months of age. Five or more separate days of hypoglycaemia increased the risk of impairment by a factor of 3.5.

Studies from Copenhagen involved 90 children born to diabetic mothers between 1980 and 1983 who were tested and examined at 4-5 years of age. The study also included a group of low birth weight children born to non-diabetic mothers and a
matched control group. They reported that children born to mothers with well controlled IDDM still incurred an increased risk of impaired psychomotor development which is comparable to that seen in low-birth weight children. Psychomotor retardation was evident in 23 out of 34 children who had demonstrated early intra-uterine growth retardation as assessed by ultrasound, their poor cognitive function suggests neurological immaturity (95, 154).

1.2.8.2 Development of Diabetes

There is a high risk of developing IDDM in the offspring of parents whose own IDDM was diagnosed before the age of 11 (155). The risk is reduced by two-thirds in the offspring of parents whose IDDM was diagnosed later in life. In addition, regardless of the mother’s age at diagnosis of IDDM, a child born after the mother is 25 years old has half the risk of developing IDDM of a child born to that mother at a younger age. The mechanisms through which maternal age reduces the risk of IDDM are unknown. Increased maternal age is associated with diminished fertility, increased spontaneous abortions and trisomic anomalies and a higher risk of complications that result from growth retardation, which presumably results from placental complications. Fetuses of older mothers may be exposed to different hormonal and immunological characteristics which may play an important role in the development of β cell function.

The children of diabetic fathers are three times more likely to develop IDDM than those born to diabetic mothers. Buschard and colleagues (156) suggested that maternal hyperglycaemia may protect the child against development of diabetes in
later life by promoting earlier maturation of fetal/neonatal β cells. Using Diabetes-Prone (DP) BB rats Buschard and colleagues stimulated the immature neonatal β cells in the first 6 days of life by administering various secretagogues - glucose, arginine, glucagon and tolbutamide, - with the intention of rendering them mature, increasing antigen expression, inducing tolerance and reducing the incidence of IDDM in these offspring (156). The data showed that neonatal treatment of DP-BB rats with combinations of either glucose and glucagon, or glucose and arginine resulted in a fall in the incidence of IDDM from 65% in untreated controls to 23% and 20% respectively in the two treated groups.

1.2.9 Overall Conclusion

In no other group of babies, (except perhaps those born prematurely to normal mothers) has the perinatal mortality rate improved so dramatically over the past 25 years as in the infants of diabetic mothers. The main factors responsible for the fall in PNMR are strict control of maternal blood glucose concentration during pregnancy (157 158) and the centralisation of cases (159). Improving metabolic control during pregnancy has resulted in a lower risk of stillbirth allowing later delivery, which along with advances in neonatal management has led to a fall in the neonatal death rate, particularly from RDS.

However even today the PNMR for diabetic pregnancy remains approximately four times that for non-diabetic women (4-8% vs 1-2%) (57). The main causes of the high PNMR in women with IDDM are prematurity resulting from either spontaneous pre-term labour or elective early delivery performed to avoid late intra-uterine death,
intra-uterine death; fetal early growth delay; infants small for gestational age; and malformation. The mechanisms underlying the teratogenic effect of IDDM, the initiation of pre-term labour, and the development of infants small for gestational age remain obscure and good metabolic control during pregnancy has not abolished these problems. It is however, well recognised that these complications tend to be associated with each other (in both diabetic and non-diabetic pregnancy) and to occur particularly in mothers with IDDM who also have clinically-evident microangiopathy and/or pre-eclampsia and/or early growth delay. Thirty to sixty percent of all perinatal deaths and a substantial proportion of the excess neonatal morbidity in IDDM pregnancy are now due to malformation. In addition, there is increasing concern about long term morbidity in the children born to mothers with IDDM.

Elucidation of the teratogenic effect of diabetes requires the study of the immunological and metabolic environment at conception, implantation and during organogenesis. Direct study of the early human embryo in IDDM pregnancies is impossible for practical and ethical reasons and so appropriate animal models provide a direct approach for studying disturbances in early IDDM pregnancy.

1.3 Animal Models of Insulin-Dependent Diabetes Mellitus

An appropriate animal model for human IDDM should include a definable genetic susceptibility linked to the MHC, evidence of both cell mediated and humoral immunity, possible initiation by environmental factors and the pathological hallmark
should include selective β cell destruction accompanied by insulitis in early stages (160). The current animal models for diabetes can be separated into two groups (i) chemically-induced and (ii) spontaneous models.

1.3.1 Chemically-Induced Animal Models

1.3.1.1 Alloxan-Induced Diabetes

The β cell toxicity of alloxan was discovered in 1943 while testing the nephrotoxicity of uric acid derivatives in rats and rabbits (161, 162). Alloxan toxicity is dose-dependent and the drug passes the placenta readily although no diabetes has been detected in the offspring. Administration of alloxan is most effective when administered by intravenous injection in a dose of 40-45 mg/kg/body weight. It produces irreversible functional β cell damage within minutes and structural damage within hours in most rodents, dogs, cats, rabbits, monkeys, cattle, birds and fish.

Alloxan is rapidly taken up by the β cells and has a direct effect on membrane permeability (163, 164). Alloxan interferes with the generation of glucose-derived energy in islets by inhibiting the glycolytic flux and pyruvate oxidation (165). Malaisse and colleagues (166) concluded that that the mechanism of alloxan toxicity was probably due to free radical formation. Alloxan is rapidly reduced within the cell to dialuric acid which autoreoxidises to alloxan and the superoxide radicals arising from this alloxan-dialuric acid cycle decompose spontaneously or through catalysis by the enzyme superoxide dismutase. Thus, hydrogen peroxide is formed which reacts with the superoxide to form, in the presence of iron ions, hydroxyl radicals, which are extremely reactive oxidising agents damaging various cellular components (167).
1.3.1.2 Streptozotocin-Induced Diabetes

The diabetogenic properties of streptozotocin (STZ) were first discovered by Rakieten, Rakieten and Nadkarni (168) during the testing of potential antibiotics from the bacterium Streptomyces achromogenes. STZ consists of a 1-methyl-1-nitrosourea moiety linked to carbon 2 of D-glucose. The glucose moiety is an essential component which specifically directs it to the β cell. Once inside the cell, STZ spontaneously decomposes to form an isocyanate compound and a methylidiazohydroxide (169), which further decomposes to form a carbonium ion. This highly reactive ion is able to alkylate various cellular components, including DNA - leading to lesions which are removed by excision repair. Part of this repair process involves the activation of the enzyme poly (ADP-ribose) synthetase to form poly (ADP-ribose) using NAD as a substrate (170, 171). In the β cell this enzyme is overactivated and NAD is critically depleted leading to cessation of cell function and cell death (172). Non-lethal but unrepaired DNA lesions may lead to abnormal gene expression and result in altered cellular function such as decreased or inappropriate insulin secretion. Moreover, the high tumour frequency in islets which is a phenomenon associated with STZ-induced diabetes is thought to be due to inhibition of DNA repair mechanisms resulting in abnormal DNA recombination with the formation of a tumour-inducing gene (173). The treatment of islets with nicotinamide, the precursor of NAD prevents depletion of NAD and protects against the diabetogenic effect of STZ (174).

Various forms of diabetes can be induced with STZ depending on the dose and the protocol employed to administer it.
1.3.1.2.1 Low-Dose STZ-Induced Diabetes

One important difference between alloxan and STZ is that only STZ in repeated small doses produces in susceptible species a delayed insulitis-related diabetes (175). Five consecutive subdiabetogenic doses (40 mg/kg/body weight) of STZ produce a delayed but progressive increase in blood glucose and islet infiltration with lymphocytes and macrophages, accompanied by β cell necrosis and hyperinsulinaemia (175). Interpretation of these experiments has also to take into account the fact that STZ has independent deleterious effects on the host defence system (176). These effects include failure of lymphocytes to release mediators involved in the recruitment of T cells, depressed numbers and functions of T cells, low phagocytic activity of macrophages and a reduced bone marrow function (177, 178, 179). This model was mainly used to study the components and progression of the pancreatic infiltrate in the early stages of β-cell destruction before the discovery of spontaneous autoimmune animal models.

The susceptibility or resistance to low dose STZ treatment involves at least three gene groups (180). One is responsible for the sex determined propensity to insulitis, since males are more sensitive to insulitits than females. Castration abolishes the sex related difference and testosterone sensitises castrated males as well as females (181, 182, 183). The reported age difference in susceptibility may also be related to changes in sex hormone levels with age. A second gene is located on the MHC of mice, H-2 and influences the development of hyperglycaemia, and the third gene is located outside the MHC and controls the susceptibility to diabetes in an opposite direction to the MHC-linked gene.
1.3.1.2.2 High-Dose STZ-Induced Diabetes

One single large dose (i.e. 50-60 mg/kg/body weight) given intravenously or intraperitoneally causes specific, acute degeneration of β cells of the pancreas and chronic hyperglycaemia. Some ketonaemia and ketonuria may accompany severe hyperglycaemia and wasting in animals with STZ-induced diabetes lethal ketoacidosis is relatively uncommon.

1.3.2 Spontaneous Autoimmune-Induced Animal Models

1.3.2.1 Non Obese Diabetic (NOD) Mouse

The non obese diabetic (NOD) mouse was derived in Japan from a cataract-prone female mouse displaying severe glycosuria. The NOD mouse spontaneously develops an insulin-dependent diabetes mellitus that has many immunological and pathological similarities to human insulin-dependent diabetes. After brother-sister mating for over 20 generations an inbred strain was established in 1980 (184). Most mice of both sexes develop insulinitis after 9 weeks of age. Conversely, the occurrence of overt diabetes is different depending on the sex : in the female the incidence rises gradually with age to approximately 80 %; in male mice however, diabetes occurs only rarely, the cumulative incidence at 30 weeks being less than 20 % (185).

1.3.2.1.1 Pathogenesis of IDDM

The autoimmune nature of the disease is suggested by lymphocytic infiltration of islets of Langerhans, preceding the destruction of insulin-producing β cells (186). Diabetes in the NOD mouse is immune-mediated since T cell deficient mice do not develop
hyperglycaemia and have very little lymphocytic infiltration. Immunosuppressive measures such as irradiation, bone marrow transplant and treatment with cyclosporin A prevent hyperglycaemia and insulitis. β cell deterioration in the NOD mouse can be prevented or attenuated by nicotinamide, scavengers of free radicals and inhibitors of poly-ADP-ribose synthetase. Therefore, the destruction of pancreatic islets shows some similarities to those of chemical cytotoxins. In addition, retrovirus-like particles are seen in the islets of NOD mice which is also the case in multiple-dose streptozotocin-induced diabetic animals.

Lymphocytes are localised in the periductular capillary spaces with a clear basement membrane boundary, without overt contact with the β cells. Later on, only the β cells are affected while the A, D and PP cells remain intact. The cellular infiltrate consists initially of macrophages and CD4+ T cells. Recruitment of CD8+ T cells occurs later coinciding with the intra-islet infiltration and overt diabetes (187). Unlike the BB rat which displays pronounced lymphopenia, NOD mice exhibit varying levels of moderate lymphocyte decline. The onset of diabetes is manifested by the presence of glucose in the urine and increasing levels of glucose in the blood. As the disease progresses the animals develop polyuria, polydypsia and muscle wasting but ketosis is variable and diabetic animals can survive for prolonged periods without insulin replacement therapy.

1.3.2.1.2 Humoral Immune Response

Humoral antibody changes such as the presence of islet cell surface antibodies (ICSA) islet cell cytoplasmic antibodies (ICA) and anti-GAD autoantibodies have been
detected in the sera of pre-diabetic NOD mice (188, 189), indicating the role of humoral immunity concomitant to cell mediated immunity.

High doses of cyclophosphamide (Cy) injected intraperitoneally induces variable diabetes incidence in NOD mice within 1 month of the injection. It has been proposed that Cy acts by targeting the regulatory/suppressor cells (190). Although this diabetes is drug induced the basic mechanism is autoimmune since macrophages and T cells are involved (191).

1.3.2.1.3 Genetics of the NOD mouse

In the NOD mouse IDDM is a polygenic trait (192, 193, 194) with the major locus Idd1 located in the MHC region on chromosome 17. Originally the action of Idd1 was believed to be recessive, but it has now been shown that the action of NOD MHC is dominant with reduced penetrance (194). The MHC genes responsible for disease susceptibility include Class II genes encoding for the I-A and I-E molecules. The NOD mouse has a unique Ab<sup>k7</sup> gene which encodes for a rare I-A<sup>k7</sup> molecule required for the disease. A deletion mutation in the promoter region of the gene encoding the Eα chain causes a lack of I-E expression, a null phenotype that is also essential for the development of the disease.

In addition to Class II alleles, there is some evidence that ClassI and II loci have some influence on disease development. Non-MHC genes are also essential but not sufficient in themselves for the development of diabetes, and at least 14 other loci contribute to the development of disease (192, 193, 194). All these genes have a
dominant mode of action with varying degrees of penetrance. These loci are $Idd2$ (on chromosome 9), $Idd3$ and $Idd10$ (on chromosome 3), $Idd4$ (on chromosome 11), $Idd5$ (on chromosome 1), $Idd6$ (on chromosome 6), $Idd7$ (on chromosome 7), $Idd8$ and $Idd12$ (on chromosome 14), $Idd9$ and $Idd11$ (on chromosome 4), $Idd13$ (on chromosome 2), $Idd14$ (on chromosome 13), and $IDD15$ (on chromosome 5) (195).

1.3.2.2 BioBreeding (BB) Rat

1.3.2.2.1 Introduction

The spontaneous autoimmune diabetic BB Wistar rat was first discovered in 1974 by Drs. Reginald and Clifford Chappel at the BioBreeding Laboratories, Ontario, Canada. The syndrome appeared in a commercial outbred colony of Wistar-derived albino rats and is thought to have been due to a spontaneous mutation. The diabetic animals were inbred to create the Diabetes Prone (DP) BB rat strain and animals from this group were subsequently supplied to establish separate breeding colonies in a number of locations including Worcester, Philadelphia and Edinburgh. Animals that failed to develop diabetes were also inbred to become a control line for non-diabetic BB rats - now designated as Diabetes Resistant (DR) BB rats. Since BB rats distributed to different centres show variations in the incidence of diabetes, it was suggested that each laboratory adopt a supplementary designation such as BB/W selected by Like and associates to designate their colony by its location in Worcester, Massachusetts (196).

Attractive features of the BB rat as a model for IDDM include a long (approximately 3 month) silent pre-diabetic period which can be used to investigate possible
intervention and primary prevention strategies; involvement of both genetic and immune factors in its aetiology; the absence of obesity; and the occurrence of functional and structural changes in the retina, kidneys and nerves of established diabetic animals. There are several dissimilarities from human IDDM in that islet cytoplasmic antibodies are only found in man; lymphopenia is only found in BB rats; BB rats are inbred and there is a higher incidence of diabetes in BB rats (30-90 %) than in humans (197).

The main problem encountered using the BB rat strain is that they are very susceptible to developing pulmonary and other infections by common varieties of bacteria, mycoplasma and viruses (198). It has therefore been necessary to develop facilities in which the rats are ‘germ-free’ using barriers to prevent such infections. There are also problems in breeding and maintaining spontaneously diabetic animals.

1.3.2.2.2 Clinical Features

The onset of diabetes in the DP-BB rat is abrupt and acute with the peak age at onset 96 days of age (range 60-120 days) in 50-80 % of an inbred colony. Diabetes develops in both sexes at a similar incidence in this model (199, 200). Clinical symptoms at onset include polyuria, polydipsia and severe weight loss and lethargy (201). At onset, hyperglycaemia is associated with undetectable circulating insulin and a high incidence of severe ketoacidosis along with hyperglucagonaemia, hyperketonaemia, hyperlipidaemia and uraemia. Ketoacidosis develops in virtually all diabetic rats and spontaneous remission is very rare. There are many similarities to human IDDM including a total dependence on insulin therapy for survival and the fact
that DP-BB rats develop IDDM at an age roughly comparable to that in diabetic patients where the highest incidence is 10-30 years old (197).

1.3.2.2.3 Aetiology of IDDM

As in human IDDM, the development of diabetes in the DP-BB rat, requires both genetic predisposition and environmental factors.

1.3.2.2.3.1 Genetic Factors

Diabetes in the DP-BB rat is inherited by an autosomal recessive transmission with approximately 50 % penetrance (200, 201). A strong association with IDDM and class II MHC genotype has been reported in the human (202) and the BB rat (203, 204, 205). The rat MHC is known as RT1 and consists of a number of class I gene loci (RT1A, RT1C, RT1E) flanking two class II gene loci, RT1B and RT1D (homologues of the human HLA-DQ and HLA-DR). The TNF-α and β loci are located between the class II loci and the telomeric class I loci which is also seen in the human and the mouse MHC (206). Susceptibility to diabetes in DP rats is linked to the class II region of the MHC and two non-MHC genes (204), one of which induces a severe T cell lymphopenia. Genetic studies crossing DP-BB rats with non-diabetic rat strains have shown that only rats expressing the RT1u haplotype develop IDDM (203) and all Diabetes-Prone BB rat lines (to date) have been shown to type as RT1u (207, 208). Furthermore, only the class II subregion of the RT1u complex appears to be necessary for disease expression (209). This is similar to human IDDM where only one copy of the permissive class II allele is sufficient to confer susceptibility.
Lymphopenia precedes clinical manifestations and involves primary and secondary lymphoid tissues and affects all subsets of T cells. Lymphopenia is controlled by a single autosomal recessive gene (lyp) (204) which maps close to the neuropeptide Y (Npy) gene on chromosome 4 (210). It is characterised by a marked decrease in the number of T lymphocytes in the peripheral blood, lymph nodes and spleen. While all T subsets are diminished, the cytotoxic supressor T cell subset (OX8+) is most affected (211). In addition, the RT6 differentiation alloantigen is absent in rats with lymphopenia (212, 213). The RT6 alloantigenic system is found on chromosome 1 and consists of at least two alleles - the RT6a gene encodes the RT6.1 alloantigen and the RT6b gene encodes RT6.2 (214, 215). The surface expression of RT6 alloantigens is restricted to T cells in the rat. It is a maturational antigen and is expressed on most but not all mature T cells in the rat, approximately 50 % of W3/25+ (CD4) and 70 % of OX8+ (CD8) peripheral T cells express RT6 (216, 217). Biochemical analysis of peripheral T lymphocytes of DP-BB/W rats failed to detect RT6 protein (218). More recent reports have demonstrated that the RT6a gene is present, transcribed and translated in DP-BB/W rat lymphocytes (219). They also show evidence of an intact phosphatidylinositol linkage of the protein to the cell surface and that the RT6.1 antigen is correctly processed and folded in the lymph node cells, therefore the lack of RT6+ T cells in DP-BB/W rats is unlikely to be caused by defects in RT6 gene expression per se. It is possible that defects in RT6 gene regulation or premature death of the T cell lineage may be responsible. Elimination of RT6+ T cells by treating in DR- BB rats with polynoinic: polycytidylic acid and an antibody against RT6 renders 50-100 % of the resistant subline susceptible to IDDM (220).
Unlike any other BB rat colony, in which only the DP-BB rats have lymphopenia, both of the BB/Edinburgh rat sublines (i.e. DP-BB/E and DR-BB/E) display profound lymphopenia and there is no difference between them in the severity of the phenotype (221). The DR-BB/E line has an apparently normal, potentially diabetogenic RT1\textsuperscript{i} haplotype at the MHC and therefore it seems likely that although the RT1\textsuperscript{i} haplotype and homozygosity for lymphopenia are necessary for the development of diabetes, these factors alone are not sufficient to create a permissive genotype in the DR-BB/E line (221). This suggests that there is an additional polymorphism required for the development of IDDM in the DP lines. One candidate gene is the pancreatic lymphocytic infiltration (Pli) alleleism (222, 223). In the DP-BB rat this is characterised by the appearance of clusters of lymphocytes around the small ductules and venules of the pancreas (224). However, more detailed analysis of this gene polymorphism is required before the exact nature of the differences between BB/E and other BB colonies can be understood.

1.3.2.2.3.2 Environmental Factors

In human IDDM there is a relatively low concordance for IDDM between monozygotic twins (36 %) (19). A similar situation exists in the BB rat where only 50-60 % of animals in DP litters develop diabetes. This indicates a role for environmental factors in the precipitation of disease in both the human and the BB rat.

(i) Diet

Diet is a crucial factor influencing the penetrance of the diabetogenic phenotype in BB rats. Elliot and Martin (225) reported that feeding DP-BB/W rats a mixture of amino
acids in place of intact proteins significantly decreased the incidence of IDDM. The addition of 1% skimmed milk powder to the amino acid mixture diet increased the risk for diabetes in the offspring, particularly when it was fed to mother and pups during the weaning period (226).

Further studies, reviewed by Scott (227), have shown that wheat and milk proteins have the strongest diabetogenic effect. These compounds have been designated ‘initiators’ of disease, since they are capable of triggering the events which result in β cell destruction but only in genetically susceptible animals. Other dietary constituents have been designated ‘modifiers’ since they appear to be able to modify the course of diabetes once it has been initiated. These compounds include vitamin E (an antioxidant) and lard, which reduces and increases the incidence of IDDM respectively. Diets either based on hydrolysed caesin, or deficient in essential fatty acids can reduce the incidence of diabetes and delay the age at onset (228, 229, 230). The mechanisms responsible for these effects are not known, whether the decrease in frequency of diabetes is secondary to the removal of a specific dietary protein or whether it is a non-specific effect of removing fibres, lectins, viral or bacterial toxins which may be present in the chow remains to be determined. None of the diets affect the lymphopenia and tend to have the greatest effect when introduced before the age of 4 weeks.

(ii) Viral and Non-Viral Infections

Certain viruses can influence the expression of IDDM in BB rats. A common rat parvovirus, Kilham’s rat virus (KRV) has been identified as a viral trigger of IDDM in
BB rats (231). This microbial agent appears to promote diabetes by acting on cells of the immune system rather than on islet β cells directly, and it has been suggested that the virus may act on effector cells or regulatory T cells to promote an autoimmune response. DR-BB/W rats housed in viral antibody-free facilities remain free of spontaneous diabetes but if infected with KRV can become diabetic (232). Recently, Ellerman and colleagues (233) demonstrated that KRV infection could induce autoimmune diabetes in three unrelated strains of rats: DR-BB/W rats, PVG.RT1u rats and LEW1.WR1 rats and they propose that for a rat strain to be susceptible to KRV-induced autoimmune diabetes it must possess beta cell specific autoreactive T cells as part of its peripheral T cell receptor repertoire.

Other observations however, have revealed that the environmental influence of microbial agents on IDDM can be protective against disease development (234). Thus some viral infections introduced into colonies of diabetes prone BB rats previously maintained in a pathogen-free environment resulted in a reduction in the incidence of diabetes. For example, experimentally induced infection with lymphocytic choriomeningitis virus (LCMV) was found to decrease the frequency of IDDM in DP-BB/W rats (234).

Bacterial and fungal preparations have also been found to exert a protective effect against diabetes development in BB rats, these include streptococcal extract OK432 (235), and complete Freud's adjuvant (CFA) comprised of killed mycobacterium tuberculosis in oil emulsion (236).
(iii) Stress

The immune system’s response to acute infection and chronic disease can be modified by environmental stress. Studies have shown that environmental stress such as restraint, rotation, crowding and resocialisation may modify the age of onset of diabetes in the DP-BB/W rat (237).

1.3.2.2.4 Pathogenesis of IDDM

Sequential pancreatic biopsies in individual animals together with cohort analysis have permitted the cellular sequence of events leading to β cell destruction to be delineated (49, 50, 238). The first change occurs prior to any cellular infiltration and consists of increased expression of MHC class I molecules on the vascular endothelium and on islet cells. The next event occurs 2-3 weeks before the onset of IDDM when there is a marked recruitment and accumulation of a population of macrophages identified by the monoclonal antibody (ED1). These ED1 macrophages are quite distinct from the resident population of ED2-positive tissue macrophages. These recruited cells proceed to infiltrate the pancreatic islets. Approximately 10 days before the onset of IDDM there is an infiltration of the islets by other activated effector cells including CD4+ and CD8+ T cells, natural killer (NK) cells and B cells appear (239). At this time the expression of MHC class II molecules is restricted to the infiltrating immunocytes and is never seen on the islet insulin-secreting cells. At the onset of IDDM, the insulin secreting cells have virtually disappeared but occasionally MHC class II antigens are expressed on the few remaining cells which contain insulin. Finally when all insulin-containing cells have been destroyed the infiltrate disappears.
Diabetes Prone BB rats show abnormal glucose tolerance and symptoms a few days before IDDM onset at approximately 60-120 days of age in 50-60 % of an inbred colony. Immediately after the development of hyperglycaemia there is pronounced insulitis with the lymphocyte infiltration (201). Insulitis precedes the onset of overt diabetes by several days and disappears soon after leaving shrunken end-stage islets devoid of β cells and composed almost entirely of cells containing glucagon (A cells), somatostatin (D cells) and pancreatic polypeptide (PP cells) (240). As in human IDDM autoantibodies reacting against various tissues and proteins are present in the BB rat, both in the pre-diabetic period and at diagnosis of diabetes. These include antibodies to gastric parietal, smooth muscle and thyroid cells as well as to lymphocytes, islet cell surface antibodies (ICSA), a 64 kDa islet cell protein and insulin (IAA) (241, 242, 243). Islet cell cytoplasmic antibodies (ICA) and antibodies to pituitary, adrenal and ovary cells have not been found in the BB rat and no mononuclear infiltration is seen in these tissues.

Cytokines are critically involved in regulating several aspects of IDDM, including the pathogenesis of the disease. This is true for human IDDM as well as spontaneous autoimmune IDDM in the DP-BB rat. Cytokines are fundamental mediators of T cell regulatory and effector functions and they have been shown to be critical for the development and maintenance of pancreatic β cell directed autoimmunity. It has been suggested that in IDDM cytokines serve as chemoattractants, generators of cytotoxic Th1 type responses, and pancreatic β cell toxins (244). Evidence for this comes mainly from in vitro studies in which interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), and γ-interferon (γ-IFN) were found to be β cell cytotoxic (245). Several
investigators have proposed that the expression of autoimmunity in BB rats depends on a balance between populations of regulatory and effector T cells, i.e. between the Th1 and Th2 subsets of the CD4+ T cell population (246, 244). Th1 subsets mediate proinflammatory reactions and secrete IFN-γ and IL-2, whereas Th2 subsets secrete IL-4 and IL-10 and can down-regulate a Th1 immune response. In the BB rat, IFN-γ and IL-12 play a major role in the expression of insulitis and thyroiditis and it has been suggested that Th1 lymphocytes predominate over Th2 lymphocytes in these inflammatory lesions (247, 248). However, it appears that the prevention of diabetes by insulin treatment is not associated with an alteration in the cytokine gene profile of islet infiltrating cells (247).

1.3.2.2.5 Vascular Disease in the BB rat with long-term IDDM

The development of widespread microvascular disease is one of the most significant long-term consequences of human IDDM, and it results in serious morbidity such as blindness, gangrene and renal failure. These microvascular alterations are characterised by a thickening of the basement membranes, deposition of extracellular matrix and scarring. These changes are also seen in the BB rat with established long-term IDDM. The exact mechanisms underlying these complications are unknown and the use of the BB rat has enabled the link between hyperglycaemia and vascular disease to be studied in more detail.

(i) Retinopathy

Diabetic retinopathy is an important cause of blindness in the world and research has been difficult owing to the limitations imposed by the use of human tissue. Although
the classical clinical features of diabetic retinopathy are not seen on ophthalmoscopy in BB rats, retinal pathology has been described (249, 250). Retinal pathology includes pericyte degeneration and loss, endothelial cell degeneration, capillary basement membrane (CBM) thickening, microthrombi and retinal pigment epitheliopathy. Retinal CBM thickening can be prevented in galactosaemic animals by aldose-reductase inhibitor therapy suggesting that augmented polyol pathway activity may play in the development of this pathology (251).

(ii) Nephropathy
Severe nephropathy as seen in human IDDM is not found in BB rats, but proteinuria and thickening of the glomerular basement membrane (GBM) and of the mesangium, occur after a few months of diabetes. The development of proteinuria and GBM thickening are correlated and are related to the degree of hyperglycaemia (252). Treatment with insulin reduces proteinuria and slows down the progression of GBM thickening (253).

(iii) Neuropathy
The first morphological change in the somatic nerves of the diabetic rat occurs approximately 3 weeks after diabetes onset and is seen as localised swellings of the large axons at the nodes of Ranvier (254). There is a reduction in nerve conduction velocity and decreased amplitude of evoked muscle potentials - indicating a loss of functioning motor units. At this stage the structural and functional abnormalities are reversible by insulin treatment or administration of aldose reductase inhibitors.
Without treatment, nerve conductivity falls and there is marked axonal atrophy and loss of myelinated fibres develops (255).

1.3.2.2.6 The BB rat as a model for human IDDM pregnancy

Although insulin therapy has resulted in a dramatic improvement in the outcome of pregnancy complicated by maternal IDDM, today, even in developed countries, the perinatal mortality and morbidity rates in these countries remains at least twice that for pregnancies in non-diabetic women. This is due to continuing high rates of (i) poor fetal growth in early pregnancy, (ii) spontaneous premature labour, (iii) congenital malformation, and (iv) low birth weight babies. The cause of these problems remains obscure. Since the development of effective preventive strategies depends on elucidation of their pathogenesis, these represent critically important areas for research.

It is very difficult to study these areas in human pregnancy, however the spontaneously diabetic insulin-dependent BB rat provides the opportunity to study the interaction of genetics and the fetal environment in utero in determining the outcome of IDDM pregnancy and the long-term development of the offspring of the diabetic mother.

The first studies of diabetic pregnancy in the BB rat by Brownscheidle and colleagues (256, 257) reported high rates of perinatal mortality and congenital malformation, particularly neural tube and skeletal defects. It has also been shown that Diabetic DP-BB/E rats maintained on continuous insulin therapy have a higher rate of fetal
resorption than non-diabetic DR rats and malformations such as sacral dysgenesis, exencephaly and omphalocoele have been described (258). In addition, these Diabetic DP-BB/E rat pregnancies were often characterised by low birth weight pups and large placentae (259).

Thus, pregnancy in the diabetic DP-BB rat is associated with many of the problems seen in human IDDM pregnancy, - namely a high PNMR and congenital malformation, which suggest that it is a valid model to study the pathogenesis of IDDM-induced embryopathy (259).

1.3.3 Concluding Remarks on Appropriate Animal Models for Human IDDM

Although some ketonaemia and ketonuria may accompany severe hyperglycaemia and wasting in animals with STZ-induced diabetes, lethal ketoacidosis is relatively uncommon and these animals are able to survive for variable periods of time (weeks/months) without insulin replacement therapy. In contrast, in properly inbred colonies of BB rats animals developing spontaneous, autoimmune-induced diabetes will usually die rapidly (within 1-2 days) in severe ketoacidosis if treatment with insulin is withheld.

This difference in clinical expression of disease reflects disparity in the degree of insulin deficiency in these two animal models of human IDDM. Thus animals with high-dose STZ-induced diabetes commonly retain a significant number of viable insulin-secreting β-cells along with a capacity for some regeneration (260) and this
results in a low level of circulating insulin at diagnosis of disease. This endogenous insulin is sufficient to exert an anti-catabolic effect (which prevents the development of lethal ketoacidosis) but insufficient to maintain an effective anabolic function: hence the severe hyperglycaemia, dehydration and wasting.

On the other hand, in BB rats no circulating endogenous insulin is detectable at diagnosis of diabetes. This means that these diabetic animals are totally dependent on rapid treatment with exogenous insulin to survive. They are therefore properly designated as having Insulin-Dependent Diabetes Mellitus (IDDM) comparable to the human disorder.

1.4 Development of the Pre- and Early Post-Implantation Embryo

Ovulation occurs in response to a surge in the level of luteinising hormone (LH) produced by the pituitary gland. After LH stimulation, the oocyte undergoes nuclear maturation when it loses its membrane (a process known as germinal vesicle breakdown) and the chromosomes assemble on the spindle and move towards the periphery of the cell where the first meiotic division takes place. One set of chromosomes surrounded by a small amount of cytoplasm is extruded as the first polar body, whereas the other set remains at metaphase II. The oocyte remains arrested at metaphase II until fertilisation triggers the second meiotic division and extrusion of the second polar body. Nuclear membranes form around the maternal and paternal chromosomes forming separate haploid male and female pronuclei. The pronuclei do not fuse but the membranes break down and the chromosomes assemble on the spindle and the first cleavage takes place soon after. The first cleavage
(beginning of the 2-cell stage) separates the embryo into two blastomeres approximately the same size, and irrespective of the species, takes place between 11 and 20 hours after fertilisation.

The initial cleavage divisions are equal, asynchronous and without any particular orientation. The blastomeres of early 8-cell embryos are spherical, evenly coated with microvilli and can easily be dissociated because cellular interactions are weak. During the 8-cell stage, cells start to flatten upon each other so that their boundaries can no longer be seen with an optical microscope. The major molecule involved in this cell flattening is the calcium-dependent cell adhesion molecule uvomorulin (also known as L-CAM or E-cadherin). Simultaneously with this flattening process, cytoplasmic and surface components become asymmetrically distributed (or polarised) - these two processes are known collectively as compaction. From the subsequent mitotic division (stage 16 in the mouse) this polarity is sufficient to produce two daughter cells which are identical when the division plane corresponds to the polar axis and different when the division separates the basolateral section of the cytoplasm (non-polar cytoplasm) of the outermost section (polar cytoplasm). The relative frequency of divisions according to the polar axis therefore determines the number of polar and non-polar cells in the subsequent division. At this stage the embryo is known as a morula. Tight junctional seals are formed in the trophoderm and the blastocoele expands as a result of fluid accumulation induced by a water transport system and a basolaterally located Na⁺K⁺ATPase enzyme. Expansion of the blastocoele generates a blastocyst containing two progressively determined cell populations - the trophoderm and the inner cell mass. The trophoderm has all the features of a
true epithelium with apical junctional complexes forming a complete permeability seal against the outside environment, and generates cells that populate the placental structures such as the ectoplacental cone, trophoblast giant cells and the chorion. The trophectoderm (TE) overlaying the inner cell mass (ICM) is known as the polar TE, and the cells surrounding the blastocyst cavity constitute the mural TE. In contrast, the ICM cells generate the entire embryo and the amnion, the yolk sac, the allantois and mesodermal components of the placenta.

During the 5th and 6th day of development (for the mouse and rat, respectively) the blastocyst hatches from the zona pellucida and is ready for implantation. The walls of the uterus become tightly opposed so that the uterine lumen is more or less occluded. The blastocyst first adheres by its abembryonic pole (the mural TE furthest away from the ICM) to the anti-mesometrial uterine wall. Blastocyst attachment induces the formation of a uterine crypt and stimulates the uterine stroma to form a spongy mass of cells known as decidual tissue. There is a rapid increase in the permeability of local capillaries causing the uterine stroma to become swollen and oedematous. The stromal cells proliferate, increase in size and establish numerous tight junctional complexes with neighbouring cells. Degeneration of the epithelium allows the trophoblast cells, which phagocytose the moribund epithelial cells, to invade the deciduum.

During the post-implantation development, the trophectoderm becomes regionally specialised with respect to morphology (51). The mural TE replicate their DNA without cell division giving rise to trophoblast giant cells which invade the uterus
during implantation. The rest of the TE grows to form the ectoplacental cone and the extra-embryonic ectoderm which both contribute to the formation of the placenta. Some cells from the primitive endoderm migrate over the whole inner surface of the mural TE - they become the parietal endoderm. The remaining primitive endoderm cells form the visceral endoderm which covers the elongating egg cylinder containing the epiblast.

By 6 days after fertilisation (in the mouse) an internal cavity has formed inside the epiblast, which becomes cup-shaped. The embryo proper develops from this curved layer of epithelium. At 6½ days, the gastrulation begins with the formation of the primitive streak. The streak starts as a localised thickening at a point on the circumference of the cup - this is the future dorsal side of the embryo. Proliferating epiblast cells migrate through the primitive streak and spread out laterally and anteriorly between the ectoderm and the visceral endoderm to form a mesodermal layer which will give rise to the gut. The development of extra-embryonic structures involves the production of extra-embryonic mesoderm at the end of the primitive streak. This eventually contributes to the amnion, the visceral yolk sac and the allantois and chorion, which are important components of the placenta.

During the final stages of gastrulation (approximately 8½ days in the mouse) organogenesis commences in the anterior part of the embryo with the formation of the heart, the cranial neural folds and the appearance of somites. Between 8½ and 9½ days the mouse embryo undergoes a complicated change in conformation in which it 'turns' so that it becomes entirely enclosed in the protective amnion and amniotic
fluid. The visceral yolk sac, a major source of nutrition, surrounds the amnion and the allantois connects the embryo to the placenta.

1.5 Apoptosis and Pre-Implantation Embryo Development

Cell death can occur by one of two distinct processes, necrosis or programmed cell death through apoptosis. Necrosis is usually associated with injury, and results in cellular swelling and rupture of the plasma and internal membranes. True programmed cell death (PCD) is genetically programmed, and requires the activation of specific genes involved in the execution of cell death. Apoptosis is triggered by external or internal stimuli, the process can be divided into four phases (389). The first phases may occur in response to an external trigger delivered through surface receptors (e.g. fas ligand and CD40 ligand), or may originate inside the cell from the action of a drug, toxin, or radiation (284). The next phase involves detection of this signal and transduction of the signal. The activation or signalling phase encompasses a great variety of signal transduction pathways including tyrosine kinases, steroid receptors, ceramide, inositol phosphates and cytokine receptors. The death effector phase is the third part of the cell death mechanism and includes the cysteine proteases that are activated during apoptosis. The fourth phase is the post-mortem phase, in which the cell’s chromatin condenses and its DNA is degraded into oligonucleosome chains (seen as DNA ‘laddering’ on gel electrophoresis) (284). Depending on their ratio, Bcl-2 and similar proteins can inhibit the action of the proteases, either by blocking their activation or by preventing them from reaching their targets. Other proteins implicated in the regulation of apoptosis include tumour suppressor protein p53 which triggers apoptosis in cells with damaged DNA, and c-Myc which can
inhibit apoptosis in the presence of factors such as insulin-like growth factor-I, insulin and epidermal growth factor (390).

Developmentally regulated cell death is widespread in embryogenesis (288) and occurs by apoptosis in both vertebrate and invertebrate species. It is a widespread feature in blastocysts of many mammals such as the cow (289); Rhesus monkey (290); mouse (2291) and the human (268). The significance of these dead cells is not fully understood, but it has been suggested that the primary function is to allow for the removal of redundant or defective cells from the blastocyst (288), or the elimination of ICM cells retaining the potential to form TE (291). Other suggestions include lack of survival factors in vitro or in vivo, and chromosomal abnormalities (387). In mouse blastocysts it has been recently reported that embryonic cells have a death-by-default mechanism that requires constant suppression by ‘survival’ factors produced both by the embryo and the maternal reproductive tract (388). However, the role of cell death in blastocyst development is unknown and the precise molecular mechanisms involved have yet to be defined.
Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals for molecular biology were obtained from BDH Chemicals Ltd., Poole, UK unless otherwise stated, and were of analytical quality. All molecular biology solutions were prepared in reverse osmosis distilled water (Elgastat Prima) and solutions for RNA work were prepared in autoclaved distilled water treated with 0.1 % diethyl pyrocarbonate (DEPC) (Sigma). Chemicals used to prepare rat embryo culture media were obtained from Sigma and were of tissue culture grade. Sterile water used to prepare cell culture media was obtained from Baxter Healthcare Ltd (Norfolk, England).

2.1.2 Animals

2.1.2.1 BB/Edinburgh (BB/E) Rats

A breeding colony of BB Wistar rats was established in Edinburgh in 1982 from a small nucleus of animals (3 male and 4 female) donated by Dr. P. Thibert from the original outbred BB rat colony in Ottawa. The BB/E colony consists of two sublines created by selectively breeding for and against diabetes. These two lines have now been through more than 30 generations of strict brother-sister mating on site thus
meeting the international criterion for designation as an inbred line, and have had their inbred status confirmed by skin allograft experiments.

In the high incidence Diabetes Prone mainline (DP-BB/E) the incidence of IDDM is 50-60 % and the mean (± SD) age at onset is 96 (± 18) days of age. In the Diabetes Resistant subline (DR-BB/E) the incidence of diabetes is zero. The lymphoid status of the two lines has been characterised in detail using FACS analysis and it has been shown that the BB/E colony is unique in that both lines are identical in all the immunological parameters examined (221). This makes the DR-BB/E subline an exceptionally good control strain for studies of the effect of diabetes on any parameter selected.

2.1.2.2 Non-BB Wistar Han Rats

Adult Wistar Han rats (out-bred on site for 5 years) were obtained from our breeding facility.

All animals were maintained at 20°C on a 12 hour light/12 hour dark cycle and allowed free access to food and water. BB/E rats were fed rat and mouse Number 1 Expanded Feed and Wistar Han rats CRM rat and mouse Breeder and Grower Diet, both from Special Diet Services (Witham, UK).

2.1.2.3 Diagnostic Criteria for IDDM in the BB/E rat

All animals are weighed twice weekly from 40 days of age. If they fail to gain weight or lose weight they are tested for glycosuria (Multistix SG reagent strips, Bayer
Diagnostics). If glycosuria is detected, the blood glucose concentration is measured (ExacTech blood glucose meter, Medisense) on a blood sample obtained by tail tipping without anaesthesia. In the Edinburgh BB rat colony a blood glucose concentration of >18 mmol/L is invariably associated with loss of weight, ketonuria, undetectable circulating endogenous insulin (J Freeman, RM Lindsay, JD Baird, J Foster, CR Elcombe, DJ Harrison, H Wolf and CR Wolf, unpublished observations) and the permanent need for daily treatment with insulin to ensure survival. These parameters constitute our criteria for classifying an animal as having autoimmune-induced insulin-dependent diabetes mellitus (IDDM).

2.1.2.4 Treatment and Management of Diabetes in the BB/E rat

(i) Conventional Insulin Therapy
Diabetic DP-BB/E rats were maintained by conventional insulin therapy i.e. a single subcutaneous injection of long acting insulin (Bovine Ultratard U40, Novo Nordisk, Denmark) each morning. The dosage of insulin (usually 2.0-2.6 units per day) was adjusted for each animal according to blood glucose measurements, glycosuria and body weight. This treatment allows the animals to be generally healthy, maintain normal body weights and live a normal lifespan but does not achieve normoglycaemia.

(ii) Sustained Release Insulin Implants
A separate group of Diabetic DP-BB/E rats with IDDM were maintained on Linplant® or LinBit™ sustained release insulin implants (SRIIs). These implants are small rods of insulin (7 mm long with a diameter of 2 mm) consisting of 14% powdered bovine insulin compressed under high pressure into a pellet with 86%
palmitic acid (Linplant SRIIs) or 5% insulin with 95% palmitic acid (LinBit SRIIs). Following implantation (using a local anaesthetic, trocar and cannula) erosion of the implant begins at once and the effect of the released insulin on the blood glucose level can be detected within 1 h of implantation. The insulin is released at a steady rate and approximately 2 units of bovine insulin is delivered per Linplant SRII or 0.4 units of bovine insulin per LinBit SRII, per 24 h per implant for >40 days. The recommended sites for implantation are subcutaneously in the dorsal neck region or the sternal area and the recommended dose for young female rats (weighing approximately 220 g) is ½ Linplant implant or two lower dose LinBit implants.

