THE EXPRESSION OF INSULIN-LIKE GROWTH FACTORS AND THEIR RECEPTORS AT PREIMPLANTATION STAGE IN REPRODUCTIVE TISSUE OF DIABETIC MOUSE

RAHIMAH BINTI ZAKARIA

UNIVERSITI SAINS MALAYSIA

2007
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by

RAHIMAH BINTI ZAKARIA

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

NOVEMBER 2006
ACKNOWLEDGEMENTS

In the name of Allah, the Most Merciful and the Most Compassionate

I would like to express my deepest gratitude and indebtedness thanks to my supervisors, Associate Professor (Dr) Nik Soriani Yaacob and Professor (Dr) Norazmi Mohd Nor for their fine and dedicated supervision throughout my studies and their thorough criticisms during my thesis writing. I am also thankful to my field supervisor, Professor (Dr) Mohd Hamim Rajikin for his encouragement and criticisms throughout my study until I completed my thesis writing. I would like to thank Professor (Dr) Harbindar Jeet Professor, Head of Department of Physiology, for his support during my study.

My thanks to our Dean of School of Medical Sciences, Professor (Dr) Abdul Aziz Baba, and Deputy Deans of School of Medical Sciences for their encouragement throughout my studies.

I would like to thank Professor (Dr) Asma Ismail, Director of INFORMM and Professor (Dr) Zainul Fadziruddin Zainuddin, Dean of School of Health Sciences for giving me the opportunity to work in their laboratories. My thanks to staff of Department of Physiology (USM and UKM, Kuala Lumpur), Animal House, Department of Pathology, INFORMM and School of Health Sciences for their kind assistance.
My special thanks to my dearest friend, Dr Asma Hayati Ahmad, for her encouragement during my study and her criticisms during my thesis writing. To all my friends in the department, Dr Mahaneem, Dr Che Badariah, Dr Wan Maliah, Dr Liza and Dr Adibah, and also my research group members, Mar, Ima, Teo, Asma, Rafezul, Dr Zul, Syam and Ayu, thank you to all of them for their assistance and support.

Finally to both my parents, my husband, my sons, Haikal, Haziq and Hadif, my sisters and brother, a very special thanks to all of them for their love, understanding and encouragement which allow me to complete my studies and thesis writing.

I would like to acknowledge Ministry of Science and Technology for financially support this research work via Fundamental Research Grant Scheme (FRGS).
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LIST OF ABBREVIATIONS

AA  arachidonic acid
ACTH  adrenocorticotropic hormone
AGE  advanced glycation end products
Akt  protein kinase B
ALS  acid-labile subunit
ATP  adenosine triphosphate
BAD  Bcl-xL/Bcl-2-associated death protein
BSA  Bovine Serum Albumin
CaCl_2  calcium chloride
cDNA  complementary DNA
cds  coding region
COX-2  cyclooxygenase-2
C-peptide  connecting peptide
cPLA_2  cytosolic phospholipase A_2
CRK  CT10 regulator of kinase
C_T  threshold cycle
Da  Dalton
DAB  3, 3'-diaminobenzidine tetrahydrochloride
DEPC  diethylpyrocarbonate
<table>
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<tbody>
<tr>
<td>DHA</td>
<td>dehydroascorbate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPX</td>
<td>distyrene plasticiser xylene</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine-tetra acetic acid</td>
</tr>
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<td>ERα</td>
<td>oestrogen receptor-α</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-related kinase</td>
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<td>EtBr</td>
<td>ethidium bromide</td>
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<tr>
<td>FBP</td>
<td>fructose 1,6-biphosphate</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>FSH</td>
<td>follicle stimulating hormone</td>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GH</td>
<td>growth hormone</td>
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<td>glucose transporters</td>
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<td>growth factor receptor-bound protein</td>
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<td>HIF-1α</td>
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<td>ICM</td>
<td>inner cell mass</td>
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<td>ICR</td>
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<tr>
<td>IGF-1</td>
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<td>mRNA</td>
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<td>MSA</td>
<td>multiplication-stimulating activity</td>
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<td>Acronym</td>
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<tr>
<td>mSOS</td>
<td>Son of sevenless</td>
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<td>NaOH</td>
<td>sodium hydroxide</td>
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<td>NIH</td>
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<td>NOD</td>
<td>non-obese diabetic</td>
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<td>SAPK</td>
<td>stress-activated protein kinase</td>
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EKSPRESI "INSULIN-LIKE GROWTH FACTORS" DAN RESEPTORNYA
PADA PERINGKAT PRAIMPLANTASI DALAM TISU REPRODUKTIF
MENCIT DIABETES

