THE EFFECT OF AN *IN UTERO* HIGH FAT DIET ON THE EXPRESSION OF TRANSCRIPTION FACTORS AND GLUCOSE SENSING IN THE DEVELOPING RAT PANCREAS

by

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"***Declaration***

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Marlon E. Cerf

Abstract

The effect of an *in utero* high fat diet on the expression of transcription factors and glucose sensing in the developing rat pancreas

Introduction

A high fat diet (HFD) reduces beta-cell mass, impairs glucose signalling and is involved in the development of Type 2 diabetes. Malnutrition during gestation is hypothesized to irreversibly damage beta-cell development. The transcription factors Pdx-1 and Pax 4 are involved in islet cell development. Pdx-1 is reported to regulate expression of GLUT-2, glucokinase (GK) and the insulin gene.

Aims

The aim of this study is to investigate, in the neonatal and weanling rat, the effect of exposure to a HFD *in utero* and/or lactation on weight, glucose and insulin concentrations, islet cell development, pancreatic transcription factors and glucose sensing genes.

Methods

Neonatal and weanling rats were exposed to a maternal HFD for defined periods of gestation and/or lactation. After termination, pups were weighed and glucose and insulin concentrations determined. mRNA expression of Pdx-1, Pax 4, GLUT-2 and GK was quantified by LightCycler PCR. Pancreatic sections were immunostained for insulin and glucagon (islet cell development), and for Pdx-1, GLUT-2 and GK (beta-cell function) followed by image analysis.

Results

Exposure to an *in utero* HFD throughout gestation resulted in hyperglycaemic pups with reduced beta-cell volume and number, Pdx-1 and GK immunoreactivity. In contrast the alpha-cell volume, number and size were augmented in neonates exposed to a HFD throughout gestation.

Most weanlings were hyperglycaemic and hypoinsulinaemic. In some weanlings, reduced beta-cell number and beta- and alpha-cell size was observed. Pdx-1 mRNA was overexpressed in weanlings exposed to a maternal HFD for the final week of gestation or throughout both gestation and lactation, but reduced in those only exposed throughout lactation. Pax 4 mRNA was reduced in weanlings exposed to a maternal HFD for the first or final week of gestation, throughout gestation or throughout lactation. In most of the weanlings, GLUT-2 mRNA expression was reduced whereas immunoreactivity for GLUT-2 was increased. Both GK mRNA expression and immunoreactivity were reduced in most of the weanlings.

Conclusions

- Exposure to an *in utero* HFD throughout gestation induced hyperglycaemia in neonates. The reduced Pdx-1 expression appears to play a role in the compromised beta-cell development, and concomitant with the reduced GK levels, contributes to the hyperglycaemia in these neonates and may make them susceptible to beta-cell failure.

- In most weanlings exposed to a HFD *in utero* and/or during lactation the hyperglycaemia and hypoinsulinaemia suggest compromised beta-cell function. The GK mRNA expression and immunoreactivity were reduced thereby impairing glycolysis which would result in reduced insulin secretion contributing to the hyperglycaemia. Furthermore, beta-cell development is adversely affected by the HFD in some weanlings. This would contribute to reduced beta-cell function and may eventually result in beta-cell failure. GLUT-2 immunoreactivity was increased in some, suggesting a compensatory adaptative mechanism to restore glucose homeostasis.

- A maternal HFD has adverse effects both in neonates and weanlings on beta-cell development, transcription factor and glucose sensing gene expression and induced hyperglycaemia and hypoinsulinaemia in some of the offspring. Ways to ameliorate the HFD-induced attenuation of key beta-cell genes to ensure normal beta-cell function are important for future research in Type 2 diabetes.

Opsomming

Inleiding

'n Höe vet diet (HVD) verminder beta-sel masse, glukose signale en speel 'n rol in Tipe 2 diabetes. Die hipothese is dat wanvoeding gedurende swangerskap lei tot onomkeerbare betasel beskadiging. Die transkripsiefaktore Pdx-1 en Pax 4 speel rolle in eilandselontwikkeling. Daar is bewyse dat Pdx-1 die uitdrukking van die GLUT-2, glucokinase (GK) en insulin gene reguleer.

Doelstelling

Die doel van hierdie studie is om, in die pasgebore en gespeende rot, die effek van 'n HVD *in utero* en/of laktasie op gewig, glukose en insulin konsentrasies, eilandselontwikkeling, pankreatiese transksripsiefaktore en op glukose-waarnemingsgene te ondersoek.