### 2.1.2.5 Assessment of Metabolic Control

Irrespective of the method of treatment it was important to assess the degree of metabolic control for each individual animal. Whole blood samples (20 μl) were taken either daily or on alternate days, by tail tipping without anaesthesia and the concentration of blood glucose was measured using a glucose meter (ExacTech, Medisense). In addition, urine samples were monitored regularly for the presence of glucose and ketone bodies (Multistix). Plasma samples were also collected intermittently by removing approximately 1.5 ml of whole blood by tail tipping into heparinised eppendorf tubes. Plasma and whole blood were separated by centrifugation using a bench top centrifuge (Eppendorf) at 10,000 x g for 5 min. The plasma was stored at -20°C before being sent to Dr. Linda Ashworth (University of Newcastle Upon Tyne) to measure the concentration of various plasma metabolites. The remaining red blood cells were washed with 0.9% sodium chloride and stored at 4°C and used to determine the percentage of glycosylated haemoglobin.
by Dr. Mark Lindsay (Western General Hospital, Edinburgh) using the Pierce Glyco.Test II (Pierce Chemical Co. Illinois, USA).

2.2 Methods

2.2.1 Preparation of Embryo Culture Media

2.2.1.1 Nutrient Mixture (Ham’s) F10

Nutrient Mixture (Ham’s) F10 (Gibco BRL) was supplemented with 0.1 % bovine serum albumin Fraction V (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). The medium was filter sterilised through a 0.22 μm bottle top filter (Falcon) into sterile glass bottles and stored at 4°C.

2.2.1.2 M2 and M16 Media

M2 (263) and M16 (264) embryo culture media were prepared from concentrated stock solutions of the component chemicals (Table 2.1). All stock solutions were filter sterilised through a 0.22 μm bottle top filter and stored at 4°C. Stock solutions A, D and E were stored for up to three months, and stock solutions B and C were changed every other week. To prepare the 1 x solutions of M2 and M16, the appropriate amounts of stock solutions (Table 2.2) were added to a conical flask and sterile distilled water was added to a volume of 50 ml. The M16 medium was gassed with 5 % CO₂ in air for 5 min to adjust the pH to 7.4. If required, 200 mg bovine serum albumin (Fraction V) (Sigma) was added and allowed to slowly dissolve. The media were then filter sterilised through a 0.22 μm bottle top filter (Falcon) into sterile glass bottles and stored at 4°C for up to 1 week.
Table 2.1: M2 and M16 culture media stock solutions

<table>
<thead>
<tr>
<th>Stock A</th>
<th>Component</th>
<th>g/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10 x concentration)</td>
<td>Sodium chloride</td>
<td>5.534</td>
</tr>
<tr>
<td></td>
<td>Potassium chloride</td>
<td>0.356</td>
</tr>
<tr>
<td></td>
<td>Potassium hydrogen phosphate</td>
<td>0.162</td>
</tr>
<tr>
<td></td>
<td>Magnesium sulphate</td>
<td>0.144</td>
</tr>
<tr>
<td></td>
<td>Sodium lactate</td>
<td>2.610</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Penicillin/streptomycin</td>
<td>2.101</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock B</th>
<th>Component</th>
<th>g/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10 x concentration)</td>
<td>Sodium bicarbonate</td>
<td>2.101</td>
</tr>
<tr>
<td></td>
<td>Phenol red</td>
<td>0.010</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock C</th>
<th>Component</th>
<th>g/10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>(100 x concentration)</td>
<td>Sodium pyruvate</td>
<td>0.036</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock D</th>
<th>Component</th>
<th>g/10 ml</th>
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</thead>
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<tr>
<td>(100 x concentration)</td>
<td>Calcium chloride</td>
<td>0.252</td>
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</table>

<table>
<thead>
<tr>
<th>Stock E</th>
<th>Component</th>
<th>g/10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10 x concentration)</td>
<td>Phenol Red</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>Hepes (Gibco BRL)</td>
<td>75 ml</td>
</tr>
</tbody>
</table>
Table 2.2: Preparation of M2 and M16 from concentrated stock solutions

<table>
<thead>
<tr>
<th>Stock</th>
<th>M2 (50 ml)</th>
<th>M16 (50 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (x 10)</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>B (x 10)</td>
<td>0.8 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>C (x 100)</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>D (x 100)</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>E (x 100)</td>
<td>4.2 ml</td>
<td>-</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>39.0 ml</td>
<td>39.0 ml</td>
</tr>
</tbody>
</table>

2.2.1.3 Minimal Essential Medium (Eagle’s)

The incomplete MEM medium (alpha modification) was prepared by dissolving the powdered media (Sigma) in 800 ml of sterile water, the pH was adjusted to 7.4 by adding 29.7 ml sodium bicarbonate (Gibco BRL) and the total volume was adjusted to 1 litre with water. The medium was filter sterilised in sterile glass bottles and stored at 4°C. Complete alpha MEM medium was prepared from 435 ml of incomplete medium, 50 ml fetal bovine serum (Advanced Protein Products), 10 mM Hepes (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma) and 5 x 10⁻⁵ M 2-mercaptoethanol.

2.2.1.4 Uterine Cell Conditioned Media

Pregnant diabetic and non-diabetic BB/E rats were killed on day 4.5 of gestation. Uterine cell conditioned media were prepared using the method described by Clark et al (265) and Lea et al (266). Uterine cells were harvested by scraping the walls of the
endometrium and passing the cells through a 60-mesh stainless steel screen (Sigma) to obtain a single cell suspension. The uterine cells from each uterus were incubated in 0.5 ml α-MEM for 48 hours in a humidified atmosphere of 5 % CO₂ at 37°C. After incubation the cell suspensions were centrifuged at 2772 x g and the supernatant was removed, filtered (0.22 μm sterile filter, Sigma) and pooled with other supernatants from either diabetic or non-diabetic rats (4 supernatants per pool).

2.2.2 Embryo Collection

Pregnancy was confirmed the morning after mating by the presence of a sperm positive vaginal smear and was designated day 0.5 of gestation. Pregnant rats were killed by cervical dislocation on day 4.5 of gestation and the uterine horns immediately removed. Embryos were gently flushed from the excised uteri with pre-warmed Nutrient Mixture (Ham’s) F10.

2.2.3 Morphological Analysis

Embryos from each individual pregnancy were transferred to an inverted microscope and classified as having reached a recognisable developmental stage, namely morula, early blastocyst with a nascent blastocoele and expanded blastocyst with a clearly demarcated trophectoderm (TE) and inner cell mass (ICM) (267).

The remaining structures could not be classified into one of these categories but were characterised by the following features: (1) having the form of a flattened disc rather than a sphere, (2) cell cytoplasm with a granular appearance, and (3) a reduced
Figure 2.1

Photograph of a typical example of an unfertilised oocyte from a Non-Diabetic DR-BBE/E rat
number of irregular shaped cells with much reduced cell-to-cell contact. This latter feature was demonstrated by the fact that when the zona pellucida was removed the ‘embryo’ separated into single cells. Figure 2.1 shows a typical example displaying these features. Structures with similar features were described by Hardy et al (268) and classified as ‘abnormal embryos’. However, since I identified similar structures in Non-Diabetic non-pregnant females which had not been mated with male rats, and in Diabetic and Non-Diabetic females mated with vasectomised male rats proven sterile, the conclusion was that in our experimental situation these abnormal structures were in fact unfertilised oocytes.

The number and percentage of (i) embryos at each developmental stage and (ii) unfertilised oocytes was determined and recorded separately for each individual pregnancy.

2.2.4 Differential Staining of Trophectoderm and Inner Cell Mass

The number of trophectoderm and inner cell mass cells was determined in expanded blastocysts using a dual fluorochrome differential staining technique originally described by Handyside and Hunter (269) and established in our laboratory by Dr. Richard Lea. Blastocysts were subjected to a 15 min incubation in 0.4 % pronase (Boehringer Mannheim, UK) in M2 medium containing 10 mg/ml BSA (Sigma) at 37°C, 5 % CO₂. The denuded blastocysts were washed four times in BSA-free M2 and incubated in 4 mM picrylsulfonic acid (trinitrobenzenesulfonic acid, Sigma) in BSA-free M16 with 1 % polyvinylpyrrolidone (Calbiochem, La Jolla, CA) for 30 min
on ice. After further washing in BSA-free M2, the blastocysts were incubated with 10 μg/ml rabbit anti-dinitrophenyl-bovine serum albumin complex polyclonal antibody (ICN Biomedicals) in BSA-free M2 for 30 min at 37°C, 5% CO₂. After a final washing step the blastocysts were incubated in 10% whole guinea pig serum (Sigma) as a source of complement in BSA-free M2 containing 10 μg/ml propidium iodide (Sigma) and 20 μg/ml bisbenzimide (Sigma) for 30 min 37°C, 5% CO₂. This sequential treatment results in the anti-DNP-BSA antibody binding to the trinitrophenol groups on the surface of the trophectoderm cells. In the presence of complement, the TE cells are partially lysed allowing access of propidium iodide (which is excluded from viable cells) and bisbenzimide to the TE cell nuclei. The ICM remains intact due to the tight junctional seal of the trophectoderm and is labelled with bisbenzimide alone. Under conditions of UV light excitation the different fluorescent spectrum of each dye results in the ICM staining blue and the TE staining red/pink. The differentially stained blastocysts were individually mounted on microscope slides in a small amount of medium in the centre of a silicone ring with a coverslip on top and incubated at room temperature in a dark box for 20 min. The number of ICM and TE cells were counted directly under a Leitz Orthoplan fluorescence microscope fitted with a Leitz A filter, after gently pressing the edges of the coverslip to convert the blastocyst into a two-dimensional form to facilitate accurate cell counting.

2.2.5 Blastocyst Culture

Expanded blastocysts flushed from the uterine horns of Diabetic and Non-Diabetic
BB/E rats on day 4.5 of pregnancy were cultured *in vitro* in Nutrient Mixture (Ham’s) F10 medium supplemented with 0.1 % BSA, 100 U/ml penicillin and 100 μg/ml streptomycin for 24 h at 37°C, 5 % CO₂. After culture, blastocysts were re-examined on an inverted microscope, washed with BSA-free M2 medium and differentially stained as described in section 2.2.4.

2.2.6 Blastocyst Culture in Uterine Cell Conditioned Media

Expanded blastocysts flushed from Diabetic DP-BB/E rats, Non-Diabetic DR-BB/E rats and Wistar Han rats were transferred into multi-well dishes (Nunc) containing 200 μl of either Diabetic DP- or Non-Diabetic DR-BB/E rat uterine cell conditioned media. Between 5 and 10 blastocysts were transferred to each well. The final volume in each well was increased to 400 μl with the addition of 200 μl Nutrient Mixture (Ham’s) F10. Blastocysts were cultured for 24 h at 37°C, 5 % CO₂. After incubation the number of trophectoderm and inner cell mass cells was determined using the dual fluorochrome method described in section 2.2.4.

2.2.7 Total Cell Counts in Early Pre-implantation Embryos

Using a giemsa staining procedure modified from the method of Tarkowski (270) the total cell number in morulae and early blastocysts was determined. Embryos were flushed from the uterus of Diabetic DP- and Non-Diabetic DR-BB/E rats at 90 h post coitum (assuming fertilisation to have occurred in the middle of the dark period preceeding the discovery of the vaginal copulation plug). The embryos were washed twice in 1 x PBS, followed by a 10 min wash in hypotonic (1 %) sodium citrate
The embryos were then incubated in freshly prepared acid : alcohol fixative (ethanol : acetic acid, 3:1 v:v) for 30 min at room temperature. Single embryos were placed in a drop of fixative on a clean glass microscope slide and before the fixative could evaporate, a small drop of 60 % acetic acid was added to separate the individual cells of the embryo. The slides were left to air dry before being stained for 20 sec in 4 % giemsa stain (Gurr, BDH). Finally, slides were washed in distilled water and air dried before counting the number of individual cells in each embryo under a light microscope.

2.2.8 Embryo Transfer Procedure

2.2.8.1 Recovery of 2-cell embryos

The standard procedure is to transfer 2-cell embryos to the oviduct on day 0.5 of pseudopregnancy (where day 0.5 is day of vaginal plug, indicative of mating the previous night). This procedure ensures that the embryos are at the blastocyst stage at the time the uterus is receptive for implantation, which is day 4.5 in the rat.

Donor females (at 1.5 days gestation) were killed by cervical dislocation and the uteri and ovaries quickly removed and placed into a petri dish containing pre-warmed M2 medium (containing 400 mg/ml BSA). The ovary was removed from one horn and under the dissecting microscope the excess fat was trimmed away and the oviduct separated from the ovary and uterus. The end of a flushing needle was inserted into the opening of the oviduct, the infundibulum, and the edge of the infundibulum was held with forceps while 0.5 ml of M2 medium was gently flushed through the oviduct into a 4-well multidish. Embryos were recovered from the second oviduct in the same
way and held in 500 µl of M2 medium at 37°C, 5 % CO₂ until recipient females were ready for transfer.

2.2.8.2 Anaesthesia

A Hypnorm/Hypnovel cocktail was prepared by mixing 1 part of Hypnorm (Janssen Animal Health, Buckinghamshire, UK; containing 0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone), 1 part of Hynovel (Hoffman La Roche, Nutley, NJ; containing 5 mg/ml midazolam) and 2 parts of sterile water.

Recipient pseudopregnant females (at day 0.5 of pseudopregnancy) were weighed and anaesthesia (0.33 ml per 100 g of body weight) administered by intraperitoneal injection. The rat was then placed in a 37°C heat box until fully anaesthetised.

2.2.8.3 Loading of the transfer pipette

Transfer pipettes were prepared from glass capillary pipettes, pulled to a fine tip. The transfer pipettes were first loaded with a small amount of light paraffin oil (BDH) followed by a small air bubble and a small amount of M2 medium containing 400 mg/ml BSA. Then, a second air bubble was drawn into the pipette followed by the embryos in a minimal volume of M2 containing 400 mg/ml BSA.

2.2.8.4 Transfer of 2-cell embryos

Once the recipient female was fully anaesthetised, a small area on her left hand side was shaved, swabbed with 70 % ethanol and a small transverse incision was made in
the skin. Once the fat pad had been located through the body wall, an incision through the abdominal muscle was made and the ovary fat pad was pulled out bringing the ovary, oviduct and uterus with it. Using a dissecting microscope the infundibulum was located and 5-10 µl drops of 1 % epinephrine (Sigma) were placed on some of the small capillaries of the ovarian bursa to prevent localised bleeding once the bursa was cut. With forceps and scissors, a small hole was made in the bursa and the transfer pipette containing the embryos was inserted through the hole and into the infundibulum, and the embryos were expelled into the ampulla of the oviduct. The ovary and uterus were carefully placed back into the body and the abdominal muscles were sutured. Finally, the skin incision was closed with 9 mm wound clips (Autoclips, Becton Dickinson and Co., Parsippany, NJ).

2.2.8.5 Post-operative care

The rat was placed on a cotton wool mat in a cage with free access to food and water and the cage put in a warm place. A post-operative analgesic, Nubain (DuPont Pharmaceuticals Ltd, UK; containing Nalbuphine hydrochloride, 10 mg/ml) was prepared by adding 0.2 ml Nubain to 0.8 ml sterile water and a dose of 0.2 ml given subcutaneously.

2.2.9 Reverse-Transcriptase Polymerase Chain Reaction

2.2.9.1 Tissue collection and RNA extraction

Pregnant rats were killed by cervical dislocation on day 4.5 of gestation and the uterine horns immediately removed. Pre-implantation embryos were gently flushed
from the excised uteri with pre-warmed Nutrient Mixture (Ham’s) F10. Single embryos, in 5 μl of medium, were flash frozen in liquid nitrogen and stored at -70°C. Total RNA was isolated using the method originally described by Chomczynski and Sacchi (271) and later modified for pre-implantation embryos by Sharkey et al (272), in which frozen tissue is homogenised with 100 μl buffer containing 4 M guanidinium thiocyanate (Fluka), 25 mM sodium citrate pH 7.0, 0.5 % sarcosyl and 0.1 M 2-mercaptoethanol. RNA precipitation was assisted with the addition of glycogen at a final concentration of 20 μg/ml (273).

The pH was lowered by the addition of 0.5 ml 2 M sodium acetate pH 4 and the lysate was extracted in phenol:chloroform with 20 μl buffer saturated phenol (Fisher Scientific) and 20 μl chloroform:isoamylalcohol (49:1, v:v) (Fisons Scientific). The tubes were placed on ice for 15 min and centrifuged at 10,000 x g at 4°C for 20 min. The aqueous phase was removed and mixed with 100 μl isopropanol and the RNA was precipitated at -20°C. The RNA was recovered by centrifugation, dried and resuspended in 30 μl of homogenisation buffer and then precipitated with 1 volume (30 μl) of isopropanol at -20°C for 1 hour. Finally, the RNA was centrifuged at 10,000 x g at 4°C for 20 min, the pellet washed in 75 % ethanol, dried and dissolved in 5 μl DEPC-treated water and stored at -20°C.

2.2.9.2 cDNA Synthesis

In a sterile RNase-free, microcentrifuge tube, 1 μl of 0.1 mM random hexamer primers (Pharmacia) was added to half (i.e. 2.5 μl) of the total RNA prepared from a single embryo and the volume was adjusted to 10 μl with DEPC-treated water. The
tube containing RNA and primers was heated to 70°C for 5 min, cooled to 25°C and centrifuged briefly to collect the liquid to the bottom of the tube. A reaction mix was prepared containing 4 µl avian myoblastosis virus (AMV)-reverse transcriptase buffer (containing 250 mM Tris HCl pH 8.8, 250 mM potassium chloride, 50 mM magnesium chloride, 50 mM DL-dithiothreitol and 2.5 mM spermidine), 2 µl dNTP mix (10 mM), 0.5 µl RNA guard (Pharmacia), 3 µl DEPC-treated water and 2 µl AMV-reverse transcriptase (Promega). The reaction mix was added to the RNA and primers. The reactions were incubated at 37°C for 60 min, followed by 95°C for 5 min and 25°C for 1 min. cDNA was also synthesised from 1 µg of ovary-derived RNA using the same method as described above.

Negative controls were prepared in which (1) the RNA was replaced with water, to check for contamination of primers, magnesium chloride and buffers, and (2) a culture medium control with added glycogen (and no embryos), which were extracted, reverse transcribed and amplified in exactly the same way as the embryo RT reactions.

2.2.9.3 Design of oligonucleotide primers for PCR

The sequence of the external and internal primers for bax, bcl-2 and β-actin genes are shown in Table 2.3. The primers were based on available sequence data for the genes of interest β-actin (274), bcl-2 (275), bax (276).

The primers were designed according to the following constraints namely: (i) between 20-30 nucleotides in length with a balanced G/C and A/T content; (ii) not have complementarity; (iii) bracket a sequence of 200-800 nucleotides in length; (iv) have
Table 2.3: Oligonucleotide primers for RT-PCR

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Primer Type</th>
<th>Sequence 5'-3'</th>
<th>Annealing Temperature</th>
<th>Amplicon size (bp)</th>
<th>Position on cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>bax</td>
<td>External (Forward)</td>
<td>TTGTTACAGGGATTTCTCCAGGAT</td>
<td>53°C</td>
<td>454</td>
<td>507-530</td>
</tr>
<tr>
<td></td>
<td>External (Reverse)</td>
<td>ACAAAGATGGTCACTGTCTGCCAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Internal (Forward)</td>
<td>AGACACCTGAGCTGACCCTGGAGC</td>
<td></td>
<td></td>
<td>122-145</td>
</tr>
<tr>
<td></td>
<td>Internal (Reverse)</td>
<td>AAGTAGGAGAGGAGCCTTTCCGGA</td>
<td>55°C</td>
<td>372</td>
<td>471-494</td>
</tr>
<tr>
<td>bcl-2</td>
<td>External (Forward)</td>
<td>TACGAGTGGGATACTGAGATGAA</td>
<td>53°C</td>
<td>426</td>
<td>719-742</td>
</tr>
<tr>
<td></td>
<td>External (Reverse)</td>
<td>ATGTTGTCCACCAGGGGTGACATC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Internal (Forward)</td>
<td>TGGGATCTTCTTCCTCCAGCCTGA</td>
<td></td>
<td></td>
<td>372-395</td>
</tr>
<tr>
<td></td>
<td>Internal (Reverse)</td>
<td>ACACACATGACCCGAACTCAAAC</td>
<td>55°C</td>
<td>329</td>
<td>678-701</td>
</tr>
<tr>
<td>β-actin</td>
<td>External (Forward)</td>
<td>ATGTTTGAGACCTTCAACACCCAGAG</td>
<td>58°C</td>
<td>747</td>
<td>1174-1195</td>
</tr>
<tr>
<td></td>
<td>External (Reverse)</td>
<td>GGTGCACGATGGAGGCGCGGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Internal (Forward)</td>
<td>GCCATCCAGGCTGTGTGTGCC</td>
<td></td>
<td></td>
<td>484-505</td>
</tr>
<tr>
<td></td>
<td>Internal (Reverse)</td>
<td>GGTGCACGATGGAGGCGCGGA</td>
<td>64°C</td>
<td>711</td>
<td>1174-1195</td>
</tr>
</tbody>
</table>
minimum homology with other members of the \textit{bcl-2} gene family, and (v) include an intron in the amplicon for discrimination of products from genomic DNA. Oligonucleotide primers for all genes were synthesised by Oswel (Southampton, UK). Lyophilised primers were dissolved in sterile water to give a concentration of 100 μM and 50 μl aliquots were stored at -20°C.

2.2.9.4 cDNA Amplification

One-tenth aliquots (i.e. 2 μl) of embryo cDNA were amplified in the presence of 10x PCR buffer (containing 50 mM Tris-HCl (pH 9.1), 16 mM ammonium sulphate and 150 μg/ml bovine serum albumin), 200 μM of dNTP mix (50 mM dATP, 50 mM dGTP, 50 mM dTTP, 50 mM dCTP), 1 μM of each primer and 0.5U \textit{Taq} polymerase (Helena BioSciences, Newcastle Upon Tyne). Magnesium chloride was added to give a final concentration of 1 mM, 1.7 mM and 2.5 mM for β-actin, bax and bcl-2 respectively. Reactions were overlaid with 30 μl of light mineral oil (Sigma) to prevent evaporation and placed in a Hybaid Omnigene PCR machine.

After 33, 30 or 20 cycles of PCR (for bax, bcl-2 and β-actin primers respectively) using the external primer pair, 1 μl of the first round reaction was transferred into a fresh tube containing the internal primer pair. PCR mix was added (as described above, with the exception that the magnesium chloride concentration was reduced to 1 mM). The reactions were amplified for further 33, 30 or 20 cycles for bax, bcl-2 and β-actin internal primers respectively.
The cycling conditions were 3 min at 94° C for one cycle, followed by 94° C for 1 min, 
\( n^\circ C \) for 1 min and 72° C for 2 min for 33, 30 or 20 cycles depending on the primers 
used, and the last cycle concluded with a 10 min extension time at 72° C. \( n^\circ C \) was the 
annealing temperature for each pair of primers as described below: bax and bcl-2 53 
and 55° C; and \( \beta \)-actin 58 and 64° C (external and internal primers respectively).

2.2.9.5 Verification of PCR products

2.2.9.5.1 Agarose gel electrophoresis

Agarose gels (2 %) were prepared in 1 x TAE buffer (40 mM Tris acetate and 1 mM 
EDTA). Agarose (Gibco BRL) was dissolved in TAE buffer and ethidium bromide 
was added to a final concentration of 0.5 \( \mu \)g/ml. The PCR samples were mixed with 
gel loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 30 % 
glycerol in water) and loaded into the wells. Since the expected sizes of the PCR 
products were 300-700 bp the size marker was Bluescript plasmid DNA (Stratagene) 
which had been digested with Sau3A, creating marker band sizes of 1000, 737, 341, 
258, 222, 105 and 78 bp. A voltage of 10 V/cm was applied to the gel for 1-1.5 h.

2.2.9.5.2 DNA Sequencing

Sequencing was performed using an ABI Prism™ 377 DNA Sequencer (Perkin Elmer) 
using the Taq DyeDeoxy™ Terminator cycle sequencing kit. The Taq polymerase, 
Ampli Taq® DNA polymerase, FS, was developed especially for fluorescent cycle 
sequencing with dye labelled primers and terminators. The kit also contains 
deoxyinosine triphosphate (dITP) in place of dGTP to minimise band compressions 
which often occur in GC-rich areas of sequences.
(i) Preparation of sequencing gel

A 36 cm 4% acrylamide gel (BioRad) containing 29:1 ratio of acrylamide to bisacrylamide and 8 M urea was made in 1X TBE (tris-borate-20mM EDTA buffer pH 8.3), and poured using 0.2 mm spacers and combs.

(ii) Preparation of cDNA

A reaction was set up for each set of primers consisting of 100 ng of purified PCR product, 3.2 pmole primers and 8 μl of Terminator Ready Reaction mix (containing A-, C-, G-, and T-Dye Terminators, dITP, dATP, dCTP, dTTP, Tris-hydrochloride pH 9), magnesium chloride, thermal stable pyrophosphatase and Ampli Taq® DNA Polymerase, FS). The reaction volume was adjusted to 10 μl with sterile distilled water, overlaid with 35 μl of mineral and amplified using a Hybaid Omnigene PCR machine for 25 cycles of 30 sec at 96°C, 15 sec at 50°C and 4 min at 60°C. The reactions were placed on ice following amplification and 1 μl 3 M sodium acetate (pH 4.6) and 20 μl 95% ethanol were added to precipitate the cDNA. The pelleted cDNA was resuspended in 6 μl of loading buffer (deionised formamide containing 25 mM EDTA (pH 8) and 50 mg/ml blue dextran in a ratio of 5:1 formamide to EDTA/blue dextran). The samples were denatured at 90°C for 2 min and placed on ice. A 1.5 μl aliquot of each sample was loaded onto a pre-warmed 4% acrylamide gel and run at 1.68 kV, 50 mA and 150 W for 7 h at a constant temperature of 51°C.

2.2.10 Preparation of Internal Standards

2.2.10.1 cDNA Amplification

One quarter of the ovary RT reaction was amplified for 30 cycles using the external
primer pair for each gene. Immediately after PCR, a 10 μl aliquot was electrophoresed on a 2 % agarose gel and the amount of product estimated by eye after ethidium bromide staining.

2.2.10.2 Ligation and cloning of PCR products

An aliquot of 2 μl of PCR product was added to 1 μl ligation buffer (containing 60 mM Tris-HCl pH 7.5, 60 mM magnesium chloride, 50 mM sodium chloride, 1 mg/ml bovine serum albumin, 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol and 10 mM spermidine) and 2 μl pCR2.1 vector at 25 ng/μl (Invitrogen) to give a molar ratio of 1:1. The volume was adjusted to 9 μl with sterile water and 1 μl T4 ligase (40 Weiss units) was added. The ligation reactions were incubated at 14°C overnight.

2.2.10.3 Preparation of Solutions for Transformation

(i) Ampicillin

A 100 mg/ml stock solution was prepared by dissolving 1 g of ampicillin (Sigma) in 10 ml of deionised water. The antibiotic was filter sterilised through a 0.2 μm filter unit and 500 μl aliquots were stored at -20°C.

(ii) LB (Luria-Bertani) Medium and Agar

To prepare 1 litre of LB broth, 10 g of bacto-tryptone (Difco Laboratories), 5 g bacto-yeast extract (Difco Laboratories) and 10 g sodium chloride (BDH) were dissolved in 950 ml of deionised water. The pH of the solution was adjusted to 7.0 with sodium hydroxide and the volume was increased to 1 litre with deionised water.
The solution was autoclaved for 20 min at 15 lb./square inch and allowed to cool to 55°C. Ampicillin was added to give a final concentration of 100 µg/ml.

LB agar plates were prepared as described above but 15 g/l agar (Difco Laboratories) was added before autoclaving. The agar was cooled to 55°C and ampicillin was added to a final concentration of 100 µg/ml. The agar was then poured into 10 cm plates allowed to set and the plates were stored at 4°C for < 1 month.

(iii) 5-bromo-4-chloro-3 indolyl-β-D-galactoside (X-Gal)
A 40 mg/ml stock solution was prepared by dissolving 400 mg X-Gal (Sigma) in 10 ml dimethylformamide. Aliquots of 500 µl were stored at -20°C protected from light.

(iv) Isopropyl-β-D-thiogalactopyranoside (IPTG)
A 100 mM stock solution was prepared by dissolving 238 mg IPTG in 10 ml deionised water. The solution was then filter sterilised and stored in 1 ml aliquots at -20°C.

(v) X-Gal / IPTG LB agar plates
To previously prepared LB agar plates containing 100 µg/ml ampicillin, 40 µl of the X-Gal stock solution and 40 µl of the IPTG stock solution were spread evenly over the surface of the agar. The plates were incubated at 37°C for 20-30 min to allow the X-gal and IPTG to diffuse into the agar. The plates were stored at 4°C protected from light.
(vi) **SOC Medium**

SOC medium (100 ml) was prepared by dissolving 2 g bacto-tryptone (Difco Laboratories), 0.5 g bacto-yeast extract (Difco Laboratories), 1 ml of 1 M sodium chloride and 0.25 ml potassium chloride in 97 ml of deionised water. A 1 ml aliquot of 2 M Mg\(^{2+}\) stock solution (1 M magnesium chloride, 1 M magnesium sulphate) and 1 ml of sterile 2 M glucose stock solution were added to give a final concentration of 20 mM. The complete medium was filter sterilised through a 0.2 μm filter unit and stored at room temperature.

**2.2.10.4 Transformation by heat shock**

One 50 μl vial of frozen TOP 10F' competent *Escherichia coli* bacteria (Invitrogen) per ligation reaction was thawed on ice. A 2 μl aliquot of 0.5 M β-mercaptoethanol was added to each vial of competent cells and mixed gently. Two μl of ligation reaction were added to each vial and incubated for 30 min on ice. The cells were heat shocked for exactly 30 sec in a 42°C waterbath and placed on ice for a further 2 min. To each vial, 250 μl of SOC medium were added and the vials were incubated at 37°C for 1 h before aliquots of 50 μl and 200 μl were spread on separate LB agar plates containing 100 μg/ml ampicillin, 0.1 M IPTG and 40 μg/ml X-Gal. Plates were inverted and incubated at 37°C overnight.

**2.2.10.5 Plasmid minipreparation**

A minimum number of 10 white colonies were picked from the above LB plates and used to inoculate 3 ml of LB medium (containing 100 μg/ml ampicillin) which was
then cultured overnight (12-16 h) at 37°C in a shaking incubator. After incubation 1.5 ml of each culture were lysed in alkali, and protein and denatured bacterial chromosomal DNA precipitated with potassium acetate (pH 4.8), using a modification of the methods originally published by Birnboin and Doly (277) and Ish-Horowicz and Burke (278), as described by Sambrook, Fritsch and Manniatis (279). The plasmid DNA was purified by phenol:chloroform extraction and precipitation. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1, v/v/v) was added and the tubes were vortexed for 30 sec followed by centrifugation at 12,000 x g for 5 min. The upper aqueous phase was transferred to a fresh tube and an equal volume of chloroform:isoamylalcohol (24:1, v/v) was added. The tubes were vortexed briefly and centrifuged at 12,000 x g for 2 min. The upper aqueous phase was removed and transferred into a fresh tube and 2.5 volumes of ice-cold 100 % ethanol was added to precipitate the DNA for 5 min. Finally the tubes were centrifuged at 12,000 x g for 5 min, rinsed with ice-cold 75 % ethanol and the pellet was allowed to air dry.

The plasmid DNA was resuspended in 50 µl of TE buffer containing RNase A (Sigma) to a final concentration of 2 µg/ml, and stored at -20°C.

2.2.10.6 Selection for recombinant plasmids

Plasmids prepared in section 2.2.10.5 were digested with appropriate restriction enzymes to confirm they contained the β-actin, bax and bcl-2 insert. The digested plasmid DNA was separated by agarose gel electrophoresis as described in section 2.2.9.5.1.
(i) Native pActPCR2.1 plasmids

In a 20 µl reaction, 2 µg of pActPCR2.1 were digested at 37°C for 1h with BglII (Boehringer Manheim) (10 units of enzyme per reaction). Digestion of plasmid pCR2.1 containing the β-actin insert yielded three fragments of 2241, 932 and 1474 bp or 2241, 285, 2121 bp (depending on the orientation of the insert). Vector (PCR2.1) which did not contain the insert yielded only two fragments of 2241 and 1659 bp after enzyme digestion.

(ii) Native pBcl-2PCR2.1 plasmids

In a 20 µl reaction, 2 µg of pBcl-2PCR2.1 plasmid was digested at 37°C for 1h with BglII (Boehringer Manheim) (10 units of enzyme per reaction). Digestion of plasmid pCR2.1 containing the bcl-2 insert yielded three fragments of 2241, 533 and 1544 bp or 2241, 365, 1722 bp (depending on the orientation of the insert). Vector which did not contain the insert yielded only two fragments of 2241 and 1659 bp after enzyme digestion.

(iii) Native pBaxPCR2.1 plasmids

In a 20 µl reaction, 2 µg of pBaxPCR2.1 plasmid was digested at 37°C for 1h with BamHI (Boehringer Manheim, Lewes, UK) (10 units of enzyme per reaction). Digestion of plasmid pCR2.1 containing the bax insert yielded two fragments of 3979 and 414 bp or 4275 and 118 bp (depending on the orientation of the insert). Vector which did not contain the insert yielded only one fragment of 3939 bp after enzyme digestion.
2.2.10.7 Mutation of pActPCR2.1, pBaxPCR2.1 and pBcl-2PCR2.1 Plasmids

(i) pActPCR2.1

In a 20 µl reaction, 2 µg of pActPCR2.1 were digested at 60°C for 1h with BstEII (Boehringer Manheim, Lewes, UK) (10 units of enzyme per reaction) for which a site existed in the insert but not in the pCR2.1 sequence. The 3'-recessed termini of the BstEII site were then filled by adding the Klenow fragment of E.coli DNA polymerase I (Boehringer Mannheim) (2 units per reaction) in the presence of 1 mM dNTPs. Enzyme activity was inactivated by adding an equal volume of TE buffer (pH 7.6) and extracting the DNA with phenol:chloroform:isoamylalcohol (as described in section 2.2.10.5). The DNA was dissolved in 20 µl TE buffer. A 20 µl aliquot (i.e. 0.2 µg) of the plasmid was then recircularised at 16°C overnight with T4 DNA ligase (Boehringer Manheim) (1 unit per reaction) before transformation.

(ii) pBcl-2PCR2.1

In a 20 µl reaction, 2 µg of pBcl-2PCR2.1 were digested with NruI (Boehringer Manheim) (10 units of enzyme per reaction) at 37°C for 1h, for which a site existed in the inserted amplicon but not in the pCR2.1 sequence. Enzyme activity was inactivated by adding an equal volume of TE buffer (pH 7.6) and extracting the DNA with phenol:chloroform:isoamylalcohol (as described in section 2.2.10.5). One µg of commercial XbaI linker (Boehringer Manheim) d(CTCTAGAG) was ligated to 1 µg of the linearised recombinant plasmid with 1 unit of T4 DNA ligase and ATP (final concentration 1 mM) in a reaction volume of 20 µl. The reaction was incubated at 16°C overnight.
(iii) pBaxPCR2.1

In a 20 µl reaction, 2 µg of pBaxPCR2.1 were digested at 37°C for 1 h with NheI (Boehringer Manheim) (10 units of enzyme per reaction) for which a site existed in the inserted amplicon but not in the pCR2.1 sequence. The 3’-recessed termini of the NheI site were then filled by adding the Klenow fragment of E.coli DNA polymerase I (Boehringer Mannheim) (2 units per reaction) in the presence of 1 mM dNTPs. Enzyme activity was inactivated by adding an equal volume of TE buffer (pH 7.6) and extracting the solution with phenol:chloroform:isoamylalcohol (see section 2.2.10.5). The DNA was dissolved in 20 µl TE buffer. A 20 µl aliquot (i.e. 0.2 µg) of the plasmid was then recircularised at 16°C overnight with T4 DNA ligase (Boehringer Manheim) (1 unit per reaction) before transformation.

2.2.10.8 Transformation by electroporation

The DNA in each ligation reaction was precipitated by adding potassium acetate pH 8.0 to a concentration of 0.25 M and 2.5 volumes of ethanol at -20°C for 2 h. DNA was pelleted at 12,000 x g at 4°C for 2 min and the pellets were washed with cold 70% ethanol and air dried before being dissolved in 5 µl sterile distilled water. A 50 µl aliquot of XL-1 Blue E.coli competent cells (Stratagene) were thawed on ice and a 2 µl aliquot recircularised recombinant plasmid was electroporated into the XL-1 Blue cells with a discharge of 2.5 KV at 25 microfarads on a Bio-Rad Gene Pulser. Immediately after completion of the pulse, 300 µl of SOC medium was added and each reaction was incubated at 37°C for 1 h on a shaking incubator. After incubation aliquots of 20 µl, 50 µl and 100 µl were spread onto LB agar plates containing 100 µg/ml ampicillin. The plates were inverted and incubated overnight at 37°C.
The following day, 12 transformants were selected and grown in 3 ml of LB medium containing 100 µg/ml ampicillin overnight at 37°C. Plasmid DNA was isolated as described in section 2.2.10.5.

2.2.10.9 Selection for Mutated pActPCR2.1, pBaxPCR2.1 and pBcl-2PCR2.1 Plasmids

In 20 µl reactions, 2 µg of mutated recombinant plasmid DNA were digested with appropriate restriction enzymes to distinguish between native and mutated recombinant plasmid DNA. pActMutPCR2.1 DNA was digested with BstEII and BamHI at 60° and 37°C (respectively) for 1 h at each temperature. pBcl-2MutPCR2.1 DNA was digested with XbaI at 37°C for 1 h, and pBaxMutPCR2.1 DNA was digested with NheI and HindIII at 37°C for 1 h. Digested plasmid DNA was separated by agarose gel electrophoresis as described in section 2.2.9.5.1.

2.2.10.10 Synthetic mRNA synthesis

For each gene of interest 25 µg of pActMutPCR2.1, pBcl-2MutPCR2.1 pBaxMutPCR2.1 DNA were digested with HindIII at 37°C for 2 hours. HindIII was chosen because it only cuts the plasmid once (upstream of the T7 promoter) and did not cut any of the inserts. An aliquot of the digestion was analysed by agarose gel electrophoresis to check complete digestion. The linearised template was purified by extraction with phenol:chloroform:isoamylalcohol as described in section 2.2.10.5. The DNA was dissolved in 25 µl RNase-free TE buffer pH 7.6 at a concentration of 1 µg/µl.
The DNA was further purified by proteinase K treatment to remove the RNase added during the plasmid minipreparation. 0.1 volume of 10 x proteinase K buffer (500 mM sodium chloride, 50 mM EDTA (pH 8) and 100 mM Tris chloride pH 8) and 0.1 volume of 5 % SDS was added to the recombinant plasmids. Proteinase K was added to a final concentration of 100 µg/ml. The reactions were incubated at 37°C for 1 hour. After the incubation, the DNA was purified by extraction with phenol:chloroform:isoamylalcohol (see section 2.2.10.5) and the DNA was dissolved in 25 µl RNase-free TE buffer pH 7.6 at a concentration of 1 µg/µl.

Five µg of purified pActMutPCR2.1, pBcl-2MutPCR2.1 pBaxMutPCR2.1 DNA were added to a transcription mix containing 20 µl transcription buffer (Promega), 10 µl of 100 mM DTT, 2.5 µl RNase inhibitor RNasin (1 unit/µl; Promega), 20 µl rNTPs at 2.5 mM each, 35 µl DEPC-treated sterile water and 2 µl T7 RNA polymerase (Promega). A transcription reaction was set up for each recombinant plasmid. The transcription reactions were incubated at 38°C for 2 hours and the DNA template was digested by incubation with RNase-free DNase (0.1 unit/µl; Promega) for 30 min at 37°C. The synthesised RNA was extracted with phenol:chloroform:isoamylalcohol (see section 2.2.10.5) and the RNA pellets were vacuum dried, dissolved in 30 µl DEPC-treated sterile water and accurately quantified by spectrophotometry at 260 nm. A precise dilution series of synthetic mRNA standards ranging from 1 ng/µl to 1 fg/µl in 500 µl total volumes were prepared and the dilutions stored at -70°C in aliquots of 20 µl.
2.2.11  Quantitative RT-PCR

2.2.11.1  cDNA Synthesis

In a sterile RNase-free microcentrifuge tube, 1 μl of 0.1 mM random hexamer primers (Pharmacia), 6.8 pg synthetic β-actin mRNA, 3.4 fg synthetic bax mRNA, 3.4 fg synthetic bcl-2 mRNA was added to 2.5 μl of the RNA prepared from a single embryo and the volume was adjusted to 10 μl with DEPC-treated water. The RNAs were denatured at 70°C for 5 min, cooled to 25°C and kept on ice. A reaction mix was prepared containing 4 μl AMV-reverse transcriptase buffer, 2 μl dNTP mix (10 mM), 0.5 μl RNA guard (Pharmacia), 3 μl DEPC-treated water and 2 μl AMV-reverse transcriptase (Promega) and added to the RNA and primers. cDNA was synthesised at 37°C for 60 min. The reaction stopped by denaturing at 95°C for 5 min.

2.2.11.2  cDNA Amplification

A 1 μl aliquot of newly synthesised embryo and standard cDNA was amplified in a total volume of 25 μl containing 10x PCR buffer (Helena BioSciences, Newcastle Upon Tyne), 200 μM of dNTP mix, 1 μM of each primer and 0.5U Taq polymerase (Helena BioSciences). Magnesium chloride was added to give a final concentration of 1 mM, 1.7 mM and 2.5 mM for β-actin, bax and bcl-2 reactions respectively. Reactions were overlaid with 30 μl of light mineral oil (Sigma) to prevent evaporation and placed in a Hybaid Omnigene PCR machine.
The cycling conditions were 3 min at 94°C and 70°C for 2 min for one cycle, followed by 94°C for 1 min, n°C for 1 min and 72°C for 2 min for 20 cycles and the last cycle concluded with a 10 min extension time at 72°C. Where n°C was the annealing temperature for the external pair of primers, namely 53°C for bax and bcl-2; and 58°C for β-actin primers.

A phosphorylation reaction was set up for each of the forward internal primers. Two-hundred pmoles of primer were added to a micro-centrifuge tube containing 2 μl T4 polynucleotide kinase (10 U/μl) (Boehringer Mannheim), 10 pmoles [γ32-P]dATP (Amersham) and 10x concentrated phosphorylation buffer (containing 500 mM tris-HCl, 100 mM magnesium chloride, 1 mM EDTA, 50 mM dithiothreitol, 1 mM spermidine, pH 8.2) (Boehringer Mannheim). The reaction was incubated at 37°C for 1-1.5 h.