ABSTRAK
Kami menghipotesis bahawa embrio praimplantasi yang berkembang dalam
persekitaran diabetes mengalami kekurangan faktor pertumbuhan tertentu.
Tujuan utama kajian ini adalah untuk menentukan kesan diabetes ke atas
perkembangan embrio praimplantasi mencit secara in vitro dan menganalisis
ekspresi mRNA dan protein IGF-1, IGF-2, IGF-1R dan IGF-2R dalam tisu
fallopio dan uterus mencit kontrol dan diabetes. Mencit ICR betina yang matang
secara seksual berusia 6 hingga 8 minggu dijadikan diabetes dengan suntikan
streptozotosin (200 mg/kg, intra peritoneum). Embrio normal dalam peringkat 2-
sel diperolehi daripada mencit kontrol dan diabetes yang disuperovulasi pada
48 jam pasca rawatan korionik gonadotropin manusia (hCG). Embrio dikultur
secara berasingan dan diperiksa di bawah mikroskop songsang selama 3 hari
berturut-turut. Tisu fallopio dan uterus diperolehi daripada mencit kontrol dan
diabetes yang disuperovulasi pada 48, 72 dan 96 jam pasca rawatan hCG.
Kuantifikasi ekspresi mRNA menggunakan tindak balas polimerase masa nyata
menggunakan piawai internal homologus yang dibangunkan secara spesifik
untuk setiap gen. Ekspresi protein menggunakan pewarnaan imunohistokimia
dijalankan ke atas tisu dan skor semikuantitatif dibuat berdasarkan sistem 5-
skala piawai. Bilangan embrio 2-sel yang diperolehi daripada mencit diabetes
THE EXPRESSION OF INSULIN-LIKE GROWTH FACTORS AND THEIR RECEPTORS AT PREIMPLANTATION STAGE IN REPRODUCTIVE TISSUES OF DIABETIC MOUSE

ABSTRACT

We hypothesized that the alteration in the expression of IGFs and their receptors may create an abnormal intrauterine environment thus affect embryos development. Therefore, the aims of the present study were to determine the effects of diabetes on in vitro development of mouse preimplantation embryos and to determine the mRNA and protein expression of IGF-1, IGF-2, IGF-1R and IGF-2R in the fallopian tube and uterine tissue of control and diabetic mice. Sexually mature female ICR mice of 6-8 weeks old were made diabetic by streptozotocin (200 mg/kg, intraperitoneal). The normal two-cell embryos were obtained from superovulated control and diabetic mice at 48 post-hCG treatment. Embryos were separately cultured and examined under an inverted microscope for 3 consecutive days. Fallopian tubes and uterine tissues were obtained from the superovulated control and diabetic mice at 48, 72 and 96 hours post-hCG treatment. The mRNA expression was measured using Real-time PCR using specifically developed homologous internal standards for each gene. Protein expression was measured by immunohistochemical staining and a semiquantitative scoring was performed using a standardized 5-scale system. The number of normal two-cell embryos obtained from diabetic mice was much reduced when compared to control mice. However, there was no significant
difference in the percentage of two-cell embryo development in control and diabetic mice. The mRNA expression of IGF-1 in the fallopian tube and uterus of diabetic mice was significantly low at 72 hours and 96 hours post-hCG treatment, respectively. The mRNA expression of IGF-1R remained high in the fallopian tube but was significantly low in the uterus of diabetic mice at 96 hours post-hCG treatment. The mRNA expression IGF-2 in the fallopian tube was significantly high at 48 and 96 hours post-hCG treatment but was significantly low in the uterus of diabetic mice at 96 hours post-hCG treatment. The mRNA expression of IGF-2R in the fallopian tube and uterus of diabetic mice was significantly high at 48 and 96 hours, and at 48 hours post-hCG treatments, respectively. For protein expression, the immunohistochemical scoring for both IGF-1 and IGF-1R was significantly decreased in the fallopian tube of diabetic mice at 96 hours post-hCG treatment. In contrast, the score for IGF-2 and IGF-2R was significantly increased in the fallopian tube of diabetic mice at 48 and 72 hours; and at 72 hours post-hCG treatment, respectively. However, there was no significant difference in the score of IGFs and their receptors in the uterus of control and diabetic mice. In conclusion, the percentage of the two-cell stage embryos which developed to blastocysts was similar in control and diabetic groups but whether the quality of these embryos were the same could not be confirmed. Both the mRNA and protein expression of IGFs and their receptors were significantly altered by maternal diabetes, which suggest their role in the pathogenesis of diabetic embryopathy.
CHAPTER ONE
GENERAL INTRODUCTION