Metodes

Pasgebore en gespeende rotte is vir bepaalde periodes van gestasie en/of laktasie blootgestel aan 'n HVD van die moeder. Na terminase, is kleinjies geweeg en die glukose- en insulienkinsentrasies bepaal. mRNA uitdrukking van Pdx-1, Pax 4, GLUT-2 en GK is geoes met LightCycler PCR. Snitte van die pankreas is gekleur met insulien en glukagon (eilandsontwikkeling) en vir Pdx-1, GLUT-2 en GK (betaselfunksie) gevolg deur beeldanalise.

Resultate

Bloodstelling aan 'n *in utero* HVD regdeur gestasie het hiperglisemie versoorsaak in pasgebore rotte met verlaagde betasel volume en aantal, Pdx-1 en GK immunoreaktiwiteit. In teenstelling daarmee was die alfasel se volume, aantal en grootte verhoog in pasgebore rotte wat regdeur gestasie aan 'n HVD blootgestel was.

Meeste van die gespeende rotte was hiperglisemies en hipoinsulinemies. In sommige gespeende rotte, was daar 'n verlaging van betasel hoeveelheid en grootte en in alfasel groote. Oormatige uitdrukking van Pdx-1 mRNA het plaasgevind in speenlinge wat aan 'n HVD van die moeder vir die laaste week van gestasie of regdeur gestasie en laktasie blootgestel was, maar dit was verlaag in die speenlinge wat net tydens laktasie blootgestel was. Pax 4 mRNA was verlaag in speenlinge wat aan 'n HVD van die moeder blootgestel was vir die eerste of laaste week van gestasie, regdeur gestasie of regdeur laktasie. In meeste van die speenlinge is onder-uitdrukking van GLUT-2 mRNA, maar 'n verhoging van GLUT-2 immunoreaktiwiteit gevind. Beide GK mRNA uitdrukking en immunoreaktiwiteit was laer in meeste van die speenlinge.

Gevolgtrekkings

- Blootstelling aan 'n *in utero* HVD regdeur gestasie lei tot hiperglisemie in pasgebore rotte. Die verlaagde Pdx-1 immunoreaktiwiteit speel klaarblyklik 'n rol by die geaffekteerde betaselontwikkeling. Dit, saam met

die verlaagde immunoreaktiwiteit vir GK, kan bydra tot die hiperglisemie in hierdie pasgebore rotte.

- In die meeste van die speenlinge wat aan 'n HVD blootgestel was, dui die hiperglisemie en hipoinsulinemie op geaffekteerde betaselfunksie. Die GK mRNA uitdrukking en immunoreaktiwiteit is verlaag, wat weer glikolise benadeel, en dit sal lei tot verminderde insulienafskeiding wat bydra tot die hiperglisemie. Betaselontwikkeling word voorts negatief beïnvloed deur die HVD, wat blyk uit die verlaagde aantal en grootte van betaselle. Dit sal bydra tot verminderde betaselfunksie. Dit kan uiteindelik tot betaselversaking lei. GLUT-2 immunoreaktiwiteit was verhoog in hierdie speenlinge, wat dui op 'n kompenserende aanpassingsmeganisme om glukose homeostase te herstel.

- 'n HVD van die moeder het 'n negatiewe uitwerking op betaselontwikkeling, transkripsiefaktor en glukosewaarneming geenuitdrukking in beide die pasgebore en gespeende rotte, en geïnduseerde hiperglisemie en hipoinsulinemie in sommige kleintjies. Dis belangrik vir toekomstige Tipe 2 diabetes navorsing dat daar na gekyk moet word om die HVD-geïnduseerde verlaging van sleutel betaselgene te verbeter vir optimale betaselfunksie.

Dedication

I dedicate this work to God;

my mother, Mrs R.M. Cerf;

my late father, Mr B.M. Cerf;

and to my siblings, Liezel, Bradley and Tracy.

Publications (in press and published)

Cerf, M.E., et al. The role of a high fat diet in the pathogenesis of Type 2 diabetes. Specialist Forum. 2005 (In press).