The [γ32-P]dATP-labelled forward internal primers were then added to their respective PCR mixes (as described in section 2.2.9.4), and 9.2 μl of this mix was added to PCR tubes containing 0.8 μl of a 1:50 dilution of the first round PCR reaction, to give a total reaction volume of 10 μl. The reactions were subjected to 27, 27 or 25 cycles of amplification at 55°C, 54°C and 64°C for bax, bcl-2 and β-actin primers respectively.

2.2.11.3 Polyacrylamide Gel Electrophoresis

(i) Preparation of polyacrylamide gel

Two glass plates and spacers were washed thoroughly in warm detergent (7X-PF
phosphate-free detergent) (ICN Flow), rinsed with tap water and then distilled water. The plates were cleaned with acetone and allowed to air dry. The smaller plate was treated with silicone solution by pouring a small quantity of Repelcote (BDH) onto the plate and wiping it over the surface of the plate with a tissue. The plate was then rinsed with distilled water and dried. The plates were assembled by inserting the two 0.4 mm spacers between the plates and securing them with tape along the sides and the bottom of the plates. For 50 ml of a casting solution of 4 % acrylamide, 8 ml of SequaGel™ concentrate (containing a ratio of 19:1 acrylamide:bisacrylamide, and 7.3 M urea), 37 ml SequaGel™ diluent (containing 7.3 M urea) and 5 ml of SequaGel™ buffer (containing 50 % urea in 1 x TBE buffer) were mixed together. A 350 µl aliquot of 10 % (w:v) ammonium persulphate (Amresco, Ohio) was added followed by 15 µl of TEMED (Sigma). The solution was poured immediately into a 50 ml syringe and injected slowly between the two plates. A comb was inserted and the gel was left in a horizontal position for approximately 1 h to polymerise.

Once polymerisation of the gel was complete, the tape was taken off and the comb carefully removed. The plates were transferred to a vertical gel electrophoresis apparatus (BRL Life Technologies). The top and bottom reservoirs were filled with 1x TBE buffer and the apparatus was connected to the power supply and pre-run for 20 min at 35 W.

(ii) Preparation of cDNA

To the [γ³²-P]-labelled PCR samples, 20 µl of loading buffer (deionised formamide containing 25 mM EDTA (pH 8) and 50 mg/ml blue dextran in a ratio of 5:1
formamide to EDTA/blue dextran) was added and the samples were denatured for 2 min at 90°C and placed on ice. A 3 μl aliquot of each sample was loaded onto the pre-electrophoresed 4 % acrylamide gel using a sequencing pipette (Sigma) and run for 2 hours at a constant power of 35 W.

(iii) Autoradiography

After electrophoresis the two plates were separated leaving the gel attached to the bottom plate and a piece of Whatman number 1 filter paper was placed on top of the gel. Gentle pressure was applied to the paper so that the gel became firmly attached to it. The excess gel was cut off and the paper was peeled away from the plate bringing the gel with it. The gel was then covered with a piece of saran wrap and dried in a vacuum dryer (Bio Rad) at 80°C for 1 h. After drying the saran wrap was removed and the gel was exposed to x-ray film (Kodak Scientific Imaging film) for 16-24 hours at room temperature or at -70°C (depending on the signal intensity on the gel). The film was developed in an automatic developer.

2.2.11.4 Phosphorimage Analysis

Quantification of RT-PCR products in the polyacrylamide gels was performed by means of a PhosphorImager (Molecular Dynamics). The intensity of the cDNA bands (measured in arbitrary units) from target template (i.e. embryo) and standard template was obtained for each of the three genes. The ratio of embryo absorbance/standard absorbance was determined, and since the number of copies of standard synthetic mRNA added to the initial RT reaction was known, the number of copies of target mRNA could be calculated.
Chapter Three

Results

3.1 Experiment I: Defining the Development of the Early Embryo in the Diabetic BB/E rat

Introduction

Recent studies of the effect of diabetes on early pre-implantation embryo development have involved NOD mice (280, 281) and STZ-diabetic rats and mice (282, 283). Neither of these animal models are analogous to diabetic patients with IDDM in that they are not dependent on insulin treatment to survive and indeed were not treated with insulin in these reported experiments. The following experiments were carried out to define the development of early pre-implantation embryos in BB/E rats with IDDM. In particular, to identify any disturbances in the development of the pre-implantation embryo which might be a presage of the pathology seen later in pregnancy in this animal, such as low birth weight pups and malformations (260). This study also provided the opportunity to observe early pregnancy in the Diabetes Resistant (DR) BB/E subline, and compare it with that of outbred Wistar Han rats.

3.1.1 Morphological Studies

Experimental Protocols

(i) Diabetic Pregnancies

All female DP-BB/E rats used for this study had established IDDM and were maintained on daily injections of long-acting insulin. One group of female DP-BB/E
rats ($n = 79$) were mated overnight with established insulin dependent Diabetic DP-BB/E male rats, and a second group of female Diabetic DP-BB/E rats ($n = 38$) were mated overnight with Non-Diabetic DR-BB/E males.

(ii) Non-Diabetic Pregnancies

Non-Diabetic DR-BB/E female rats ($n = 73$) were mated overnight with Non-Diabetic DR-BB/E male rats. A second group of Non-Diabetic DR-BB/E females ($n = 17$) were mated with Diabetic DP-BB/E male rats with established IDDM.

(iii) Wistar Han Pregnancies

Female outbred Wistar Han rats ($n = 40$) were mated overnight with outbred Wistar Han males.

All male rats used for these studies were of proven fertility. Embryos were collected from each individual pregnancy (see Methods section 2.2.1), transferred to an inverted microscope and classified as having reached a recognisable developmental stage, namely morula, early blastocyst with a nascent blastocele and expanded blastocyst with a clearly demarcated trophectoderm (TE) and inner cell mass (ICM). The number and percentage of (i) embryos at each developmental stage and (ii) unfertilised oocytes was determined and recorded for each pregnancy.

Results

Table 3.1 shows the number of morulae, early blastocysts, expanded blastocysts and unfertilised oocytes recovered from each type of pregnancy at 4.5 days gestation.
Table 3.1: Number of embryos recovered from Diabetic DP-BB/E, Non-Diabetic DR-BB/E and Wistar Han rats on day 4.5 of pregnancy

<table>
<thead>
<tr>
<th>Pregnancy Type: Mother x Father</th>
<th>Pregnancies</th>
<th>Total Embryos</th>
<th>Unfertilised Oocytes</th>
<th>Morulae</th>
<th>Early Blastocysts</th>
<th>Expanded Blastocysts</th>
<th>Embryos per pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic (DP) x Diabetic (DP)</td>
<td>79</td>
<td>592</td>
<td>119</td>
<td>62</td>
<td>87</td>
<td>324</td>
<td>6.0 ± 0.4 *</td>
</tr>
<tr>
<td>Diabetic (DP) x Non-Diabetic (DR)</td>
<td>38</td>
<td>283</td>
<td>39</td>
<td>31</td>
<td>33</td>
<td>180</td>
<td>6.4 ± 0.6</td>
</tr>
<tr>
<td>Non-Diabetic (DR) x Non-Diabetic (DR)</td>
<td>73</td>
<td>527</td>
<td>21</td>
<td>46</td>
<td>86</td>
<td>374</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>Non-Diabetic (DR) x Diabetic (DP)</td>
<td>17</td>
<td>130</td>
<td>7</td>
<td>17</td>
<td>19</td>
<td>87</td>
<td>7.2 ± 0.5</td>
</tr>
<tr>
<td>Wistar Han x Wistar Han</td>
<td>40</td>
<td>400</td>
<td>27</td>
<td>31</td>
<td>33</td>
<td>309</td>
<td>9.3 ± 0.4 **</td>
</tr>
</tbody>
</table>

Data are n or means ± SEM. * P < 0.05 : significantly less than Non-Diabetic DR x Non-Diabetic DR pregnancies, and Non-Diabetic DR x Diabetic DP pregnancies, and ** P < 0.0001 : significantly greater than all BB/E pregnancies (Student's t test)
All of the BB/E rats had significantly fewer embryos per pregnancy than Wistar Han rats ($P < 0.0001$), which may be explained as strain differences between outbred Wistar Han and inbred BB/E rats. Diabetic DP- rats had fewer embryos per pregnancy than Non-Diabetic DR-BB/E rats but this difference only reached statistical significance for the Diabetic DP mother x Diabetic DP father group, when compared to both types of Non-Diabetic DR-BB/E pregnancies ($P < 0.05$). Significance was probably lost in the Diabetic DP mother x Non-Diabetic DR father group because (i) it was a much smaller group compared with the other Diabetic pregnancy group (38 vs 79 pregnancies), and (ii) there was greater variation within the smaller group. When the number of unfertilised oocytes was added to the total embryo number in each pregnancy, the mean number all structures per pregnancy was not significantly different for any of the BB/E pregnancies, indicating that all BB/E groups had approximately the same ovulation rate.

Diabetic DP-BB/E pregnancies had fewer expanded blastocysts and more unfertilised oocytes per pregnancy than Non-Diabetic DR-BB/E pregnancies. This effect appeared to be a function of maternal diabetes with no input from the father, since (i) pregnancies in which both parents had established diabetes had the same low number of expanded blastocysts and high number of unfertilised oocytes as diabetic pregnancies where only the mother had diabetes - showing no evidence of a synergistic effect when both parents had established diabetes, and (ii) all non-diabetic pregnancies had the same morphological distribution of embryos irrespective of the metabolic status of the father.
Figure 3.1:
Mean ± SEM percentage of embryos at each morphological stage of development recovered from 40 Wistar Han (light bars), 73 Non-Diabetic DR-BB/E (grey bars), and 79 Diabetic DP-BB/E (black bars) rats on day 4.5 of pregnancy.

Significant differences in the percentage of embryos at each developmental stage is indicated by * (as assessed by the Student’s t-test)

Morulae: not significantly different

Early Blastocysts: P < 0.01 for Diabetic DP vs Wistar Han and P < 0.001 for Non-Diabetic DR vs Wistar Han

Expanded Blastocysts: P < 0.0001 for both Diabetic DP- vs Non-Diabetic DR-BB/E and Wistar Han rats

Unfertilised Oocytes: P < 0.0001 for both Diabetic DP- vs Non-Diabetic DR-BB/E and Wistar Han rats

NB. Statistical differences between the percentage of expanded blastocysts per Diabetic DP and Non-Diabetic DR-BB/E pregnancies remain significant when calculations exclude the number of unfertilised oocytes.
Figure 3.1 depicts the data from Table 3.1 expressed as the mean percentage of embryos in each developmental category for the Diabetic and Non-Diabetic groups. Having already established that the paternal metabolic status has no effect on the morphological distribution of embryos at the pre-implantation stage, only pregnancies in which both parents have the same metabolic status (i.e. Diabetic mother, Diabetic father pregnancies; and Non-Diabetic DR mother, Non-Diabetic DR father pregnancies) have been compared with Wistar Han pregnancies for simplicity. The percentage of morulae per pregnancy did not differ significantly between the three groups of animals. The percentage of early blastocysts was significantly greater in BB/E rat pregnancies (both Diabetic DP and Non-Diabetic DR) than Wistar Han pregnancies at 4.5 days gestation (P < 0.01 for Diabetic DP-BB/E vs Wistar Han, and P < 0.001 for Non-Diabetic DR-BB/E vs Wistar Han). Diabetic DP- and Non-Diabetic DR-BB/E pregnancies did not differ significantly in this regard. The percentage of embryos reaching expanded blastocyst stage did not differ significantly in Wistar Han and Non-Diabetic DR-BB/E pregnancies. However, significantly fewer embryos reached the expanded blastocyst stage in Diabetic DP-BB/E than Non-Diabetic DR-BB/E and Wistar Han pregnancies (P < 0.0001 for both differences) and the percentage of unfertilised oocytes was significantly higher in Diabetic than both groups of Non-Diabetic pregnancies (P < 0.0001 for both differences).

Figure 3.2A shows that in the 73 Non-Diabetic DR- BB/E pregnancies the number of expanded blastocysts per pregnancy is normally distributed at day 4.5 of gestation and there are only 3 pregnancies without expanded blastocysts. In contrast, the distribution of expanded blastocysts recovered from Diabetic DP-BB/E rats is
Figure 3.2A

Distribution of the number of expanded blastocysts in each pregnancy. Non-Diabetic DR-BB/E rat pregnancies (n = 73) are indicated by hatched bars (Fig. 3.2A); Diabetic DP-BB/E rat pregnancies (n = 79) are indicated by solid bars (Fig. 3.2B).
Figure 3.3A

The relationship between the number of expanded blastocysts and unfertilised oocytes in individual Non-Diabetic DR- and Diabetic DP-BB/E rat pregnancies. Each dot (●) represents a single pregnancy with one or more unfertilised oocyte. The right-hand axis indicates the number of unfertilised oocytes in each individual pregnancy. The bars represent the same data shown in Figure 3.2A and B.
markedly disturbed (Figure 3.2B) with 16 out of 79 pregnancies having no expanded blastocysts at all. Moreover, two thirds (67 %) of Non-Diabetic DR-BB/E pregnancies had 5 or more expanded blastocysts per pregnancy compared with just under half (46 %) of the Diabetic DP pregnancies.

Further analysis (Figure 3.3A) shows that although unfertilised oocytes occurred in Non-Diabetic DR-BB/E pregnancies they were few in number and randomly distributed. Diabetic DP pregnancies were very different, firstly, there were more pregnancies with unfertilised oocytes; secondly, they were not randomly distributed but were clustered in pregnancies with few or no expanded blastocysts; and thirdly, there were some apparently normal pregnancies with no unfertilised oocytes (Figure 3.3B). There was no significant difference between diabetic mothers with and without unfertilised oocytes with respect to age at pregnancy (130.3 ± 4.5 days vs 132 ± 2.1 days, respectively), duration of diabetes (41 ± 4.6 days vs 43 ± 2.3 days, respectively) or blood glucose concentration measured at 10.00 hours on the day that the embryos were retrieved - using an ExacTech meter, the majority being > 20 mmol/L.

3.1.2 Expanded Blastocyst Cell Lineage Analysis

Blastocysts from these apparently ‘normal’ Diabetic DP-BB/E pregnancies were analysed more closely using the dual fluorochrome staining method described above which allows the number of inner cell mass (ICM) and trophectoderm (TE) cells to be counted accurately.
Figure 3.4:

A: Micrograph of a three-dimensional BB/E rat blastocyst differentially stained by immunofluorescence to show the Inner Cell Mass cells (blue) and the Trophectoderm cells (pink)

B: The same blastocyst after slight pressure has been applied to facilitate cell counting
Results

Figure 3.4A is a photograph of a healthy expanded blastocyst stained (as described in Methods section 2.2.3) to show the clearly polarised inner cell mass (blue) and surrounding trophectoderm (red). Figure 3.4B shows the same blastocyst after slight pressure has been applied to facilitate cell counting.

Table 3.2 shows ICM and TE cell numbers in expanded blastocysts with apparently normal morphology isolated from Diabetic DP- and Non-Diabetic DR-BB/E rats on day 4.5 of pregnancy. The number of cells in the ICM was significantly less in blastocysts from Diabetic DP- compared with Non-Diabetic DR-BB/E rats (P < 0.0001); this represents a -20 % ICM cellular deficit. In contrast, there was no difference in the number of TE cells in blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats.

Table 3.2:  ICM and TE cell number in blastocysts from Diabetic DP and Non-Diabetic DR-BB/E rats on day 4.5 of pregnancy

<table>
<thead>
<tr>
<th>Type of pregnancy</th>
<th>Number of Blastocysts</th>
<th>Cell lineage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ICM</td>
</tr>
<tr>
<td>Diabetic</td>
<td>57</td>
<td>8.35 ± 0.34 *</td>
</tr>
<tr>
<td>Non-Diabetic</td>
<td>43</td>
<td>10.40 ± 0.25</td>
</tr>
<tr>
<td>% increase / decrease</td>
<td>-19.7</td>
<td>-0.7</td>
</tr>
</tbody>
</table>

* P < 0.0001: significantly less than blastocysts from Non-Diabetic DR-BB/E rats (Student’s t test). Data expressed as mean ± SEM.
Figure 3.5:

A: Differentially stained BB/E rat blastocyst showing dead cells and fragmented nuclei, the appearances being characteristic of apoptosis in the ICM

B: Dead cells located at the edge of the ICM

C: Dead ICM cells on the outer edge of the blastocyst TE
Closer examination of stained blastocysts revealed that cells with fragmented nuclei were seen in blastocysts from both Diabetic DP and Non-Diabetic DR rats. Although this technique did not allow accurate quantitation, it appeared that cells with fragmented nuclei were observed more often in the ICM than in the TE (Figure 3.5A). At high magnification (x 500) these cells showed the features characteristic of apoptosis such as cell surface blebbing, nuclear condensation and fragmentation, and finally splitting of the cell itself into multiple membrane bound ‘apoptotic bodies’, some of which contained nuclear fragments (285). Dead cells were often seen at the edge of the ICM (Figure 3.5B) and in some cases appeared to be in process of extrusion (Figure 3.5C).

3.1.2.1 Effect of Paternal Metabolic Status on Blastocyst Cell Number

Since there is evidence that paternal genes can influence the development of early embryos and blastocysts (286) Diabetic DP-BB/E rat pregnancies were generated by mating Diabetic (DP-BB/E) females with Non-Diabetic (DR-BB/E) males. Similarly, Non-Diabetic pregnancies were generated by mating Non-Diabetic (DR-BB/E) females with Diabetic (DP-BB/E) males. Embryos were recovered on day 4.5 of pregnancy as described above, the expanded blastocysts were differentially stained and the number of TE and ICM cells in each blastocyst counted.

As shown in Table 3.3, blastocysts from Diabetic DP pregnancies where the father is Non-Diabetic (DR-BB/E) exhibited the same -20 % deficit in the number ICM cells as
that previously observed in blastocysts from Diabetic DP pregnancies where both parents were diabetic. The number of TE cells remained unaffected.

Table 3.3: Effect of paternal metabolic status on ICM and TE cell number in blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats on day 4.5 of pregnancy

<table>
<thead>
<tr>
<th>Type of Pregnancy</th>
<th>Number of Blastocysts</th>
<th>Inner Cell Mass</th>
<th>Trophectoderm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic (DP) mother x Non-Diabetic (DR) father</td>
<td>57</td>
<td>8.00 ± 0.30 *</td>
<td>19.61 ± 0.38</td>
</tr>
<tr>
<td>Non-Diabetic (DR) mother x Diabetic (DP) father</td>
<td>21</td>
<td>10.48 ± 0.43</td>
<td>18.62 ± 0.55</td>
</tr>
</tbody>
</table>

% increase/decrease

-23.6 +5

* P < 0.0001: significantly less than blastocysts from Non-Diabetic DR-BB/E rats (Student’s t test) Data expressed as mean ± SEM.

3.1.2.2 Effect of 24h Culture on Blastocyst Cell Number

To investigate whether these blastocysts with an ICM cellular deficit were capable of restorative ‘catch-up’ growth when removed from the maternal diabetic environment, blastocysts were flushed from Diabetic DP- and Non-Diabetic DR-BB/E pregnancies (generated from Diabetic DP- and Non-Diabetic DR-BB/E males, respectively) and cultured in vitro for 24 hours in Nutrient Mix (Ham’s) F10 growth medium supplemented with 0.1 % BSA.
Table 3.4 shows that although proliferation of both the ICM and TE cells occurred in blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats during the culture period, this was insufficient to restore the ICM cell number to that observed in blastocysts from Non-Diabetic DR rats. Thus, an ICM cellular deficit of -18 % (P < 0.001) remained which was approximately the same as that found in vivo at day 4.5 of gestation. In contrast, in vitro culture resulted in a 25 % increase in the number of TE cells in blastocysts from Diabetic DP rats compared to those from Non-Diabetic DR-BB/E rats (P < 0.0001).

Table 3.4: ICM and TE cell number in blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats after 24 h in vitro culture.

<table>
<thead>
<tr>
<th>Type of Pregnancy</th>
<th>Number of Blastocysts</th>
<th>Cell Lineage</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inner Cell Mass</td>
<td>Trophoderm</td>
<td></td>
</tr>
<tr>
<td>Diabetic (DP)mother x Non-Diabetic (DP) father</td>
<td>33</td>
<td>10.61 ± 0.54*</td>
<td>37.88 ± 1.47 #</td>
</tr>
<tr>
<td>Non-Diabetic (DR) mother x Diabetic (DR) father</td>
<td>40</td>
<td>12.93 ± 0.50</td>
<td>30.18 ± 0.94</td>
</tr>
</tbody>
</table>

% increase/decrease |
-17.9 | +25.5 |

Data expressed as mean cell number ± SEM

* P < 0.001 : significantly less than blastocysts from Non-Diabetic DR rats

# P < 0.0001 : significantly more than blastocysts from Non-Diabetic DR rats

(Student’s t test)
In addition, a group of pregnancies were generated from (a) Diabetic DP- females and Non-Diabetic DR-BB/E males, and (b) Non-Diabetic DR- females and Diabetic DP-BB/E males, to determine whether the TE cellular accretion observed after 24h in vitro culture could be related to paternal or maternal metabolic status. The results, shown in Table 3.5 demonstrate that the TE cell accretion is entirely due to the presence of established diabetes in the mother, and that blastocysts from pregnancies where the mother is Non-Diabetic (and the father is Diabetic), develop normally.

Table 3.5: Effect of paternal metabolic status on ICM and TE cell number in blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats after 24 h in vitro culture.

<table>
<thead>
<tr>
<th>Type of Pregnancy</th>
<th>Number of Blastocysts</th>
<th>Inner Cell Mass</th>
<th>Trophoderm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic (DP) mother x Non-Diabetic (DR) father</td>
<td>33</td>
<td>10.52 ± 0.55*</td>
<td>37.67 ± 1.18#</td>
</tr>
<tr>
<td>Non-Diabetic (DR) mother x Diabetic (DP) father</td>
<td>32</td>
<td>12.28 ± 0.34</td>
<td>31.38 ± 1.01</td>
</tr>
</tbody>
</table>

% increase/decrease

[| Data expressed as mean cell number ± SEM |

* P < 0.01 : significantly less than blastocysts from Non-Diabetic DR rats

# P < 0.0001 : significantly more than blastocysts from Non-Diabetic DR rats (Student’s t test)
Blastocysts from Diabetic DP pregnancies (with Non-Diabetic DR fathers) showed an ICM cellular deficit of -14% which was not significantly different from the deficit of -18% observed in the previous group of Diabetic DP pregnancies which had Diabetic DP fathers. The ICM cell deficit was however, significantly different from blastocysts from Non-Diabetic DR pregnancies (P < 0.01). In addition, there was a 20% increase in TE cell number in blastocysts from Diabetic DP pregnancies (with Non-Diabetic DR fathers) compared to those from Non-Diabetic DR pregnancies (P < 0.0001).

3.1.2.3 Effect of 24h Culture in Conditioned Media

In an attempt to mimic the intra-uterine milieu more closely, uterine cell conditioned media were prepared by scraping uterine cells from the lining of the uterus of pregnant Diabetic DP- and Non-Diabetic DR-BB/E rats on day 4.5 of gestation, and allowing the uterine cells to condition the growth medium for 48 hours. During the incubation period the uterine cells release cytokines and growth factors into the medium and supernatants from these cultures were used as an incubation medium for 24h culture of blastocysts (see Methods section 2.2.6).

In this study, blastocysts were removed from Diabetic DP- and Non-Diabetic DR-BB/E pregnancies (both generated from Non-Diabetic DR-BB/E male rats) on day 4.5 of gestation. The number of inner cell mass and trophectoderm cells was determined in the blastocysts and the results are shown in Tables 3.6 and 3.7 respectively.
Table 3.6: Number of ICM cells in blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats after 24h culture in uterine cell conditioned media.

<table>
<thead>
<tr>
<th>Pregnancies used to prepare conditioned media:</th>
<th>ICM cell number in blastocysts from:</th>
<th>Non-Diabetic Pregnancies</th>
<th>Diabetic Pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Diabetic (DR)</td>
<td>16.85 ± 0.14</td>
<td></td>
<td>14.06 ± 0.50 *</td>
</tr>
<tr>
<td></td>
<td>(n = 33)</td>
<td></td>
<td>(n = 33)</td>
</tr>
<tr>
<td>Diabetic (DP)</td>
<td>17.06 ± 0.50</td>
<td></td>
<td>13.43 ± 0.52 *</td>
</tr>
<tr>
<td></td>
<td>(n = 32)</td>
<td></td>
<td>(n = 37)</td>
</tr>
<tr>
<td>Alpha MEM alone</td>
<td>16.07 ± 0.41</td>
<td></td>
<td>14.33 ± 0.39 #</td>
</tr>
<tr>
<td></td>
<td>(n = 39)</td>
<td></td>
<td>(n = 36)</td>
</tr>
</tbody>
</table>

Significantly less than blastocysts from Non-Diabetic DR-BB/E rats (by Student’s t test) where * P < 0.0001 # P < 0.01 and n is the number of stained blastocysts

When blastocysts from Diabetic DP mothers were removed from the diabetic intra-uterine environment and cultured in conditioned media prepared from Non-Diabetic DR rats, they did not show any sign of ICM ‘catch up’ growth and they still developed a TE cell accretion. Moreover, when blastocysts from Non-Diabetic DR rats were cultured in conditioned media prepared from Diabetic DP rats, they did not develop an ICM cellular deficit or a TE cell accretion.
Table 3.7: Number of TE cells in blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats after 24h culture in uterine cell conditioned media

<table>
<thead>
<tr>
<th>Pregnancies used to prepare conditioned media:</th>
<th>TE cell number in blastocysts from:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-Diabetic Pregnancies</td>
<td>Diabetic Pregnancies</td>
</tr>
<tr>
<td>Non-Diabetic (DR)</td>
<td>43.55 ± 1.32 (n = 33)</td>
<td>47.24 ± 1.23 # (n = 33)</td>
</tr>
<tr>
<td>Diabetic (DP)</td>
<td>35.72 ± 1.33 (n = 32)</td>
<td>43.78 ± 1.30 * (n = 37)</td>
</tr>
<tr>
<td>Alpha MEM alone</td>
<td>37.90 ± 1.26 (n = 39)</td>
<td>42.81 ± 1.46 # (n = 36)</td>
</tr>
</tbody>
</table>

Significantly more than blastocysts from Non-Diabetic DR-BB/E rats (by Student’s t test) where * P < 0.0001, and # P < 0.05 and n is the number of stained blastocysts.

Comparing blastocysts obtained from Diabetic DP rats with those from Non-Diabetic DR rats the data suggest that regardless of the culture medium used, blastocysts from Diabetic DP rats invariably exhibited an ICM cellular deficit and a TE cellular accretion. Furthermore, these differences were greatest when conditioned media prepared from Diabetic DP rats were used as the incubation medium: the ICM cell deficit after culture in Non-Diabetic DR and Diabetic DP conditioned media was -16.55 % and -21.28 % respectively; and the TE cell accretion in Non-Diabetic DR media and Diabetic DP conditioned media was +8.47 % and +22.56 % respectively. Thus blastocysts from Diabetic DP mothers were unable to restore their ICM cell...
number to normal even when conditioned medium prepared from Non-Diabetic DR uterine cells was used as the culture medium.

3.1.2.4 Total Cell Counts in Early Embryos

Morulae and early blastocysts were flushed from Diabetic DP and Non-Diabetic DR pregnancies at approximately 90 h post coitum (p.c.). The total cell number was determined using a giemsa staining method (see 2.2.7 Methods section). The results presented in Table 3.8 show that the mean total cell number per morula or early blastocyst was not significantly different for Diabetic DP- and Non-Diabetic DR-BB/E rat pregnancies.

These results suggest that the ICM cellular deficit develops between the early blastocyst and the expanded blastocyst stage, - a period of approximately 6-8 hours.

Table 3.8: Total cell number in morulae and early blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats at 90 h p.c

<table>
<thead>
<tr>
<th>Pregnancy Type</th>
<th>Mean total cell number per embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(i) Morulae</td>
</tr>
<tr>
<td>Diabetic DP (DP mother x DR father)</td>
<td>14.1 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>(n = 27)</td>
</tr>
<tr>
<td>Non-Diabetic DR (DR mother x DR father)</td>
<td>14.0 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>(n = 30)</td>
</tr>
</tbody>
</table>

Where n is the number of stained embryos
Conclusions from Experiment I

These results demonstrate that pregnancy in the DP-BB/E rat with established IDDM is associated with a severe disturbance in embryo development, which can be detected as early as the pre-implantation stage. This was indicated by (i) a five fold increase in the number of unfertilised oocytes, (ii) a reduced percentage of expanded blastocysts, and (iii) a -20 % ICM cellular deficit in embryos which reached the expanded blastocyst stage.

Although there is evidence that paternal genes exert an influence on the development of the pre-implantation embryo and the formation of the blastocyst (285), the occurrence of unfertilised oocytes could not be correlated with any particular male rat(s). Moreover, the ICM cellular deficit appeared to be solely a function of maternal diabetes with no input from the father, since Non-Diabetic DR pregnancies with Diabetic DP fathers were normal in respect to the number of unfertilised oocytes and expanded blastocysts, and ICM cell number.

The mean total cell number per morula or early blastocyst was not significantly different for Diabetic DP- and Non-Diabetic DR-BB/E rat pregnancies at 90 h p.c. These results suggest that the ICM cellular deficit develops between the early blastocyst and the expanded blastocyst stage, - a period of approximately 6-8 hours.

Blastocysts from Diabetic DP-BB/E mothers failed to show any sign of ‘catch up’ growth in the ICM after 24 h in culture media or conditioned media prepared from uterine cells. Under these conditions however, a TE cellular accretion occurred.
Since it is the inner cell mass which forms all of the fetal germ layers, whilst the
trophectoderm forms the placenta and the extraembryonic membranes (286), it is
tempting to speculate that these phenomena are a presage of the characteristic
combination of fetal growth delay and large placentae which are a feature of both
BB/E rat (258) and human IDDM pregnancy (287).

When differentially stained blastocysts were examined under the fluorescence
microscope, cells with fragmented nuclei characteristic of apoptosis were observed in
blastocysts from both Diabetic DP- and Non-Diabetic DR-BB/E rats. It appeared that
apoptotic cells were observed more often in the ICM than in the TE although it was
not possible to accurately quantitate this phenomenon using differential staining.
Developmentally regulated cell death is widespread in embryogenesis (288) and
occurs by apoptosis in both vertebrate and invertebrate species. It is a widespread
feature in blastocysts of many mammals such as the cow (289); Rhesus monkey (290);
mouse (291); rat (292) and the human (268). The significance of these dead cells is
not fully understood and there is clearly a need to examine how apoptosis is regulated
in the blastocyst with respect to the balance of endogenous gene products and
exogenous signals.
3.2 Experiment II: The Relative Influence of Maternal Genetic Background and Metabolic Status on the Development of the Pre-Implantation Embryo

Introduction

Earlier experiments (Section 3.1) have shown that pregnancy in the Diabetes Prone BB/E rat with established IDDM is associated with severe disturbance in the development of the pre-implantation embryo. The complexity of the intra-uterine environment makes it difficult to identify the mechanism(s) leading to this embryopathy. The aim of this study therefore, was to assess the relative contribution of maternal genetic background and metabolic status on pre-implantation embryo development. This was achieved by (1) studying pregnancy in the Diabetes Prone BB/E rat during the pre-diabetic period, and (2) embryo transfer experiments.

3.2.1 Effect of Pre-Diabetes in the Mother

Introduction

As described earlier (Methods section 2.1.2.1), the BB/Edinburgh colony consists of two selectively bred sublines: the Diabetes Prone and the Diabetes Resistant subline. The incidence of diabetes is 50-60 % in the DP and zero in the DR subline. Since the mean (± SD) age of onset of IDDM in the BB/E rat colony is 96 ± 18 days, and the female rat is fertile at approximately 60 days of age, it is possible to achieve pregnancy in the pre-diabetic period in these rats.
Experimental Protocol

A small group (n = 12) of young female Diabetes Prone BB/E rats were studied during the pre-diabetic period before pregnancy and during the first four days of pregnancy.

Plasma samples were taken from all rats at three specific time points: (1) at the beginning of the study (non-pregnant), (2) the morning after conception - day 0.5 of pregnancy, and (3) the morning of day 4.5 of pregnancy. In this study males used for mating were Non-Diabetic DR-BB/E rats. The concentration of plasma metabolites was measured and compared with samples from pregnant non-diabetic DR-BB/E (n = 6) and established Diabetic DP-BB/E rat pregnancies (n = 10).

On day 4.5 of pregnancy, the blastocysts were flushed from all three groups of pregnancies and taken through a differential staining technique to determine the number of ICM and TE cells.

Results

Table 3.9 shows the concentration of plasma metabolites for the three groups of rats: pre-diabetic DP-, Non-Diabetic DR- and established Diabetic DP-BB/E at the beginning of the study prior to pregnancy. Pre-diabetic DP rats were not significantly different from Non-Diabetic DR rats for any of the metabolic parameters measured, showing that when the study began these rats did not have diabetes. Established Diabetic DP rats were however, significantly different from both Non-Diabetic DR and pre-diabetic DP rats for glucose (P < 0.0001 for both), pyruvate (P < 0.01 and
P < 0.0001, respectively), glycerol (P < 0.05 and P < 0.01, respectively) and β-hydroxybutyrate (BOHB) (P < 0.05 for both).

Table 3.9: Concentration of plasma metabolites in non-pregnant pre-diabetic DP-, Non-Diabetic DR- and Diabetic DP-BB/E rats

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pre-Diabetic DP-BB/E</th>
<th>Non-Diabetic DR-BB/E</th>
<th>Diabetic DP-BB/E</th>
<th>P-value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>7.20 ± 0.13</td>
<td>7.01 ± 0.3</td>
<td>32.18 ± 1.98</td>
<td>0.0001</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.18 ± 0.007</td>
<td>0.18 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>0.0001</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.84 ± 0.18</td>
<td>3.93 ± 1.3</td>
<td>3.49 ± 0.64</td>
<td>NS</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.50 ± 0.01</td>
<td>0.45 ± 0.02</td>
<td>0.47 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.22 ± 0.02</td>
<td>0.20 ± 0.05</td>
<td>0.40 ± 0.46</td>
<td>0.001</td>
</tr>
<tr>
<td>β-hydroxybutyrate</td>
<td>0.11 ± 0.01</td>
<td>0.09 ± 0.008</td>
<td>0.81 ± 0.24</td>
<td>0.007</td>
</tr>
<tr>
<td>Lactate/Pyruvate ratio</td>
<td>15.18 ± 0.8</td>
<td>20.62 ± 5.4</td>
<td>58.24 ± 7.61</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Where NS denotes not significantly different by one-way analysis of variance (ANOVA) and n is the number rats in each group

All three groups of rats were mated with Non-Diabetic DR male rats of proven fertility. The concentration of plasma metabolites on day 0.5 of pregnancy (i.e. at conception), is shown in Table 3.10 for Non-Diabetic DR, Diabetic DP and pre-diabetic DP rats. Once again, pre-diabetic DP rats were not significantly different from Non-Diabetic DR rats for any of the metabolites measured. Diabetic DP rats
however, had significantly higher plasma glucose (P < 0.001), glycerol (P < 0.001) and BOHB (P < 0.01) measurements, but significantly lower levels of plasma pyruvate (P < 0.001) than both Non-Diabetic DR- and pre-diabetic DP-BB/E rats.

Table 3.10: Concentration of plasma metabolites in pre-diabetic DP-, Non-Diabetic DR- and Diabetic DP-BB/E rats on day 0.5 of pregnancy

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pre-Diabetic DP-BB/E</th>
<th>Non-Diabetic DR-BB/E</th>
<th>Diabetic DP-BB/E</th>
<th>P-value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>6.77 ± 0.13</td>
<td>6.47 ± 0.4</td>
<td>19.63 ± 2.44</td>
<td>0.0001</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.19 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.0001</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.84 ± 0.19</td>
<td>3.63 ± 0.72</td>
<td>3.40 ± 0.43</td>
<td>NS</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.45 ± 0.03</td>
<td>0.43 ± 0.03</td>
<td>0.50 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.17 ± 0.008</td>
<td>0.18 ± 0.03</td>
<td>0.33 ± 0.02</td>
<td>0.0001</td>
</tr>
<tr>
<td>β-hydroxybutyrate</td>
<td>0.08 ± 0.01</td>
<td>0.04 ± 0.009</td>
<td>0.32 ± 0.06</td>
<td>0.0001</td>
</tr>
<tr>
<td>Lactate/Pyruvate ratio</td>
<td>15.50 ± 0.8</td>
<td>19.45 ± 2.8</td>
<td>110.0 ± 57.1</td>
<td>NS</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>6</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Where NS denotes not significantly different by one-way analysis of variance (ANOVA) and n is the number rats in each group.

Pregnancies were continued until day 4.5 when the final plasma sample was taken. The concentration of plasma metabolites at day 4.5 of pregnancy for the three groups of rats is shown in Table 3.11. The concentration of plasma glucose remained
significantly higher in Diabetic DP rats than in either pre-diabetic DP or Non-Diabetic DR rats (P < 0.0001 for both). The concentration of plasma pyruvate and alanine fell significantly in Non-Diabetic DR rats at day 4.5 of gestation when compared to pre-diabetic and diabetic DP rats (P < 0.0001 for both). There was no significant difference between the three groups of rats for lactate, glycerol or β-hydroxybutyrate at this stage of pregnancy.

Table 3.11: Concentration of plasma metabolites in pre-diabetic DP-, Non-Diabetic DR- and Diabetic DP-BB/E rats on day 4.5 of pregnancy

<table>
<thead>
<tr>
<th>Metabolite (mmol/L)</th>
<th>Pre-Diabetic DP-BB/E</th>
<th>Non-Diabetic DR-BB/E</th>
<th>Diabetic DP-BB/E</th>
<th>P-value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>6.55 ± 0.09</td>
<td>6.53 ± 0.13</td>
<td>21.38 ± 2.79</td>
<td>0.0001</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.19 ± 0.01</td>
<td>0.03 ± 0.008</td>
<td>0.18 ± 0.01</td>
<td>0.0001</td>
</tr>
<tr>
<td>Lactate</td>
<td>3.86 ± 0.25</td>
<td>3.18 ± 0.22</td>
<td>4.45 ± 0.59</td>
<td>NS</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.54 ± 0.02</td>
<td>0.41 ± 0.02</td>
<td>0.62 ± 0.02</td>
<td>0.0001</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.18 ± 0.01</td>
<td>0.23 ± 0.02</td>
<td>0.22 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>β-hydroxybutyrate</td>
<td>0.05 ± 0.005</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Lactate/Pyruvate</td>
<td>132.9 ± 23.6</td>
<td>19.88 ± 0.7</td>
<td>24.74 ± 1.93</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Where NS denotes not significantly different by one-way analysis of variance (ANOVA) and n is the number rats in each group

129
Table 3.12 shows the effect of early pregnancy on the maternal metabolic profile of pre-diabetic DP-BB/E rats. These metabolic changes reflect the normal maternal metabolic adaptations to pregnancy, such as a decrease in plasma glucose (P < 0.01), an increase in plasma lactate (P < 0.01), accompanied by a significant fall in β-hydroxybutyrate levels (P < 0.0001). The change in plasma lactate was reflected in the increased lactate/pyruvate ratio (P < 0.0001).

Table 3.12: Effect of early pregnancy on maternal metabolic profile of pre-diabetic DP-BB/E rats

<table>
<thead>
<tr>
<th>Metabolite (mmol/L)</th>
<th>Non-Pregnant</th>
<th>Day 0.5 of Pregnancy</th>
<th>Day 4.5 of Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>7.20 ± 0.13</td>
<td>6.77 ± 0.13</td>
<td>6.55 ± 0.09 *</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.18 ± 0.007</td>
<td>0.19 ± 0.01</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.84 ± 0.18</td>
<td>2.84 ± 0.19</td>
<td>3.86 ± 0.25 *</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.50 ± 0.01</td>
<td>0.45 ± 0.03</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.22 ± 0.02</td>
<td>0.17 ± 0.008</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>β-hydroxybutyrate Lactate/Pyruvate ratio</td>
<td>0.11 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.05 ± 0.005 *</td>
</tr>
<tr>
<td>Lactate/Pyruvate ratio</td>
<td>15.18 ± 0.8</td>
<td>15.50 ± 0.8</td>
<td>19.88 ± 0.73 *</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

Where * denotes significant difference between non-pregnant and 4.5 pregnant pre-diabetic rats (Student’s t test): glucose and lactate: P < 0.01; β-hydroxybutyrate and lactate/pyruvate ratio P < 0.0001
Table 3.13: Mean percentage of embryos per pregnancy (± SEM) recovered from pre-diabetic DP, Non-Diabetic DR and Diabetic DP-BB/E rats on day 4.5 of pregnancy

<table>
<thead>
<tr>
<th>Pregnancy Type: Mother x Father</th>
<th>Pregnancies</th>
<th>Total Embryos</th>
<th>Unfertilised Oocytes</th>
<th>Morulae</th>
<th>Early Blastocysts</th>
<th>Expanded Blastocysts</th>
<th>Embryos per pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Diabetic (DP) x Non-Diabetic (DR)</td>
<td>12</td>
<td>79</td>
<td>4.2 ± 4.2 *</td>
<td>10.6 ± 3.7</td>
<td>4.3 ± 1.9 *</td>
<td>80.9 ± 5.6 *</td>
<td>6.58 ± 0.7</td>
</tr>
<tr>
<td>Non-Diabetic (DR) x Non-Diabetic (DR)</td>
<td>6</td>
<td>53</td>
<td>0.8 ± 0.8</td>
<td>5.7 ± 2.6</td>
<td>20.4 ± 5.7</td>
<td>73.0 ± 4.8</td>
<td>8.42 ± 0.4</td>
</tr>
<tr>
<td>Diabetic (DP) x Non-Diabetic (DR)</td>
<td>10</td>
<td>65</td>
<td>24.4 ± 8.1</td>
<td>13.4 ± 7.7</td>
<td>10.0 ± 4.0</td>
<td>52.2 ± 8.4</td>
<td>7.47 ± 0.4</td>
</tr>
</tbody>
</table>

Data are $n$ or means ± SEM

Significant differences in the percentage of embryos at each developmental stage from pre-diabetic rats are indicated by *

**Unfertilised oocytes**: Pre-diabetic DP vs Diabetic DP: $P < 0.05$

**Morulae**: not significantly different

**Early Blastocysts**: Pre-diabetic DP vs Non-Diabetic DR: $P < 0.05$

**Expanded Blastocysts**: Pre-diabetic DP vs Diabetic DP: $P < 0.01$
Embryos were flushed from day 4.5 pregnant pre-diabetic DP rats and the number of embryos at each morphological stage of development was recorded and compared with those from Diabetic DP rats with established diabetes (Table 3.13). Diabetes Prone BB/E rats in the pre-diabetic period had fewer unfertilised oocytes (P < 0.05) and more expanded blastocysts (P < 0.01) per pregnancy than their DP counterparts who had developed diabetes. These results suggested that the fertility of DP-BB/E rats is normal prior to disease onset. Although the pre-diabetic DP-BB/E females were considerably younger than the DP-BB/E rats with established IDDM (60.42 ± 3.4 vs 131.54 ± 1.9 days of age for pre-diabetic vs Diabetic DP rats; P < 0.0001) it is unlikely that age alone was sufficient to cause such a reduction in fertility since the age of DP-BB/E rats with established diabetes at pregnancy was not significantly different from that of the Non-Diabetic (DR-BB/E) rats (125.4 ± 4.2 vs 131.54 ± 1.9 days of age for Non-Diabetic vs Diabetic DP rats, NS different).