1.1 PHYSIOLOGY OF PREIMPLANTATION EMBRYO DEVELOPMENT

1.1.1 Proliferation and differentiation of preimplantation embryo

Fertilization of the mouse ovum by sperm occurs in the ampullary region of the fallopian tube. Subsequent development occurs as the embryo moves down the fallopian tube and into the uterus over a period of about four days in mice, as compared to five and seven days in rats and humans, respectively (Figure 1.1). Approximately 24 hours after fertilization, the embryo undergoes relatively synchronous cell division resulting in the formation of two cells or blastomers. There are no junctions established between the individual cells, which are held together by ionic attractions on the opposing plasma membranes (Chavez, 1984). The cells are constrained within a physical shell, the zona pellucida, which is a matrix of four glycoproteins (Wassarman and Mortillo, 1991). During this period, each blastomere is totipotent, retaining the capacity to form a complete fetus. Further mitotic divisions occur asynchronously at progressively shorter intervals so that from the eight-cell stage onwards, one cell cycle is approximately six hours. At the eight-cell stage, generation of two distinct lineages commences with the process of compaction when individual blastomers polarize, become epithelial-like and flatten on each other,
The fertilization takes place in the ampullary region of the fallopian tube and the developing embryo traverse down to the site of implantation in the uterine cavity.
maximising cell contact and forming tight and gap junctions (Ducibella et al., 1977; Magnuson and Epstein, 1981; Chavez, 1984; Fleming et al., 1992). This compacted morula stage coincides with the arrival of the embryo at the uterotubal junction. In the compacted morula, fluid is transported across the newly formed epithelium to form a blastocoel and at this point the embryo is referred to as a blastocyst. At this stage, two distinct cell populations can be recognized (Johnson, 1981). The eccentrically placed inner cell mass (ICM) eventually forms the embryo proper and some extraembryonic tissues. The trophectoderm (TE), which is a single epithelial layer of flattened cells surrounding the blastocoel and ICM, establishes the foci of adhesion with the uterine epithelium and gives rise to the fetal component of the placenta (Hogan et al., 1986).

1.1.2 Metabolic activity of preimplantation embryo

Mouse oocyte and zygote have an absolute requirement for pyruvate (Biggers et al., 1967); i.e. glucose cannot support early embryo development until the eight-cell stage (Biggers, 1971). From the two-cell to the blastocyst stage, the embryos experience an increase in the tricarboxylic acid (TCA) cycle metabolites and a dramatic increase in fructose 1, 6-biphosphate (FBP). The dramatic switch from a dependence on the TCA to a metabolism based on glycolysis occurs at the time of compaction. The only source of adenosine triphosphate (ATP) for the preimplantation embryo would be conversion of glucose to pyruvate and lactate via glycolysis.