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International Seminars

Invited seminars, "The effect of a high fat diet on GLUT-2 expression in the developing rat pancreas", 9-16 September 2002, Spain:

Department of Biochemistry and Molecular Biology, University of Complutense (Madrid),

Department of Nutrition, Metabolism, and Hormones, Fundacion Jiminez Diaz (Madrid),

Faculty of Experimental Science and Technology, University of San Pablo (Madrid),

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International Conferences

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Local Conferences

Society for Endocrinology, Metabolism and Diabetes of South African (SEMDSA), "Effects of a maternal high fat diet at different stages of gestation on beta cell volume and function of pups at 3 weeks of age", "The effect of a high fat diet on GLUT-2 expression in the developing rat pancreas", 28 March-2 April 2003, Drakensberg

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Abbreviations

ANOVA one-way analysis of variance

AMV Avian Myeloblastosis Virus

APES aminopropyltriethoxysilane

bp base pair

BSA bovine serum albumin

cDNA complementary deoxyribonucleic acid

DAB dimethylaminobenzaldehdye

dH_20 distilled water

DNA deoxyribonucleic acid

dNTP deoxynucleotide triphosphate

EDTA ethylenediaminetetraacetic

e embryonic day (or day of gestation)

EtBr ethidium bromide

Foxa2 forkhead box A2

FFA free fatty acids

GK glucokinase

GLP-1 glucagon-like peptide-1

GSIS glucose-stimulated insulin secretion

GLUT-2 glucose transporter-2

Hnf hepatocyte nuclear factor

HFD high fat diet

HF/HP high fat/high protein

IGF-1R insulin-like growth factor-1 receptor

IL-6 interleukin-6

IR insulin receptor

IRS insulin receptor substrate

K_m Michalis Menten constant

LPL lipoprotein lipase

$MgCl_2$ magnesium chloride

mRNA messenger RNA

MODY maturity-onset diabetes of the young

n sample number

Na_2 sodium

NeuroD1 neurogenic differentiation 1

NGS normal goat serum

Nkx Nk homeobox protein

PAGE/HPLC Polyacrylamide gel electrophoresis/High performance liquid chromatography

Pax paired domain homeobox

PBGD porphobilinogen deaminase

PCR polymerase chain reaction

Pdx-1 pancreatic duodenal homeobox-1

PPAR-γ peroxisomal proliferator-activated receptor-gamma

RIA radio immunoassay

RNA ribonucleic acid

RT room temperature

RT-PCR reverse transcriptase PCR

SEM standard error of the mean

TAE tris-acetate

Taq Taq polymerase

TNF-α tumor necrosis factor-alpha

TBS tris-buffered saline

TZDs thiazolidinediones

UV ultra-violet

WHO World Health Organization

CHAPTER 1

INTRODUCTION AND HISTORICAL REVIEW

1.1 Overview of Type 2 diabetes mellitus

The clinical hallmark of diabetes mellitus is chronic hyperglycaemia. According to WHO a random venous plasma glucose concentration that exceeds 11.1 mmol/l or a fasting value of ≥7.8 mmol/l establishes the diagnosis of diabetes. Diabetes can be subdivided into two main classifications: Type 1 diabetes, the more severe form characterized by the autoimmune destruction of pancreatic beta-cells and a young age of onset; and Type 2 diabetes, which accounts for approximately 90% of all diabetes cases and predominantly occurred in the middle-aged and elderly but is now occurring at a much younger age. Type 1 diabetes can be distinguished from Type 2 diabetes by a sudden onset of severe hyperglycaemic symptoms, in the former, with marked weight loss, spontaneous sustained ketosis or ketonuria, an inadequate C-peptide response to stimulation by glucagon and by markers of autoimmune activity (1). Symptoms of Type 2 diabetes include hunger, thirst, excessive urination and weight loss.

Type 2 diabetes is primarily associated with beta-cell failure and insulin resistance. Beta-cell failure can be defined as the reduced capacity of beta-cells to secrete sufficient insulin in response to glucose. Insulin resistance is the inability of insulin to exert its usual biological effects at circulating concentrations that are effective in normal subjects. Obesity is the most common cause of insulin resistance in children (2). A recent study has shown that insulin resistance deteriorates with increasing obesity in children and adolescents (3). A Type 2 diabetes epidemic is arising in developing countries and their migrants, with an