**Cell Lineage Analysis**

Embryos were flushed from pre-diabetic DP-BB/E rats and those which had reached the expanded blastocyst stage and taken through the differential staining procedure to determine the number of ICM and TE cells. To examine whether blastocysts from pre-diabetic rats were normally developed with respect to their ICM and TE cell number, the mean cell number of each lineage was compared with blastocysts from Diabetic DP and Non-Diabetic DR rats. The results are shown in Table 3.14.
Table 3.14: Mean ICM and TE cell number in blastocysts from pre-diabetic DP-, Non-Diabetic DR- and Diabetic DP-BB/E rats on day 4.5 of pregnancy

<table>
<thead>
<tr>
<th>Type of pregnancy</th>
<th>Number of Blastocysts</th>
<th>Number of ICM</th>
<th>Number of TE</th>
<th>Number of Trophoderm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Diabetic DP</td>
<td>40</td>
<td>10.0 ± 0.27</td>
<td>19.43 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>Non-Diabetic DR</td>
<td>43</td>
<td>10.40 ± 0.25</td>
<td>20.23 ± 0.41</td>
<td></td>
</tr>
<tr>
<td>Diabetic DP</td>
<td>33</td>
<td>8.25 ± 0.30*</td>
<td>19.80 ± 0.52</td>
<td></td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM.

* P < 0.0001: significantly less than blastocysts from pre-diabetic DP and Non-Diabetic DR-BB/E rats (Student’s t test).

The data presented in Table 3.14 demonstrate that blastocysts from DP-BB/E rats during the pre-diabetic stage were indeed normal as there was no evidence of an ICM cellular deficit in these blastocysts. Blastocysts from Diabetic DP rats exhibited an ICM cellular deficit which was significantly different from both pre-diabetic DP and Non-Diabetic DR rat blastocysts (P < 0.0001).

The concentration of blood glucose was measured (using an ExacTech blood glucose meter) in a small sample of tail blood taken from the three groups of rats 30 min before the embryos were collected. Blood glucose at 4.5 days gestation in pre-diabetic DP rats was within the normal range and not significantly different from values obtained from Non-Diabetic DR rats (mean ± SEM blood glucose: pre-diabetic
rats 4.13 \pm 0.1 \text{ mmol/L} vs Non-Diabetic DR rats 4.26 \pm 0.4 \text{ mmol/L}), which contrasted with the blood glucose measurements taken from Diabetic DP rats (mean \pm SEM, 21.93 \pm 1.6 \text{ mmol/L}). These results were later confirmed when the concentration of plasma glucose was measured in these rats (see Table 3.11).

3.2.2 Embryo Transfer Experiments

Introduction

The effect of maternal metabolic status on early embryo development was examined more closely using embryo transfer experiments. Embryos may be transferred either at the blastocyst stage, directly into the uterus, which is a relatively quick and simple procedure; or at the 2-cell stage requiring transfer into the oviduct of the recipient animal. Since the ICM cellular deficit is already present in embryos at the blastocyst stage, it was necessary to perform embryo transfers at the 2 cell stage.

Experimental Protocol

The aim of these experiments was to investigate whether (1) the diabetic uterine environment could induce an ICM cellular deficit in embryos from normal non-diabetic mothers, and (2) removing embryos from a potentially harmful diabetic environment could 'rescue' the embryos and allow them to develop normally. Embryos at the 2-cell stage were transferred into the oviduct of pseudopregnant females and the pregnancy was continued until day 4.5. At this time the blastocysts were flushed from the recipient females and differentially stained to determine the number of ICM and TE cells.
Results

In vitro and in vivo evidence exists to suggest that certain stimuli can induce unfertilised oocytes to undergo parthenogenic activation, these include ether (293) and avertin anaesthesia (294), heat shock (295), electric stimulation of the oviduct (296) and in vitro treatment with hyaluronidase (297). These findings stress the importance of taking into account the possible consequences of anaesthesia, when operative procedures are carried out in the post-ovulatory period. Although the rate of oocyte activation with anaesthesia is low, it is possible that the anaesthetic used during the embryo transfer procedure (a cocktail of hypnorm and hypnovel) could activate some oocytes to develop parthenogenetically into structures that resemble blastocysts. Therefore it was important to test this phenomenon before embryo transfer experiments were carried out.

Six female BB/E rats (three Diabetic DP and three Non-Diabetic DR) were mated with vasectomised males rats. On day 0.5 of pseudopregnancy the females were anaesthetised with the cocktail of hypnorm and hypnovel (as described in section 2.2.8.2 of Materials and Methods). The rats were sacrificed at day 4.5 of pseudopregnancy and the uterine horns flushed with culture medium. No parthenogenetic blastocysts were found in any of the BB/E rats, suggesting that unlike avertin (a commonly used anaesthetic) the mix of hypnorm and hypnovel did not induce rat oocytes to develop parthenogenetically.
Table 3.15: Mean ICM and TE cell number in blastocysts flushed from embryo transfer recipient Diabetic DP- and Non-Diabetic DR-BB/E rats

<table>
<thead>
<tr>
<th>Donor Type</th>
<th>Recipient Type</th>
<th>Number of Embryos</th>
<th>ICM cell number</th>
<th>TE cell number</th>
<th>ICM cell Deficit (%)</th>
<th>TE cell Accretion (%)</th>
<th>Success rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Diabetic</td>
<td>Non-Diabetic</td>
<td>30</td>
<td>17.6 ± 0.4</td>
<td>34.0 ± 0.9</td>
<td>-</td>
<td>-</td>
<td>54%</td>
</tr>
<tr>
<td>Non-Diabetic</td>
<td>Diabetic</td>
<td>28</td>
<td>16.0 ± 0.4*</td>
<td>40.5 ± 1.1&quot;</td>
<td>9.09</td>
<td>19.1</td>
<td>39%</td>
</tr>
<tr>
<td>Diabetic</td>
<td>Non-Diabetic</td>
<td>32</td>
<td>14.6 ± 0.5*</td>
<td>37.2 ± 1.2&quot;</td>
<td>17.0</td>
<td>9.4</td>
<td>62%</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM. Significant differences indicated by * and "

ICM cells: * P < 0.01 for Non-Diabetic/Diabetic vs Non-Diabetic/Non-Diabetic transfers; and P < 0.0001 for Diabetic/Non-Diabetic vs Non-Diabetic/Non-Diabetic transfers

TE cells: " P < 0.001 for Non-Diabetic/Diabetic vs Non-Diabetic/Non-Diabetic transfers and P < 0.05 for Diabetic/Non-Diabetic vs Non-Diabetic/Non-Diabetic transfers (Student's t test).
The outcome of the embryo transfer experiments is shown in Table 3.15. Two cell embryos from Non-Diabetic DR donor females were transferred into Non-Diabetic DR recipient females to provide the control ICM and TE cell numbers. The blastocysts flushed from these pregnancies were slightly larger than blastocysts from previous pregnancies at 4.5 days gestation. This was to be expected because it is standard practice to transfer 2 cell embryos from 1.5 day pregnant mothers into 0.5 day pseudopregnant recipients, this additional day allows the transferred embryos time to adjust to the transfer procedure which can slow down cell proliferation. The success rate for this procedure was approximately 54 % which is quite acceptable for rat embryo transfers, which generally have lower rates of success than embryo transfer in the mouse [50-75 % success rate, from Hogan et al (298)].

Transfer of embryos from Non-Diabetic DR donors into Diabetic DP recipients however, had a much lower success rate (39 %). This was mainly due to problems with the conventional insulin therapy the diabetic rats received. Nevertheless, blastocysts from these transfers showed a significant decrease in the number of ICM cells compared with those from Non-Diabetic DR recipients (P < 0.01). In addition, these blastocysts also demonstrated a significant TE cellular accretion (P < 0.001). Therefore it appeared that the oviductal/uterine environment of the Diabetic DP recipient induced not only an ICM deficit but a TE cell accretion in blastocysts from normal, Non-Diabetic DR parents. These results are consistent with those seen after 24 h blastocyst culture in growth media and uterine cell conditioned media (section 3.1.2.3), ruling out the possibility that the TE cell accretion is only seen in vitro and could have been a cell culture artefact.
The success rate for transfer of embryos from Diabetic DP donors into Non-Diabetic DR-BB/E recipients was 62%. Problems encountered with these transfers were mainly due to poor embryo quality and lack of fertilised oocytes. Transfer of embryos from Diabetic DP mothers into Non-Diabetic DR mothers did not prevent an ICM deficit or a TE cell accretion occurring, as these blastocysts had significantly fewer ICM cells ($P < 0.001$) and significantly more TE cells ($P < 0.05$) than the controls.

**Discussion of embryo transfer data**

Diabetic DP-BB/E rats treated with conventional insulin therapy i.e. a single daily subcutaneous injection of long acting insulin (Bovine Ultratard U40, Novo Nordisk, Denmark) each morning show marked diurnal fluctuations in their plasma glucose concentrations with values ranging between 2.4 and 18 mmol/L (299). It was necessary to perform the transfer procedures during the early afternoon so that the embryos could be harvested at the correct stage but this was also the time when most of the rats were becoming hypoglycaemic. When deciding the dose of insulin to be given on the morning of the experiment, it was necessary to achieve a balance between reducing the already present hyperglycaemia and the hypoglycaemia which would develop later that afternoon. It was assumed initially, that the stress of the procedure would cause the blood glucose to rise and so the mid-afternoon hypoglycaemia would not be a problem, but this was not always the case since several of the Diabetic DP rats responded differently to the effects of the anaesthetic (see Table 3.16).
Table 3.16: Effect of anaesthesia on Diabetic DP-BB/E rats used as recipients for embryo transfer

<table>
<thead>
<tr>
<th>Rat Number</th>
<th>Blood glucose 9 am (mmol/L)</th>
<th>Insulin dose 9 am (U)</th>
<th>Pre-anaesthetic blood glucose 2 p.m (mmol/L)</th>
<th>Post-anaesthetic blood glucose 5 p.m (mmol/L)</th>
<th>Blood glucose next morning 9 a.m (mmol/L)</th>
<th>Success rate (no. embryos recovered / no. transferred)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2414</td>
<td>&gt; 25</td>
<td>2.0</td>
<td>6.1</td>
<td>&gt; 25</td>
<td>18.9</td>
<td>73 % (11/15)</td>
</tr>
<tr>
<td>2470</td>
<td>20</td>
<td>2.0</td>
<td>6.0</td>
<td>4.1</td>
<td>-</td>
<td>86 % (6/7)</td>
</tr>
<tr>
<td>2384</td>
<td>18.4</td>
<td>2.0</td>
<td>5.4</td>
<td>2.2</td>
<td>Died o/n</td>
<td>0 % (0/5)</td>
</tr>
<tr>
<td>2320</td>
<td>&gt;25</td>
<td>2.0</td>
<td>2.5</td>
<td>2.2</td>
<td>&gt; 25</td>
<td>0 % (0/13)</td>
</tr>
<tr>
<td>2419</td>
<td>10.2</td>
<td>1.6</td>
<td>2.2</td>
<td>2.2</td>
<td>&gt; 25</td>
<td>0 % (0/15)</td>
</tr>
<tr>
<td>2415</td>
<td>&gt; 25</td>
<td>2.0</td>
<td>&gt;25</td>
<td>&gt; 25</td>
<td>19.7</td>
<td>0 % (0/6)</td>
</tr>
<tr>
<td>2498</td>
<td>15.1</td>
<td>1.0</td>
<td>2.6</td>
<td>2.2</td>
<td>3.9</td>
<td>55% (5/9)</td>
</tr>
<tr>
<td>2473</td>
<td>&gt;25</td>
<td>1.0</td>
<td>3.9</td>
<td>3.3</td>
<td>19.7</td>
<td>80 % (8/10)</td>
</tr>
<tr>
<td>2572</td>
<td>&gt; 25</td>
<td>1.1</td>
<td>3.8</td>
<td>10.5</td>
<td>9.7</td>
<td>55 % (5/9)</td>
</tr>
</tbody>
</table>
For some, the stress of the operation did cause their blood glucose to rise, but in others the blood glucose level fell post-anaesthetic. This made the decision regarding insulin dosage very difficult.

One female in particular (rat number 2415), was significantly hyperglycaemic at the time of transfer (despite having been given 2.0 units of long acting insulin, Bovine Ultratard U40, that morning), after the operation she remained hyperglycaemic and although fully conscious she became very lethargic and dehydrated. She was given another dose of insulin and fed water with a syringe, and although she survived no blastocyst were obtained from this pregnancy. The first two embryo transfers into Diabetic DP recipients were highly successful with 73 % and 86 % embryo recovery rates, however it became clear that in some rats the normal morning insulin dose had to be almost halved to prevent severe post-anaesthetic hypoglycaemia. For the final three transfers this protocol proved to be successful.

Conclusions from Experiment II

These experiments were designed to assess the relative influence of maternal genetic background and metabolic status on pre-implantation embryo development. These experiments clearly demonstrate that Diabetes Prone BB/E rats during the pre-diabetic period have normal pregnancies and that embryo development up until the blastocyst stage at least, proceeds normally. There was no evidence of an ICM cellular deficit and all metabolic parameters measured throughout the first 4.5 days of pregnancy were normal. From this it can be concluded that the full complement of
IDDM susceptibility genes was not in itself, sufficient to disturb early embryo development and/or give rise to an ICM cell deficit.

Experiments involving the transfer of two-cell embryos examined the influence of maternal metabolic status more closely. The data show that the oviductal/uterine environment of the Diabetic DP recipients could induce not only an ICM deficit (P < 0.01) but a TE cell accretion (P < 0.001) in normal embryos from Non-Diabetic DR donor rats. These results were consistent with those seen after 24 h blastocyst culture in growth media and uterine cell conditioned media (section 3.1.2.3) when blastocysts from Diabetic DP mothers developed an ICM cell deficit in association with a TE cell accretion. The data confirm therefore, that the TE cellular accretion was not simply the result of in vitro culture conditions. Transfer of 2-cell embryos from Diabetic DP mothers into Non-Diabetic DR mothers however, did not prevent an ICM deficit or a TE cell accretion since these blastocysts had significantly fewer ICM cells (P < 0.001) and more TE cells (P < 0.05) than the controls. These 2-cell embryos, flushed from diabetic donor rats, had only been in the oviduct of the diabetic mother for 1.5 days prior to transfer. Whether this short exposure to a disturbed metabolic environment was sufficient to cause embryo damage is uncertain.

Comparison of maternal metabolic profiles in 0.5 day pregnant pre-diabetic DP-, Non-Diabetic DR- and Diabetic DP-BB/E rats (Table 3.10) showed that DP rats with established IDDM had significantly lower levels of plasma pyruvate than either pre-diabetic DP and Non-Diabetic DR-BB/E rats (0.07 ± 0.01 vs 0.19 ± 0.01 and 0.18 ± 0.01; P < 0.001, for Diabetic DP vs pre-diabetic DP and Non-Diabetic DR rats
respectively). Pyruvate and lactate are the two main energy sources for early pre-implantation embryos, as embryos are unable to use glucose as their principal nutrient until the blastocyst stage. Whether poor nutritional status accompanied by high levels of inhibitory substrates such as glucose and β-hydroxybutyrate in the diabetic oviduct could result in disturbed embryo development is not fully known.

In conclusion, these results suggest that early damage to the pre-implantation embryo is due to a disturbed maternal metabolic state, and that the full complement of IDDM susceptibility genes is not in itself, sufficient to cause an ICM cellular deficit. This suggests that the embryonic damage seen in diabetic pregnancies is secondary to a metabolic disturbance and is therefore potentially preventable by improved insulin delivery systems.
3.3 Experiment III: Is IDDM-Induced Embryopathy Preventable?

Introduction

The data presented so far clearly demonstrate that it is difficult to achieve good metabolic control in BB/E rats with IDDM in the earliest stages of pregnancy using conventional insulin therapy i.e. one injection of long-acting insulin each morning. This difficulty cannot be accounted for solely by pregnancy since it has previously been shown that male BB/E rats with IDDM maintained on CIT exhibit marked diurnal fluctuation in plasma glucose concentration (range 2.4-25 mmol/L), and are only rarely normoglycaemic (299). The usual pattern is that between 6 and 10 a.m. these rats are markedly hyperglycaemic, following the insulin injection at 10 a.m. the plasma glucose falls rapidly to hypoglycaemic levels between 2 and 10 p.m. Thereafter the concentration of circulating insulin declines slowly overnight resulting in a concomitant steady rise in the plasma glucose concentration (299).

Sustained release insulin implants (SRIIs) represent an alternative insulin delivery system (300). These are small rods (2 x 7mm) consisting of powdered bovine insulin compressed with palmitic acid. Following implantation subcutaneously, erosion of the implant begins at once and the effect of the released insulin on the blood glucose level can be detected within 1 h of implantation. The SRII releases a steady basal dose of insulin continuously throughout the day and night for up to 40-60 days, delivering approximately 2 units of bovine insulin per 24 h per implant.
SRIIs are easy to insert using local anaesthetic and a trocar and cannula, and animals need no special aftercare. The initial starting dose can be augmented (either by subcutaneous injections of insulin or insertion of additional pieces of SRII) if required. Excellent metabolic control (without significant fluctuation in the blood glucose profile) has been achieved previously in male BB/E rats with IDDM maintained on SRIIs (299).

Such stable control can be achieved by a steady basal dose of insulin because rats with IDDM eat more or less continuously, rather than mainly through the night as normal rats do.

**Experimental Protocol**

A group of 21 young female Diabetic DP-BB/E rats were randomly selected for this study. Plasma glucose concentrations at diabetes diagnosis ranged from 21.8 to 44.2 mmol/L.

The aim of treatment was to achieve a target blood glucose concentration of 3-10 mmol/L as quickly as possible, using various combinations of SRIIs and subcutaneous injections of insulin.

Blood glucose measurements were taken at 10 a.m. every two days to assess metabolic control. If the blood glucose concentration was found to be >15 mmol/L an additional 1/4 piece of the regular dose Linplant® or a whole LinBit™ SRII was inserted.
Table 3.17: Concentration of plasma metabolites on the day of diabetes detection in 21 female Diabetes Prone BB/E rats

<table>
<thead>
<tr>
<th>Rat Number</th>
<th>Glucose (mmol/L)</th>
<th>Pyruvate</th>
<th>Lactate (mmol/L)</th>
<th>Alanine (mmol/L)</th>
<th>Glycerol</th>
<th>BOHB</th>
<th>Ratio of Lactate/Pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1747</td>
<td>44.2</td>
<td>0.005</td>
<td>3.34</td>
<td>0.45</td>
<td>0.19</td>
<td>0.63</td>
<td>668.0</td>
</tr>
<tr>
<td>1641</td>
<td>40.8</td>
<td>0.07</td>
<td>3.09</td>
<td>0.63</td>
<td>0.36</td>
<td>0.51</td>
<td>44.1</td>
</tr>
<tr>
<td>1675</td>
<td>40.3</td>
<td>0.17</td>
<td>3.08</td>
<td>0.54</td>
<td>0.38</td>
<td>1.22</td>
<td>18.2</td>
</tr>
<tr>
<td>1745</td>
<td>38.5</td>
<td>0.12</td>
<td>2.87</td>
<td>0.66</td>
<td>0.29</td>
<td>0.28</td>
<td>24.9</td>
</tr>
<tr>
<td>1633</td>
<td>34.4</td>
<td>0.06</td>
<td>4.14</td>
<td>0.57</td>
<td>0.35</td>
<td>0.45</td>
<td>75.2</td>
</tr>
<tr>
<td>1812</td>
<td>34.3</td>
<td>0.14</td>
<td>4.07</td>
<td>0.54</td>
<td>0.28</td>
<td>0.25</td>
<td>29.34</td>
</tr>
<tr>
<td>2306</td>
<td>33.5</td>
<td>0.02</td>
<td>4.68</td>
<td>0.56</td>
<td>0.20</td>
<td>0.38</td>
<td>23.3</td>
</tr>
<tr>
<td>2271</td>
<td>33.2</td>
<td>0.28</td>
<td>6.61</td>
<td>0.59</td>
<td>0.26</td>
<td>0.38</td>
<td>23.5</td>
</tr>
<tr>
<td>1542</td>
<td>32.8</td>
<td>0.16</td>
<td>3.37</td>
<td>0.95</td>
<td>0.22</td>
<td>0.22</td>
<td>21.1</td>
</tr>
<tr>
<td>1737</td>
<td>32.3</td>
<td>0.005</td>
<td>2.79</td>
<td>0.59</td>
<td>0.27</td>
<td>0.26</td>
<td>558.0</td>
</tr>
<tr>
<td>1748</td>
<td>31.5</td>
<td>0.17</td>
<td>3.29</td>
<td>0.60</td>
<td>0.33</td>
<td>0.47</td>
<td>19.91</td>
</tr>
<tr>
<td>1608</td>
<td>31.0</td>
<td>0.01</td>
<td>3.52</td>
<td>0.56</td>
<td>0.32</td>
<td>0.40</td>
<td>351.0</td>
</tr>
<tr>
<td>2313</td>
<td>30.2</td>
<td>0.15</td>
<td>2.88</td>
<td>0.40</td>
<td>0.48</td>
<td>1.45</td>
<td>19.2</td>
</tr>
<tr>
<td>2333</td>
<td>29.0</td>
<td>0.003</td>
<td>2.14</td>
<td>0.40</td>
<td>0.54</td>
<td>0.39</td>
<td>712.0</td>
</tr>
<tr>
<td>1669</td>
<td>28.3</td>
<td>0.04</td>
<td>4.18</td>
<td>0.71</td>
<td>0.23</td>
<td>0.10</td>
<td>119.3</td>
</tr>
<tr>
<td>2314</td>
<td>26.7</td>
<td>0.21</td>
<td>3.27</td>
<td>0.52</td>
<td>0.20</td>
<td>0.13</td>
<td>15.4</td>
</tr>
<tr>
<td>2309</td>
<td>25.9</td>
<td>0.22</td>
<td>3.05</td>
<td>0.57</td>
<td>0.19</td>
<td>0.14</td>
<td>14.1</td>
</tr>
<tr>
<td>2335</td>
<td>24.8</td>
<td>0.12</td>
<td>2.75</td>
<td>0.44</td>
<td>0.51</td>
<td>0.36</td>
<td>22.4</td>
</tr>
<tr>
<td>1625</td>
<td>24.5</td>
<td>0.10</td>
<td>6.85</td>
<td>0.65</td>
<td>0.23</td>
<td>0.004</td>
<td>72.4</td>
</tr>
<tr>
<td>2310</td>
<td>23.5</td>
<td>0.18</td>
<td>2.98</td>
<td>0.47</td>
<td>0.26</td>
<td>0.14</td>
<td>16.6</td>
</tr>
<tr>
<td>2270</td>
<td>21.8</td>
<td>0.20</td>
<td>3.85</td>
<td>0.57</td>
<td>0.18</td>
<td>0.05</td>
<td>19.2</td>
</tr>
<tr>
<td>mean</td>
<td>31.5</td>
<td>0.13</td>
<td>3.66</td>
<td>0.57</td>
<td>0.42</td>
<td>0.51</td>
<td>86.4</td>
</tr>
<tr>
<td>± SEM</td>
<td>± 1.3</td>
<td>± 0.02</td>
<td>± 0.26</td>
<td>± 0.03</td>
<td>± 0.13</td>
<td>± 0.14</td>
<td>± 39.0</td>
</tr>
</tbody>
</table>
The rats were allowed to stabilise for 2-3 weeks before being mated with Non-Diabetic DR-BB/E males of proven fertility. On the morning of conception a second plasma sample was taken for metabolite analysis. The pregnancies were allowed to continue until day 4.5 when the embryos were collected for analysis.

Results

3.3.1 Establishing pre-pregnancy metabolic control

Table 3.17 shows the concentration of plasma metabolites on the day of diabetes detection in the 21 female Diabetic DP-BB/E rats. In Table 3.18 the group mean for each metabolite in diabetic rats is compared with that of a group of non-pregnant Non-Diabetic DR-BB/E rats.

The data in Table 3.18 show that the onset of diabetes in the DP-BB/E rat is associated with significant hyperglycaemia ($P < 0.0001$), ketonaemia ($P < 0.001$) and increased lipolysis ($P < 0.01$) when compared to Non-Diabetic DR rats. This acute metabolic decompensation reflects the absolute lack of endogenous insulin seen in these animals at diagnosis of diabetes (160). Plasma alanine was also significantly raised in the diabetic animals due to increased muscle protein catabolism. The level of plasma lactate was lower in the diabetic group although statistical significance was not achieved. The ratio of lactate to pyruvate reflects the tissue free NADH : NAD$^+$ ratio. If the plasma lactate concentration is increased but the pyruvate concentration is unchanged or decreased it would suggest an increase in the cytosol NADH : NAD$^+$ ratio, and under most circumstances this indicates cellular hypoxia. Although the lactate : pyruvate ratio was greater in the diabetic rats it was not significantly different
from the non-diabetic group. These metabolic disturbances demonstrate that at the beginning of the study all 21 DP rats had permanent insulin-dependent diabetes mellitus (IDDM).

Table 3.18: Comparison of plasma metabolites in Diabetic DP at diabetes detection and non-pregnant Non-Diabetic DR-BB/E rats

<table>
<thead>
<tr>
<th>Metabolite (mmol/L)</th>
<th>Diabetic DP</th>
<th>Non-diabetic DR</th>
<th>P-value (student's t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>31.50 ± 1.3</td>
<td>7.02 ± 0.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.13 ± 0.02</td>
<td>0.19 ± 0.08</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Lactate</td>
<td>3.66 ± 0.26</td>
<td>3.93 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.57 ± 0.03</td>
<td>0.45 ± 0.02</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.42 ± 0.13</td>
<td>0.18 ± 0.02</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>BOHB</td>
<td>0.51 ± 0.14</td>
<td>0.09 ± 0.008</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lactate/Pyruvate</td>
<td>86.4 ± 39.0</td>
<td>20.62 ± 5.4</td>
<td>NS</td>
</tr>
<tr>
<td>n</td>
<td>21</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM and n is the number rats in each group

Where NS denotes not significantly different from Non-Diabetic DR-BB/E rats
Table 3.19: Comparison of plasma metabolites in SRII-treated Diabetic DP-, CIT-treated Diabetic DP-, and Non-Diabetic DR-BB/E rats at day 0.5 of pregnancy

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CIT-Diabetic</th>
<th>SRII-Diabetic</th>
<th>Non-Diabetic</th>
<th>P-value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>19.6 ± 2.4</td>
<td>9.3 ± 1.4</td>
<td>6.47 ± 0.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.07 ± 0.001</td>
<td>0.12 ± 0.02</td>
<td>0.18 ± 0.04</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Lactate</td>
<td>3.40 ± 0.4</td>
<td>3.34 ± 0.15</td>
<td>3.63 ± 0.72</td>
<td>NS</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.50 ± 0.02</td>
<td>0.52 ± 0.02</td>
<td>0.43 ± 0.03</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.33 ± 0.06</td>
<td>0.20 ± 0.008</td>
<td>0.18 ± 0.03</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BOHB</td>
<td>0.32 ± 0.05</td>
<td>0.05 ± 0.007</td>
<td>0.04 ± 0.009</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lactate/Pyruvate ratio</td>
<td>110 ± 57.1</td>
<td>61.2 ± 20.4</td>
<td>19.45 ± 2.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM

Where n is the number of animals in each group and a indicates same data as shown in Table 3.10.

Six rats were excluded from further study because target blood glucose levels were not consistently achieved during the 2-3 week stabilisation period (rat number 1745, 1625, 2333 and 2271) or episodes of severe and prolonged hypoglycaemia (rat number 2313 and 1542). Blood glucose measurements consistently within the target range were achieved in the remaining 15 rats.
3.3.2 Assessing maternal metabolic control at conception

These 15 rats were mated with Non-Diabetic DR-BB/E male rats of proven fertility. Plasma samples were taken on the morning of day 0.5 of pregnancy (conception day) and the profile of circulating metabolites was compared with those from Diabetic DP rats treated conventionally with subcutaneous insulin injections and Non-Diabetic DR-BB/E rats (Table 3.19). In order to assess the success of SRIIs as an alternative delivery system two questions were asked:

(1) Did Diabetic DP rats treated with SRIIs achieve better metabolic control than those treated with CIT?

Although conventional insulin therapy did improve the plasma glucose concentration (plasma glucose at diabetes detection 31.5 mmol/L vs 19.6 mmol/L at conception; \( P < 0.01 \)) the rats were still significantly hyperglycaemic compared to Non-Diabetic DR-BB/E rats at this time. In addition, CIT did not have any effect on the hyperketonaemia or increased lipolysis in these diabetic rats, as shown in Table 3.19 (\( P < 0.001 \) for glucose, glycerol and BOHB in CIT-treated Diabetic DP rats vs Non-Diabetic DR BB/E rats).

However, Diabetic DP rats treated with SRIIs had significantly lower concentrations of plasma glucose (\( P < 0.01 \)), glycerol (\( P < 0.001 \)) and BOHB (\( P < 0.001 \)) than CIT-treated Diabetic DP rats. There was no significant difference in plasma concentrations of pyruvate, alanine or lactate between SRII- and CIT-treated Diabetic DP rats. All differences were assessed by the student’s t-test.
(2) Did SRII-treatment achieve normal control?

SRII-treatment significantly improved maternal metabolic control, seen as a reduction in plasma glucose, glycerol and BOHB closely approaching but not quite achieving the normal range for Non-Diabetic DR rats (although there was no difference statistically). Pyruvate remained significantly lower, and alanine significantly higher (P < 0.05 for both differences) in SRII-treated rats than in Non-Diabetic DR-BB/E rats. All differences were assessed by the student’s t-test.

3.3.3 Assessing maternal metabolic control during pregnancy

Scrutiny of daily blood glucose readings for each individual animal showed that prior to pregnancy the degree of metabolic control established in these SRII-treated females was much more variable than that previously achieved in male SRII-treated rats (299). Closer examination of blood glucose measurements revealed that at conception there were two distinct blood glucose patterns: (i) in 5 rats the blood glucose concentration remained within the target range during and following conception; and (ii) in the remaining 10 rats there was a dramatic rise in the blood glucose level (range 20-25 mmol/L) at or following conception (mean blood glucose concentration pre-pregnancy vs day 2.5 of pregnancy 6.20 ± 0.4 vs 20.43 ± 1.17 mmol/L, respectively; P < 0.0001).

The individual blood glucose profiles for the 15 SRII-treated rats are shown in Figures 3.6 and 3.7. Figure 3.6 shows the profiles of the 10 rats which displayed a dramatic rise in blood glucose concentration at or following conception. Figure 3.7
shows the profiles of the 5 rats which did not show a rise in blood glucose concentration at or following conception.

Figure 3.6: Blood glucose profiles of SRII-treated Diabetic rats showing good metabolic control pre-conception followed by a post-conception rise in blood glucose.

![Graph 1]

Rat number 1669

![Graph 2]

Rat number 1812

Where day of conception is indicated by [■]
Figure 3.6 (continued): Blood glucose profiles of SRII-treated Diabetic rats showing good metabolic control pre-conception followed by a post-conception rise in blood glucose.

Rat number 1675

Rat number 1608

Where day of conception is indicated by ■

152
Figure 3.6 (continued): Blood glucose profiles of SRII-treated Diabetic rats showing good metabolic control pre-conception followed by a post-conception rise in blood glucose.

Rat number 1641

![Graph showing blood glucose concentration over time for Rat number 1641.]

Rat number 1747

![Graph showing blood glucose concentration over time for Rat number 1747.]

Where day of conception is indicated by ■
Figure 3.6 (continued): Blood glucose profiles of SRII-treated Diabetic rats showing good metabolic control pre-conception followed by a post-conception rise in blood glucose

Rat number 1633

Rat number 2335

Where day of conception is indicated by [■] and * indicates additional SRII given
Figure 3.6 (continued): Blood glucose profiles of SRII-treated Diabetic rats showing good metabolic control pre-conception followed by a post-conception rise in blood glucose

Rat number 2314

Rat number 2310

Where day of conception is indicated by [■] and * indicates additional SRII given
Figure 3.7: Blood glucose profiles of SRII-treated Diabetic rats showing good metabolic control pre-conception maintained through conception and during early pregnancy with no blood glucose rise post conception.

Rat number 1748

![Blood glucose profile for Rat number 1748](image)

Duration of SRII treatment (days)

Rat number 1737

![Blood glucose profile for Rat number 1737](image)

Duration of SRII treatment (days)

Where day of conception is indicated by [□] and * indicates additional SRII given.
Blood glucose profiles of SRII-treated Diabetic rats showing good metabolic control pre-conception maintained through conception and during early pregnancy with no blood glucose rise post conception.

Rat number 2270

Rat number 2309

Where day of conception is indicated by □
Figure 3.7 (continued): Blood glucose profiles of SRII-treated Diabetic rats showing good metabolic control pre-conception maintained through conception and during early pregnancy with no blood glucose rise post conception.

Rat number 2306

Where day of conception is indicated by □ and * indicates additional SRII given

The mean blood glucose profiles for the two profile types are shown in Figure 3.8A and 3.8B.
Figure 3.8:

Mean blood glucose profiles of SRII-treated Diabetic DP-BB/E rats before pregnancy, at conception and during the first four days of pregnancy.

Panel A: shows the group profile of the 10 rats which displayed a significant rise in blood glucose concentration at or following conception, indicated by * (P < 0.0001).

Panel B: shows the group profile of the five rats which did not show a rise in blood glucose concentration at or following conception.
A mean blood glucose profile for non-diabetic DR BB/E rats (n = 8) one week before pregnancy, at conception and during the first four days of pregnancy is shown in Figure 3.9. As expected, there was no post-conception blood glucose rise in these rats.

**Figure 3.9:** Mean blood glucose concentrations in Non-Diabetic DR-BB/E rats (n = 8) before pregnancy, at conception and during the first four days of pregnancy

Maternal metabolic control during pregnancy was also assessed by HbA1 measurement. Whole blood was taken by tail tipping (without anaesthesia) on day 4.5 of pregnancy and used to measure the blood glucose concentration and the percentage of glycosylated haemoglobin. The mean blood glucose and HbA1 values for SRII-treated Diabetic DP-, CIT-treated Diabetic DP- and Non-Diabetic DR-BB/E rats are shown in Table 3.20.
Table 3.20: Mean blood glucose (BG) concentration and HbA1 in SRII-treated Diabetic DP-, CIT-treated Diabetic DP- and Non-Diabetic DR-BB/E rats on day 4.5 of pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Mean blood glucose (mmol/L)</th>
<th>Mean HbA1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRII-treated Diabetic DP-BB/E (n = 15)</td>
<td>14.90 ± 2.2</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>(i) Post-conception BG rise (n = 10)</td>
<td>20.24 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6 ± 0.5</td>
</tr>
<tr>
<td>(ii) No Post-conception BG rise (n = 5)</td>
<td>4.12 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>CIT-treated Diabetic DP-BB/E (n = 10)</td>
<td>21.93 ± 1.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.3 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-Diabetic DR-BB/E (n = 8)</td>
<td>4.26 ± 0.4</td>
<td>3.1 ± 0.2</td>
</tr>
</tbody>
</table>

Data are means ± SEM. Statistical differences by Student’s t-test:

**Blood glucose concentration:**

<sup>a</sup> SRII-Diabetic rats with post conception (p.c.) BG rise significantly different from SRII-Diabetic with no p.c. BG rise (P < 0.0001), CIT-Diabetic (P < 0.05) and Non-Diabetic (P < 0.0001) rats.

<sup>b</sup> SRII-Diabetic rats with no p.c. BG rise significantly different from CIT-Diabetic rats (P < 0.0001)

<sup>c</sup> CIT-Diabetic significantly different from Non-Diabetic rats (P < 0.0001)

**HbA1:**

<sup>d</sup> Non-Diabetic rats significantly different from SRII-Diabetic (P < 0.001) and CIT-Diabetic (P < 0.0001) rats.
Table 3.21: Total number of morulae, early blastocysts, expanded blastocysts and unfertilised oocytes recovered from SRII-treated and CIT-treated Diabetic DP- and Non-Diabetic DR-BB/E rats at day 4.5 of gestation.

<table>
<thead>
<tr>
<th>Pregnancy Type</th>
<th>Morulae</th>
<th>Early Blastocysts</th>
<th>Expanded Blastocysts</th>
<th>Unfertilised Oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRII-Diabetic (n = 15)</td>
<td>7</td>
<td>14</td>
<td>86</td>
<td>19</td>
</tr>
<tr>
<td>(i) Post-conception BG rise (n =10)</td>
<td>6</td>
<td>10</td>
<td>45</td>
<td>19</td>
</tr>
<tr>
<td>(ii) No Post-conception BG rise (n =5)</td>
<td>1</td>
<td>4</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>CIT-Diabetic (n = 20)</td>
<td>13</td>
<td>26</td>
<td>110</td>
<td>1</td>
</tr>
<tr>
<td>Non-Diabetic (n = 20)</td>
<td>14</td>
<td>17</td>
<td>80</td>
<td>25</td>
</tr>
</tbody>
</table>
Despite achieving good maternal metabolic control prior to pregnancy, the mean HbA1 value for the SRII-treated Diabetic DP rats was not significantly different from CIT-treated Diabetic DP rats. When the HbA1 values for the SRII-treated rats were separated into those which had a post-conception blood glucose rise and those which did not, statistical difference was still not achieved. Thus, both types of insulin therapy, - SRII and CIT resulted in HbA1 values significantly greater than those for Non-Diabetic DR BB/E rats (P < 0.001 and P < 0.0001, respectively).

3.3.4 Morphology analysis

Pregnancy was continued until day 4.5 when the embryos were collected and the number of embryos at each morphological stage of development recorded (Table 3.21).

There was no significant difference in the morphological distribution of embryos at day 4.5 of pregnancy between SRII- and CIT-treated Diabetic DP rats. However, when only those SRII-treated rats with good metabolic control at conception and during pregnancy were compared with CIT-treated Diabetic DP rats there was a significant improvement in fertility (reflected as a decrease in the number of unfertilised oocytes). In addition, there was a significant increase in the number of expanded blastocysts per pregnancy (P < 0.001). Comparing SRII-treated Diabetic DP rats with and without the post-conception blood glucose rise there were significantly more expanded blastocysts per pregnancy in SRII-Diabetic DP rats with no post-conception blood glucose rise (45 expanded blastocysts from 10 pregnancies with post-conception blood glucose rise vs 41 expanded blastocysts from 5
pregnancies with no post-conception blood glucose rise, \( P < 0.05 \). Moreover, pregnancies from SRII-treated Diabetic DP rats were not significantly different from Non-Diabetic DR pregnancies with respect to the number of morulae, early blastocysts, expanded blastocysts or unfertilised oocytes.

### 3.3.5 Cell lineage analysis

Expanded blastocysts were collected from each individual pregnancy on day 4.5 of gestation and differentially labelled to determine the number of inner cell mass and trophectoderm cells (Table 3.22)

<table>
<thead>
<tr>
<th>Type of Pregnancy</th>
<th>No. of Embryos</th>
<th>ICM cell number</th>
<th>TE cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRII-treated Diabetic DP (n = 15)</td>
<td>74</td>
<td>8.00 ± 0.45 *</td>
<td>19.43 ± 0.48</td>
</tr>
<tr>
<td>(i) Post-conception BG rise (n = 10)</td>
<td>38</td>
<td>8.00 ± 0.41 *</td>
<td>19.66 ± 0.44</td>
</tr>
<tr>
<td>(ii) No Post-conception BG rise (n = 5)</td>
<td>36</td>
<td>8.00 ± 0.48 *</td>
<td>19.19 ± 0.51</td>
</tr>
<tr>
<td>CIT-treated Diabetic DP a</td>
<td>57</td>
<td>8.00 ± 0.25 *</td>
<td>20.23 ± 0.41</td>
</tr>
<tr>
<td>Non-Diabetic DR b</td>
<td>43</td>
<td>10.40 ± 0.25</td>
<td>19.77 ± 0.59</td>
</tr>
</tbody>
</table>

Where a indicates same data as Table 3.3 and b same data as shown in Table 3.2.

Data expressed as mean ± SEM. * \( P < 0.0001 \): significantly less than blastocysts from Non-Diabetic rats (Student’s t test)
Blastocysts from SRII-treated Diabetic DP rats were found to exhibit the same disturbed development as that observed previously in poorly controlled CIT-treated Diabetic DP rats, namely a 20% cellular deficit in the ICM, despite achieving good maternal metabolic control (blood glucose 3-10 mmol/L) for at least one week prior to conception. Moreover, this 20% ICM cellular deficit was also found in blastocysts from pregnancies which did not exhibit a post-conception blood glucose rise.

3.3.5 Measurement of Progesterone

The hyperglycaemic peak which develops over the period of conception could be associated with the physiological rise in progesterone which occurs at this time. A third group of young female Diabetic DP-BB/E rats (n = 19) were treated with SRII from the day of diabetes onset. Metabolic control was assessed by daily blood glucose measurements (taken at 10 a.m. each morning), and as described earlier, good metabolic control was defined as blood glucose readings consistently between 3-10 mmol/L. Rats which did not achieve good metabolic control a minimum of two weeks prior to conception were rejected from the study. Ten of the SRII-treated Diabetic DP rats were excluded because of failure to achieve good metabolic control. The remaining nine females with good metabolic control were mated with fertile Non-Diabetic DR rats. Serum samples were taken at day 0.5 and 2.5 of pregnancy and the concentration of progesterone was measured by the laboratory of Professor S. Hillier, Centre for Reproductive Biology, University of Edinburgh. There was a four-fold increase in progesterone between day 0.5 and day 2.5 of pregnancy (26.0 vs 108.4 nmol/L, P < 0.001) (Table 3.23).
Table 3.23: Serum progesterone concentration in SRII-treated Diabetic DP-BB/E rats

<table>
<thead>
<tr>
<th>Pregnancy Type</th>
<th>Progesterone concentration (nmol/L)</th>
<th>P value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 0.5</td>
<td>day 2.5</td>
</tr>
<tr>
<td>SRII-Diabetic (n = 9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) Post-conception BG rise (n = 6)</td>
<td>26.0 ± 3.9</td>
<td>108.4 ± 15</td>
</tr>
<tr>
<td>(ii) No Post-conception BG rise (n = 3)</td>
<td>28.3 ± 5.7</td>
<td>106.7 ± 14</td>
</tr>
<tr>
<td>Pseudopregnant (n = 5)</td>
<td>21.4 ± 1.8</td>
<td>111.9 ± 43</td>
</tr>
</tbody>
</table>

Data expressed as means ± SEM and where n is the number of animals in each group and BG denotes blood glucose.