The blastocyst stage marks a new peak in cellular proliferation and growth. These changes create new biosynthetic demands on the embryos.
Maintenance of a high rate of glycolysis is important for providing a “dynamic buffer” of metabolic intermediates for the biosynthesis of macromolecules (Newsholme and Newsholme, 1989) and increasing amount of glucose are converted to lactate at this stage in humans and rodents (Leese and Barton, 1984). Interspecies variations in the rate of glycolysis have been reported, higher in human blastocysts (Leese et al., 1993) and lower in mouse blastocysts (Leese, 1991) compared to rat embryos.

Blastocysts are actively engaged in the uptake and metabolism of maternally derived nutrients such as glucose (Leese, 1991). The major site of uptake regulation is likely to be the system of facilitative glucose transporters (GLUT) situated at the basolateral surface of the TE in mouse blastocysts (Aghayan et al., 1992).

1.1.3 Influence of maternal factors on preimplantation embryo development

Although the activation of the embryonic genome provides the conceptus with a number of vital developmental signals (Kidder, 1992; Schultz and Heyner, 1992), its progression through the preimplantation period is also influenced by maternal factors present in the oviductal and uterine environment.

Biggers (1981) proposed a theoretical model summarizing the physiological processes that influence the microenvironment of the preimplantation embryo. The composition of the microenvironment is determined by several transport mechanisms: between the embryo and the
secretions in which it is bathed, between the fallopian tube and uterus and the bathing secretions and further mixing is produced as the secretions flow up and down the reproductive tract as shown in Figure 1.2.

Maternally-derived nutrients/factors can either be transudates from the maternal circulation such as glucose (Leese et al., 1979, Wales and Edirisinghe, 1989; Gonzalez et al., 1994) and insulin (Heyner et al., 1989; Smith et al., 1993), or secretions by various uterine cells into the lumen such as growth factors (Pollard, 1990; Song et al., 2000) and cytokines (Pampfer et al., 1991; Robertson et al., 2001) during the preimplantation period.

1.2 DIABETIC PREGNANCY

The association between maternal diabetes and the increased risk of congenital malformations has a long history and was first reported by LeCorche (1885). It is generally accepted that congenital malformations are the leading cause of death in the offspring of diabetic women (Kitzmiller et al., 1978). The incidence of congenital malformations is approximately 6 to 9% in diabetic pregnancies, which is three- to four-fold higher than in the general population, and accounts for 33 to 66% of perinatal deaths (Reece and Hobbins, 1986). The congenital malformations most commonly associated with maternal diabetes are listed in Table 1.1 (Reece and Hobbins, 1986). However, none of the reported congenital malformations is pathognomonic for the diagnosis of diabetic embryopathy.
Figure 1.2  A theoretical model summarising the physiological processes that influence the microenvironment of the preimplantation embryo (Adapted from Biggers, 1981).
1.3 DEFINITION OF “DIABETIC EMBRYOPATHY”

The term “embryopathia diabetica” was coined by Mayer (1952) and was later replaced by the term “diabetic embryopathy” (Passarge and Lenz, 1966). The concept of diabetic embryopathy initially encompassed the long-recognized newborn features such as macrosomia and organomegaly but was later broadened to include congenital malformations (Mayer and Camara, 1964). Presently, the same concept was separated into two different entities, diabetic embryopathy and diabetic fetopathy. Diabetic embryopathy occurs during embryogenesis, mainly from the end of blastogenesis until the period of organogenesis (between the 3rd and 7th week of gestation) and is associated with congenital malformations (Kousseff, 1999). In contrast, diabetic fetopathy occurs during fetal development, after the 10th week of gestation, and is not associated with malformations (Kousseff, 1999). Occasionally, diabetic embryopathy is associated with diabetic fetopathy. However, these two entities, both induced by maternal diabetes mellitus, have different windows of vulnerability and perhaps, different pathogenesis.