expected increase of up to 200 million people being affected worldwide by 2010. Diabetes is a major health problem, especially in developing countries with poor economies, as it causes high rates of morbidity and mortality and therefore has a great economic impact on national health systems. The prevalence of Type 2 diabetes is low (<1%) in many developing societies such as rural Melanesians in Papua New Guinea (4), and significantly higher in certain population groups that have undergone rapid westernization, such as the Pimas and Nauruans in whom the incidence can be as high as 40-50% concomitant with an earlier age of onset (5;6). In South Africa, Type 2 diabetes is increasing in incidence particularly in migrants. The highest incidence of Type 2 diabetes is described in individuals who have moved from severe malnutrition and a traditional way of life to an urbanized setting. This has been demonstrated in Ethiopian Jews who migrated to Israel where the prevalence of Type 2 diabetes was 9% compared to an incidence of only 1-2% in Ethiopia (7). Similarly, the prevalence of Type 2 diabetes is expected to increase significantly over the next decade in South Africa with its increasing urbanization, sedentary lifestyles, and wider variety of food choices with higher dietary fat contents.

The thrifty genotype hypothesis suggests that Type 2 diabetes susceptibility genes favour energy storage, perhaps by inducing insulin resistance selectively in the liver and skeletal muscle, resulting in raised insulin levels which would promote triglyceride deposition in fat (8). These thrifty genes confer survival in harsh conditions such as famine but could lead to obesity, insulin resistance and

Type 2 diabetes in conditions of excessive availability of food, much of which is from unhealthy fast food outlets. This has been suggested to explain the emergence and spread of Type 2 diabetes in societies that adopt a westernized lifestyle (4).

1.2 Pancreatic development

The pancreas is composed of the endocrine hormone-producing islets, the exocrine acini that produce various digestive enzymes (such as amylases, proteases, nucleases), and the ductal tree comprising the centroacinar cells, ductules and ducts that transport these enzymes into the intestine. The normal human adult pancreas is highly vascularized, richly innervated and contains about 1 million islets, which constitute 2% of the organ's total weight. The main cell types are the beta-, alpha-, delta- and PP-cells that produce the hormones insulin, glucagon, somatostatin and pancreatic polypeptide, respectively, with the core of the islet containing the beta-cells and the remaining cell types dispersed in the periphery of the islets.

Pancreas organogenesis involves a sequential cascade of inductive events in association with the activation of specific transcription factors (9). During early embryogenesis, the pancreas arises from an evagination of the foregut to form a dorsal and then a ventral epithelial bud (9). Subsequently both of these buds proliferate to form multiple branches and fuse together to form a functional organ (9). The ontogenesis of the pancreas starts with the appearance of protodifferentiated epithelial cells, which form ductules from which both acinar and islet cells originate (10).

All islet cells are believed to originate from pluripotent progenitor cells both during development and later in life (11). Islet progenitor cells, which arise from the

pancreatic ductal epithelium, undergo a series of cytodifferentiation steps that lead to the formation of mature islets (12). During embryogenesis in rodents, presumptive islet precursor cells are first observed about halfway through the gestational period. These cells proliferate, differentiate and then aggregate into mature islets which are not seen until later in gestation (13). New islets continue to form postnatally, but at a lower rate than during embryogenesis (13;14). Islet progenitor cells are thought to reside within pancreatic ductal epithelium and serve as a reservoir for islet neogenesis (14). Hence it may be possible that they express molecular markers shared between both the foregut endoderm and the developing islet cells, that they express some of the transcription factors and other proteins that distinguish mature beta-cells, and that they are able to differentiate into islet hormone-expressing cells (15). Islet cell differentiation depends on multiple transcription factors that display highly restricted cell-specific expression patterns (16-18).

Histomorphometric studies have revealed that, during late fetal gestation (19-21) and early neonatal life (22;23), most new beta-cells are formed by neogenesis, a process by which endocrine cell buds develop from duct epithelium (10). In the rat, the various endocrine cell types appear independently during different times of gestation and both the size and number of islets continue to increase as the animal develops (10). There is continued growth of each islet cell population throughout the prenatal and postnatal development of the rat (21). On the final day of gestation, the arrangement of the islet cells in the neonate appears to be

similar to the adult rat, with most of the growth occurring in the last 2-3 days before birth (21). The most abundant endocrine cell type at day 18 of gestation is reported to be the beta-cell (21). New beta-cells can be formed by either mitotic division of a pre-existing differentiated beta-cell (replication), or by differentiation from an undifferentiated precursor or stem cell (neogenesis) (24).