Of the nine SRII-treated Diabetic DP rats with good metabolic control, six had a dramatic rise in the blood glucose level (range 20-25 mmol/L) at or following conception previously observed in SRII treatment, whilst the remaining three rats had blood glucose readings within the target range during and following conception. As shown in Table 3.23 there was a four-fold increase in progesterone in response to conception in all groups of SRII-treated Diabetic DP rats, although statistical significance was not achieved in the smallest group because of large standard errors. These data demonstrate that the reason for the hyperglycaemic peak in some of the SRII-treated Diabetic DP rats was not simply due to these rats producing more progesterone, - but presumably due to insufficient insulin.
Serum progesterone was also measured in SRII-treated Diabetic DP rats mated with vasectomised males. Although statistical significance was less (P < 0.05) there was still a four-fold increase in progesterone between day 0.5 and 2.5 in these rats, demonstrating that there was a hormonal response even in the absence of fertilisation.

**Conclusions from Experiment III**

(i) **Assessing SRII as an alternative insulin-delivery system**

Establishing good metabolic control from diabetes onset in female rats proved to be very difficult. Despite the fact that all of the rats had approximately the same body weight at diabetes detection (200 g) their insulin requirements were quite variable, with some animals requiring additional SRII. Occasionally too much insulin was given and the animals experienced long periods of hypoglycaemia (up to one week) and had to be excluded from the study.

Some rats had very erratic blood glucose readings, changing from 2.5 to 18 mmol/L over a period of 24 h for no apparent reason. The low dose LinBit implants were easier to use in young female rats than the regular dose Linplant as it was possible to ‘fine tune’ the dose of insulin by adding variably sized pieces of insulin rod.

Despite the problems the degree of metabolic control achieved in 15 female rats treated with SRII was significantly improved compared with CIT with respect to reducing the concentration of plasma glucose, ketones and glycerol closely approaching but not quite achieving the normal range for Non-Diabetic DR rats.
(ii) Assessing SRII as an alternative insulin-delivery system to prevent IDDM-induced embryopathy in pregnancy

As described earlier, pregnancy in the Diabetic DP-BB/E rat treated with conventional insulin therapy is associated with a severe disturbance in embryo development, namely (i) a five-fold increase in the number of unfertilised oocytes, (ii) a reduced number of expanded blastocysts, and (iii) a -20 % ICM cellular deficit in embryos which reached the expanded blastocyst stage. The aim of this study was to determine whether this IDDM-induced embryopathy could be prevented using the sustained release insulin implant.

Comparing the morphological distribution at day 4.5 of pregnancy in SRII- and CIT-treated Diabetic DP rats there was no significant improvement. There was however, a significant improvement in fertility and the number of expanded blastocysts per pregnancy when only those SRII-treated rats with good metabolic control at conception and during pregnancy were compared with CIT-treated Diabetic DP rats ($P < 0.001$). Since all 15 rats had good metabolic control for at least one week before conception, this suggests that normal metabolic control at the time of conception and during the first few days of pregnancy was essential in determining successful pregnancy outcome.

However, blastocysts from all SRII-treated rats exhibited a 20 % cellular deficit in the inner cell mass, irrespective of maternal metabolic control at the time of conception. This suggests that either there are different aetiologies for these two pathologies, or
that the degree of metabolic control achieved in these rats was sufficient to restore fertility to normal but not sufficient to prevent the ICM-specific embryopathy.

In conclusion, these data suggest that despite vastly improved maternal metabolic control SRII-treatment was unable to prevent all of the IDDM-induced embryopathy observed in Diabetic DP-BB/E rats in early pregnancy. Given the fact that the blood glucose concentration during pregnancy in the Non-Diabetic DR-BB/E rat rarely falls below 3.0 or rises above 5.5 mmol/L, failure of SRII-treatment in preventing embryopathy may be due to the fact that the SRII-treated rats were not normoglycaemic. Alternatively, an additional unknown factor may be responsible for the ICM cellular deficit which although is associated with IDDM, is not directly related to the degree of hyperglycaemia.
3.4 Experiment IV: Investigating one possible mechanism of the ICM Cellular Deficit

Introduction

As described in section 3.1, cells with features characteristic of apoptosis were observed in differentially stained blastocysts from both Diabetic DP- and Non-Diabetic DR-BB/E rats. It appeared that these cells with fragmented nuclei were observed more often in the ICM than in the TE, and more often in Diabetic DP than Non-Diabetic DR rats, although it was not possible to accurately quantitate this phenomenon because of the limitations of using fluorescence microscopy (see Discussion).

The following experiments were designed to investigate whether the reduction in ICM cell number seen in blastocysts from Diabetic DP rats was due to disturbed regulation of apoptosis. Conventional methods of mRNA analysis such as Northern and dot blot hybridisation and nuclease mapping are not sensitive enough to detect mRNA in samples limited by either low cell number or low copy number per cell. *In situ* hybridisation allows detection of mRNA in single cells but is non-quantitative. The reverse-transcriptase polymerase chain reaction (RT-PCR) however, is a very powerful technique, and its unique sensitivity allows large amounts of complementary DNA (cDNA) for the gene of interest to be rapidly produced from very small amounts of starting material (302). The specificity and sensitivity of the PCR can be greatly enhanced by using a nested PCR protocol where a fraction of the first round of amplification is re-amplified with a new set of primers, complementary to the
sequence contained within the initial product. Therefore, the non-specific priming associated with the high number of PCR cycles required to amplify cDNA from single embryos can be avoided.

The first gene shown to be specifically involved in the process of physiological cell death was bcl-2 (303). bcl-2 is now known to belong to a growing family of apoptosis-regulating genes, which may either be death antagonists (bcl-2, bcl-XL, bcl-W, bfl-1, mcl-1 and aI) or death agonists (bax, bak, bcl-Xs, bad, bid, bik and hrk). The ratio of death antagonists to agonists is believed to determine whether a cell will respond to an apoptotic signal. This death-life rheostat is mediated, at least in part, by competitive dimerisation between selective pairs of antagonists and agonists (304, 305, 306, 307, 308).

The ratio of bcl-2 (a cell death antagonist gene) to bax (a cell death agonist gene) expression was determined in blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats using quantitative RT-PCR. This technique has the potential to reveal some of the earliest molecular events in the development of the rat embryo.

Quantitative RT-PCR is very difficult to achieve mainly because PCR amplification is an exponential process, and minute differences in any of the parameters that control the efficiency of the reaction can substantially affect the final yield of PCR product, making it difficult to quantitate the amount of mRNA in the original material (309). The use of RT-PCR to quantify the level of expression of a gene in terms of copy number requires the availability of a standard with characteristics as close as possible
to the target cDNA sequence - in particular, with regard to base composition and amplified product length.

Quantitative RT-PCR Strategy

Internal standards for each gene of interest were created by inserting the external primer PCR product into a commercial plasmid transcription vector - PCR2.1 (from Invitrogen), and modifying the insert by increasing its length by 5-8 nucleotides. The recombinant plasmid DNA was transcribed \textit{in vitro} to produce synthetic mRNA. Synthetic mRNA internal standards were created for the two genes of interest: \textit{bax} and \textit{bcl}-2, and \textit{\beta}-actin which served as a positive control for RNA quality. Figure 3.10 shows a schematic diagram outlining the creation of the synthetic \textit{\beta}-actin mRNA internal standard.

Total RNA was prepared from single pre-implantation embryos using a scaled-down protocol based on the method of Chomczynski and Sacchi (272). Known amounts of \textit{bax}, \textit{bcl}-2 and \textit{\beta}-actin synthetic mRNA internal standard were added to the embryo reverse transcription reaction and cDNA was then synthesised. One-tenth of this cDNA was amplified using the external pair of oligonucleotide primers, followed by a second round of amplification with the internal primer pair. The forward internal primers used for the second round of amplification were labelled at the 5' end using polynucleotide kinase and [\textit{\gamma}^{32}\text{P}]-ATP. After amplification, the PCR products were separated by polyacrylamide gel electrophoresis and visualised by autoradiography. Radioactive signals were quantified by a phosphorimager (Molecular Dynamics) by measuring intensity of each cDNA band.

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Figure 3.10:  Construction of the β-actin internal standard

(i)  PCR Amplification

Forward external primer

5' ... 3'

Reverse external primer

(ii) Cloning

Insert  Vector (PCR2.1)

(iii) Digestion with BstEII

(iv) Filling

(v) Ligation

(vi)  *In vitro* transcription  mRNA

<table>
<thead>
<tr>
<th>mRNA standards</th>
<th>mRNA size</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>873 bp</td>
<td>204 bp</td>
</tr>
<tr>
<td>Mutant</td>
<td>868 bp</td>
<td>209 bp</td>
</tr>
</tbody>
</table>
Results

3.4.1 RT-PCR of single pre-implantation embryos

Before the embryo mRNA quantification experiments could begin the RT-PCR protocol had to be optimised for the external and internal pairs of bax, bcl-2 and β-actin primers. β-actin served as a positive control for RNA quality. Initially all optimisation was carried out using ovary RNA from normal Sprague-Dawley rats, reported to have high levels of bax and bcl-2 gene expression (310). Conditions which required optimisation included concentrations of enzyme, dNTPs, magnesium ions, and primers; amount of starting material (cDNA); temperatures of denaturation, annealing, and extension; and cycle number. Once the PCR conditions had been set total RNA was prepared from single pre-implantation embryos (morula, early blastocyst and expanded blastocyst) from Non-Diabetic DR-BB/E rats. Half of the total RNA was reverse transcribed and amplified using the nested PCR protocol described in section 2.2.9 of Materials and Methods. After amplification one-fifth of the reaction mixture was separated through a 2% agarose gel and visualised by ethidium bromide staining. A single band of the predicted size was observed for each reaction (bax 372 bp; bcl-2 329 bp; β-actin 747 bp) (Figure 3.11). The negative controls used were amplification of ‘extracted’ embryo culture media, or amplification without added template. No cDNA product was observed in these lanes indicating that there was no contamination with extraneous cDNA or RNA.
Figure 3.11:

Agarose gel showing the reverse transcription (RT)-PCR amplified products from RNA derived from single pre-implantation embryos from Non-Diabetic DR-BB/E rats using bax, bcl-2 and β-actin specific primers.

where:  

- m = molecular weight marker (1000, 740, 341, 258, 219 and 105 bp)  
- a = ‘No RNA’ (negative control)  
- b = ‘No RNA’ + glycogen (negative control)  
- c = ovary (positive control)  
- d = single morula  
- e = single early blastocyst  
- f = single expanded blastocyst

The size of the expected RT-PCR products are shown in base pairs (bp)
The RT-PCR experiments using bax, bcl-2 and β-actin primers were repeated a minimum of three times with pre-implantation embryos at each stage. These data demonstrate for the first time that bax and bcl-2 messages are present in pre-implantation embryos.

The identity of the specific RT-PCR products was confirmed by DNA sequencing and matched the published sequences in the GenBank database (276, 277, 275) for bcl-2, bax and β-actin sequences respectively (see Appendix Figure 5.1, 5.2 and 5.3).

3.4.2 Preparation of internal standards

A 'TA' cloning kit (Invitrogen) was used to clone the bax, bcl-2 and β-actin RT-PCR products directly from PCR reactions into the vector PCR2.1. Ligation took advantage of the template-independent addition of a single deoxyadenosine (A) to the 3' ends of PCR products by thermostable polymerases such as Taq polymerase. The linearised vector with single deoxythymidine (T) residues at each end allowed the PCR products to ligate efficiently with the vector.

The recombinant colonies (i.e. those which contained the plasmid and insert) were selected by insertional inactivation. The enzyme β-galactosidase which breaks down lactose into glucose and galactose, is normally coded by the gene lac Z on the E.coli chromosome. The E.coli strain TOP 10F' had a modified lac Z gene referred to as lac Z' which codes for the α-peptide portion of the β-galactosidase. These mutants can synthesise the enzyme only when they harbour a plasmid that carries the missing lac Z' segment of the gene. The vector pCR2.1 contained the lacZα complementation
in addition to ampicillin and kanamycin resistant genes for selection. Insertional inactivation of the α-peptide allowed recombinant clones to be distinguished by their inability to synthesise β-galactosidase. Thus, the addition of ampicillin, X-gal (a lactose analogue which forms a blue product when broken down by β-galactosidase) and IPTG to the agar allowed distinction of colonies which were able to synthesise β-galactosidase (they were coloured blue) and recombinant colonies with a disrupted lac Z’ gene and unable to make β-galactosidase which were white.

Several white colonies were grown overnight in LB broth and plasmid DNA was prepared. The presence of the insert synthesised by RT-PCR in the plasmid (PCR2.1) was confirmed by restriction enzyme digestion at 37°C for 1 hour. Digestion of bax recombinant plasmids (pBaxPCR2.1) with BamHI yielded fragments of either 3979 and 414 bp or 4275 and 118 bp (depending on the orientation of the insert). Vector which did not contain the bax insert was distinguished from pBaxPCR2.1 as it yielded only one DNA band of 3939 bp after digestion. Digestion of bcl-2 recombinant plasmids (pBcl-2PCR2.1) with BglII yielded fragments of either 2241, 365 and 1722 bp or 2241, 533 and 1544 bp (depending on the orientation of the insert), vector which did not contain the bcl-2 insert yielded only two DNA bands of 2241 and 1659 bp after digestion. Similarly, digestion of β-actin recombinant plasmids (pActPCR2.1) with BglII yielded fragments of either 2241, 932 and 1474 bp or 2241, 285 and 2121 bp (depending on the orientation of the insert). Vector which did not contain the β-actin insert yielded only two DNA bands of 2241 and 1659 bp after digestion.
The products of enzyme digestion were analysed by agarose gel electrophoresis and the results shown in Figure 3.12 demonstrate the presence of the β-actin, bax or bcl-2 insert in representative examples of pActPCR2.1, pBaxPCR2.1 and pBcl-2PCR2.1 DNA digests.

As described in section 2.2.10.7 and shown in Figure 3.10 the size of the insert in the recombinant plasmids was altered either by adding a new restriction enzyme site or by filling in the 3' recessed ends after enzyme digestion. The mutated pBaxMutPCR2.1, pActMutPCR2.1 and pBcl-2MutPCR2.1 DNA was digested with appropriate restriction enzymes to distinguish between native and mutated recombinant plasmid DNA. Plasmids which contained the mutated β-actin insert (pActMutPCR2.1) no longer contained the BstEII restriction site and yielded only one DNA fragment after digestion, whilst the native plasmid (pActPCR2.1) yielded two fragments of 4509 and 139 bp after enzyme digestion.

Plasmids which contained the mutated bcl-2 insert (pBcl-2MutPCR2.1) were identified by the presence of a new restriction site in the insert. Thus, digestion of the plasmids with XbaI yielded two DNA fragments (4037 and 299 bp) from pBcl-2MutPCR2.1 and only one from native pBcl-2PCR2.1 plasmids (4328 bp).

The presence of the mutated insert in pActMutPCR2.1 and pBcl-2MutPCR2.1 was also verified by DNA sequencing. Partial nucleotide sequences of these plasmids are shown in the Appendix (Figure 5.4 and 5.5).
Figur 3.12:

Agarose gel showing the verification of recombinant plasmid DNA.

Panel A: Plasmid PCR2.1 containing the bax insert digested with BamHI, yielding two fragments of 3979 bp and 414 bp (lane 1) or 4275 bp and 118 bp (lane 2).
Panel B: Plasmid PCR2.1 containing the bcl 2 insert digested with BglII, yielding three fragments of 2241 bp, 1722 bp and 365 bp (lane 1).
Panel C: Plasmid PCR2.1 containing the β-actin insert digested with BglI, yielding three fragments of 2241 bp, 221 bp and 285 bp (lane 1).

Lane M: Molecular weight marker (1000, 740, 341, 258, 219, 105 bp)
To distinguish between plasmids which contained the mutated bax insert (pBaxMutPCR2.1) and native plasmids (pBaxPCR2.1), plasmid DNA was amplified with bax internal primers and the products were separated by polyacrylamide gel electrophoresis. As shown in Figure 3.13A, PCR amplification of several clones containing pBaxMutPCR2.1 resulted in PCR products of a slightly smaller or larger size than the native pBaxPCR2.1. Plasmid DNA prepared from bacterial clone number 6 was chosen to become the bax DNA internal standard as there was a good separation between the mutant and native plasmid PCR products.

3.4.3 Quantitative RT-PCR

Quantitative RT-PCR is based on the assumption that cDNA synthesis efficiency is identical for both the target mRNA (a fragment of specific mRNA from the gene to be analysed, contained within the preparation of total RNA) and standard mRNA (a fragment of recombinant synthesised RNA sharing identical primer binding sequences with the target RNA, used as an internal control) (311). Thus, after reverse transcription the ratio of target to standard cDNA molecules should be equal to the initial ratio of target to standard cDNA molecules, and this ratio should remain unchanged throughout the PCR amplification process. To achieve real quantification by this method many of the variables of RT-PCR which can affect reaction efficiency have to be controlled as much as possible (concentrations of dNTPs, cDNA, magnesium ions, and primers and temperatures of denaturation, annealing, and extension). Those variables particularly critical to quantitative RT-PCR relate to the amount of starting material - mRNA, cDNA, and the ratio of standard to target RNA.
**Figure 3.13:** Screening pBaxMutPCR2.1 mutant recombinant plasmids by PCR

**A**

In the autoradiograph shown here, lanes 1-11 correspond to PCR of pBaxMutPCR2.1 plasmid clones 1-11 and lane 12: PCR of Native bax plasmid (pBaxPCR2.1) DNA.

**B**

Autoradiograph showing results of PCR amplification of pBaxPCR2.1 DNA and three pBaxMutPCR2.1 plasmid clones. Lane 1 corresponds to pBaxPCR2.1 and pBaxMutPCR2.1 clone #9; lane 2: pBaxPCR2.1 plasmid DNA and pBaxMutPCR2.1 clone #5; lane 3: pBaxPCR2.1 plasmid DNA and pBaxMutPCR2.1 clone #6.
It was also necessary to determine the number of PCR cycles which would generate a product proportional to the cDNA added to the reaction, and ensure that there was good separation of the standard and target RT-PCR products by polyacrylamide gel electrophoresis. Non-specific PCR products were prevented by increasing the primer annealing temperature and the concentration of primers to 100 pmoles. Other conditions requiring optimisation included gel running time, amount of product loaded onto the gel and gel exposure time to X-ray film.

3.4.3.1 Validation of quantitative RT-PCR method

(i) Using DNA internal standards

In order to verify that the efficiency of amplification was equivalent for the standard and the target, preliminary experiments were carried out demonstrate that (1) the internal standard could be titrated over a range of concentrations, and (2) the presence of target cDNA did not affect the amplification of the internal standard.

In Figure 3.14A a nested PCR using primers specific for bax is shown. Each reaction contains a constant amount of ovary (target) cDNA amplified with increasing concentrations of DNA internal standard (pBaxMutPCR2.1) in triplicate. The PCR products were separated by denaturing polyacrylamide gel electrophoresis and visualised by autoradiography. The upper bands are amplified from ovary cDNA and the lower bands from the standard pBaxMutPCR2.1 DNA. The amount of $^{32}$P-labelled primer in each reaction was quantified by means of a Molecular Dynamics Phosphorimager system (as described in section 2.2.11.4 of Materials and Methods) and the intensity (measured in arbitrary units) of the target cDNA and standard DNA
Figure 3.14A:

Autoradiograph showing results of PCR amplification of both target (ovary) cDNA templates and pBaxMutPCR2.1 standard DNA templates with increasing amounts of pBaxMutPCR2.1 standard DNA using bax specific primers. Amounts of pBaxMutPCR2.1DNA were 1.92 pg (lanes 1-3); 3.84 pg (lanes 4-6); 7.68 pg (lanes 16-18).
bands were calculated (shown in Table 3.24) and plotted against the amount of standard DNA (pBaxMutPCR2.1) added to the reaction (Figure 3.14B). Regression analysis of the curves showed that for the pBaxMutPCR2.1 DNA standard curve $R^2 = 0.9871$ and for the ovary cDNA $R^2 = 0.0024$.

The ratios of standard DNA/ovary cDNA band intensity were plotted against amount of standard pBaxMutPCR2.1 DNA and an exponential curve was produced (Figure 3.14C). These data confirm that the efficiencies of amplification of the target and standard were equal under these conditions and there was a linear relationship with increasing amounts of standard pBaxMutPCR2.1 between 7 pg and 20 pg.

<table>
<thead>
<tr>
<th>pBaxMutPCR2.1 DNA concentration (pg)</th>
<th>pBaxMutPCR2.1 DNA band intensity</th>
<th>Ovary cDNA band intensity</th>
<th>Ratio DNA standard/ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.92</td>
<td>38.06 ± 2.45</td>
<td>160.3 ± 8.5</td>
<td>0.24</td>
</tr>
<tr>
<td>3.84</td>
<td>60.66 ± 2.5</td>
<td>182.8 ± 2.5</td>
<td>0.33</td>
</tr>
<tr>
<td>7.68</td>
<td>122.3 ± 11.5</td>
<td>221.7 ± 2.5</td>
<td>0.55</td>
</tr>
<tr>
<td>11.52</td>
<td>249.8 ± 5.1</td>
<td>203.3 ± 6.3</td>
<td>1.23</td>
</tr>
<tr>
<td>15.36</td>
<td>306.8 ± 9.7</td>
<td>186.5 ± 9.4</td>
<td>1.65</td>
</tr>
<tr>
<td>19.20</td>
<td>409.4 ± 4.3</td>
<td>171.0 ± 4.3</td>
<td>2.40</td>
</tr>
</tbody>
</table>
Figure 3.14B:

Analysis of bax PCR with constant ovary cDNA and variable concentrations of pBaxMutPCR2.1 DNA standard. Each reaction contains a constant amount of ovary (target) cDNA amplified with increasing concentrations of pBaxMutPCR2.1 DNA standard. Regression analysis of the curves showed that for the DNA standard curve $R^2 = 0.9871$ and for the ovary cDNA $R^2 = 0.0024$. 
Figure 3.14C:
Ratios of standard pBaxMutPCR2.1 DNA/ovary cDNA band intensity plotted against pBaxMutPCR2.1 DNA concentration.

Figure 3.15A shows a similar nested PCR, using β-actin specific primers. A constant amount of target (ovary cDNA) was amplified with increasing amounts of pActMutPCR2.1 DNA standard. The upper bands are amplified from the standard plasmid DNA and the lower bands from ovary cDNA. The intensity of each band was calculated (Table 3.25) and plotted against the concentration of standard pActMutPCR2.1 DNA (Figure 3.15B).
Figure 3.15 A:

Autoradiograph showing results of PCR amplification of both target (ovary) cDNA templates and pActMutPCR2.1 standard DNA templates with increasing amounts of pActMutPCR2.1 standard DNA using β-actin specific primers. Amounts of pActMutPCR2.1 DNA were 19.20 pg (lanes 1-3); 9.50 pg (lanes 4-6); 3.84 pg (lanes 7-9); 1.92 pg (lanes 10-12); 0.38 pg (lanes 13-15).
Table 3.25: Mean intensity of pActMutPCR2.1 DNA and ovary cDNA after nested PCR using β-actin specific primers

<table>
<thead>
<tr>
<th>pActMutPCR2.1 DNA concentration (pg)</th>
<th>pActMutPCR2.1 DNA band intensity</th>
<th>Ovary cDNA band intensity</th>
<th>Ratio DNA standard/ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.38</td>
<td>26.08 ± 2.9</td>
<td>43.9 ± 6.8</td>
<td>0.59</td>
</tr>
<tr>
<td>1.92</td>
<td>120.13 ± 9.3</td>
<td>46.1 ± 8.1</td>
<td>2.60</td>
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<tr>
<td>3.84</td>
<td>249.47 ± 37</td>
<td>47.45 ± 3.9</td>
<td>5.26</td>
</tr>
<tr>
<td>9.50</td>
<td>390.63 ± 24.3</td>
<td>40.83 ± 1.1</td>
<td>9.57</td>
</tr>
<tr>
<td>19.20</td>
<td>485.93 ± 37.3</td>
<td>26.1 ± 2.6</td>
<td>18.62</td>
</tr>
</tbody>
</table>

Figure 3.15B: Analysis of β-actin PCR with constant ovary cDNA and variable concentrations of pActMutPCR2.1 DNA standard. Each reaction contains a constant amount of ovary (target) cDNA amplified with increasing concentrations of pActMutPCR2.1 DNA standard. Regression analysis of the curves showed that for the DNA standard curve $R^2 = 0.954$ and for the ovary cDNA $R^2 = 0.109$. 

Concentration of pActMutPCR2.1 DNA (pg)
Figure 3.15C:

Ratios of standard DNA/ovary cDNA band intensity plotted against pActMutPCR2.1 DNA concentration. A linear curve was produced where $R^2 = 0.9946$.

The results of a nested PCR, using bcl-2 specific primers is shown in Table 3.26 (autoradiograph not shown because signal was very weak). As described above, a constant amount of target (ovary) was amplified with increasing amounts of standard pBcl-2MutPCR2.1 DNA. The intensity of each band was plotted against the concentration of standard pBcl-2MutPCR2.1 DNA (Figure 3.16A).
Table 3.26: Mean intensity of pBcl-2MutPCR2.1 DNA and ovary cDNA after nested PCR using bcl-2 specific primers

<table>
<thead>
<tr>
<th>pBcl-2MutPCR2.1 DNA concentration (pg)</th>
<th>pBcl-2MutPCR2.1 DNA band intensity</th>
<th>Ovary cDNA band intensity</th>
<th>Ratio DNA standard/ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.92</td>
<td>2.68 ± 1.1</td>
<td>9.48 ± 2.8</td>
<td>0.28</td>
</tr>
<tr>
<td>3.84</td>
<td>8.05 ± 3.5</td>
<td>16.50 ± 4.3</td>
<td>0.49</td>
</tr>
<tr>
<td>7.68</td>
<td>30.1 ± 5.2</td>
<td>16.44 ± 1.8</td>
<td>1.83</td>
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<tr>
<td>11.52</td>
<td>57.86 ± 4.3</td>
<td>15.42 ± 3.5</td>
<td>3.75</td>
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<tr>
<td>15.36</td>
<td>63.19 ± 1.2</td>
<td>11.10 ± 2.0</td>
<td>6.00</td>
</tr>
<tr>
<td>19.20</td>
<td>88.84 ± 3.2</td>
<td>11.35 ± 0.8</td>
<td>7.83</td>
</tr>
</tbody>
</table>
Concentration of pBcl-2MutPCR2.1 standard DNA (pg)

Figure 3.16A:
Analysis of bcl-2 PCR with constant ovary cDNA and variable concentrations of pBcl-2MutPCR2.1 standard DNA. Each reaction contains a constant amount of ovary (target) cDNA amplified with increasing concentrations of pBcl-2MutPCR2.1 standard DNA. Regression analysis of the curves showed that for the DNA standard curve $R^2 = 0.981$ and for the ovary cDNA $R^2 = 0.048$.

The ratios of standard/ovary band intensity were plotted against amount of pBcl-2MutPCR2.1 plasmid DNA and an linear curve was produced, and are shown in Figure 3.16B.
Figure 3.16B:

Ratios of band intensity (standard pBcl-2MutPCR2.1 DNA/ ovary cDNA) plotted against pBcl-2MutPCR2.1 DNA concentration. An exponential curve was produced where $R^2 = 0.981$.

These figures (3.14, 3.15 and 3.16) are representative examples of at least two independent repeat experiments. These results confirm that for each of the genes the internal standard could be titrated over a range of concentrations, and the presence of target cDNA did not affect the amplification of the internal DNA standard.
(ii) Using mRNA internal standards

The plasmids pActMutPCR2.1, pBaxMutPCR2.1 and pBcl-2MutPCR2.1 were transcribed in vitro to produce synthetic mRNA. To be certain that no contaminating template DNA remained after transcription and isolation of the synthetic message, an aliquot of the transcription reaction (after the DNase treatment) was subjected to electrophoresis through agarose. No template DNA was detected after ethidium bromide staining.

To determine the optimal amount of mRNA internal standard to add to embryo RNA, RT-PCR was carried out using a constant amount of blastocyst (target) total RNA and variable amounts of internal standard mRNA. When the synthetic internal standard/endogenous RNA signal is plotted against the concentration of the standard a hyperbolic curve is seen. The most accurate measurement is taken in the linear portion of the curve, where the signal ratios are most similar (309). A representative example of this is shown in Figure 3.17A. Five different concentrations of β-actin synthetic mRNA standard (854 fg, 1.7 pg, 3.4 pg, 6.8 pg, and 13.6 pg) were reverse transcribed and amplified with embryo total RNA (prepared from 5 pooled blastocysts so that each RT reaction contained embryo RNA equivalent to half a single blastocyst). Each reaction was performed in duplicate. Since one single blastocyst provides sufficient RNA for only two RT reactions it was necessary to use pooled blastocysts for this part of the procedure, mainly to ensure that each RT reaction contained RNA of equal quality and quantity. Table 3.27 shows the phosphorimage analysis data and Figure 3.17B shows the standard/embryo RNA ratios plotted against the concentration of standard mRNA.
Figure 3.17A:

Results of RT-PCR showing co-amplification of β-actin mRNA internal standard and blastocyst Actin mRNA. Amounts of Actin mRNA internal standard added were 854 fg (lanes 1 and 2); 1.7 pg (lanes 3 and 4); 3.4 pg (lanes 5 and 6); 6.8 pg (lanes 7 and 8); 13.6 pg (lanes 9 and 10).
### Table 3.27: Results of phosphorimager analysis of β-actin RT-PCR using synthetic β-actin mRNA internal standard co-amplified with blastocyst actin mRNA

<table>
<thead>
<tr>
<th>pg of mRNA standard added</th>
<th>mRNA standard band intensity</th>
<th>Embryo band intensity</th>
<th>Ratio of standard/embryo</th>
<th>Mean standard/embryo ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.854</td>
<td>14.49</td>
<td>126.8</td>
<td>0.114</td>
<td>0.1085</td>
</tr>
<tr>
<td>0.854</td>
<td>15.62</td>
<td>151.4</td>
<td>0.103</td>
<td></td>
</tr>
<tr>
<td>1.70</td>
<td>13.41</td>
<td>76.71</td>
<td>0.175</td>
<td>0.1795</td>
</tr>
<tr>
<td>1.70</td>
<td>22.64</td>
<td>123.1</td>
<td>0.184</td>
<td></td>
</tr>
<tr>
<td>3.4</td>
<td>89.14</td>
<td>161.9</td>
<td>0.551</td>
<td>0.5515</td>
</tr>
<tr>
<td>3.4</td>
<td>94.42</td>
<td>171.0</td>
<td>0.552</td>
<td></td>
</tr>
<tr>
<td>6.8</td>
<td>131.2</td>
<td>179.4</td>
<td>0.731</td>
<td>0.764</td>
</tr>
<tr>
<td>6.8</td>
<td>176.8</td>
<td>221.7</td>
<td>0.797</td>
<td></td>
</tr>
<tr>
<td>13.6</td>
<td>226.5</td>
<td>238.9</td>
<td>1.116</td>
<td>1.1055</td>
</tr>
<tr>
<td>13.6</td>
<td>251.8</td>
<td>230.0</td>
<td>1.095</td>
<td></td>
</tr>
</tbody>
</table>

From the phosphorimager data calculated in Table 3.28, the concentration of synthetic β-actin mRNA standard appropriate for quantitative RT-PCR of single blastocysts was calculated to be between 6.8 and 13.6 pg.
Figure 3.17B:

Synthetic β-actin mRNA standard/embryo mRNA band intensity ratios plotted against the concentration of synthetic β-actin standard mRNA. Five different concentrations of synthetic β-actin mRNA standard (854 fg, 1.7 pg, 3.4 pg, 6.8 pg, and 13.6 pg) were reverse transcribed and amplified with embryo total RNA and the autoradiograph band intensity ratios were plotted against synthetic β-actin standard mRNA concentration.

Since expression levels differ between genes, unique amounts of standard RNA were required for each gene under investigation. β-actin mRNA is a relatively abundant mRNA species and so it was likely that the amount of bax and bcl-2 internal standard mRNA required to give an endogenous/standard RNA ratio of approximately 1.0 would be considerably less. Four concentrations of synthetic bax mRNA standard
(68 fg, 6.8 fg, 3.4 fg and 1.7 fg i.e. 100x, 1000x, 2000x and 4000x less than β-actin mRNA standard respectively) were reverse transcribed and amplified with embryo total RNA (prepared from 5 pooled blastocysts - each RT reaction contained embryo RNA equivalent to half a single blastocyst). Each reaction was performed in duplicate. From the phosphorimager data calculated in Table 3.28, Figure 3.18A was plotted and the concentration of synthetic bax mRNA standard appropriate for quantitative RT-PCR of single blastocysts was calculated to be approximately 3.4 fg. A representative autoradiograph is shown in Figure 3.18B.

Table 3.28:  Results of phosphorimager analysis of bax RT-PCR using synthetic bax mRNA internal standard co-amplified with blastocyst bax mRNA

<table>
<thead>
<tr>
<th>fg of mRNA standard added</th>
<th>bax mRNA standard band intensity</th>
<th>Embryo band intensity</th>
<th>Ratio of standard/embryo</th>
<th>Mean standard/embryo ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7</td>
<td>2.8</td>
<td>12.0</td>
<td>0.23</td>
<td>0.3</td>
</tr>
<tr>
<td>1.7</td>
<td>6.9</td>
<td>18.7</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>3.4</td>
<td>14.3</td>
<td>15.7</td>
<td>0.91</td>
<td>0.85</td>
</tr>
<tr>
<td>3.4</td>
<td>12.9</td>
<td>16.3</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>6.8</td>
<td>19.2</td>
<td>12.3</td>
<td>1.56</td>
<td>1.73</td>
</tr>
<tr>
<td>6.8</td>
<td>28.3</td>
<td>14.9</td>
<td>1.90</td>
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</tr>
<tr>
<td>68</td>
<td>55.1</td>
<td>17.5</td>
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<td>3.64</td>
</tr>
<tr>
<td>68</td>
<td>68.1</td>
<td>16.5</td>
<td>4.13</td>
<td></td>
</tr>
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</table>
Figure 3.18A:
Synthetic bax mRNA standard/embryo mRNA band intensity ratios plotted against the concentration of synthetic bax standard mRNA. Four concentrations of synthetic bax mRNA standard (68 fg, 6.8 fg, 3.4 fg and 1.7 fg) were reverse transcribed and amplified with embryo total RNA and the autoradiograph band intensity ratios were plotted against synthetic bax standard mRNA concentration.

The same procedure was repeated using four concentrations of synthetic bcl-2 mRNA standard (0.4 fg, 0.8 fg, 2.0 fg and 5.0 fg). Each internal standard was reverse transcribed and amplified with the bcl-2 primer pairs. From the phosphorimager data shown in Table 3.29 Figure 3.19A was plotted and the concentration of synthetic bcl-2 mRNA standard appropriate for quantitative RT-PCR of single blastocysts was calculated to be approximately 5.0 fg. A representative autoradiograph is shown in Figure 3.19B.
Figure 3.18 B:

Results of RT-PCR showing co-amplification of bax mRNA internal standard and blastocyst bax mRNA. Amounts of bax mRNA internal standard added were:

Panel (A): 68 fg (lanes 1 and 2); 6.8 fg (lanes 3 and 4); 3.4 fg (lanes 5 and 6); 1.7 fg (lanes 7 and 8).

Panel (B): 3.4 fg (lanes 1 and 2)

Figure 3.19B:

Results of RT-PCR showing amplification of bcl.2 mRNA internal standard. Lane 1 corresponds to 0.4 fg standard mRNA; lane 2: 0.8 fg standard mRNA; lane 3: 2.0 fg standard mRNA; lane 4: 5.0 fg standard mRNA; lanes 5 and 6: 5.0 fg standard mRNA co-amplified with blastocyst bcl.2 mRNA.
Table 3.29: Results of phosphorimager analysis of bcl-2 RT-PCR using synthetic bcl-2 mRNA internal standard co-amplified with blastocyst bcl-2 mRNA

<table>
<thead>
<tr>
<th>fg of bcl-2 mRNA standard added</th>
<th>bcl-2 mRNA standard band intensity</th>
<th>Embryo band intensity</th>
<th>Ratio of standard/embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>1.2 ± 0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.8</td>
<td>7.8 ± 1.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.0</td>
<td>50.5 ± 1.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>162.8 ± 4.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>176.1 ± 1.9</td>
<td>145.9 ± 5.9</td>
<td>1.22</td>
</tr>
</tbody>
</table>

Figure 3.19A:

Analysis of bcl-2 RT-PCR with variable concentrations of synthetic bcl-2 mRNA internal standard. Four concentrations of synthetic bcl-2 mRNA standard (0.4 fg, 0.8 fg, 2.0 fg and 5.0 fg) were reverse transcribed and amplified with the bcl-2 primer pairs. From the phosphorimager data shown in Table 3.29 Figure 3.19A was plotted and the concentration of synthetic bcl-2 mRNA standard appropriate for quantitative RT-PCR of single blastocysts was calculated to be approximately 5.0 fg.
3.4.3.2 Quantification of bax and bcl-2 gene expression in the single blastocyst

The preliminary experiments described above have demonstrated that under the described RT-PCR conditions the levels of bax, bcl-2 and β-actin mRNA can be detected in blastocysts and ovaries from non-diabetic rats. Moreover, experiments which involved the titration of standard mRNA for the three genes has identified the optimum amount of standard to add to each RT reaction giving a ratio of standard : endogenous embryo RNA ratio of approximately 1.0, and additional analysis has shown that the amplification of target mRNA was not compromised by the presence of standard mRNA within a range of concentrations. Having established this, experiments could then be carried out comparing the expression of bax, bcl-2 and β-actin genes in single blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats.

Experimental Protocol

To reduce the inter-assay variation, each stage of the analysis of the embryos from Diabetic DP- and Non-Diabetic DR-BB/E rats was carried out on the same day. Total RNA was prepared from eight single blastocysts (four from Diabetic DP- and four from Non-Diabetic DR-BB/E rats). Ten individual RT reactions were set up: eight contained half of the RNA from a single embryo plus the three synthetic mRNA internal standards (6.8 pg β-actin, 3.4 fg bax and 5.0 fg bcl-2 synthetic mRNA). Parallel reactions, containing (1) embryo RNA alone (no internal standard mRNA), and (2) no RNA and no standard mRNA, as negative controls were amplified simultaneously. The RNA was reverse transcribed and amplified firstly using the β-actin nested PCR protocol. The RT-PCR products were separated by polyacrylamide gel electrophoresis. Once the RNA quality had been checked with β-actin, the same
cDNAs were amplified using the bax primers, and the products separated by polyacrylamide gel electrophoresis and analysed by means of a phosphorimager. The same procedure was followed using the bcl-2 specific primers.

**Results**

The RT-PCR products were visualised by autoradiography and are shown in Figure 3.20 (panels A, B and C: β-actin, bax and bcl-2 RT-PCR experiments respectively). The intensity of the bands on the gels was analysed by means of a phosphorimager and since the amount of internal standard added to the starting reaction (i.e. the RT reaction) is known, the amount of endogenous β-actin, bax and bcl-2 mRNA was calculated. Tables 3.30, 3.31 and 3.32 show the amount of endogenous β-actin, bax and bcl-2 mRNA in single blastocysts for the four Diabetic DP- and four Non-Diabetic DR-BB/E rats. An example of how the data were calculated is shown at the bottom of each table.
Figure 3.20: Quantification of β-actin, bax and bcl-2 mRNA in single pre-implantation embryos from Non-Diabetic DR and Diabetic DP-BB/E rats

A

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

B

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

C

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Autoradiographs showing the detection of β-actin mRNA (Panel A), bax mRNA (Panel B) and bcl-2 mRNA in single blastocysts from Diabetic DP-BB/E rats (lanes 1-8) and Non-Diabetic DR-BB/E rats (lanes 9-16) on day 4.5 of gestation. Lane 17: blastocyst mRNA amplified without internal standard; lane 18: no RNA negative control.
The data presented in Table 3.30 show that the level of β-actin mRNA expression in the single blastocysts ranged from 10.52 to 19.0 pg. These data can also be expressed as the number of copies of mRNA per nanogram of total RNA (1 mol = 6.02 x 10^{23} Avagadro’s constant). Copy numbers ranging from 2.4 x 10^{7} to 4.4 x 10^{7} were calculated.

**Table 3.30: Analysis of β-actin gene expression in single blastocysts from Non-Diabetic DR- and Diabetic DP- BB/E rats**

<table>
<thead>
<tr>
<th>Blastocysts from:</th>
<th>Embryo (E) Peak height</th>
<th>Standard (S) Peak height</th>
<th>Ratio (E/S)</th>
<th>Mean E/S ratio</th>
<th>β-actin mRNA (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Diabetic DR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>N/D</td>
<td>82.12</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
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<tr>
<td>#2</td>
<td>110.8</td>
<td>144.5</td>
<td>0.767</td>
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<tr>
<td>#3</td>
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<td>137.0</td>
<td>170.1</td>
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</tr>
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<td>#1</td>
<td>147.8</td>
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<td>159.5</td>
<td>1.373</td>
<td>1.396</td>
<td>19.0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>203.6</td>
<td>143.5</td>
<td>1.419</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#2</td>
<td>127.9</td>
<td>110.0</td>
<td>1.163</td>
<td>1.185</td>
<td>16.12</td>
</tr>
<tr>
<td>#4</td>
<td>163.1</td>
<td>135.2</td>
<td>1.206</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Embryo alone</td>
<td>213.2</td>
<td>11.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\( ^a \) where N/D denotes not detected

**Calculation of β-actin mRNA in single blastocysts:**

6.8 pg of synthetic mRNA standard was added to ½ blastocyst RNA

For blastocyst #1 (from a Non-Diabetic DR rat) Embryo/Standard ratio = 0.781

\( \therefore 0.781 \times 6.8 = 5.31 \) pg per half blastocyst or 10.6 pg per whole blastocyst
The data presented in Table 3.31 show that the level of bax mRNA expression in the single blastocysts ranged from 4.1 to 6.02 fg. These data can also be expressed as the number of copies of mRNA per nanogram of total RNA, copy numbers ranging from $1.5 \times 10^4$ to $2.1 \times 10^4$ were calculated.