1.4 AETIOLOGICAL FACTORS ASSOCIATED WITH DIABETIC EMBRYOPATHY

It has been suggested that the absence of a specific malformation pattern for diabetic embryopathy signals the presence of several aetiological factors and mechanisms in diabetic pregnancy (Khoury et al., 1989). Likewise, the number of different teratogenic agents identified indicates that diabetic embryopathy is of complex aetiology (Sadler et al., 1989; Zusman et al., 1989; Buchanan et al., 1994).
1.4.1 Maternal Hyperglycemia

Hyperglycemia-induced teratogenic effects have been demonstrated in animal studies both in vivo and in vitro (Cockroft and Coppola, 1977; Baker et al., 1981; Horton and Sadler, 1983; Kalter and Warkany, 1983a & 1983b; Freinkel et al., 1986; Reece and Hobbins, 1986). The percentage of congenital malformations correlated with blood glucose levels (Reece et al., 1985) and glycosylated hemoglobin levels (Rose et al., 1988).

The period and time of exposure to hyperglycemia as well as the level of hyperglycemia are all important for dysmorphogenesis to occur. The critical period of exposure to hyperglycemia is during organogenesis, which is considered to be between days 9.5 to 11.5 in rats and 8.0 to 9.6 in mice (Freinkel, 1988), corresponding to the first 5 or 6 weeks of human pregnancy. A minimum exposure time of two or more hours is needed to induce these malformations. A 20% malformation rate was induced at glucose levels that were approximately two-fold above normal concentrations; an almost 50% malformation rate was seen at glucose levels three-fold above normal concentrations; and approximately 100% rate at six times above normal concentrations (Reece et al., 1985).

Maternal hyperglycemia adversely affects not only the postimplantation embryos but also preimplantation progression from one-cell to the blastocyst stage in a streptozotocin (STZ)-induced or a non-obese diabetic (NOD) mouse model (Diamond et al., 1989; Moley et al., 1991 & 1994). In the NOD model at 96 hours after superovulation and mating, only 20% of the recovered embryos
reached blastocyst stage in the diabetic compared to 90% among the non-diabetic. This developmental delay is reversible by treating the mothers with insulin before superovulation and mating and during the first 96 hours of gestation. This early preimplantation delay may be manifested later in gestation as fetal loss, early growth delay or congenital malformation. There is now convincing evidence that severe developmental anomalies leading to fetal resorption or malformation can occur as a consequence of subtle damage inflicted to the embryos before or at the time of implantation (Rutledge, 1997).

The mechanism of hyperglycemia-induced congenital anomalies remains unclear. Hyperglycemia-induced reduction in GLUT has been proposed to be one of the possible mechanisms. A paradoxical reaction to hyperglycemia has been demonstrated in preimplantation embryos (Moley, 1999). In embryos of diabetic mice, a pronounced intracellular hypoglycemia was found despite maternal hyperglycemia (Moley et al., 1998b). The decreased intracellular glucose concentration was associated with decreased GLUT namely, GLUT-1, GLUT-2 and GLUT-3 isoforms; both at the protein and mRNA levels (Moley et al., 1998b).

Reduced availability of glucose associated with decreased GLUT in diabetic embryos, results in significantly lower FBP and higher pyruvate, indicating decreased glycolysis and increased pyruvate uptake by the embryos, respectively. The glycolytic changes lead to dysfunction of the outer mitochondrial membrane and subsequently trigger the apoptotic cascade (Chi et al., 2002).
The decrease in GLUT especially GLUT-1 and -3 isoforms also explains the elevated extracellular dehydroascorbate (DHA) and reduced intracellular ascorbic acid (Rumsey et al., 1997) related to hyperglycemia as described previously (Ely, 1981). Ely (1981) proposed that reduced intracellular ascorbic acid resulted in decreased hexose monophosphate shunt activity (DeChatelet et al., 1972), which might suppress deoxyribonucleic acid (DNA) synthesis as shown in Figure 1.3. Reduced DNA synthesis may slow cell division, leading to impaired cell proliferation or anomalies. It has also been suggested that elevated extracellular DHA may inhibit mitosis or cell proliferation (Edgar, 1970).