1.3 *In utero* programming

The thrifty phenotype hypothesis states that malnutrition *in utero* causes beta-cell development to be irreversibly damaged during critical periods of foetal development (25). Epidemiological studies, using archival records of measurements related to human foetal growth, have shown strong statistical links between birth weight and/or maternal nutrition and Type 2 diabetes and the insulin resistance syndrome in adult life (25). It has been hypothesized that these associations involve adaptive alterations of foetal organogenesis in response to maternal nutrition (25). Foetal growth and development are determined primarily by the genetic potential of the foetus and this can be influenced by environmental factors such as the nutritional status of the mother and the capacity of the placenta to transport these nutrients to the foetus (26). Exposure to an *in utero* high fat diet (HFD) could programme the foetus metabolically for continued HFD consumption postnatally. Poor nutrition *in utero*, followed by normal or supranormal nutrition after birth could result in impaired growth of beta-cells and a predisposition of the individual to develop Type 2 diabetes later in life (25). This was supported by the finding that intrauterine low protein exposure, in rats, reduces pancreatic islet size, islet vascularization, number of beta-cells and insulin content (27;28).

An isocaloric diet, containing 8% instead of the recommended 20% protein required during pregnancy, leads to a profound impairment in the structural and functional development of the rat foetal endocrine pancreas, reduced foetal

growth and a low birth weight (29). However, provision of a diet containing the normal protein content, from weaning to adulthood leads to recovery in insulin secretory capacity (29). A high energy/HFD led to a markedly lower insulin secretory response to glucose in previously protein malnourished rats (30). The transfer of rats that previously experienced sub-optimal protein nutrition, to a high energy/HFD was associated with a modest (18%) decline in the rate of glucose disappearance after intravenous glucose tolerance test (30). High fat feeding dramatically enhanced glucose-stimulated insulin secretion (GSIS) after intravenous glucose infusion in control rats, but not in rats previously maintained on a low protein (8%) diet (30). Impaired insulin secretion after protein malnutrition is exacerbated by a HFD, but this response is not linked to whole body insulin resistance (30). A recent study showed that newborn and weaned rats that were exposed to low protein diets had low body weights and high free fatty acid (FFA) levels (31). Islets of rats exposed to a low protein diet showed reduced GSIS in the presence of a high glucose concentration of 16.7 mmol/l (31).

1.4 A HFD, obesity, insulin resistance and Type 2 diabetes

A HFD has been shown to be linked to the development of obesity, insulin resistance, and Type 2 diabetes and to impair the glucose-signalling system in the beta-cell thereby suppressing insulin secretion (32-34). A HFD in human subjects and nonhuman primates has been shown to induce impaired glucose tolerance, supporting the hypothesis that an increase in dietary fat intake could be associated with the development of Type 2 diabetes (35;36). Reduced beta-cell volume has been reported in Vervet monkeys maintained on a HFD for 1.5 years (37), and significantly high plasma LDL and raised glucagon levels and weight have been recorded in Vervet monkeys after 2.5 years of a HFD (38).

Obesity is characterized by increased body fat stores resulting from an imbalance between energy intake and energy expenditure. Increased adiposity is strongly correlated with Type 2 diabetes (39) and different fat depots have different effects on reducing insulin sensitivity, with intra-abdominal fat having a greater impact than peripheral fat (40;41). FFA from central depots have direct access, via the portal vein to the liver, where they enhance hepatic glucose production by increasing the rate of gluconeogenesis (42;43). Increased levels of FFA are also thought to contribute to insulin resistance via the Randle cycle (44). This glucose-FFA cycle involves a reciprocal relationship between glucose and FFA oxidation. During states of excess FFA, FFA oxidation is increased and glucose oxidation reduced, thereby affecting glucose utilization (45). Other factors whereby excess fat may contribute to insulin resistance include increased

production of tumor necrosis factor-α (46) and interleukin-6 (47), excess muscle triglyceride accumulation (48), elevated leptin levels (49) and the production of resistin (50).