**Table 3.31: Analysis of bax gene expression in single blastocysts from Non-Diabetic DR- and Diabetic DP- BB/E rats**

<table>
<thead>
<tr>
<th>Blastocysts from:</th>
<th>Embryo (E) Peak height</th>
<th>Standard (S) Peak height</th>
<th>Ratio (E/S)</th>
<th>Mean E/S ratio</th>
<th>bax mRNA (fg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Diabetic DR #1</td>
<td>N/D</td>
<td>28.13</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>Non-Diabetic DR #2</td>
<td>62.68</td>
<td>79.03</td>
<td>0.793</td>
<td>0.841</td>
<td>5.72</td>
</tr>
<tr>
<td>Non-Diabetic DR #3</td>
<td>54.45</td>
<td>90.71</td>
<td>0.600</td>
<td>0.636</td>
<td>4.32</td>
</tr>
<tr>
<td>Non-Diabetic DR #4</td>
<td>59.66</td>
<td>71.49</td>
<td>0.835</td>
<td>0.882</td>
<td>6.0</td>
</tr>
<tr>
<td>Diabetic DP #1</td>
<td>87.58</td>
<td>134.9</td>
<td>0.650</td>
<td>0.614</td>
<td>4.18</td>
</tr>
<tr>
<td>Diabetic DP #2</td>
<td>77.56</td>
<td>113.7</td>
<td>0.682</td>
<td>0.680</td>
<td>4.62</td>
</tr>
<tr>
<td>Diabetic DP #3</td>
<td>91.24</td>
<td>96.99</td>
<td>0.941</td>
<td>0.886</td>
<td>6.02</td>
</tr>
<tr>
<td>Diabetic DP #4</td>
<td>86.30</td>
<td>96.45</td>
<td>0.895</td>
<td>0.886</td>
<td>6.02</td>
</tr>
<tr>
<td>Embryo alone</td>
<td>94.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* where N/D denotes not detected

**Calculation of bax mRNA in single blastocysts:**

3.4 fg of synthetic mRNA standard was added to $\frac{1}{2}$ blastocyst RNA

For blastocyst #1 (from a Non-Diabetic DR rat) Embryo/Standard ratio = 0.841

$\therefore 0.841 \times 3.4 = 2.86$ fg per half blastocyst or $5.72$ fg per whole blastocyst
The data presented in Table 3.32 show that the level of bcl-2 mRNA expression in the single blastocysts ranged from 3.05 to 7.76 fg. These data can also be expressed as the number of copies of mRNA per nanogram of total RNA, copy numbers ranging from $1.1 \times 10^4$ to $3.2 \times 10^4$ were calculated.

**Table 3.32: Analysis of bcl-2 gene expression in single blastocysts from Non-Diabetic DR- and Diabetic DP- BB/E rats**

<table>
<thead>
<tr>
<th>Blastocysts from:</th>
<th>Embryo (E) Peak height</th>
<th>Standard (S) Peak height</th>
<th>Ratio (E/S)</th>
<th>Mean E/S ratio</th>
<th>bcl-2 mRNA (fg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Diabetic DR</td>
<td>N/D 12.5</td>
<td>N/D 30.6</td>
<td>N/D 0.884</td>
<td>N/D 0.761</td>
<td>N/D 7.76</td>
</tr>
<tr>
<td>#1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Diabetic DR</td>
<td>40.2 45.5</td>
<td>8.8 13.8</td>
<td>0.638</td>
<td>0.903</td>
<td>9.21</td>
</tr>
<tr>
<td>#2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Diabetic DR</td>
<td>15.8 18.4</td>
<td>38.2 40.4</td>
<td>0.946</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Diabetic DR</td>
<td>10.5 22.9</td>
<td>8.9 25.6</td>
<td>0.348</td>
<td>0.404</td>
<td>4.12</td>
</tr>
<tr>
<td>#4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic DP</td>
<td>13.7 46.8</td>
<td>14.9 48.9</td>
<td>0.293</td>
<td>0.299</td>
<td>3.05</td>
</tr>
<tr>
<td>#1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic DP</td>
<td>15.5 36.9</td>
<td>16.3 38.4</td>
<td>0.420</td>
<td>0.422</td>
<td>4.30</td>
</tr>
<tr>
<td>#2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic DP</td>
<td>20.4 32.9</td>
<td>40.3 45.7</td>
<td>0.882</td>
<td>0.751</td>
<td>7.66</td>
</tr>
<tr>
<td>#3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic DP</td>
<td>35.1 48.1</td>
<td>32.9 47.8</td>
<td>0.688</td>
<td>0.709</td>
<td>7.23</td>
</tr>
<tr>
<td>#4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Embryo alone</td>
<td>40.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* where N/D denotes not detected

**Calculation of bcl-2 mRNA in single blastocysts:**

5.1 fg of synthetic mRNA standard was added to ½ blastocyst RNA

For blastocyst #1 (from a Non-Diabetic DR rat) Embryo/Standard ratio = 0.761

\[
0.761 \times 5.1 = 3.88 \text{ fg per half blastocyst or } 7.46 \text{ fg per whole blastocyst}
\]
From the level of bax and bcl-2 mRNA expression the ratio of bcl-2 to bax in the seven blastocysts was determined, and is presented in Table 3.33.

Table 3.33: Ratio of bcl-2 to bax mRNA in single blastocysts from Non-Diabetic DR- and Diabetic DP-BB/E rats at 4.5 days gestation.

<table>
<thead>
<tr>
<th>Blastocysts from:</th>
<th>Amount of bcl-2 mRNA (fg) per blastocyst</th>
<th>Amount of bax mRNA (fg) per blastocyst</th>
<th>Ratio (bcl-2 to bax)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Diabetic DR #1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Non-Diabetic DR #2</td>
<td>7.76</td>
<td>5.72</td>
<td>1.36</td>
</tr>
<tr>
<td>Non-Diabetic DR #3</td>
<td>9.21</td>
<td>4.32</td>
<td>2.13</td>
</tr>
<tr>
<td>Non-Diabetic DR #4</td>
<td>4.12</td>
<td>6.0</td>
<td>0.69</td>
</tr>
<tr>
<td>Diabetic DP #1</td>
<td>3.05</td>
<td>4.18</td>
<td>0.73</td>
</tr>
<tr>
<td>Diabetic DP #2</td>
<td>4.30</td>
<td>4.62</td>
<td>0.93</td>
</tr>
<tr>
<td>Diabetic DP #3</td>
<td>7.66</td>
<td>6.02</td>
<td>1.27</td>
</tr>
<tr>
<td>Diabetic DP #4</td>
<td>7.23</td>
<td>6.02</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Although the mean bcl-2 to bax ratio was slightly higher in the blastocysts from non-diabetic BB/E rats (1.393 ± 0.42 vs 1.033 ± 0.12 for Non-Diabetic DR- vs Diabetic DP-BB/E rats respectively) statistical significance was not achieved.
Conclusions from Experiment IV

RT-PCR analysis of pre-implantation embryos revealed the expression of \( bax \) and \( bcl-2 \) genes at all stages examined (morula through to expanded blastocyst stage). This is the first time the expression of \( bax \) and \( bcl-2 \) genes has been measured in pre-implantation embryos, or indeed at any stage of embryo development. However, there was no statistical difference between the mean \( bcl-2 : bax \) ratio in blastocysts from Diabetic DP- and Non-Diabetic DR-BB/E rats (1.033 ± 0.12 vs 1.393 ± 0.42, respectively). Data published by Oltvai et al (304) have shown that overexpressed Bax protein accelerates apoptotic death induced by cytokine deprivation in an IL-3-dependent cell line (RL7). Cells with a high \( Bcl-2 : Bax \) protein ratio of 2.04 possessed viable cells over 2 weeks following IL-3 deprivation, whereas cell lines with the lowest ratios (0.55) lost all viability by day 7. Although these results are calculated from levels of expressed protein in cultured cell lines and no direct comparison can be made, it is interesting that the ratio of \( bcl-2 \) to \( bax \) mRNA in single blastocysts was within the same range. Since only a few individual blastocysts were assayed for \( bax \) and \( bcl-2 \) gene expression statistical difference may be achieved with larger numbers of blastocysts from the Diabetic DP- and Non-Diabetic DR-BB/E groups.

There was however, a considerable difference in the \( \beta \)-actin copy number calculated from this study (2x10\(^7\)) and that previously reported by Gaudette and Crain (312) (1.4x10\(^5\)). The reasons for this discrepancy may lie in differences between the methods of quantification, the amount of \( \beta \)-actin in rat and mouse blastocysts, or errors in calculating the concentration of synthetic mRNA internal standard. The
standards prepared in the present work were prepared in large volumes, quantitated by spectroscopy, aliquoted and frozen in RNase free microcentrifuge tubes at -70°C.

Nevertheless, these data demonstrate for the first time that bax and bcl-2 messages are present in pre-implantation embryos and the method for quantifying the level of their expression has been established and can easily be applied to other systems where there is very little starting material.
Chapter Four

General Discussion

The mechanisms underlying the phenomena of (i) fetal early growth delay, (ii) congenital malformation, (iii) prematurity due to spontaneous pre-term labour, and (iv) babies small for gestational age in human autoimmune-induced IDDM pregnancy have yet to be precisely defined but circumstantial evidence suggests that they may result primarily from embryonic damage in the earliest stages of pregnancy. Experimental approaches in the study of human IDDM pregnancy are limited by practical and ethical considerations and this hypothesis has therefore been examined directly using the BioBreeding/Edinburgh (BB/E) rat,- which spontaneously develops autoimmune-induced IDDM,- as a model for the human disorder.

Pregnancy in the Diabetic DP-BB/E rat was associated with a five-fold increase in the number of unfertilised oocytes. This phenomenon could not be correlated with any particular male rat, indeed all the males used in this study had previously been shown to be consistently highly fertile when mated with female Non-Diabetic DR-BB/E rats. However, this finding in the Diabetic DP-BB/E rat supports the data published by Diamond et al (312) who reported that STZ-diabetic mice had a lower percentage of 2-cell zygotes (48 h after superovulation) than non-diabetic control mice. It has been suggested that appropriate metabolic control during the period of folliculogenesis may be necessary to avoid the development of embryopathy in the offspring of the diabetic mother (313). Diamond et al (312) also reported that oocytes from STZ-diabetic mice show significantly less germinal vesicle breakdown (GVB) (which signals the
resumption of meiosis I and oocyte maturation) when compared with non-diabetic mice (P < 0.001). When near normoglycaemia was achieved in these mice, the delay in oocyte maturation was reversed. No attempt was made to assess the degree of GVB in oocytes from Diabetic DP-BB/E rats but it is possible that poor or delayed oocyte maturation could be responsible for the high number of unfertilised oocytes in rats with established IDDM. Infertility is often a problem in poorly controlled diabetic women but fertility can be restored when metabolic control is improved (314). The importance of this and the work published by Diamond et al (312) is that the deleterious effects of poor metabolic control on oocyte maturation are potentially reversible, suggesting that the outcome of pregnancy in the Diabetic DP-BB/E rat could also be improved by better diabetic management prior to conception.

Studies have shown that non-diabetic human pregnancies, where the father has IDDM, have normal rates of perinatal mortality (approximately 2 %) (315). Similar findings were seen in experiments designed to investigate the effect of paternal diabetes on blastocyst development in BB/E rats. Non-Diabetic DR-BB/E rat pregnancies with Diabetic DP fathers demonstrated a normal morphological distribution of embryos at day 4.5, and normal numbers of ICM and TE cells in blastocysts from these pregnancies. In addition, there was no evidence to suggest that pregnancies generated from two Diabetic DP-BB/E parents had any more developmental disturbance than those where only the mother had diabetes.

Within the group of Diabetic DP-BB/E rats studied at 4.5 days gestation there appeared to be two distinct populations of pregnancies - one with a high incidence of
unfertilised oocytes and a reduced number of expanded blastocysts, and the other with no unfertilised oocytes and a normal complement (more than 6) of expanded blastocysts. The reasons for this phenomenon are as yet unknown. Comparing mothers from these two populations there was no difference in age at pregnancy, duration of diabetes and blood glucose concentration measured at 10 a.m. on the day when embryos were retrieved, the majority being >20 mmol/L. Closer examination showed that expanded blastocysts from these apparently normal pregnancies were in fact not normal since they exhibited an ICM cellular deficit similar to that described previously in rats with STZ-induced diabetes (316). The ICM cellular deficit was similar in pregnancies with and without unfertilised oocytes, suggesting a different aetiology for these two pathologies.

At 4.5 days gestation, the expanded blastocyst has two distinct cell lineages: the inner cell mass which forms all three germ layers of the fetus as well as complementary contributions to the extraembryonic membranes, and the trophectoderm which forms the placenta and the extraembryonic membranes (286). There is substantial in vitro evidence that the cells of the ICM are more sensitive than TE cells to damage inflicted by a range of chemicals, drugs and antimetabolites, such as [3H]-thymidine (317); X-rays (318); cyclophosphamide (319) and mitomycin-C (320) and that this is usually manifest as loss of ICM cells and suppression of differentiation of the ICM into primitive egg cylinders in vitro. Only the highest doses of metabolic inhibitors affect TE cell integrity and differentiation (320). The underlying cause of the differential sensitivity of the ICM to damage is not fully understood but it has been proposed that the high proliferative activity (321, 322) and the active metabolic status (323, 324)
needed to support its own growth and to control TE development may make the ICM cells more vulnerable. Normal blastocyst formation depends on adequate cellular interaction between ICM and TE cells (325). Thus, a diabetes-associated disturbance in any component of the intra-uterine milieu during the pre-implantation period is likely to affect the ICM more than TE cells, and to disturb the ICM-TE cell interaction.

The nature of the ICM cellular deficit was investigated further by culturing blastocysts in growth medium or conditioned medium, for 24 h *in vitro*. Blastocysts from Diabetic DP-BB/E mothers failed to show any sign of ‘catch up’ growth in the ICM, but under these conditions a TE cellular accretion also occurred. This is the first time that trophectoderm cellular accretion has been described in blastocysts from any species. However these results differ from *in vitro* culture studies reported by Pampfer et al (282) who used blastocysts from rats with STZ-induced diabetes to demonstrate a similar lack of ‘catch up’ growth in the ICM but also describe a ‐8.5 % decrease in TE cell number. There are however, several obvious differences between the two animal models which are listed below: (i) the rats used by Pampfer and colleagues were outbred Wistar rats with a much greater biological variation than the inbred BB/E rat colony, (ii) the outbred Wistar rats rendered diabetic by STZ injection were diabetic for approximately 14 days before pregnancy, whereas the Diabetic DP-BB/E rats had a mean duration of diabetes of 39 ± 2.7 days prior to pregnancy, (iii) the blood glucose concentration used to diagnose diabetes was lower for the STZ-induced diabetic rats at 11 mmol/L than for the DP-BB/E rats at 18 mmol/L (a blood glucose concentration of >18 mmol/L in the BB/E rat is invariably
associated with loss of weight, ketonuria, undetectable circulating endogenous insulin and the permanent need for daily treatment with insulin to ensure survival, the STZ-induced diabetic rats were not treated with insulin at any point prior to or during pregnancy), (iv) the preferred method of killing BB/E rats, particularly pregnant rats, is cervical dislocation,- Pampfer and colleagues however chose to ether anaesthetise their pregnant rats. Thus the difference in results obtained from the two models is likely to be related to be animal/model related as all of the experimental procedures used were identical. Since it is the inner cell mass which forms all three of the fetal germ layers, whilst the trophectoderm forms the placenta and the extra-embryonic membranes (286), it is possible that this combination of a reduction in ICM cell number and an increase in TE cell number in pre-implantation blastocysts could be a presage of the low birth weight pups and large placentae which has been previously described in Diabetic DP-BB/E rat pregnancies (258). These features also occur commonly in human IDDM pregnancy (287).

Although the method of preparing uterine or decidual cell conditioned media has been described several times (326, 327, 328) these authors have largely focused on later stages of pregnancy in the mouse (day 9.5 - 14.5 of pregnancy) and have assayed the concentration and activity of only one growth factor, transforming growth factor β-2 (TGFβ-2) in these conditioned media. Since this growth factor is involved in immunosuppression in later stages of post-implantation embryo development and is first detected in the mouse at day 9.5 of pregnancy, it is unlikely that significant amounts of TGFβ-2 were present in the uterine conditioned media from diabetic and non-diabetic BB/E rats in the present study. No attempt was made to assay the
uterine cell conditioned media for any growth factors or cytokines for several reasons: (i) the volume of conditioned media prepared from each uterus was very small and most assays required a minimum volume of 100 μl, (ii) a paper published by Bienvenu and colleagues (329) outlined substantial differences in sensitivity and behaviour of commercial ELISA-kits for cytokine analysis, and (iii) most of the commercially available kits for cytokine measurement are designed for mouse proteins and in the few kits that were tried there was poor cross-reactivity between the mouse monoclonal antibody and the rat protein (J.E McCracken and R.G Lea, unpublished observations).

The conditioned media prepared from BB/E rat uterine cells were unable to stimulate ‘catch-up’ growth of the ICM in blastocysts from diabetic rats, or to induce a deficit in the ICM of blastocysts from non-diabetic rats. This suggests that either the embryo damage which results in an ICM cell deficit occurs much earlier on in pregnancy and it is too late at 4.5 days gestation for the conditioned media to affect ICM development, or that the conditioned media did not contain growth factors and cytokines able to cause a developmental disturbance. Since the total cell counts of morulae and early blastocysts from diabetic rats were not different from embryos from Non-Diabetic DR rats, culturing morulae or early blastocysts in the uterine cell conditioned media may have tested the activity of the media to induce or prevent an ICM cell deficit/TE cell accretion more efficiently than culturing blastocysts.

The present study provided the opportunity to observe early pregnancy in the Diabetes Resistant BB/E subline, and compare it with that of outbred Wistar Han rats.
Despite the immunological abnormalities of the DR-BB/E subline, early experiments (Experiment I) showed that pregnancies in these rats were similar to those of standard outbred Wistar Han rats with respect to the morphological distribution of embryos at 4.5 days gestation, indicating that the DR-BB/E subline was an excellent control for studying the effects of IDDM on rat pregnancy.

Further evidence to support this came from data obtained from pregnant Diabetes Prone (DP) BB/E rats during the pre-diabetic period (Experiment II). Since the mean (± SD) age of onset of IDDM in the BB/E rat colony is 96 ± 18 days, and the female rat is fertile at approximately 60 days of age, pregnancy was achieved in the pre-diabetic period. These experiments are simply not possible in either the chemically-induced rodent models or the human. The data show that these rats had normal numbers of unfertilised oocytes and expanded blastocysts and the expanded blastocysts from these pregnancies were completely normal with respect to the number of ICM and TE cells. Maternal metabolic profiles were identical to those of Non-Diabetic DR-BB/E rats at all stages of pregnancy. These results suggest that early damage to the pre-implantation embryo is due to a disturbed maternal metabolic state, and that the full complement of IDDM susceptibility genes is not in itself, sufficient to cause an ICM cellular deficit. It is likely that the embryonic damage is secondary to a metabolic disturbance and is therefore potentially preventable by improved insulin delivery systems.

It was clear from Experiment III that the degree of metabolic control established in female Diabetic DP-BB/E rats treated with sustained release insulin implants was
much more variable in female rats than that previously achieved in male Diabetic DP-BB/E rats (299). There are several explanations for this apparent sex-related difference in response to SRII-treatment. Firstly, assessment of metabolic control and SRII-treatment began in the female diabetic BB/E rats from day of diabetes detection, whereas all of the previous SRII studies have used male rats previously treated with subcutaneous insulin injections for some time before SRII-treatment. It is unrealistic to expect an insulin-deficient individual be they a newly diagnosed patient with IDDM or a BB/E rat, to have well controlled diabetes from day of diagnosis. Indeed, the degree of hyperglycaemia at diabetes diagnosis varied considerably in the female BB/E rats in this study (plasma glucose concentrations ranging from 21.8 to 44.2 mmol/L) and as in the human, their insulin regime was tailored for each individual rat to achieve metabolic control as near normal as possible.

Secondly, the female rats used in Experiment III were considerably younger and smaller than the males which had been studied previously (299). In the treatment of children with IDDM the insulin requirement will alter considerably as they grow. Although the diabetic female BB/E rats were only maintained on SRII-treatment for a maximum period of 4-5 weeks the effect of growth (as seen as an increase in body weight over the period of treatment) cannot be ignored.

Thirdly, and perhaps the most striking difference between the male and female Diabetic DP-BB/E rats treated with SRIIs was the effect of circulating female sex hormones during the oestrus cycle. This appeared to have a major influence on blood glucose concentration both during the pre-conception period and particularly during
the first two days of pregnancy. Although SRII treatment resulted in improved conception rates (as indicated by a reduction in the number of unfertilised oocytes to normal levels) and reduced the concentration of maternal plasma metabolites to almost normal levels, this degree of metabolic control was insufficient to prevent the ICM-specific embryopathy.

With one recent exception (330), there are no published data using SRIIs to control diabetes in female mice or rats. These authors used SRII-treatment to achieve normal metabolic control (target blood glucose concentration 4-6 mmol/L) in ICR mice with STZ-induced diabetes, and make no mention of any difficulty in achieving good metabolic control. This may be related to the fact that animals with STZ-induced diabetes commonly have some endogenous insulin and are not therefore completely dependent on insulin for survival. Interestingly, the STZ-diabetic mice treated with SRIIs became hyperglycaemic at the time of embryo implantation (although no information is given regarding maternal blood glucose profiles between conception and implantation). This early pregnancy blood glucose rise is similar to that found in ten out of the fifteen diabetic BB/E rats treated with SRIIs.

It is difficult to explain why some of the SRII-treated rats had a post-conception rise in blood glucose concentration and some did not. There was no difference in maternal age, weight, duration of diabetes, dose of insulin or results of daily urine tests for glucose and ketone bodies between these two groups of SRII-treated Diabetic DP-BB/E rats. Indeed, given (i) the inbred status of the BB/E colony, (ii) the fact that it is held in a barrier unit under strictly controlled conditions and screens
negative for all the common rat viruses, and (iii) that all the Diabetic rats involved in
the study had established IDDM as defined by strict criteria, these Diabetic rat
mothers were a remarkably homogeneous group. Nevertheless, subtle differences
may exist in these two populations of pregnancies. In the normal animal with an
intact glucose responsive insulin delivery system, the hyperglycaemia caused by the
anti-insulin action of progesterone is prevented by an increase in the production of
endogenous insulin. Although there was a four-fold increase in progesterone in
response to conception in all groups of SRII-treated Diabetic DP rats, those rats with
a post-conception blood glucose rise did not have an abnormally high progesterone
response to conception. These data suggest that the hyperglycaemic peak in some of
the SRII-treated Diabetic DP rats was not simply due to these rats producing more
progesterone. Progesterone is essential for endometrial preparation for blastocyst
implantation in most mammals and inadequate endometrial maturation and
progesterone insufficiency are well known causes of infertility. Progesterone also
inhibits superoxide radical formation (331) and tumour necrosis factor-α production
in the endometrium (332). Both these factors induce degenerative changes in the
uterine tissue. Superoxide dismutase is a scavenging system for superoxide radicals,
has been detected in human uterine fluid during the pre- and peri-implantation stages
(333) and may play a role in protecting blastocysts from superoxide radical damage.
Under the influence of progesterone various endometrial cellular compartments
differentiate and undergo cell-cell interaction, which allows the endometrium to
secrete into the luminal milieu a variety of active biomolecules which support embryo
growth (334). The viable embryo in turn secretes different types of biomolecules
which influence embryo growth (autocrine effect) and endometrial receptivity (paracrine effect).

One possible explanation as to why some of the SRII-treated rats had a post-conception rise in blood glucose concentration may simply be due to the fact that rats without the post-conception blood glucose rise were given sufficient insulin (prior to pregnancy in the form of a SRII insulin rod) to prevent a progesterone-associated blood glucose rise during early pregnancy.

With almost 75 years' experience using exogenous insulin in the treatment of IDDM, it has become increasingly clear that insulin treatment via the subcutaneous tissue, either by intermitent injection (CIT) or slow continual release from an implant (SRII), is far from ideal. It is unphysiological and plagued by slow and erratically varying absorption. Furthermore, this method of delivering insulin is not subject to the natural pancreatic feedback control by the prevailing blood glucose concentration. A normal metabolic state will only be achieved using an insulin delivery system which is glucose-responsive.

The first pancreas transplants in patients with IDDM were reported by Kelly et al in 1966 (335). Two patients with long-standing diabetes and end-stage renal disease underwent simultaneous kidney and renal transplantation. The procedure was associated with limited success. Less than 10 % of early transplant recipients achieved and maintained normoglycaemia, and only a fraction were insulin-independent beyond one year post-transplant (336). By the late 1970's significant
improvements in surgical technique, treatment of acute rejection, and the management of immunosuppression prompted renewed interest in pancreas transplantation. Today, patients undergoing simultaneous pancreas-kidney transplantation have a 75% chance of pancreas graft survival and a 92% chance of renal graft survival one year following the procedure (337). However, the risks of immunosuppressive therapy remain significant, and as a consequence routine use of pancreas transplantation is not recommended by the American Diabetes Association (338). Thus, pancreatic transplantation is only considered as an acceptable therapeutic alternative to insulin treatment in patients with end-stage renal disease who have had or plan to have a kidney transplant.

The future of IDDM treatment lies in the development of a bioartificial pancreas able to secrete insulin in response to the concentration of blood glucose. The principle of the bioartificial pancreas is to permit non-syngeneic islet transplantation by separating the islets from the recipient's immune system using an artificial membrane (permeable to glucose and insulin but not immunoglobulins and cells). One area of active investigation is the search for alternative sources of islet tissue. For islet transplantation, sources of tissue could include human allogeneic tissue, xenografts from other mammalian species (such as porcine or bovine islets), fetal tissue or manufactured insulin-releasing cells. It is necessary to surround the transplanted islets with a semi-permeable membrane since it is clear that in both rat and man, the immune system retains the ability to recognise and destroy transplanted islet β cells long after IDDM has developed and the infiltrate has disappeared. Previous work carried out in our laboratory by C. Lynch et al (unpublished observations)
demonstrated that microencapsulation and transplantation of rat islets into Diabetic DP-BB/E rats successfully resulted in normoglycaemia. It would be of particular interest and relevance to this project to follow on these experiments, transplanting rat islets into Diabetic DP females prior to pregnancy and monitoring their blood glucose concentration at conception and through early pregnancy, and observing the effect on pregnancy outcome, with particular reference to the ICM cellular deficit.

Transport or secretion of growth factors into the blastocoel may be essential for the formation of a micro-environment critical for the development of the ICM since the ICM is sequestered from the maternal environment by the tight epithelium formed by the TE. Lack of ICM cells in blastocysts from diabetic rats may be due to poor development of the ICM as a result of either abnormally low levels of maternally derived growth factors in the uterine environment, or poor function of the TE to transport or secrete the necessary growth factors.

The development of pre-implantation embryos to the blastocyst stage is, at least partially, a function of autoregulation. In vitro and in vivo studies indicate that a number of peptide growth factors contribute in an autocrine fashion to pre-implantation development. Other growth factors are maternally derived and act in a paracrine manner on the embryo. Some of these factors such as insulin-related factors stimulate growth preferentially, whilst others such as EGF play more important roles in differentiation. Cytokines are generally involved in the later stages of pre- or peri-implantation embryo development, as the embryo meets the challenge of the mother’s immune system.
The insulin-like growth factor family consists of insulin, insulin-like growth factors I and II (IGF-I and IGF-II), the insulin receptor, IGF-I receptor (IGF-IR) and IGF-II receptor (IGF-IIR) and at least six insulin-like growth factor binding proteins (IGFBP1-6) that are all involved in metabolism, growth and development. IGF-II mRNA has been demonstrated in pre-implantation embryos from the 2-cell to the blastocyst stage (339) but only the maternal IGF-II receptor gene is active in pre-implantation embryos as the paternal copy is inactivated by genomic imprinting (340). IGF-I however, is not produced by the embryo during the pre- or post-implantation stage and is only produced by the maternal tissues. IGF-I has been described as a survival factor, able to prevent apoptosis (341). In vitro culture of mouse blastocysts in IGF-I and II has been shown to stimulate ICM cell proliferation without any visible effect on TE cell development (342, 343). The role of IGF-I in anomalies of embryo development remains to be clarified. However, the observation that diabetes decreases concentrations of circulating IGF-I in the human (reviewed in 344) provides an interesting link with respect to IDDM-induced embryopathy of the pre-implantation embryo.

EGF and TGF-α (which bind to the same receptor, the EGF-R) have been reported to increase the frequency of oocyte germinal vesicle breakdown (343), increase the number of blastocysts and the blastocyst hatching rate (345) and to stimulate blastocele expansion (346). In the mouse, the location of the EGF-receptor in the morula stage is predominantly apical. At blastocyst formation, the receptor is found mainly on the basal surfaces of the TE with less on the apical surfaces and on the ICM (347). It has been suggested that the function of the EGF-receptor in the early
embryo is to receive signals from maternal EGF in the oviduct, and when the receptors begin to receive signals from blastocyst-derived endogenous TGF-α they shift to an internal location. In a clinical study, Oehninger et al (348) demonstrated that women with IDDM had a follicular milieu resembling that of non-diabetic women, except for a lack of epidermal growth factor.

The expression of LIF fluctuates with the oestrus cycle and is highest after ovulation and on the day of implantation in the mouse (349) and human (350). Furthermore, female mice lacking both copies of the LIF gene fail to implant their blastocysts. If these blastocysts are transferred into normal pseudopregnant recipient female mice they implant normally (351). LIF may also preserve the high rate of cell proliferation in the ICM. The major source of LIF is the uterus as the blastocyst has only low expression (352).

The expression of TNF-α in the mouse, has been detected in oviductal and uterine tissues throughout the pre-implantation period (353, 354). In the rat, during the peri-implantation period TNF-α is synthesised by epithelial cells lining the uterine lumen and the decidualized cells adjacent to the implanted embryo (355). Mouse and rat pre-implantation blastocysts have been shown to express TNF-α receptors and in vitro culture of these blastocysts in culture medium containing TNF-α significantly downregulates the proliferation of the ICM cells of the blastocyst (356, 357). The selective inhibitory action of TNF-α on the ICM cell lineage supports the hypothesis that TNF-α could contribute to the aetiology of many unexplained reproductive failures in women with high concentrations of TNF-α in the reproductive tract (358,
359). Thus it appears that high concentrations of TNF-α in the local uterine milieu is not beneficial for pre-implantation growth or development.

Culture of pre-implantation embryos in vitro has several limitations, particularly between the late morula and expanded blastocyst stage when the culture requirements become more complex and the growth factors and cytokines described above come into play. Rather than continue with in vitro culture experiments it was possible to examine the same hypothesis using embryo transfer experiments. The aim of these experiments was to answer two questions: (i) can the diabetic uterine environment induce an ICM cell deficit in embryos from a Non-Diabetic DR-BB/E mother? and (ii) can removal of embryos from a potentially harmful diabetic environment ‘rescue’ the embryos and allow them to develop normally? The data shown in Experiment II demonstrate that the maternal diabetic uterine environment is able to induce both an ICM cell deficit (-9%) and a TE cell accretion (+19%) in blastocysts from Non- Diabetic DR-BB/E rats transferred at the 2-cell stage. However, the transfer of 2-cell embryos from Diabetic DP mothers to Non-Diabetic DR recipients did not prevent the development of an ICM cell deficit (17%) or a TE cell accretion (9%) implying earlier damage to germ cells. These findings indicate that maternal IDDM in the BB/E rat has deleterious effects on pre-implantation embryo development which persists despite the removal of embryos from the diabetic mother and transfer to a non-diabetic milieu. To date, there is only one other publication of data relating to the transfer of embryos from diabetic animals into non-diabetic recipients (360). Veselá et al (360) transferred 2-cell embryos from BALB/c donor mice (given a sub-diabetogenic dose of streptozotocin) into non-diabetic pseudopregnant ICR recipient
mice, and 2-cell embryos from control (non-diabetic) BALB/c mice into non-diabetic recipient ICR mice. The embryos were recovered from the recipient mice 24-28 h later and morphological evaluation revealed that almost half of the embryos from the streptozotocin-treated donor females were incapable of development to the eight-cell stage even when transferred to a non-diabetic maternal environment. Although the authors made no attempt to transfer embryos from non-diabetic mice into STZ-diabetic recipient mice, their data support the findings observed in BB/E rat embryo transfers.

The embryo transfer experiments showed that maternal diabetes had a major effect on embryonic development. The embryo transfers were carried out at the 2-cell stage, the embryos had been exposed to a diabetic maternal environment (the oviduct) for 1.5 days before transfer. Although a direct effect of the oviductal environment on early pre-implantation embryo development (i.e. day 0.5 to day 1.5 of pregnancy) cannot be excluded however unlikely, the most likely explanation is that maternal diabetes has affected oocyte development and/or fertilisation. Reasons for this are based on the fact that (i) early pre-implantation embryos are undifferentiated, non-vascularised entities which divide rapidly but show no net growth (361); (ii) genetic control of development is, to begin with, dependent on maternal mRNA before the embryonic genome is expressed (362), and (iii) embryos at this stage are largely insensitive to hormones and growth factors. It is late pre-implantation embryos which exhibit net growth (363), differentiate into ICM and TE cells, and increase their sensitivity to growth factors and hormones at the blastocyst stage. The oviduct provides mainly physical and chemical factors to facilitate embryo development.
Physical factors include the action of the cilia lining the oviduct and the contraction of the myosalpinx, used to transport the embryos down the oviduct towards the uterus. Chemical factors include oxygen tension, electrolytes and nutrients (pyruvate, lactate, glucose and amino acids). Pyruvate and lactate are the main regulatory substrates for early pre-implantation embryo growth. Glucose however, has been shown to be inhibitory to the *in vitro* development of early pre-implantation embryos in the hamster (364), mouse (365), rat (366), cow (367, 368), sheep (369) and human (370). Studies by Nichol et al (371) measuring the concentration of glucose in samples of oviductal fluid from anaesthetised, unmated pigs, pre- and post-ovulatory, and from mated pigs, post-ovulatory, have shown that the concentration of glucose in the unmated ampulla fell sharply from pre-ovulatory values of 0.97 mM to 0.47 mM post-ovulation. After mating, the glucose concentration was even lower at 0.25 mM. The reasons for these data are unclear but it is tempting to speculate that these findings in the pig represent a strategy adopted by the female reproductive tract to protect embryos from exposure to glucose at too high a concentration. The degree to which oviductal fluid glucose levels are elevated in pregnant diabetic women has not been established, but it has been reported in normal subjects that oviductal fluid glucose levels are approximately half that of serum levels (372). Therefore, the only way to determine definitively the exact effect of the maternal metabolic environment on the development of the pre-implantation embryo would be to carry out *in vitro* fertilisation followed by embryo transfer. This would remove all possible effects of a damaging oviductal environment on donor embryo development.
The growth and maturation of oocytes depend on their surrounding somatic cells. Many *in vitro* studies have shown that up to the formation of the antral cavity the growth of the oocyte is paralleled by the proliferation of the granulosa and thecal cells. During the first phase of follicular growth, that is the pre-antral phase, the granulosa cells are largely independent of the antral cavity, oocyte growth ceases and the follicles become dependent on the presence of Follicle Stimulating Hormone (FSH) for further growth and differentiation. Apart from FSH, the major specific hormonal stimulus for granulosa cells, many other locally produced factors determine the fate of the follicle unit. Circulating growth factors, theca- and interstitial cell-derived factors, and stimuli originating in the oocyte itself are able to condition the granulosa cells toward proliferation and/or differentiation. These factors include IGF’s and their binding proteins, EGF, TGFs, oestrogens, inhibins and activins (373). Activin βA has been localised in rat oocytes, suggesting a role for activin in meiotic maturation (374). There is a potential role for growth factors in the intrafollicular control of meiosis. IGF-I is a potent mitogen for granulosa cells (375) and enhances nuclear maturation in oocytes surrounded by cumulus cells in the cow (376) and human (377). IGF-I is also known to stimulate oocyte maturation in *Xenopus* (378), rabbit (379) and buffalo (380). The addition of IGF-I during *in vitro* maturation of buffalo oocytes not only helped in achieving nuclear maturation but also influenced cytoplasmic maturation and improved early embryonic development (380). Thus it is possible that the stimulatory effect of IGF-I on cumulus and granulosa cell proliferation might enhance the syncytial network and nutrient uptake by oocytes during cytoplasmic maturation and result in beneficial effects on embryo development. Since the only source of IGF-I throughout the pre-implantation period is a maternal
one, and IGF-I is known to be a survival factor, able to prevent apoptosis (341), it is also possible that low maternal IGF-I levels in the Diabetic DP-BB/E rat may be responsible for poor oocyte development and the ICM cellular deficit (possibly due to increased apoptosis). No data are currently available in relation to the levels of IGF-I and II in animals with chemically-induced or spontaneous diabetes during the pre-implantation stage.

Many studies of the pathogenesis of diabetic embryopathy have involved animals with chemically-induced diabetes and have largely focused on the period of organogenesis. However, the relevance of these animal models to human IDDM (an autoimmun-induced disorder) is clearly very limited, particularly in relation to disease aetiology. The dosage of alloxan and streptozotocin required to induce insulin-dependence may have additional widespread extra pancreatic toxic effects, on the parental germ cells for example. Many of the STZ-diabetic rodents used for pregnancy studies are not insulin-dependent. Although they have severe hyperglycaemia they are rarely treated with insulin and are able to survive without developing lethal ketoacidosis. Indeed, some workers regard a single random blood glucose measurement of >11 mmol/L sufficient to diagnose IDDM (292, 381, 281). Even studies using the NOD mouse (281) use ill-defined criteria for diabetes diagnosis and these mice do not require insulin treatment for survival, casting further doubt on the relevance of these models to human insulin-dependent diabetes mellitus. It has been suggested that conventional treatment with a daily subcutaneous injection of insulin completely prevents diabetic embryopathy in rats and mice with STZ-induced diabetes and in NOD mice (283, 381, 382). This contrasts with the data presented here since all the DP-BB/E rats with
IDDM are completely and irreversibly insulin deficient and require daily injections of insulin to survive and still develop severe embryopathy. In this respect they differ from NOD mice and rats with STZ-induced diabetes, which are not totally insulin deficient and are capable of spontaneous regeneration of islet β cells (260).

The clinical relevance of many of the in vitro studies reported in the literature is questionable. For example, many studies have shown that various components of serum, glucose and ketone bodies are toxic to pre- and post-implantation embryo development. However, the concentration of these compounds is so high that they would only be observed in women with life-threatening ketoacidosis. Three particular examples include (i) Eriksson and Borg (383) who cultured 11.5 day old rat embryos in media containing 50 mmol/L glucose (ii) Diamond et al (384) who used media containing 53 mmol/L glucose to inhibit mouse pre-implantation embryo development, and (iii) Shum and Sadler (385) who used media containing 32 mmol/L β-hydroxybutyrate for their in vitro culture studies when the concentration of β-hydroxybutyrate in normal rat serum is approximately 0.25 mmol/L.

When differentially stained blastocysts were examined under the fluorescence microscope, apoptotic cells were observed in blastocysts from both Diabetic DP- and Non-Diabetic DR-BB/E rats. It appeared that cells with fragmented nuclei were observed more often in the ICM than in the TE although it was not possible to accurately quantitate this phenomenon because of the limitations of using fluorescence microscopy, unless a confocal microscope was used. Particular problems included the speed of apoptosis and the rapidity of clearance of apoptotic cells; identifying
apoptotic cells in a three-dimensional structure such as a blastocyst; and the fragility of cells undergoing apoptosis, which are effectively lost when the blastocyst is flattened to facilitate accurate counting of cells. However, it is possible that the reduction in ICM cell number seen in blastocysts from Diabetic DP-BB/E rats may be due to disturbed regulation of apoptosis. It has been suggested that apoptosis occurs in the ICM of the blastocyst during the transition from early blastocyst (when the ICM has the potential to form trophectoderm cells) to expanded blastocyst (when the ICM no longer has the potential to form trophectoderm cells) and that this cell death is designed to eliminate redundant ICM cells with trophectodermal potential (291). In addition, it has been proposed that the mechanism of action of apoptosis in the blastocyst is mediated by epigenetic factors in the fluid of the blastocoele (386). The data from total cell counts of morulae and early blastocysts from Diabetic DP-BB/E rats, shows that at this stage of development there is no difference in embryos from Diabetic DP and Non-Diabetic DR rats, and is consistent with the hypothesis that maximum cell death in the blastocyst occurs between the early and expanded blastocyst stage.

Cell death can occur by one of two distinct processes, necrosis or programmed cell death through apoptosis. Necrosis is usually associated with injury, and results in cellular swelling and rupture of the plasma and internal membranes. True programmed cell death (PCD) is genetically programmed, and requires the activation of specific genes involved in the execution of cell death. Developmentally regulated cell death is widespread in embryogenesis (288) and occurs by apoptosis in both vertebrate and invertebrate species. It is a widespread feature in blastocysts of many
mammals such as the cow (289); Rhesus monkey (290); mouse (2291) and the human (268). The significance of these dead cells is not fully understood, but it has been suggested that the primary function is to allow for the removal of redundant or defective cells from the blastocyst (288), or the elimination of ICM cells retaining the potential to form TE (291). Other suggestions include lack of survival factors in vitro or in vivo, and chromosomal abnormalities (387). In mouse blastocysts it has been recently reported that embryonic cells have a death-by-default mechanism that requires constant suppression (388). Apoptosis is a physiological cell death process triggered by external or internal stimuli, the process can be divided into four phases (389). The first phases may occur in response to an external trigger delivered through surface receptors (e.g. fas ligand and CD40 ligand), or may originate inside the cell from the action of a drug, toxin, or radiation (284). The next phase involves detection of this signal and transduction of the signal. The activation or signalling phase encompasses a great variety of signal transduction pathways including tyrosine kinases, steroid receptors, ceramide, inositol phosphates and cytokine receptors. The death effector phase is the third part of the cell death mechanism and includes the cysteine proteases that are activated during apoptosis. The fourth phase is the post-mortem phase, in which the cell’s chromatin condenses and its DNA is degraded into oligonucleosome chains (seen as DNA ‘laddering’ on gel electrophoresis) (284). Depending on their ratio, Bcl-2 and similar proteins can inhibit the action of the proteases, either by blocking their activation or by preventing them from reaching their targets. Other proteins implicated in the regulation of apoptosis include tumour suppressor protein p53 which triggers apoptosis in cells with damaged DNA, and c-Myc which can inhibit apoptosis in the presence of factors such as IGF-I, insulin and
EGF (390). This suggests that the increase in ICM cell numbers in blastocysts grown in IGF-I and insulin in vitro could be due to these factors reducing the level of cell death rather than having a direct mitogenic effect (reviewed in 391).

Prior to the introduction of sensitive molecular biology techniques such as RT-PCR, the quantification of dead cells was limited to the histological localisation and analysis of serial sections of blastocysts which was both laborious and time-consuming. For these reasons only a small number of blastocysts could be analysed this way (392, 393). In addition, the initial stages of apoptosis appear to be extremely rapid with cytoplasmic fragmentation occurring within minutes (394) therefore histological or differential labelling techniques provide only a 'snapshot' of the embryo at a specific point in time during the final phase of the cell death process. Using a quantitative RT-PCR protocol with its unique sensitivity, the level of expression of apoptosis regulating genes can be quantified before the cells undergo the final morphological changes.

Several methods of quantitative PCR have been described (308, 311, 395, 396, 397) all of which use different strategies to quantify PCR product yield. Some methods base quantitation upon co-amplification of a reporter gene which differs from the target gene (396, 398). This method however, is not a particularly reliable strategy since differences in primer efficiencies can lead to differences in the amount of amplified product. Others such as Wang et al (397) have compared the co-amplification of target cDNA to cloned genomic sequences (containing introns). The disadvantage of this method is that the effects of reverse transcription and the possible
differences in the amplification efficiencies of large genomic DNA and cDNAs are not
taken into consideration. Methods based on large size differences between the target
and standard sequences must be applied and interpreted with caution.