This decrease in intracellular glucose concentration leads to a lower cell number in the ICM, either by increased apoptotic rate (Pampfer et al., 1997b; Moley et al., 1998a), or by diminished proliferation of these cells (Pampfer et al., 1990). Decrease in glucose transport and metabolism is not only related to progressive decrease in embryo viability but served as important regulatory points in the early apoptotic cascade (Johnson et al., 1996; Li et al., 1998; Shim et al., 1998; Bialik et al., 1999, Lin et al., 2000). Maternal hyperglycemia, moreover, can cause direct disruption of the highly regulated gene program that controls the expression pattern of crucial developmental determinants during early embryogenesis, including apoptosis (Phelan et al., 1997; Cai et al., 1998; Moley et al., 1998a; Pampfer et al., 2001).

Three cell death paradigms that are linked to decreased GLUT include, (i) induction of ATP depletion and stimulation of the mitochondrial death cascade or (ii) induction of oxidative stress and triggering of Bax-associated
Hyperglycemia

Reduced DHA cellular uptake

Increased extracellular DHA

Reduced intracellular ascorbate

Reduced Hexose Monophosphate shunt activity

Reduced DNA synthesis

Decreased cell division

Congenital Anomaly

Figure 1.3  Mechanism of hyperglycemia-induced alterations in cell division and congenital anomalies

(Adapted from Ely, 1981)
events including the c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase (MAPK) signalling pathways or (iii) regulation of expression of the gene encoding hypoxia-inducible factor 1α (HIF-1α) and the stabilization of p53 by HIF-1α binding, leading to an increase in p53-associated apoptosis and, in turn, increased expression of Bax, and thus exaggerated apoptosis within blastocysts during glucose deprivation (Chi et al., 2000; Moley and Mueckler, 2000; Keim et al., 2001).

The outcome of apoptosis during preimplantation stage will depend on the percentage of cell death, if more than 60% of ICM undergo cell death, the pregnancy may result in fetal loss and resorption. However, the death of fewer cells (e.g. 40-45%) can result in either fetal resorption or malformation if this cell death involves key progenitor cells in development (Tam, 1988; Moley, 2001).

Another possible mechanism of hyperglycemia-induced congenital anomalies that has been put forward is related to dysregulation in the uterine cytokine secretion (Pampfer, 2001). Diabetes-induced modifications in the oviductal and uterine concentrations of nutrients (such as increased glucose levels), hormones (such as decreased insulin levels), growth factors, and cytokines (increased local synthesis of inhibitory factors or decreased local synthesis of stimulatory factors) are likely to elicit alterations in embryo development before implantation and organogenesis.

Studies in STZ- and alloxan-treated diabetic mice exhibit an increased amount of tumour necrosis factor-α (TNF-α) messenger ribonucleic acid
(mRNA) and protein in the uterus and placenta of diabetic mice (Pampfer et al., 1995; Flein et al., 2001) as well as a marked reduction in pregnancy rate and a high incidence of litters with severely malformed fetuses (Torchinsky et al., 1997, Machado et al., 2001). In addition, overexpression and excessive secretion of TNF-α by uterine cells in diabetic pregnancy may induce a decrease in cell number of the ICM as reported in an earlier study (Pampfer et al., 1997b; Wuu et al., 1999).

TNF-α acts in a cell type- and stimulus-dependent manner, to generate apoptotic-signalling pathways (Baud and Karin, 2001; Gupta, 2001). The apoptotic action of TNF-α could occur mainly through its binding to the type 1 receptor TNF-α, followed by the activation of caspase 8 (Slee et al., 1999; Mohr et al., 2002; Torchinsky et al., 2003), which is considered to be among the main mediators of apoptosis (Baud and Karin, 2001; Gupta, 2001). The apoptotic action of TNF-α could be mediated through interleukin-1β (IL-1β) secreted by macrophages (Pampfer et al., 1999) localized at the subepithelial region of the uterine stroma (Takacs et al., 1988).

The mechanism of hyperglycemia-induced IL-1β secretion has been proposed to be via the formation and interaction of advanced glycation end products, AGE (Vlassara et al., 1988). Macrophages have a receptor that recognizes the AGE moiety and mediates the uptake and degradation of AGE proteins. This removal process is associated with the production and secretion of TNF-α and IL-1. The localized release and action of these cytokines may play