A long-term (48 weeks) challenge of C57BL/6J mice with a HFD (58% fat on a caloric base compared with 11% in the control diet), led to hyperglycaemia, hyperinsulinaemia, hyperlipidaemia and hyperleptinaemia (51). These mice were also found to have increased beta-cell mass but reduced insulin mRNA expression and an intraperitoneal glucose tolerance test revealed marked impairment of glucose disposal (51). Therefore glucose intolerance after long-term high fat feeding is accompanied by reduced cellular expression of insulin (51). A mechanism which may be involved in islet dysfunction is elevated levels of circulating FFA, which increase the beta-cell content of triglycerides, reduce insulin biosynthesis and secretion and induce apoptosis (52-54). The increase in beta-cell mass in HFD-fed mice suggests that it evolves as an adaptation to hyperlipidaemia and insulin resistance.

Although insulin resistance often accompanies obesity, glucose homeostasis is usually maintained by compensatory hypersecretion of insulin by the beta-cells (55;56). This hyperinsulinaemic state promotes lipogenesis, inhibits lipolysis and increases lipoprotein lipase (LPL) activity in adipose tissue (57;58), further aggravating the obese state. LPL catalyzes the hydrolysis of lipoprotein-associated triglycerides into fatty acids and glycerol. These FFA become

available for local uptake (59), thereby increasing insulin resistance. Ultimately, in some individuals, beta-cells are unable to meet the demand for insulin production and secretion and hyperglycemia and Type 2 diabetes develop.

Pathophysiological events, that relate a HFD to Type 2 diabetes include obesity-induced alterations in glucose metabolism; insulin resistance induced by hyperglycemia, which results from elevations in FFA levels via the Randle cycle, and alterations in insulin action that arise from changes in plasma membrane fluidity because of their dietary fat composition (60). The Type 2 diabetes lipotoxicity hypothesis (52) states that peripheral insulin resistance and islet cell dysfunction result from increased triglycerides in the peripheral and islet tissue where, for example, lipid induced nitric oxide-mediated apoptosis of beta-cells has been demonstrated (54).

1.5 An overview of the transcription factors involved in pancreatic development and beta-cell maintenance

Most new beta-cells are formed by neogenesis when endocrine cell buds develop from ductal epithelium during late gestation and early neonatal life (10;21;22). Factors that a HFD could affect are duct cell proliferation, budding or beta-cell differentiation. Cell differentiation depends on multiple transcription factors that display highly restricted cell-specific expression patterns. These transcription factors are required for the initiation of transcription and control mechanisms for cell differentiation. During early pancreatic development, differentiation of endocrine progenitor cells into hormone-producing islet cells is tightly regulated by the sequential expression of specific transcription factors.

1.5.1 MODY

Maturity-onset diabetes of the young (MODY) is a subtype of diabetes characterized by autosomal dominant inheritance, non-ketotic diabetes mellitus, an age of onset of less than 25 years and a major defect in pancreatic beta-cell function. In humans, mutations in specific genes lead to the various forms of MODY. Mutations in glucokinase (GK), the glucose sensing enzyme, lead to MODY2, with the remaining 5 types of MODY resulting from defects in genes encoding for transcription factors: HNF-4α (MODY1), HNF-1α (MODY3), Pdx-1 (MODY4), HNF-1β (MODY5) and NeuroD1 (MODY6) (61).