Standards prepared for quantitative RT-PCR can be internal or external, and be added
to the reaction as mRNA (added prior to the reverse transcriptase step) or cDNA
(added prior to amplification step). Internal standards must allow the distinction of
amplified products in order to be useful. This can be based on differences in size
between the target and standard, the main disadvantage of which as described above,
is that even small differences in size may affect amplification efficiency. Alternatively,
an internal standard can be designed so that its PCR product contains a unique
restriction site not found on the target amplified product (399). The standard PCR
products are differentiated from that of the target by gel electrophoresis of the
restriction enzyme digest of the amplified products. One caveat of this method is that
under conditions in which primer is rate limiting, annealing may occur between
heterologous strands of mutant and target templates, forming heteroduplexes which
cannot be cleaved by the restriction enzymes.

External standards can be prepared by cloning the PCR product into a transcription
vector. mRNA is synthesised, purified and quantified in the same way as internal
standards. The biggest concern when using this method is tube-to-tube variability.
The parameters that are particularly important are magnesium ion concentration and
the proportion of primer to template cDNA.
Given the stringent requirements in quantitative PCR to relate the precise yield of PCR product to the abundance of the target template to be quantified, the reliability of any quantitative PCR procedure is susceptible to minor variations in PCR reagents and/or amplification conditions which can substantially affect the yield of product. Internal standard procedures have the intrinsic advantage that the method avoids variability of reagents and conditions because the internal standard and target are in the same tube and any variables affecting the PCR conditions in those tubes will equally affect the amplification of standard and target.

Many of the disadvantages of other available quantitative PCR and RT-PCR methods were taken into consideration when designing an appropriate and sensitive method of quantitative RT-PCR in single rat blastocysts. This technique was used to investigate whether the reduction in ICM cell number seen in blastocysts from diabetic rats was due to disturbed regulation of apoptosis. Synthetic mRNA transcribed in vitro from mutated cloned PCR sequences was used as the internal mRNA standard. mRNA rather than cDNA standards were chosen so that differences in reverse transcription could be taken into consideration. The increase in insert length was limited to a maximum of eight nucleotides so that both target and standard cDNAs would have approximately the same reverse transcription and PCR amplification efficiencies. Such small differences in size between the target and standard PCR products required separation by polyacrylamide rather than agarose gel electrophoresis. Since separation of the $^{32}$P-labelled PCR products by polyacrylamide gel electrophoresis is much more sensitive than agarose gel electrophoresis with ethidium bromide staining, the PCR can be optimised more stringently and the presence of non-specific PCR
products (which would not be detected with agarose gel separation) can be seen and conditions changed accordingly to prevent them, thus increasing the accuracy of quantification.

The quantitative RT-PCR method used in the present study however, did not detect a significant difference in the ratio of bcl-2 : bax mRNA in 8 single expanded blastocysts from diabetic and non-diabetic BB/E rats. There may be several reasons for this: firstly, the Bcl-2 family has many members and it may be necessary to look at the expression levels of several other genes in addition to bax and bcl-2. This was not possible in this study because of time limitations but now that the methods of RNA extraction, reverse transcription and PCR amplification have been established for the single blastocyst it would be possible to observe the ratio of death antagonist and agonist genes in much more detail. Secondly it appears that members of the Bcl-2 family interact with each other to form a dynamic equilibrium between homo- and heterodimers at the protein level and so it would be interesting to complement the RT-PCR data with immunohistochemistry to localise the proteins with particularly high gene expression to the ICM or TE. It was important to establish a method which was sensitive enough to detect small changes in mRNA levels in the single pre-implantation embryo. Pooling large numbers of blastocysts together is not only expensive in terms of animal numbers but may also mask subtle changes in gene expression in individual blastocysts, as it is clear from the data presented in Experiment I that in any one pregnancy each embryo is a completely separate entity.
Since derivatives of the ICM give rise to all germ layers of the fetus, it is likely that the ICM cellular deficit could be an underlying cause of the high rate of fetal resorptions and malformations reported by Eriksson et al (258) in Diabetic DP-BB/E rats maintained on conventional insulin therapy. To date, no post-implantation studies have been carried out using embryos from BB rats so speculation about the future consequences of the ICM cellular deficit is difficult. However, it has been shown that normal mouse blastocysts with a reduced ICM implant normally (400) but organogenesis is disturbed (320). While these blastocysts might eventually achieve normal future growth and development, this impairment is consistent with the retardation of growth observed in human pregnancies with poorly controlled IDDM, which may lead to congenital malformation or IUGR.

There is a 3-4 fold increase in major congenital malformation in infants of mothers with IDDM. Congenital malformations are now the most common cause of neonatal death in these infants. Cardiac anomalies affect up to 4 % of IDDM pregnancies presenting as ventricular-septal defects, complex lesions of the great vessels and coarctation of the aorta. IDDM pregnancy also carries an especially high risk for neural tube defects (NTDs): 19.5 per 1000 pregnancies compared to 2 per 1000 for normal pregnancies (104). High rates of perinatal mortality and congenital malformation, in particular neural tube and skeletal defects, have also been reported in the BB rat (256, 257, 258, 259). The association between early growth delay (EGD) and congenital malformation has been reported by Pedersen and Molsted-Pedersen (92). This is a controversial area since one centre has suggested that EGD disappears when the correct time of conception is determined (99). However, the time of
conception in the BB/E rat studies was precisely known as a sperm positive vaginal plug was found the morning after mating and designated day 0.5 of pregnancy. Thus the data show that at day 4.5 of pregnancy blastocysts from rats with IDDM are already exhibiting signs of early growth delay. Whether it is these blastocysts which develop into fetuses with the types of congenital malformations previously described in diabetic BB/E rats is at present unknown. It would be interesting for future studies to investigate the fate of the ICM deficit with particular reference to malformations such as cardiac and neural tube defects. In addition, clinical studies have shown that EGD is associated with poorer psychomotor development postnatally (95) and it would be of particular relevance to the clinical situation to study psychomotor development in the offspring of diabetic BB/E rats, a phenomenon which has not yet been described in either the BB rat or rodents with STZ-induced diabetes.

Most non-genetic and non-toxic IUGR is believed to be due to a reduction in nutrient or oxygen delivery to the fetus either because of maternal illness or due to placental dysfunction (401). IGF-I, IGF-II and their respective receptors are synthesised by a number of fetal tissues and are believed to be involved in an autocrine/paracrine stimulation of cellular proliferation and differentiation in development (402). Alterations in these growth factors may result in developmental abnormalities such as macrosomia or intra-uterine growth retardation. Much of what is known of the effects of the IGF's and their receptors has been obtained from mice knockout experiments. Mice null mutants for IGF-I, IGF-II, insulin receptor and IGF-IR all exhibit reduced birthweight (40-60 %) and are associated with neonatal lethality (403, 404, 405).
Using *in situ* hybridisation Chernicky et al (406) found that some embryos from STZ-induced diabetic mice at day 6 of gestation were growth retarded and had significantly low levels of IGF-II gene expression. Those embryos from diabetic mice which were not growth retarded had normal IGF-II expression. These studies complement that of Shen et al (407) who previously demonstrated that an increase in placental IGF-II levels in term placentae from diabetic mothers, is often associated with macrosomia. IGF-I levels are highest in first- and second-trimester placentae compared with term placentae, indicating that it may be more important in early gestation as a placental growth factor, when organogenesis and rapid cell proliferation occur (408).

A recent paper by Abu-Amero et al (409) has described a quantitative PCR method used to compare transcription levels of the IGF family in human term placentae from normal and IUGR births. They found no significant difference in the levels of transcripts for *IGF-I*, *insulin receptor*, or *IGF-II* between normal and IUGR placentae. However, the IUGR placentae had significantly higher levels of *IGF-II* and *IGF-IR* expression compared with the normal term placentae. The increase in the transcription of *IGF-II* and *IGF-IR* in IUGR term placentae may represent a counter regulatory mechanism in response to the growth retardation.

Clinical observations in the human suggest that IUGR is associated with developmental alterations in the ‘programming’ of a number of key homeostatic mechanisms leading to the development of disease in adulthood such as NIDDM, cardiovascular disease and hypertension (410, 411, 412). Direct experimental
evidence to support this model has shown that the progeny of undernourished pregnant rats became hypertensive postnatally (413, 414). In these rats catch-up growth is delayed postnatally and appears to be associated with a delayed development of responsivity to growth hormone or IGF-I therapy. Patients with poorly controlled / uncontrolled IDDM are very similar metabolically to individuals suffering from prolonged starvation. During periods of starvation there is an increased secretion of glucagon, gluconeogenesis is increased in the liver and glycolysis is switched off. As a result, muscle shifts from using glucose as fuel to using fatty acids, and there is a large increase in the amount of acetoacetate and β-hydroxybutyrate (ketone bodies) in the blood - produced by the liver and used by the brain for fuel. Clearly these metabolic changes are very similar to those described in section 1.1.1 of the Introduction (and Figure 1.1) the metabolic consequences of insulin-deficiency. Thus, the diabetic BB rat can be also seen as a model for pregnancy under conditions of relative starvation and used to investigate the phenomenon of small for gestational age babies and the effect of IUGR later on in adult life.

In conclusion, the data presented here clearly demonstrate that autoimmune-induced IDDM which develops spontaneously in the DP-BB/E rat was associated with a severe disturbance in pre-implantation embryo development. Perhaps the most significant data of all came from the embryo transfer experiments. The maternal uterine environment of the Diabetic DP recipient was able to induce a small ICM cellular deficit (9 %) accompanied by a substantial TE cellular accretion (19 %) in blastocysts transferred from Non-Diabetic DR donor rats at the 2-cell stage. It has
been suggested that normal blastocyst formation depends on adequate cellular interaction between ICM and TE cells. The increase TE cell number may represent a counter regulatory mechanism in response to the ICM cellular deficit. However, transfer of two-cell embryos from Diabetic DP- into Non-Diabetic DR-BB/E mothers did not prevent the development of an ICM cellular deficit or a TE cell suggesting that maternal IDDM in the BB/E rat may negatively influence oocyte maturation and/or the oviductal milieu where fertilisation occurs.

IGF-I and IGF-II are synthesised by a number of maternal, embryonic and fetal tissues and are believed to be involved in an autocrine/paracrine stimulation of cellular proliferation and differentiation in development (404). Although no definitive evidence is presently available for the exact role of IGF’s in diabetes-induced embryopathy, STZ-induced diabetic rats with IUGR have been shown to express low levels of IGF-II. It is possible therefore that maternal IDDM may affect the balance of these growth factors resulting in the formation of some of the developmental abnormalities of the pre-implantation blastocyst seen in Diabetic DP-BB/E rats.

The aims of these studies were (i) to elucidate the mechanism of the teratogenic effect of IDDM by studying the interaction of maternal genetic background and metabolic profile on pre-implantation embryo development using the BB/E rat as a model for human IDDM, and (ii) to examine the effect of the degree of diabetic metabolic control achieved before and at various points after conception on embryonic development. Although the exact mechanisms underlying the teratogenic effect of maternal IDDM have not been precisely defined, these studies using the BB/E rat
support the hypothesis that a detrimental effect induced by maternal IDDM occurs on oocytes during folliculogenesis and on early pre-implantation embryos during progression from the two-cell stage to the blastocyst stage of development. These findings and those obtained from Diabetic DP rats treated with SRIIs indicate that in order to achieve normal pregnancy outcome exceptionally good metabolic control (i.e. normoglycaemia) may need to be established from diabetes onset.
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Chapter Five

Appendix

Figure 5.1: Partial nucleotide sequence of rat bax cDNA aligned with the published rat bax cDNA sequence from nucleotide number 115 to 518 (GenBank accession number U49729). The bax cDNA fragment was isolated using RT-PCR with total RNA prepared from Non-Diabetic DR-BB/E rat blastocysts (internal primer sequences are shown in boxes).

Genbank sequence:
TTGTACAGGGTTTTCATCCAGGATGGGCAGCAGGGAGGATGG

BB/E rat consensus:
GGGGGACACCTGACCTGACCTTGGAGCGACAGCCGCCAGGATGCATCCACCA

AAGAGCTGACGCGGATCTCCGCGGAAATTGAGATGAACCTGACAGCAATATTG

AGAAGCTGACTCGATCTCGACGAGGAATTATAAGATGAACTGGACACTGGACA ACAAATATTG

GAGCTGACAGGGATGATTGTGAGCTGACGGACTCCCGCCAGAGGAGGTCTTCC

TTCCGGGTGGCAGCTGACATGTTTGCTGAGCTGACCTGACCTTGGAGGAGGATG

ATCCACCAAGAAGCTGATCGAGTGTCTCGGCGAATTGGAGATGAACTGGACACTG

ACAATATGGAGCTGCAGAGGATGATTGCTGACGTGGACACGGACTCCCCCCGAGAGGT

CTTC

GGGACCCCCCATGGCAGACAGTGACCATCTTTGT

Differences between the published Rattus rattus cDNA sequence and the BB/E rat bax cDNA sequence are shown in red.
Figure 5.2: Partial nucleotide sequence of BB/E rat bcl-2 cDNA aligned with the published rat bcl-2 cDNA sequence from nucleotide number 372 to 662 (GenBank accession number L14680). The bcl-2 cDNA fragment was isolated using RT-PCR with total RNA prepared from Non-Diabetic DR-BB/E rat blastocysts (internal primer sequences are shown in boxes).

Genbank sequence: TGGCATCTTCTCCTCTCCAGTCAGAGCAACCGAACGCCC 372
BB/E rat consensus: TGGCATCTTCTCCTCCAGTGCAGACCGAACGCCC

GCTGTGCAACGAGAACAGGCTGCGCCAGACGTCCTCTACGGCCCCTTTGGTCGCA 427
GCTGTGCAACGAGAACAGGCTGCGCCAGACGTCCTCTACGGCCCCTTTGGTCGCA

ACGCTGGGCCCTGCTGCTCAGCTGCCACCTGACCTGACCGCCCGCTGACCGCCCGACTTTGCAGAGATGTCCAAGT 482
ACGCTGGGCCCTGCTGCTCAGCTGCCACCTGACCGCCCGCTGACCGCCCGACTTTGCAGAGATGTCCAAGT

GACTGCGCTGCCATGGCTGCCAGACGTCGCTCTACGGCCCCTTGTCGCCACGGCTGGGCCTTCAGGGGTAG 537
GACTGCGCTGCCATGGCTGCCAGACGTCGCTCTACGGCCCCTTGTCGCCACGGCTGGGCCTTCAGGGGTAG

CAGCTGCACCTGAGGCCCTTCACCGAGGGGACGCTTTGCCACGGTGGTGGAG 591
CAGCTGCACCTGAGGCCCTTCACCGAGGGGACGCTTTGCCACGGTGGTGGAG

GAACTCTTCAGGGATGGGGTGAACTGGGGGAGGATTGTGGCCTTCTTTGAGTTAC 645
GAACTCTTCAGGGATGGGGTGAACTGGGGGAGGATTGTGGCCTTCTTTGAGTTAC

GGTTGGGGTCATGTGTGTGTTGCTTTGGCTCTTCTTGAATTG 662
GGTTGGGGTCATGTGTGTGTTGCTTTGGCTCTTCTTGAATTG

Differences between the published Rattus norvegicus cDNA sequence and the BB/E rat bcl-2 cDNA sequence are shown in red.
Figure 5.3: Partial nucleotide sequence of rat β-actin cDNA aligned with the published rat β-actin cDNA sequence from nucleotide number 2235 to 2907 (Genbank accession number V01217 J00691). The β-actin cDNA fragment was isolated using RT-PCR with total RNA prepared from Non-Diabetic DR-BB/E rat blastocysts (internal primer sequences are shown in boxes).

Genbank sequence:  
```
GCCATCCAGGCTGTGTTGTCTCCGTATGCCTCTGGTCGTA
```

BB/E rat consensus:  
```
CCCATTTAGGCTGTGTTGTCCCTGTATGCCTCTGGTCGTA
```

Differences between the published *Rattus norvegicus* cDNA sequence and the BB/E rat β-actin cDNA sequence are shown in red.
Figure 5.4: Partial nucleotide sequence of pBcl-2Mut.PCR2.1. The size of the insert in the recombinant plasmid was increased by eight nucleotides by inserting an XbaI linker (shown in red) into the NheI site.

BB/E rat consensus : TGGCATCTTTCCTCTCCAGCCTGAGAGCAACCGAACGCCC
GCTGTGCAACGGAGACACGGGCTGCCAGGACGTGCTGCTCTACGCCCCTCTTGTGCCCA
ACGCTGGGCTCGCTCAGGCCCCTGTCGCCACCTGTCGTCCACCTCGCCCG
GGCTGGGATGACTTCTCTCGCTGCTACCGTCGCTGCTCTAGAGCGACTTTTGCAAGAG
TGTCAGACGTGCTCGACCCGCTGACCGGCCCTTCACCGCAGGAGGACGCTTTTGCAACCAGG
GGTGGAGAACTCTTCCAGGGATGGGGTGAACTGGGGGAGGATTGTGGCCTTCTT
TGAGTTCGGTGGGGGTACATGTGTTG

Figure 5.5: Partial nucleotide sequence of pActMut.PCR2.1. The size of the insert in the recombinant plasmid was increased by five nucleotides by filling in the 3' recessed ends after BstEII digestion, as shown in red.

BB/E rat consensus : CCCATTTAGGCTGTGTTTGGCTCCTGTATGCCCTCTGGTGTA
CCTCTGGCATTTGATGGACTCCCGGAGACGGGGTCAAGTCAGTCACCCACACTGGCC
CATCTATGGGTACCGGTCTCCCTCACATCCATGCCTGCTGTGGACCTGGGCTTTT
CCGGGGACCTGAGAAGCTACCTGCACTATGAGATGGAGTGGCTGCATCCTTT
CCGGAGCAAGCATACCTGAGATGGGAAACCTGTCGCTGACATTAAAGAGAAGGCTGCT
TGTTGCCCTAGACTTCGAGCAAGAGATGGCCCACTGCGCATCCTCTCCCTCG
GAGAAA

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Diabetes, Vol. 45, No. 11, November 1996

**Original Articles**

**Disturbed Development of the Preimplantation Embryo in the Insulin-Dependent Diabetic BB/E Rat**

Richard G. Lea, Jane E. McCracken, Susan S. McIntyre, William Smith, and Joyce D. Baird

Although improved regulation of maternal IDDM during pregnancy has resulted in a major fall in the stillbirth rate, the rates for other problems, such as spontaneous preterm labor, fetuses small for gestational age, congenital malformation, and the incidence of large placentas, remain raised. This has suggested the possibility that the damaging effect of conventionally treated but poorly regulated IDDM may operate primarily at the earliest stages of gestation, even before the diagnosis of pregnancy has been made. This study shows that spontaneous autoimmune IDDM in the Bio Breeding/Edinburgh (BB/E) rat is associated with severe disturbance in the development of the preimplantation embryo in a majority of pregnancies, as indicated by a fivefold increase in the incidence of degenerate fragmented embryos and a 33% reduction in the number of expanded blastocysts and in those blastocysts that reach the expanded stage a 20% cellular deficit in the inner-cell mass without any change in trophectoderm cell number. In addition, we find that blastocysts removed from diabetic rats and cultured in vitro for 24 h show no sign of “catch-up” growth of the inner-cell mass, although under these conditions, the trophectoderm exhibits a 25% cellular accretion. It is tempting to speculate that these phenomena are a presage of the characteristic combination of fetal growth retardation and large placentas, which are a feature of both BB/E rat and human IDDM pregnancy. *Diabetes* 45:1463-1470, 1996

The main causes of the high perinatal mortality rate (PNMR) in women with IDDM are sudden intrauterine death, particularly in the 3rd trimester of pregnancy; prematurity, resulting from either spontaneous preterm labor or elective early delivery performed to avoid late intrauterine death; infants small for gestational age; and malformation. Intrauterine death (the “classical accident” in diabetic pregnancy) has been shown to be due to fetal hypoxia, attributable to a 23% increase in the oxidative metabolism of glucose and lactate consequent on fetal hyperglycemia/insulinemia induced by maternal hyperglycemia. The mechanisms underlying the teratogenic effect of IDDM, initiation of preterm labor, and the development of infants small for gestational age remain obscure. It is, however, recognized that these complications tend to be associated with each other (in both diabetic and nondiabetic pregnancy) and occur particularly in mothers with IDDM, who also have clinically evident microangiopathy, preeclampsia, and/or early growth delay (EGD). The latter can be demonstrated by ultrasound scanning at 7-14 weeks gestation in 30-40% of IDDM pregnancies and has been shown to be a marker for low birth weight (LBW) and malformation (1).

There is a general agreement that 1) the main factor responsible for the improved outcome of pregnancy in women with IDDM in the past 2 decades is better regulation of maternal diabetes during pregnancy; 2) the main impact of good metabolic control is the reduction of the number of late intrauterine deaths, and 3) the effect of improved diabetic control during pregnancy on the rates of malformation, LBW, and spontaneous preterm labor is much less impressive (2). This has suggested that the damaging effect of IDDM may operate at the very earliest stages of pregnancy. However, prospective studies designed to test this hypothesis have yielded conflicting results (3,4), and today, even in “centers of excellence,” the PNMR for diabetic pregnancy remains approximately four times that for nondiabetic women (4-8% vs. 1-2%) (5), and 15-20% of the infants born to mothers with IDDM experience neonatal morbidity severe enough to require a stay of >10 days’ duration in intensive care (6). A substantial proportion of the excess neonatal morbidity in IDDM pregnancy and 30-60% of all perinatal deaths are now due to malformation. In addition, there is increasing concern about long-term morbidity in the children born to mothers with IDDM. Careful studies from Copenhagen are particularly important in that they confirm and extend earlier less-substantial reports by showing that 1) 30% of the children born to IDDM mothers have a range of problems (7,8), indicating cerebral dysfunction, which is comparable to those seen in LBW children born to nondiabetic mothers; and 2) children who have had EGD, premature birth, and/or LBW are at greatest risk of developing long-term problems.

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BB/E, Bio Breeding/Edinburgh; BSA, bovine serum albumin; DNP, dinitrophenyl; DP, diabetes-prone; DR, diabetes-resistant; EGD, early growth delay; ICN, inner-cell mass; LBW, low birth weight; PNMR, perinatal mortality rate; STZ, streptozotocin; TE, trophectoderm.
Our hypothesis is that the noxious effect of IDDM, which results in malformation, EGD, LBW, and preterm labor, operates primarily at the very earliest stages of pregnancy.

Obviously, direct study of the embryo in the earliest stages of human pregnancy is impossible, and prospective epidemiological studies have major problems and limitations (4). Appropriate animal models provide a direct approach for studying disturbances early in pregnancy and the effect of various interventions can be investigated. With few exceptions (9–14), in vivo studies of the pathogenesis of diabetic embryopathy in IDDM pregnancy have involved animals with chemically induced diabetes and have largely focused attention on the period of organogenesis (15). Studies of the preimplantation period are less common (16–18). In this study, we have used the spontaneously diabetic Bio Breeding/Edinburgh (BB/E) rat as a model for human IDDM pregnancy to study the effects of maternal diabetes on the development of the preimplantation embryo.

RESEARCH DESIGN AND METHODS

Animals

BB/E. A breeding colony of BB Wistar rats was established in Edinburgh in 1982 from a small nucleus of animals (three male and four female) donated by Dr. P. Thibert from the original outbred BB rat colony in Ottawa. The BB/E colony consists of two sublines created by selectively breeding for and against diabetes. These two lines have now been through 24 generations of strict brother-sister mating on site, thus meeting the international criteria for designation as an inbred line, and we have also confirmed their inbred status by skin alllograft experiments. All animals are weighed twice weekly from 40 days of age. If they fail to gain weight or lose weight, they are tested for glycosuria (Multistix SG reagent strips, Bayer Diagnostics). If glycosuria is detected, the blood glucose concentration is measured (ExacTech blood glucose meter, Medisense) from a blood sample obtained by tail nicking without anesthesia. In the Edinburgh BB colony, a blood glucose concentration of >18 mmol/l is invariably associated with loss of weight, ketonuria, undetectable circulating endogenous insulin, and the need to treat with a daily injection of insulin (Bovine Ultralard U40, Novo Nordenmark, Denmark) to ensure survival; and these parameters constitute our criterion for classifying an animal as having IDDM. In the high incidence diabetes-prone mainline (DP BB/E), the incidence of IDDM is 50–60% and the mean ± SD age at onset is 96 ± 18 days. In the diabetes-resistant subline (DR BB/E), the incidence of diabetes is zero. The lymphoid status of the two lines has been characterized in detail using fluorescence-activated cell sorter analysis, and it has been shown that the BB/E colony is unique in that both lines are identical in all the immunological parameters examined (19). This makes the DR BB/E subline an exceptionally good control strain for studies of the effect of diabetes on any parameter selected.

Non-BB Wistar Han rats. Adult Wistar Han rats (outbred on site for 5 years) were obtained from our breeding facility.

All animals were maintained at 20°C on a 12-h light/12-h dark cycle and allowed free access to food and water. BB/E rats were fed rat and mouse Number 1 Expanded Feed, and Wistar Han rats were fed CRM rat and mouse Breeder and Grower Diet, both from Special Diet Services (Witham, U.K.).

Experimental protocol

Diabetic pregnancies. Female DP BB/E rats (n = 52; age 129 ± 2.4 [mean ± SE] days) with established IDDM (duration of diabetes, 30 ± 2.7 days) were mated overnight with established insulin-dependent diabetic BB/E male rats of proven fertility.

Nondiabetic pregnancies. Nondiabetic female DR BB/E rats (n = 51; age 124 ± 4.1 days; not significantly different from diabetic group) were mated overnight with nondiabetic DR BB/E males.

Wistar Han pregnancies. Female Wistar Han rats (n = 40; 150 days of age) were mated overnight with Wistar Han males. Pregnancy was confirmed the following morning by the presence of a sperm-positive vaginal smear and was designated day 0.5 of gestation.

Pregnant rats were killed by cervical dislocation on day 4.5 of gestation and the uterine horns immediately removed. Embryos were gently flushed from the excised uteri with prewarmed Nutrient Mixture (Ham's) F10 (Gibco BRL) supplemented with 0.1% bovine serum albumin (BSA) Fraction V (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma). Embryos from each individual pregnancy were transferred to an inverted microscope (Nikon) and classified as having reached a recognized and well-defined stage of development (20), namely morula, early blastocyst with a nascent blastocoele, and expanded blastocyst with a clearly demarcated trophoderm (TE) and inner-cell mass (ICM). The remaining structures were classified as abnormal embryos and defined as those exhibiting cytoplasmic fragmentation, a collapsed blastocoele, and/or blastomeres of unequal size (21). The number and percentage of J embryos at each developmental stage and for each individual pregnancy were determined and recorded for each pregnancy. All embryos classified as expanded blastocysts appeared to be normal and were collected for further study.

Differential staining of TE and ICM. The number of TE cells (the cell lineage giving rise to the placenta) and ICM cells (the cell lineage giving rise to the fetus) was determined in expanded blastocysts from diabetic and nondiabetic BB/E rats using a dual fluorochrome differential staining technique originally described by Handyside and Hunter (22) and modified by Pampfer et al. (23). Blastocysts were subjected to a 15-min incubation in 0.4% pronase (Boehringer Mannheim, U.K.) in M2 medium containing 10 mg/ml BSA (Sigma) at 37°C, 5% CO2. The denuded blastocysts were washed four times in BSA-free M2 and incubated in 4 mmo/l picrosulfonyl acid (trinitrobenzenesulfonic acid, Sigma) in BSA-free M16 with 1% polyvinylpyrrolidone (Calbiochem, La Jolla, CA) for 30 min on ice. After washing in BSA-free M2, the blastocysts were incubated with 10 μg/ml rabbit anti-dinitrophenyl (DNP) X (DNP) monoclonal antibody (ICN Biomedicals) in BSA-free M2 for 30 min at 37°C, 5% CO2. After a final washing step, the blastocysts were incubated in 10% whole guinea pig serum (Sigma) as a source of complement in BSA-free M2 containing 10 μg/ml propidium iodide (Sigma) and 20 μg/ml bisbenzimide (Sigma) for 30 min at 37°C, 5% CO2. This sequential treatment results in the anti-DNP-BSA antibody binding to the trimetaphenyl groups on the surface of the TE cells. In the presence of complement, the TE cells are partially lysed allowing access to the cytoplasm (which is excluded from viable cells) and bisbenzimide to the TE cell nuclei. The ICM remains intact because of the tight junctional seal of the trophoderm and is labeled with bisbenzimide alone. Under our conditions of ultraviolet light excitation, the different fluorescence spectrum of each dye results in the ICM staining blue and the TE staining red/pink. The differentially stained blastocysts were individually mounted on microscope slides in a small amount of medium in the center of a silicone ring with a coverslip on top and incubated at room temperature in a dark box for 20 min. The numbers of ICM and TE cells were counted directly under a Leitz Orthoplan fluorescence microscope fitted with a Leitz A filter after gently pressing the edges of the coverslip to convert the blastocyst into a two-dimensional form to facilitate accurate cell counting.

The cell counts were done over a period of 4 months with three people being involved (J.E.M., S.S.M., and R.G.L.). Initial counting was done by J.E.M. and, later, J.E.M. and S.S.M. were mainly involved. Random analysis of cell counts obtained during these two periods of time shows identical results.

Embryo culture. Expanded blastocysts flushed from the uterine horns of diabetic and nondiabetic BB/E rats on day 4.5 of pregnancy were cultured in vitro in Nutrient Mixture (Ham's) F10 medium supplemented with 0.1% BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin for 24 h at 37°C, 5% CO2. After culture, blastocysts were reexamined on an inverted microscope, washed with BSA-free M2 medium, and differentially stained.

Statistical analysis. Mean ± SE is used throughout. Differences were assessed by unpaired Student's t test.

RESULTS

Embryo morphology. Table 1 shows the number of morulae, early blastocysts, expanded blastocysts, and abnormal embryos recovered from BB diabetic, BB nondiabetic, and outbred non–BB Wistar Han rats at 4.5 days' gestation. The mean number of embryos (viable + nonviable) per pregnancy for BB diabetic and BB nondiabetic was not significantly different (6.94 ± 0.35 vs. 7.19 ± 0.29 for diabetic and nondiabetic BB/E rats, respectively); however, both BB diabetic and BB nondiabetic rats had significantly fewer embryos per pregnancy than Wistar Han rats (P < 0.0001). This low fertility is characteristic of a highly inbred line. Figure 1 depicts the same
data expressed as the mean percentage of embryos in each developmental category for the diabetic and the two nondiabetic groups. The percentage of morulae per pregnancy did not differ significantly between the three groups of animals. The percentage of early blastocysts was significantly greater in BB rat pregnancies (both diabetic and nondiabetic) than Wistar Han pregnancies at 4.5 days’ gestation (P < 0.05 for BB diabetic vs. Wistar Han; P < 0.01 for BB nondiabetic vs. Wistar Han). Diabetic and nondiabetic BB pregnancies did not differ significantly in this regard. The percentage of embryos reaching expanded blastocyst stage did not differ significantly in Wistar Han and BB nondiabetic pregnancies. However, significantly fewer embryos reached the expanded blastocyst stage in BB diabetic pregnancies than in BB nondiabetic and Wistar Han pregnancies (P < 0.0001 for both differences), and the percentage of abnormal embryos was significantly higher in diabetic pregnancies than in both groups of nondiabetic pregnancies (P < 0.0001 for both differences).

Figure 2A shows that in the 51 BB nondiabetic pregnancies, the number of expanded blastocysts per pregnancy is normally distributed at day 4.5 of gestation. In contrast, the distribution of expanded blastocysts recovered from BB diabetic rats (n = 52) is markedly disturbed (Fig. 2B), with 62% of diabetic pregnancies showing fewer than five expanded blastocysts per pregnancy compared with only 31% of nondiabetic pregnancies. Because the number of embryos per pregnancy is variable in both nondiabetic and diabetic pregnancies, the same data have also been expressed as the percentage of expanded blastocysts in each pregnancy (nondiabetic Fig. 2C and diabetic Fig. 2D). The effect of this is to emphasize the outstanding difference between diabetic and nondiabetic pregnancies, namely the high proportion of diabetic pregnancies with <10% expanded blastocysts. Further analysis (Fig. 3) shows that these same diabetic pregnancies with few expanded blastocysts were also characterized by a high number of abnormal embryos (Fig. 3B). Figure 4 depicts the close inverse correlation between these two parameters (r = 0.866). Figure 3A shows that although abnormal embryos also occurred in BB nondiabetic pregnancies, they were few in number and randomly distributed. Comparing diabetic mothers with and without abnormal embryos, we found no difference in age at pregnancy (132 ± 4.7 vs. 130 ± 3.0 days, respectively; NS), duration of diabetes (38 ± 4.9 vs. 39 ± 3.6 days, respectively; NS), and blood glucose concentration measured at 1000 on the day when embryos were retrieved, using an ExacTech meter, the majority being >20 mmol/l.

**Expanded blastocyst cell lineage analysis**

**Cell numbers.** Figure 5A is a photograph of a healthy expanded blastocyst stained as described above to show the clearly polarized ICM (blue) and surrounding TE (red). Figure 5B shows the same blastocyst after slight pressure has been applied to facilitate cell counting. Table 2 shows ICM and TE cell numbers in expanded blastocysts with apparently normal morphology isolated from diabetic and nondiabetic BB/E rats on day 4.5 of gestation. The number of cells in the ICM was significantly less in blastocysts from diabetic compared with nondiabetic BB/E rats (P < 0.0001); this represents a -20% ICM cellular deficit. The ICM cellular deficit seen in diabetic pregnancies occurred to the same extent in expanded blastocysts from pregnancies with and without abnormal embryos. In contrast, there was no difference in the number of TE cells in blastocysts from diabetic and nondiabetic rats.

**Effect of 24-h culture on cell numbers.** To determine if growth-retarded embryos were capable of catch-up growth when removed from the maternal diabetic environment, blastocysts were flushed from diabetic and nondiabetic rats and cultured in vitro for 24 h in Ham’s F10 growth medium supplemented with 0.1% BSA. Table 3 shows that blastocysts from diabetic rats did not

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### Table 1

<table>
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<th>Pregnancies</th>
<th>Total embryos</th>
<th>Abnormal embryos</th>
<th>Morulae</th>
<th>Early blastocysts</th>
<th>Expanded blastocysts</th>
<th>Embryos per pregnancy</th>
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<td>Diabetic</td>
<td>52</td>
<td>361</td>
<td>81</td>
<td>38</td>
<td>57</td>
<td>187</td>
<td>6.94 ± 0.35*</td>
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<tr>
<td>Nondiabetic</td>
<td>51</td>
<td>372</td>
<td>15</td>
<td>30</td>
<td>58</td>
<td>269</td>
<td>7.19 ± 0.29*</td>
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<tr>
<td>Wistar Han</td>
<td>40</td>
<td>400</td>
<td>28</td>
<td>31</td>
<td>33</td>
<td>308</td>
<td>10.00 ± 0.39</td>
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Data are n or means ± SD. *P < 0.0001: significantly less than Wistar Han (Student’s t test).
exhibit any sign of ICM catch-up growth in vitro, with the ICM cellular deficit virtually the same as that found in vivo at -18% ($P < 0.001$). In contrast, in culture TE cellular accretion was 25% greater in blastocysts from diabetic mothers than from nondiabetic mothers ($P < 0.0001$).

**Cell death in the blastocyst.** Closer examination of stained blastocysts revealed that cells with fragmented nuclei were observed more often in the ICM than in the TE (Fig. 6A). At high magnification ($\times500$), these cells show the features characteristic of apoptosis, including cell surface blebbing, nuclear condensation and fragmentation, and, finally, splitting of the cell itself into multiple membrane bound “apoptotic bodies,” some of which contain nuclear fragments (24). Dead cells were often seen at the edge of the ICM (Fig. 6B) and, in some cases, appeared to be in the process of extrusion (Fig. 6C).

**FIG. 2.** Distribution of the number of expanded blastocysts in each pregnancy (A and B). BB nondiabetic pregnancies ($n = 51$) are indicated by hatched bars; BB diabetic pregnancies ($n = 52$) are indicated by solid bars. Same data are expressed as a percentage of expanded blastocysts in each pregnancy (C and D).

**FIG. 3.** The relationship between the number of expanded blastocysts and abnormal embryos in individual nondiabetic and diabetic pregnancies. Each dot (●) represents a single pregnancy with one or more abnormal embryos. The right-hand axis indicates the number of abnormal embryos in each individual pregnancy. The bars represent the same data shown in Figs. 2A and B.
DISCUSSION

We have used the BB/E rat to demonstrate that pregnancy in established diabetic rats maintained on daily injections of insulin is associated with a severe disturbance in the development of the preimplantation embryo in vivo as indicated by 1) a high incidence of abnormal (that is, degenerate and fragmented) embryos, 2) a reduced percentage of expanded blastocysts, and 3) a -20% ICM cellular deficit in blastocysts that reach the expanded blastocyst stage (with no alteration in TE cell number) compared with pregnancy in both nondiabetic BB and Wistar Han rats. Moreover, blastocysts removed from the maternal diabetic environment and cultured in vitro for 24 h showed no sign of catch-up growth in the ICM, although under these conditions, a TE cellular accretion of +25% occurred.

Women with IDDM have been shown to have an increased rate of early fetal loss (25,26). Perhaps the most striking feature of the disturbance associated with IDDM pregnancy in our study is the high incidence of degenerate and fragmented embryos at 4.5 days' gestation. This phenomenon has been reported previously in hormonally superovulated mice with both chemically induced (27) and spontaneous (12) diabetes and also in rats, which were not superovulated, with streptozotocin (STZ)-induced diabetes (28). The latter paper is important because superovulation per se has been shown to increase the incidence of abnormal embryos (29); however, streptozotocin-induced damage to oocytes cannot be ruled out in this study. Although abnormal preimplantation embryos are not specific to diabetic pregnancy, it appears that diabetes is associated with a five- to eightfold increase in their incidence. In our studies, the incidence of abnormal embryos in the two nondiabetic groups, namely DR BB/E and non-BB Wistar Han, was not significantly different (Fig. 1), ruling out a BB strain-related effect in the diabetic BB/E group.

TABLE 2

<table>
<thead>
<tr>
<th>Type of pregnancy</th>
<th>Number of blastocysts</th>
<th>Cell lineage</th>
<th>Inner cell mass</th>
<th>Trophoderm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic</td>
<td>57</td>
<td>8.35 ± 0.34*</td>
<td>20.09 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>Nondiabetic</td>
<td>43</td>
<td>10.40 ± 0.25</td>
<td>20.23 ± 0.41</td>
<td>-19.7</td>
</tr>
<tr>
<td>% increase/decrease</td>
<td></td>
<td></td>
<td></td>
<td>-0.7</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SE. *P < 0.0001; significantly less than blastocysts from nondiabetic rats (Student's t test).
pregnancies have one or more abnormal embryo, the number of abnormal embryos per pregnancy can be as high as seven, and abnormal embryos do not occur randomly but rather are concentrated in pregnancies with no or few expanded blastocysts. Indeed, the data suggest that the lack of expanded blastocysts is a direct result of the high incidence of abnormal embryos and that this also accounts for our inability to demonstrate the retardation of embryonic development (as measured by distribution of developmental stages) that has been reported by others (12,28).

While our data suggest two distinct populations of pregnancies within the BB diabetic group—one with a high incidence of abnormal embryos and a reduced number of expanded blastocysts and the other with no abnormal embryos and a normal complement (more than six) of expanded blastocysts—we show that the morphologically normal expanded blastocysts from both populations of diabetic pregnancies are not in fact normal, since they exhibit an ICM cellular deficit similar to that described previously in rats with STZ-induced diabetes (28). This suggests either that the mechanisms underlying the development of abnormal embryos and the ICM cellular deficit are different or, more probably, that these two pathological features result from either variation in the degree of genetic susceptibility and/or damaging environmental factors to which the gametes and/or embryos are exposed.

Although there is evidence that paternal genes exert an influence on the development of the preimplantation embryo and formation of the blastocyst (30), we cannot correlate the occurrence of abnormal embryos with any particular male rat(s), and, indeed, all the male diabetic rats used in this study had previously been shown to be consistently highly fertile when mated with female nondiabetic DR BB/E rats over a considerable period of time before this study began. Moreover, the ICM cellular deficit appears to be solely a function of maternal diabetes with no input from the father (ICM cell number: diabetic DP BB/E mother × diabetic DP BB/E father, 8.35 ± 0.344; diabetic DP BB/E mother × nondiabetic DR BB/E father, 8.00 ± 0.304; nondiabetic DR BB/E mother × diabetic DP BB/E father, 10.48 ± 0.43; nondiabetic DR BB/E mother × nondiabetic DR BB/E father, 10.40 ± 0.25 [P < 0.0001 for all differences]).

So far, we have been unable to identify any difference between diabetic pregnancies with and without abnormal embryos in relation to maternal features, such as age, duration of diabetes, daily dose of insulin, results of daily urine tests for glucose and ketone bodies, and blood glucose measured at 1000 on day 4.5 of gestation. Indeed, given 1) the inbred status of the BB/E colony, 2) the fact that it is held in a barrier unit under strictly controlled conditions and screens negative for all the common rat viruses, and 3) that all the diabetic rats involved in the study had established IDDM as defined by strict criteria detailed above, these diabetic rat mothers were a remarkably homogeneous group. Nevertheless, subtle differences may exist in the diurnal metabolic profile of these two populations of pregnancies—perhaps including secondary alterations in growth factors and/or cytokines, which have been shown to play important

![Image](https://example.com/image.png)

**FIG. 6.** A: differentially stained BB/E rat expanded blastocysts showing dead cells and fragmented nuclei, the appearances being characteristic of apoptosis (see text) in the ICM. B: dead cells located at the edge of the ICM. C: dead ICM cells on the outer edge of the embryo TE.

Analysis of the distribution of pregnancies with abnormal embryos (Fig. 3) not only emphasizes the low number of pregnancies with abnormal embryos in the nondiabetic group (8 out of 51 = 16%) but also shows that in this group, the number of abnormal embryos per pregnancy is never more than two and the distribution of pregnancies with abnormal embryos is random. In contrast, in the diabetic group, 58% (30 out of 52) of all
regulatory roles in the development of the preimplantation embryo and in implantation (31–33)—and further work is planned.

Our results showing a diabetes-related cellular deficit specific to the ICM are in agreement with previous studies in rats with STZ-induced diabetes (28). Pampfer et al. (34) also cultured blastocysts in vitro for 24 h and, like us, found no sign of catch-up growth in the ICM during this period. This concordance with the results obtained for TE cells in culture, since we find a highly significant TE cellular accretion (+25%; P < 0.0001), while Pampfer et al. describe a −8.5% decrease in cell number (P < 0.05). We are unable to explain this difference in the results obtained from these two models, but we have previously shown that pregnancy in diabetic BB/E rats is characterized by low birth weight pups and large placentas (11) and these features also occur commonly in human IDDM pregnancy.

We have also previously observed that diabetic BB/E rats maintained on continuous conventional insulin therapy have a higher rate of fetal resorption than nondiabetic DR BB/E rats (11). This could be related to the ICM cellular deficit seen in preimplantation embryos from diabetic BB/E rats, since it has been shown that although mouse blastocysts with a reduced or even absent ICM implant normally (35), organogenesis is disturbed (36). In addition, normal blastocyst formation depends on adequate cellular interaction between ICM and TE cells and in vitro studies have shown that ICM cells are more susceptible than TE cells to damage by a range of chemicals, drugs, and antimetabolites (36). Thus, a diabetes-associated disturbance in any component of the intrauterine milieu during the preimplantation period is likely to affect ICM more than TE cells and to disturb ICM-TE cell interaction. Indeed, it is possible that the abnormal fetal growth and development characteristic of human IDDM pregnancy has its origin in the damage incurred by the preimplantation embryo.

Other groups have suggested that conventional treatment with subcutaneous injection of insulin completely prevents diabetic embryopathy in rats and mice with STZ-induced diabetes and in NOD mice. This contrasts with our results in the BB/E rat, since all our rats with IDDM (as described above) are completely and irreversibly insulin deficient and require daily injections of insulin to survive (J. Freeman, R.M. Lindsay, J.D. Baird, J. Foster, C.R. Elcombe, D.J. Harrison, H. Wolf, C.R. Wolf, unpublished observations). In this respect, they differ from NOD mice and rats with STZ-induced diabetes, which are not always totally insulin deficient and are capable of spontaneous regeneration of islet β-cells (37). We have previously documented the metabolic status of BB/E rats with IDDM maintained on conventional insulin therapy in detail, both during pregnancy (11) and when not pregnant (38,39).

Coordinated apoptosis is a crucial factor in normal morphogenesis. The reduction in ICM cell number seen in blastocysts from diabetic BB/E rats may be due to disturbed regulation of apoptosis. Apoptosis may occur either as a programmed “cell-autonomous” event, the activating switch for which is unknown, or in response to an external trigger. Drugs, toxins, radiation, cellular ligands (e.g., fas ligand and CD40 ligand), cytokines (e.g., tumor growth factor β and tumor necrosis factor α), and matrix attachment factors are among those identified (24). At this stage, we are unable to say whether or not apoptosis is upregulated in blastocysts from diabetic mothers because there are major difficulties in quantitating the phenomenon using microscopy. Problems include the speed of apoptosis and the rapidity of clearance of apoptotic cells; identifying apoptotic cells in a threedimensional structure, such as a blastocyst; and the fragility of cells undergoing apoptosis, which are effectively lost when the blastocyst is flattened to facilitate accurate counting of cells.

In conclusion, we have demonstrated major disturbances in embryo development at the earliest stages of pregnancy in an animal model with spontaneous autoimmune IDDM. It is not known whether improved diabetic control at conception can prevent or reverse the pathology documented in this study. Further studies are in progress in which development of the preimplantation embryo is examined in relation to the maternal metabolic profile at various levels of maternal blood glucose control established before and after conception.

ACKNOWLEDGMENTS

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REFERENCES


Increased cell death in rat blastocysts exposed to maternal diabetes in utero and to high glucose or tumor necrosis factor-α in vitro

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SUMMARY

The morphogenetic function of the transient phase of cell death that occurs during blastocyst maturation is not known but it is thought that its regulation results from a delicate balance between survival and lethal signals in the uterine milieu. In this paper, we show that blastocysts from diabetic rats have a higher incidence of dead cells than control embryos. Differential lineage staining indicated that increased nuclear fragmentation occurred mainly in the inner cell mass. In addition, terminal transferase-mediated dUTP nick end labeling (TUNEL) demonstrated an increase in the incidence of non-fragmented DNA-damaged nuclei in these blastocysts. Analysis of the expression of clusterin, a gene associated with apoptosis, by quantitative reverse transcription-polymerase chain reaction detected an increase in the steady-state level of its transcripts in blastocysts from diabetic rats. In situ hybridization revealed that about half the cells identified as expressing clusterin mRNA exhibited signs of nuclear fragmentation. In vitro experiments demonstrated that high D-glucose increased nuclear fragmentation, TUNEL labeling and clusterin transcription. Tumor necrosis factor-α (TNF-α), a cytokine whose synthesis is up-regulated in the diabetic uterus, did not induce nuclear fragmentation nor clusterin expression but increased the incidence of TUNEL-positive nuclei. The data suggest that excessive cell death in the blastocyst, most probably resulting from the overstimulation of a basal suicidal program by such inducers as glucose and TNF-α, may be a contributing factor of the early embryopathy associated with maternal diabetes.

Key words: blastocyst, cell death, clusterin, diabetes, glucose, rat, TNF-α

INTRODUCTION

It has long been recognized that many critical developmental events occur through the death of selected cells in predictable places and at predictable times (Glucksman, 1951; Sanders and Wride, 1995). The fact that these cells appear to die naturally led to the proposal that their elimination results from the operation of a physiological procedure which was designated 'programmed cell death' (Wyllie et al., 1980). Active cell elimination appears to be engaged very early in development since the process of proamniotic cavity formation already relies on the destruction of selected cells (Coucouvanis and Martin, 1995). Several reports also suggest that this eliminative process may be activated even earlier, at the blastocyst stage. Following the initial description of dead cells in preimplantation embryos (Potts and Wilson, 1967), additional evidence has been based on electron microscopy (El-Shershaby and Hinchliffe, 1974), reconstruction of serial sections (Copp, 1978) and differential labeling with DNA fluorochromes (Handyside and Hunter, 1986). In mouse blastocysts, cell death occurs as they reach maximal expansion, with most disintegrating cells located in the inner cell mass (ICM) lineage. It has been hypothesized that the primary function of this process is to allow for the removal of redundant or defective cells from the blastocyst, hence eliminating the risk of maintaining a subgroup of cells with unwanted developmental potential within the embryonic germ layers (Pierce et al., 1989).

It is not clear whether the elimination process observed in blastocysts is cell autonomous or controlled by extraembryonic signals. Mouse blastocysts rendered unresponsive to both intracellular and extracellular survival signals simultaneously have been shown to engage in massive cell death, suggesting that embryonic cells are equipped with a death-by-default mechanism that requires constant suppression (Weil et al., 1996). One uterine survival agent may be transforming growth factor-α. This effector is expressed in the pregnant mouse uterus at the time of implantation (Paria et al., 1994) and its addition to the composition of culture medium reduces the incidence of dead cells in mouse blastocysts in vitro (Brison and Schultz, 1997). Thus, if the normal programming of developmental genes, including those involved in cell death, depends on the correct integration of multiple signals, it seems reasonable to speculate that, under certain pathological conditions, an imbalance in these signals would lead to inappropriate cell determination (Kimmel et al., 1993). In addition, because the correct allocation of cells to the ICM is of funda-
mental importance for the subsequent formation of the embryonic germ layers (Tam, 1988), it can be postulated that excessive cell death in this lineage at the blastocyst stage would predispose to later developmental deficiencies.

Experiments on animal models have shown that the developmental status of embryos recovered at the blastocyst stage from diabetic females is markedly altered (Pampfer and De Hertogh, 1996). Rat blastocysts from streptozotocin-induced diabetic rats contain fewer cells than control embryos (Vereheval et al., 1990) and this cellular loss is predominantly located in the ICM (Pampfer et al., 1990a). Recent investigations on blastocysts from genetically prone diabetic BB/E rats have confirmed both the cellular deficit and the higher susceptibility of the ICM (Lea et al., 1996). In addition, when blastocysts from streptozotocin-induced diabetic rats are incubated in vitro, their viability continues to decline in contrast to control blastocysts (Pampfer et al., 1994a), suggesting that the impact of the maternal disorder is long-lasting. Because diabetes is associated with a myriad of hormonal, metabolic and other alterations, identifying which imbalanced factor(s) are responsible for the developmental anomalies detected before implantation may prove difficult. Although high glucose concentrations are likely to be accumulated in the uterine lumen of the diabetic female (Nilsson et al., 1980) and high glucose is detrimental to rat blastocysts (De Hertogh et al., 1991), recent data suggest that more indirect effects may be involved. Previous observations that tumor necrosis factor-α (TNF-α) is up-regulated in the diabetic uterus (Pampfer et al., 1995a, 1995b) combined with findings that TNF-α inhibits rat blastocyst growth (Pampfer et al., 1994b) support the hypothesis that inappropriate release of this cytokine may be integral in inducing the early embryopathy associated with maternal diabetes.

In the present work, we used different cellular and molecular techniques to detect an increase in the incidence of cell death in blastocysts recovered from diabetic rats and in normal blastocysts incubated with high glucose and/or TNF-α.

**MATERIALS AND METHODS**

**Animals and embryos**

In experiments involving diabetic rats, adult females were administered a single injection of streptozotocin and tested 24 hours later for their glycosuria (Vereheval et al., 1990). 2-4 weeks after drug treatment, diabetic females were mated to normal males and pregnancy was determined on the next morning by the presence of a vaginal plug (gestational day 1). Normal pregnant females were used in parallel as controls. On day 5 or day 6, the uterine horns from diabetic and control rats were flushed to recover the embryos. Only those embryos displaying the morphology of mid-expanded blastocyst on day 5 or hatched blastocyst on day 6 were selected for further study. Whole blood glycerol was measured using a glucose test strip meter. Serum levels of β-hydroxybutyrate were measured using a colorimetric assay (ref. 310-A, Sigma, St Louis, USA) and serum concentrations of TNF-α were measured by means of an enzyme-linked immunosorbent assay (ref. 80-3905-01, Genzyme, Boston, USA). For in vitro experiments, blastocysts were recovered from normal rats on day 5 and incubated at 37°C in a humidified atmosphere with 5% CO2 for 24 hours. The basal culture medium was Ham’s F-10 (ref. 04-1040-20, Gibco, Buckinghamshire, UK) supplemented with 1 mM glutamine, 0.1% bovine serum albumin, 100 U/ml penicillin and 100 μg/ml streptomycin. Depending on the experiments, various combinations of glucose (control 6 mM D-glucose, 17 mM D-glucose, 6 mM D-glucose + 11 mM L-glucose) and/or 3000 U/ml of rat recombinant TNF-α (ref. CY-044, Innogenetics, Zwijnaarde, Belgium) were added to the culture medium. The specificity of TNF-α action was confirmed using a neutralizing rabbit anti-rat TNF-α antibody (ref. CY-051, Innogenetics) in 100-fold molar excess.

**Differential nuclear staining**

The number of cells per embryo and their distribution between the inner cell mass (ICM) and the trophectoderm (TE) were counted using a differential staining technique based on the partial immunolysis of the outer TE layer and the exposure of the embryo to two DNA dyes with different permeability and fluorescent properties (Pampfer et al., 1990b). Partial complement-mediated lysis of the peripheral TE layer resulted in the permeabilization of that lineage to propidium iodide (PI, which is normally excluded from membrane-intact cells) whereas bisbenzimide (Hoechst 33258, HO) entered into all the cells of the embryo, including the ICM. Nuclei in the ICM were found clustered at the center of the embryo and stained blue under u.v. light whereas nuclei of TE cells looked more spread, larger in size and stained pink. In both cell lineages, some nuclei appeared fragmented or scattered into highly fluorescent chromatid particles which were still comprised within intact cell boundaries as determined under visible light. For each blastocyst, the incidence of disintegrating nuclei in the two cell lineages was expressed as percentage of the corresponding numbers of ICM and TE cells (nuclear fragmentation index). In a limited set of experiments, rat blastocysts were exposed to the two DNA dyes without prior treatment with promace and complement. The observation of cells containing either HO-stained or PI-stained fragmented nuclei was restricted to the TE layer in these embryos.

**Terminal transferase-mediated dUTP nick end labeling (TUNEL)**

Nuclei containing abundant 3'-hydroxyl termini were detected using the TUNEL technique (Gavrieli et al., 1992). Blastocysts were exposed to 0.4% pronase, fixed in 4% paraformaldehyde in PBS, exposed to 0.3% hydrogen peroxide in methanol and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. Following a rinse in PBS supplemented with 0.5% Tween-20 (PBS-T), the embryos were pretreated with 25 μg/ml of HO in PBS-T. Incubation with 50 units/ml of terminal transferase and 15 μM of fluorescein-dUTP was performed for 35 minutes at 37°C in 50 μl drops. The embryos were exposed to sheep anti-fluorescein conjugated to peroxidase and nuclear TUNEL staining was developed in a solution of diaminobenzamide and nickel chloride. Exposure of the embryos to u.v. light allowed to visualize and count the total number of HO-nuclei and the proportion of fragmented nuclei. Observation under visible light allowed for the counting of TUNEL-positive nuclei. For each blastocyst, the incidence of fragmented nuclei (F-index), TUNEL-positive nuclei (T-index) and nuclei with both labeling features (F+T-index) was calculated as percentage of the total cell number. For negative control experiments, the terminal deoxynucleotidyl transferase was omitted from the labeling reaction. For positive control reactions, blastocysts were pretreated with 15 units/ml of deoxynuclease I before labeling.

**Quantitative reverse transcription-polymerase chain reaction (RT-PCR)**

The steady-state level of clusterin transcripts was assessed by means of a quantitative RT-PCR method based on the co-amplification of the unknown amount of clusterin cDNA in each sample with a known amount of internal standard. To construct the standard, a 564 bp-long amplicon was generated from rat placental cDNA with two primers (SP1 and SP2) specific for clusterin (Fig. 1). The genomic structure of rat clusterin and its cDNA sequence (GenBank M64723) have been reported (Wong et al., 1993). The amplicon was inserted
into a pCRII plasmid (clone p16rCLUm) and modified to increase its length by 4 nucleotides. One of the mutated constructs (p9rCLUm) was used as internal standard for PCR. To study clusterin mRNA expression in blastocysts, total RNA was extracted from pools of 30 embryos and retro-transcribed with 200 μM random hexamers and MMLV reverse transcriptase (100 U per reaction) (Pampfer et al., 1994b). A fixed amount of internal standard (5×10^6 molecules) was added to each cDNA sample which was then serially diluted in PCR reaction mixture. Each dilution series (1:1, 1:3 and 1:9) was amplified with 1 μM of primers SP28 and SP29 (Fig. 1) and Taq DNA polymerase (5 U per reaction). PCR reactions were terminated after 32 cycles when the amplification process was still within the exponential phase so that the ratio of unknown target to known internal standard at the initiation of the reaction was conserved. Aliquots of PCR reactions were electrophoresed on 5% polyacrylamide gel containing 8 M urea. Silver-stained ampiclons produced from the target cDNA (459 bp) and the internal standard (463 bp) were quantitated by direct scanning densitometry. For each cDNA sample, the ratio between the integrated intensities of the target and the internal standard was calculated at the three dilutions tested and the average ratio value was expressed in arbitrary units. Two control cDNAs, one from a group of clusterin negative rat preblastocyst embryos (morula stage) and one from clusterin positive rat ovaries, were serially diluted after addition of internal standard and run in parallel in each assay, together with a mock sample prepared without RNA input. The blastocyst cDNAs were also tested for variations in the retro-transcription step by amplifying the samples with primers complementary to nucleotide positions 273-292 (SP30) and 552-571 (SP23) of the rat β-actin cDNA sequence (Nudel et al., 1993). Corrections made to the clusterin expression values never exceeded 10% of the original result.

In situ hybridization (ISH)

Localization of clusterin mRNA expression in rat blastocysts was studied by ISH using specific 5′-end biotinated probes. A combination of two oligonucleotides (antisense ISHB and ISHD) complementary to distinct regions of the clusterin cDNA sequence was used against a combination of corresponding reverse oligonucleotides (sense ISHA and ISHC) as controls (Fig. 1). Blastocystes were fixed in 3% paraformaldehyde/0.5% glutaraldehyde in PBS, rinsed in PBS containing 0.1% Triton X-100 (PBS-TR), exposed to 10 μg/ml proteinase K in PBS-TR and washed in 2 mg/ml glycine in PBS-TR before fixation in 4% paraformaldehyde/0.2% glutaraldehyde in PBS. The blastocystes were then incubated in 0.1% sodium borohydride and prehybridized in a solution described elsewhere (MacPhee et al., 1994). The embryos were hybridized in the same solution containing the probes ISHB+ISHD or ISHA+ISHC at 0.5 μM of each oligonucleotide for 34 hours at 50°C with agitation. Post-hybridization consisted of successive washes in 300 mM NaCl, 1 mM EDTA, 10 mM Pipes and 1% SDS; in 50 mM NaCl, 1 mM EDTA, 10 mM Pipes and 0.1% SDS; in 500 mM NaCl, 10 mM Pipes and 0.1% Triton X-100 before transfer into the latter buffer with 100 μg/ml ribonuclease A and 100 U/ml ribonuclease T1. Post-hybridization was continued in 300 mM NaCl, 1 mM EDTA, 10 mM Pipes, 50% formamide and 1% SDS; in 150 mM NaCl, 1 mM EDTA, 10 mM Pipes, 50% formamide and 0.1% Triton X-100 and in 500 mM NaCl with 0.1% Triton X-100. Following a rinse in PBS supplemented with 0.5% Tween-20 (PBS-T), the embryos were treated in 0.3% hydrogen peroxide in PBS-T, incubated in 0.1% sodium citrate and 0.1% Triton X-100 and then exposed to a streptavidin-biotin-peroxidase complex. The embryos were prestained with 25 μg/ml of HO in PBS-T and transferred into a solution of diaminobenzamide and nickel chloride. Exposure of the embryos to u.v. light allowed to visualize intact and fragmented nuclei whereas visible light allowed for the detection of clusterin mRNA-positive cells.

Statistical analysis

Except for the data in Table 1, which were compared by either Student’s t-test or Mann-Whitney U-test, differences between control values and experimental values were compared by one-way analysis of variance coupled with Scheffe’s F-test. Except in Fig. 9, the data are given as mean±s.e.m. For each set of experiments, blastocysts from 4 to 8 females (normal or diabetic) were pooled before either direct observation or assignment to different incubation groups. Inter-litter variations, if any, have been previously found to be neutralized by this randomization process. In addition, the results in Figs 3, 4, 6 and 7 as well as in Table 2 were based on reproducible data collected from at least three independent and ‘blind’ repeats.

RESULTS

Embryo collection

On both day 5 and day 6, hyperglycemia and ketosis were confirmed in the diabetic group (P<0.05) (Table 1). Although the average serum TNF-α value in diabetic rats on day 6 was twice the control value, on neither day was the difference statistically significant. On day 5, both the total number of embryos recovered per rat (9.4±0.2 versus 7.7±0.3) and the number of blastocysts per rat (Table 1) were decreased in the diabetic group. No difference was found on day 6, however, probably because the most advanced blastocysts in the control group had already implanted at the time of uterine flushing.

Nuclear fragmentation in blastocysts in vivo

The occurrence of cell death was analyzed by means of a dual staining technique that discriminates between ICM and TE
nuclei (Fig. 2). When blastocysts from diabetic rats were collected on day 5 and compared to control embryos, a 18.3% deficit in the total cell number was found in the diabetic group (P<0.01) (Fig. 3), with about 4/5 of this loss located to the ICM. Closer examination revealed that the percentage of OH-stained fragmented nuclei increased to 8.4±1.7% in the ICM of blastocysts in the diabetic group from 1.1±0.3% in the ICM of control embryos (P<0.01). In contrast, the percentage of PI-stained fragmented nuclei, which was below 1.0% in the TE lineage of control blastocysts, was not statistically different in embryos from diabetic rats. Altogether, 48.9% of the blastocysts in the day 5 diabetic group contained at least one disintegrating nucleus versus 18.2% in the control group. These observations were repeated on blastocysts recovered on day 6. Compared with control embryos, blastocysts in the diabetic group contained fewer cells (16.8% loss) on the average (P<0.01) (Fig. 3) with the deficiency again predominantly located to the ICM. The nuclear fragmentation index was increased in the ICM of blastocysts from diabetic rats, 7.1±1.1% against the control value of 2.2±0.9% (P<0.05), whereas it remained low in TE cells. Exactly 50% of the blastocysts from day 6 diabetic rats had at least one fragmented nucleus, irrespective of its location, versus 31.4% in the control group.

Table 1. Recovery of rat blastocysts on gestational day 5 and day 6

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
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<tbody>
<tr>
<td>Blood glycemia (mM)</td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>n</td>
<td>83</td>
<td>19</td>
</tr>
<tr>
<td>Serum β-hydroxybutyrate (µM)</td>
<td>1.7±0.2</td>
<td>2.7±0.4</td>
</tr>
<tr>
<td>Serum TNF-α (U/ml)</td>
<td>323.4</td>
<td>266.7</td>
</tr>
<tr>
<td>Confidence interval</td>
<td>95.1±11.7</td>
<td>132.9±400.4</td>
</tr>
<tr>
<td>Number of blastocysts per rat</td>
<td>8.2±0.3</td>
<td>4.9±0.3</td>
</tr>
</tbody>
</table>

Data are means ± s.e.m. with n representing the number of values. (*) and (**) indicate P<0.05 or 0.01 respectively.

Fig. 2. Nuclear fragmentation in blastocysts. Differential staining with HO and PI was used to distinguish ICM (blue) and TE (pink) nuclei (A). Closer examination revealed the presence of fragmented HO-stained ICM nuclei (B; arrow) and fragmented PI-stained nuclei (C; arrowhead) in some blastocysts. In some blastocysts, expelled cells with HO-stained nuclear fragments were observed in the perivitelline space (D; arrow). Blastocysts were from a control rat (A), from diabetic rats (B, C) or following incubation in high D-glucose (D). The scale bar represents 50 µm in A, D and 10 µm in B, C.

Fig. 3. Nuclear fragmentation in blastocysts exposed to maternal diabetes. Embryos were recovered from control (CTR) and diabetic (DIAB) rats on day 5 (upper panels) or day 6 (lower panels) and counted for the total number of cells per embryo (A, C) and for the frequency of nuclear fragmentation (B, D) in the inner cell mass (ICM) and trophectoderm (TE) lineages. More than 45 embryos were examined in each group on day 5 and more than 30 embryos on day 6. (**) indicates a statistically significant difference from corresponding control value (P<0.01).
Nuclear fragmentation in blastocysts in vitro

Blastocysts from normal rats were incubated under different conditions. At the initiation of the culture, the mean cell number per blastocyst was 37.6±1.3 with 13.5±0.5 of ICM cells and 24.1±0.9 TE cells. After 24 hours, there was a 2.3-fold increase in the mean cell number per embryo in control culture medium. Compared with control blastocysts, fewer cells were counted in embryos incubated in the presence of 17 mM D-glucose (13.3% loss), 3000 U/ml rat recombinant TNF-α (13.8% loss) or in a combination of high D-glucose and TNF-α (17.7% loss) (P≤0.01) (Fig. 4). In all these groups, the cell deficiency was more pronounced in the ICM than in the TE. Closer examination showed that the percentage of fragmented ICM nuclei was increased to 8.4±1.4% in high D-glucose versus 1.6±0.4% in control culture medium (P≤0.01) whereas exposure to TNF-α alone did not induce any significant difference. Combining high D-glucose and TNF-α raised the nuclear fragmentation index in the ICM to 9.5±1.8% (P≤0.01). None of the treatments tested increased nuclear fragmentation in the TE. At the start of the incubation period, average frequencies of disintegrating nuclei were below 1.0% in both ICM and TE lineages. In additional experiments, blastocysts were incubated in control medium or in high D-glucose or L-glucose for 24 hours and directly transferred into a solution of HO and PI. The incidence of HO-stained fragmented nuclei remained below 0.5% in the peripheral TE layer of all the blastocysts examined. The frequencies of PI-stained intact and fragmented nuclei remained also at a very low level (0.1%). Omission of enzymatic digestion allowed for the observation of extruded cells in the perivitelline space of about 15% of the blastocysts exposed to high D-glucose (Fig. 2). Most of these cells contained HO-stained nuclear fragments.

TUNEL-positive nuclei in blastocysts in vivo

The occurrence of cell death was further analyzed using a technique that allowed for the simultaneous detection of fragmented nuclei in u.v. light and nuclei with abundant free DNA 3'-OH ends (TUNEL-positive nuclei) in visible light (Fig. 5). A comparison between blastocysts from control and diabetic rats on day 5 confirmed that the mean total cell number was decreased in the diabetic group (P≤0.01) (Fig. 6). In these blastocysts, the F-index and T-index were both increased (P≤0.01) whereas the presence of nuclei combining signs of fragmentation with TUNEL-staining was not detected (below 0.1%). Only 21.4% of the blastocysts collected from diabetic rats showed no signs of cell death against 73.2% in the control group. The frequency of nuclei in metaphase was not statistically different in embryos from the control and diabetic groups (1.5±0.3% versus 1.2±0.3%). Close examination of 12 blastocysts from diabetic rats in which the blastocoele was clearly delineated showed that 82% of the TUNEL-positive nuclei detected in these embryos were localized in the ICM region. Blastocysts recovered on day 6 were examined using the same technique. All the three nuclear labeling indexes were increased in blastocysts in the diabetic group (P≤0.01) (Fig. 6). Only 13.2% of the blastocysts from diabetic rats were without signs of nuclear damage, relative to 42.2% in the control group. The percentage of nuclei in metaphase was low (60.3%) and

Fig. 4. Nuclear fragmentation in blastocysts exposed to high glucose and TNF-α. Embryos were recovered from normal rats and incubated in control medium (CTR, n=55 embryos) or in high D-glucose (HIGH GLUCOSE, n=36), TNF-α (TNF, n=32) or a combination of high D-glucose and TNF-α (TNF + HIGH GLUCOSE, n=32) before examination for the total number of cells per embryo (A) and for the frequency of nuclear fragmentation (B) in the inner cell mass (ICM) and trophectoderm (TE) lineages. (***) indicates a statistically significant difference from corresponding control value (P≤0.01).

Fig. 5. TUNEL staining in blastocysts. HO staining coupled with TUNEL staining were used to visualize different types of nuclear degradation. Damaged nuclei were classified in three categories according to their labeling pattern: HO-stained fragmented nucleus without TUNEL signal (F-labeling) (A,B; arrow), HO-stained intact nucleus with TUNEL signal (T-labeling) (C; arrow, D) or HO-stained fragmented nucleus with TUNEL signal (F+T labeling) (E,F; arrow). Blastocysts shown were from control (A,B and C,D) and diabetic (E,F) rats. The scale bar in F represents 15 μm.
identical in the two groups. Because the blastocoele had collapsed in almost all the blastocysts recovered on day 6, it was more difficult to confirm that TUNEL-positive nuclei were still predominantly located in the ICM at that later stage.

**TUNEL-positive nuclei in blastocysts in vitro**

Normal blastocysts cultured in the presence of either 17 mM D-glucose, 3000 U/ml TNF-α or high D-glucose and TNF-α combined for 24 hours contained fewer cells than blastocysts in control medium (P<0.05) (Fig. 7). Compared with control embryos, F-index and T-index were both increased in embryos exposed to high D-glucose (P<0.01) although there was no difference in the F+T index. Both T-index and F+T-index, but not nuclear fragmentation alone, were increased in embryos exposed to TNF-α (P<0.05). Exposure to high D-glucose and TNF-α simultaneously resulted in an increase in all three nuclear labeling indexes (P<0.05). The percentage of blastocysts showing no signs of cell death was 12.5% in the control group versus 12.1% in high D-glucose, 2.9% in TNF-α and 2.3% in high D-glucose combined with TNF-α. The frequency of metaphasic nuclei (≤1.0%) was not influenced by the culture conditions. Careful examination of 19 blastocysts exposed to high D-glucose and 18 blastocysts treated with TNF-α in which the limits of the blastocoele were clearly visible showed that, respectively, 81% and 73% of the TUNEL-positive nuclei detected in these embryos were localized in the ICM region. In 16 control blastocysts, 77% of the TUNEL-positive nuclei were found in the ICM. Addition of 11 mM L-glucose to the control medium did not influence the mean total number of cells per embryo nor the percentages of nuclei showing signs of fragmentation and/or TUNEL-staining (Fig. 7). Preincubation of a culture medium containing 3000 U/ml TNF-α with a 100-fold molar excess of neutralizing rabbit anti-rat TNF-α antibody before embryo culture prevented the impact of the cytokine on the mean number of cells and on the nuclear labeling indexes. When an equivalent molar excess of normal rabbit IgG was substituted for anti-TNF-α antibody, the mean total number of cells per embryo as well as the average T-index

![Fig. 6](image-url)

**Fig. 6.** TUNEL staining in blastocysts exposed to maternal diabetes. Embryos were recovered from control (CTR) and diabetic (DIAB) rats on day 5 (upper panels) or day 6 (lower panels) and counted for the total number of cells per embryo (A,C) and for the frequencies of fragmented nuclei (F), TUNEL-positive nuclei (T) and nuclei displaying both nuclear labels (F+T). More than 40 embryos were examined in each group on day 5 and more than 30 embryos on day 6. (***) indicates statistically significant difference from corresponding control value (P<0.01).
and F+T-index were altered to the same extent as these values were in the presence of TNF-α alone (P≤0.05). In a series of negative control reactions, blastocysts were incubated in 17 mM D-glucose for 24 hours and subjected to the TUNEL-staining procedure without terminal deoxyribonucleotidyl transferase. Nine embryos, with a mean total number of cells of 49.1±2.3 and an average frequency of fragmented nuclei of 5.7±0.6%, were found completely free of TUNEL staining. In a series of positive control reactions, blastocysts were cultured in control medium for 24 hours and then treated with deoxyribonuclease I for 2 hours before TUNEL staining. Examination of 5 blastocysts showed that the proportion of TUNEL-positive nuclei was increased to 49.3±5.9%. The high embryotoxicity of the enzyme buffer made it difficult to test whether a longer exposure time would result in an even higher T-index.

Clusterin mRNA expression in blastocysts

Amplification of cDNA preparations from blastocysts recovered from both control and diabetic rats with clusterin primers SP1 and SP2 generated an amplicon of the expected size (564 bp) (Fig. 8). Positive control amplifications performed on cDNAs from either rat ovaries or limb buds obtained from day 15 fetuses (a developing organ rich in clusterin mRNA; Buttyan et al., 1989) produced the same amplicon. In contrast, amplification of cDNA from morulae was negative. When day 5 blastocysts from control or diabetic rats were compared by means of a quantitative RT-PCR method, the average level of clusterin mRNA was 57.7±9.1 arbitrary units in the control group versus 101.1±5.5 arbitrary units in the diabetic group (P≤0.01) (Fig. 9). In a second series of experiments, the presence of clusterin transcripts was analyzed in normal blastocysts incubated for 24 hours in either 17 mM D-glucose, 3000 U/ml TNF-α or a combination of high D-glucose and TNF-α. Compared with embryos maintained in control medium (50.6±2.5 arbitrary units), the average level of clusterin mRNA was increased to 136.5±11.4 arbitrary units in blastocysts exposed to high D-glucose alone and 158.2±14.1 arbitrary units in high D-glucose with TNF-α (P≤0.01) (Fig. 9). Exposure to TNF-α alone did not influence clusterin mRNA expression. In addition, there was no significant difference between blastocysts treated with high D-glucose alone or with high D-glucose and TNF-α combined.

Fig. 8. Detection of clusterin transcripts in blastocysts by RT-PCR. Total cDNA from rat fetal limb (LM), adult ovary (OV), morula (MC) and blastocysts (BC) from control rats as well as blastocysts from diabetic rats (BD) were tested for clusterin/TRPM-2 mRNA expression using specific primers expected to generate a 564 bp-long amplicon (upper lane). The same cDNAs were amplified for β-actin in control reactions (lower lane). DNA size markers (MK) were run in the first gel lane.

Table 2. In situ hybridization for clusterin mRNA expression in blastocysts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Diabetic group</th>
<th>Incubation in high glucose</th>
<th>Incubation in TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell number</td>
<td>59.2±1.9</td>
<td>42.1±1.5**</td>
<td>51.8±1.4**</td>
<td>50.4±1.9**</td>
</tr>
<tr>
<td>Nuclear fragmentation index (%)</td>
<td>1.2±0.3</td>
<td>6.9±0.7**</td>
<td>4.7±0.7**</td>
<td>2.5±0.4</td>
</tr>
<tr>
<td>Clusterin-positive cell frequency (%)</td>
<td>0.6±0.2</td>
<td>3.9±0.6**</td>
<td>2.9±0.4**</td>
<td>1.4±0.4</td>
</tr>
<tr>
<td>n</td>
<td>18</td>
<td>21</td>
<td>24</td>
<td>21</td>
</tr>
</tbody>
</table>

Data are means ± s.e.m. with n representing the number of values. (**) indicates P<0.01.

Localization of clusterin mRNA expression

Control and diabetic blastocysts were collected on day 5 and maintained overnight in normal culture medium before being analyzed by ISH. Although 81% of the blastocysts from diabetic rats were found to contain at least one clusterin mRNA-positive cell, the frequency of these cells per embryo was low (1.6±0.2 such cell per embryo). Simultaneous staining with HO demonstrated that, in 46% of the clusterin mRNA-positive cells, the ISH labeling in the cytoplasm correlated with nuclear fragmentation (Fig. 10). The same pattern was found in blastocysts incubated for 24 hours in high D-glucose, with 92% of the embryos containing at least one clusterin-expressing cell, an average of 1.5±0.2 such cell per embryo and 48% of these cells displaying signs of nuclear disintegration. In these two blastocysts groups, the incidence of clusterin-expressing cells was higher than in control embryos (P<0.01) (Table 2). In contrast, ISH analysis of blastocysts exposed to TNF-α did not detect an increase in the proportion of clusterin-expressing cells. About half the TNF-α-treated blastocysts contained at least one clusterin mRNA-positive cell, with an average of 0.6±0.2 such cell per embryo, and 49% of these cells also displayed signs of nuclear fragmentation. Overall, the ISH
Fig. 10. Localization of cells expressing clusterin transcripts by ISH. HO staining coupled with ISH were used to identify the cells overexpressing clusterin in blastocysts. In about half the cells found to contain high levels of clusterin transcripts, the nucleus was in fragmentation, as exemplified in the blastocyst from a diabetic rat presented here (A,B; arrows). Blastocysts exposed to high D-glucose and hybridized to the control ISH probes did not show any ISH signal (C). The scale bar represents 10 μm in A,B and 30 μm in C.

data were consistent with the RT-PCR results. Experiments on 20 blastocysts exposed to high D-glucose and hybridized to the control sense probes demonstrated the complete lack of non-specific signal (Fig. 10).

DISCUSSION

Cell death in blastocysts exposed to diabetes in utero

Exposing blastocysts to the DNA fluorochromes HO and PI following TE immunolysis showed that a high proportion of cells identified as ICM cells showed signs of nuclear fragmentation in blastocysts from day 5 and day 6 diabetic rats. The fact that these dying cells were stained only with HO indicated that they were still delimited by intact membrane boundaries. Although ICM cells in advanced degradation stages would take PI when membrane integrity is lost and risk being miscounted as TE cells, this possibility is considered as unlikely because the proportion of cells with PI-stained nuclear fragments was equally low in both embryo groups. Because cells in very early stages of the suicidal process may already have subtle chromatin alterations before nuclear fragmentation occurs, blastocysts were also examined using the TUNEL method coupled with HO staining. This technique revealed the presence in blastocysts from day 5 diabetic rats of a population of dying cells distinct from those identified on the basis of HO-stained nuclear fragments. This distinction was confirmed when blastocysts were investigated on day 6, with the additional observation of yet another population of dying cells whose nuclei were displaying the two markers simultaneously (HO-stained DNA particles within a TUNEL-positive nucleus). The observation that not all TUNEL-positive nuclei were in the process of dis-integration, a phenomenon also recently described in mouse blastocysts cultured under suboptimal conditions (Brison and Schulitz, 1997), is still unexplained, mostly because it is unclear whether these two features form a directly coupled sequence of nuclear events. Uncertainties as to the nature of the initial event(s) leading to the DNA damages identified by the TUNEL technique as well as the duration of fragmentation and TUNEL detection in cells undergoing cell death also complicate the interpretation (Gold et al., 1994; Didenko and Hornsby, 1996). Although no attempt was made to combine TUNEL staining with ICM-TE differential staining, it was clear that most of the TUNEL-positive and/or fragmented nuclei were in cells situated within the ICM region. To further describe the occurrence of cell death in embryos, the mRNA expression of clusterin was quantitated by RT-PCR and localized by ISH in blastocysts from diabetic rats. Production of clusterin has been associated with cell death in several systems (Buttyan et al., 1989). One of its many proposed functions is the capture and neutralization of cellular debris resulting from the degradation of dying cells (Koch-Brandt and Morgans, 1996), a function clusterin might perform for instance during the remodeling process of blastocyst implantation (Brown et al., 1996). According to the present study, control rat embryos were found to start expressing clusterin mRNA at the blastocyst stage. Previous studies have demonstrated that cells in the TE lineage selectively expressed the gp330 clusterin receptor in the rat (Sahali et al., 1993) as well as in the mouse (Guet-Hallonet et al., 1994) blastocyst, findings that together with our data support the concept that a clusterin / gp330-based system may participate in the clearance of dead cell fragments inside embryos. Compared with control blastocysts, the relative amount of clusterin transcripts was increased two-fold in blastocysts from diabetic rats on day 5. Up-regulated clusterin expression was confined to a very limited number of cells, many of them displaying signs of nuclear fragmentation. Thus analysis of clusterin expression on an individual basis revealed a close correlation with the process of cell death in blastocysts. Controversial results exist about whether cells accounting for an elevation in clusterin expression within a site of cellular elimination are those undergoing cell death or neighboring cells destined to survive. Studies aimed at identifying clusterin-expressing cells and dying cells in the human thymus (French et al., 1992) and in various lympho-hematopoietic cell lines (French et al., 1994) support the idea that only viable cells produce clusterin. In contrast, simultaneous display of nuclear degradation and clusterin transcription has been reported in individual glandular epithelial cells in the mouse uterus (Ahuja et al., 1994). A reconciling view would be that, in certain situations, such as in blastocysts, dying cells may engage into the altruistic process of up-regulating their synthesis of clusterin in order to facilitate the removal of their own debris by surrounding clusterin receptor-expressing cells.

Cell death in blastocysts exposed to high glucose

Previous reports have shown that hyperglycemia per se is directly toxic to certain cell populations, such as human endothelial cells (Lorensi et al., 1985), and increases the expression of several markers of active cell death (Baumgartner-Parzer et al., 1995). Rat blastocysts incubated in high glucose were found to contain fewer cells than control embryos, with the cell
loss occurring mainly at the expense of the ICM. High glucose also induced an five-fold increase of the proportion of ICM cells displaying nuclear fragmentation within intact cell membranes. When blastocysts were subjected to the TUNEL technique paired with HO staining, both the F-labeling and T-labeling indexes were found increased following culture in high glucose. Substituting non-metabolizable L-glucose for D-glucose in cultures demonstrated the specificity of the active form of the nutrient. Additional experiments aimed at assessing the expression of clusterin showed an increase in the relative abundance of transcripts in blastocysts incubated in high glucose. About half the cells expressing high levels of clusterin transcripts also contain a fragmented nucleus, indicating again a close relationship between individual cell death and clusterin up-regulation. The mechanism by which moribund cells may be cleared from within a blastocyst is not known. In contrast with most situations where resident macrophages could rapidly phagocytose cell fragments, the phagocytic potential of embryonic cells is probably limited (El-Shershaby and Hinchliffe, 1974). Embryonic dead cells that can not be phagocytosed by adjacent viable cells would therefore undergo in situ degeneration or expulsion into the perivitelline space. Our observations revealed the presence of such extruded cells (containing a fragmented nucleus within an intact cell membrane) on the outer edge of several blastocysts. This was consistent with a previous description of dead cell extrusion in blastocysts from diabetic BB/E rats (Lea et al., 1996).

Cell death in blastocysts exposed to TNF-α

Previous studies have shown that the synthesis of TNF-α is up-regulated in the uterus of the pregnant diabetic rat (Pampfer et al., 1995a) as well as in primary cultures of rat uterine cells incubated in high glucose (Pampfer et al., 1997). These data suggest that blastocysts developing in diabetic rats may be exposed to abnormally high concentrations of TNF-α. Cell death induction is clearly one of the prominent biological activities exerted by TNF-α (Nagata, 1997) and several reproductive cell types, such as rat trophoblasts (Hunt et al., 1989; Roby et al., 1994), have been shown to be sensitive to the cytotoxic action of the cytokine. When rat recombinant TNF-α was tested at a concentration about 5 times higher than the circulating concentration of TNF-α in day 6 diabetic rats, no significant increase was observed in the incidence of nuclear fragmentation in blastocysts. These data confirmed earlier results showing that mouse recombinant TNF-α could induce a decrease in the total number of cells per rat blastocyst without increasing the rate of nuclear fragmentation in these embryos (Pampfer et al., 1994b) and were consistent with other observations that DNA fragmentation is not always involved in the cytotoxic process triggered by the cytokine (Mirkina et al., 1996). This lack of nuclear fragmentation was tentatively explained by the fact that rat blastocyst cells, like several other cell types similarly resistant to TNF-α-induced DNA fragmentation (Higuchi and Aggarwal, 1994), express only the p60 isoform of TNF-α receptor (Pampfer et al., 1995b). In contrast, the cytokine was found to double the T-index and to triple the F+T-index, thus confirming the ability of the cytokine to up-regulate the expression of some markers of cell death in embryos. The extent of the increase in T-index in rat blastocysts was consistent with the recently described 2.5-fold increase in the proportion of TUNEL-positive cells among TNF-α-treated human trophoblasts (Yui et al., 1994). Because of previous indications that TNF-α can up-regulate clusterin expression in mouse fibrosarcoma cells (Kyprianou et al., 1991) and in human prostatic cells (Sensibar et al., 1995), it was expected that TNF-α would increase the relative mRNA level of clusterin in rat embryos. The data showed that the cytokine did not influence the steady-state transcription level of clusterin in blastocysts.

Cell death in blastocysts exposed to high glucose and TNF-α

Co-exposure of the blastocysts to high glucose and TNF-α was found to recapitulate the different effects observed in embryos recovered from diabetic rats on day 6. Increases in all three nuclear labeling indexes were detected after treatment with high glucose and TNF-α. Clusterin mRNA expression was also stimulated following this combined exposure. No additive effect was seen in the frequency of TUNEL-positive nuclei, a cell death parameter that was up-regulated by both glucose and TNF-α, suggesting that the cell death mechanisms triggered by the two effectors might converge at some limiting point.

CONCLUSION

Overall, the data demonstrate that several markers of active cell death are increased in blastocysts exposed to maternal diabetes. Excessive cell death occurred predominantly in the ICM. Incubation of normal blastocysts in high D-glucose, and to a lesser extent in TNF-α, also increased the expression of these markers. Because cell death is constitutively detected at a very low level in control blastocysts, it is concluded that the deleterious impact of maternal diabetes on embryo development (Pampfer and De Hertogh, 1996) may be mediated by the inappropriate up-regulation of this regular elimination process. The observation of clusterin overexpression in blastocysts from diabetic rats indicates that, in addition to suffering from a cell deficiency in their vital ICM lineage, these embryos may be affected by subtle disruptions in the expression pattern of critical developmental genes.

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