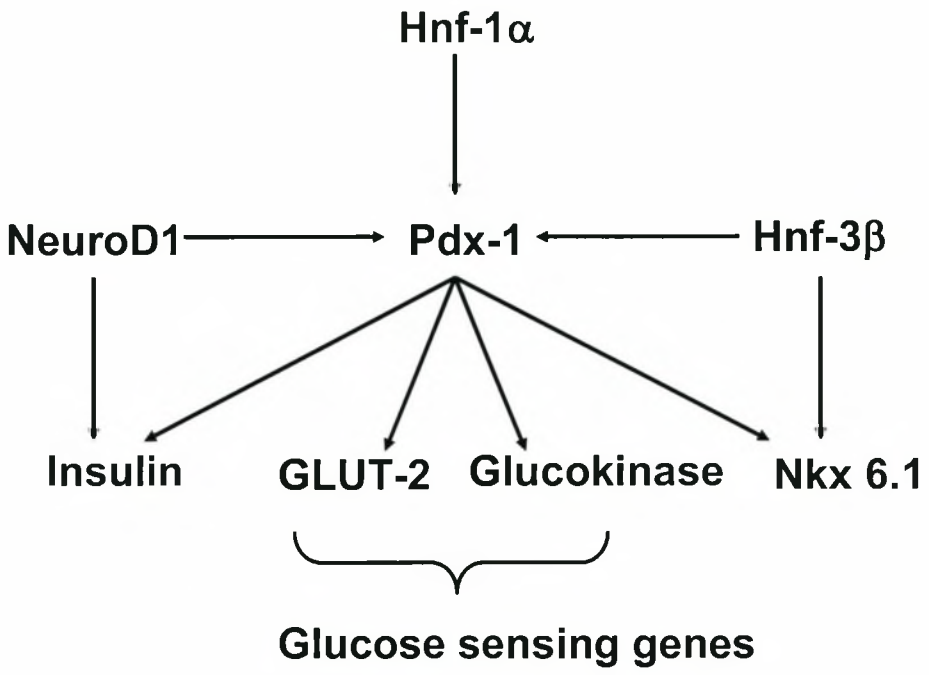
1.5.2 Pdx-1

Pdx-1 (pancreatic duodenal homeobox-1), which is expressed in adult beta-cells, has been reported to regulate the transcription of the insulin gene (62), GLUT-2 (63), GK (64) and Nkx 6.1 (65) (Fig.1). It first appears in the digestive tract of the mouse at embryonic day (e) 8.5, a day before the dorsal bud of the pancreas develops. Pdx-1 is considered to be the master regulator of pancreatic development and beta-cell differentiation. It has a dual role as an inducer of the endocrine lineage from ductal epithelial cells and in the maturation of beta-cells (66). Pdx-1 may be directly activated by the transcription factors NeuroD1 (67), Hnf-1α and Hnf-3β (68). Pancreatic agenesis occurs in Pdx-1 null mutant mice (16).

A characteristic feature of Pdx-1 is the highly conserved homeodomain, a 61 amino acid domain that builds a helix-turn-helix motif with three α-helical segments, the third helix being the DNA-recognition helix (69). Other conserved sequences, which mediate transactivation, are found in the N-terminal (69). This transactivation domain is situated within the first 79 amino acids, with amino acids 32-38 and 60-73 being of crucial importance (70;71).

Fig. 1 Regulation of pancreatic islet transcription factors and glucose sensing genes

Pdx-1 is the major transcription factor involved in early pancreatic development and maintenance of beta-cell function. The transcription factors, NeuroD1, Hnf-1α and Hnf-3β are proposed to activate Pdx-1 gene expression directly. Both Pdx-1 and NeuroD1 can regulate the transcription of the insulin gene. Pdx-1, however, also activates Nkx 6.1 gene expression, a role it shares with Hnf-3β. In addition, the expression of the glucose transporter, GLUT-2, and the glycolytic enzyme, glucokinase, which are both involved in glucose sensing, has been reported to be regulated by Pdx-1. Regulation of the expression of specific transcription factors is especially important during pancreatic development as any mutational defects would lead to the development of the MODY subtypes of diabetes.



Pdx-1 links glucose metabolism to the regulation of insulin gene transcription (72). Glucose activates Pdx-1 through an insulin-dependent cell signalling pathway involving phosphotidylinositol 3-kinase, and the stimulation of this pathway leads to phosphorylation and activation of a cytoplasmic form of Pdx-1 that translocates to the nucleus (73). Thus lower levels of circulating glucose, in rats exposed to a low protein diet, could contribute to the reduced Pdx-1 expression, and this indicates that a low protein diet during the foetal and lactation periods leads to alterations in islet size and functionality. These effects are linked to a reduction in Pdx-1 protein, but not Pdx-1 mRNA expression. The reintroduction of a normal diet, immediately after birth, normalizes the expression of Pdx-1 protein and restores islet cell mass and beta-cell secretory capacity (31).

A recent study showed that by inhibiting glucose-stimulated insulin secretion (GSIS) through the inactivation of insulin-like growth factor-1 receptor (IGF-1R), elevated glucose concentrations regulate preproinsulin, Pdx-1 and GK gene expression in beta-cells. These studies showed that released insulin, acting via insulin receptor (IR), is likely to be an important mediator of the effects of glucose on Pdx-1 gene expression in normal MIN6 beta-cells. However, insulin-independent mechanisms are sufficient to allow the control by glucose of GK gene expression in wild type cells and the regulation of the Pdx-1 gene in the absence of IGF-1R (74).

Pdx-1 regulates the expression of GLUT-2 in a dosage dependent manner, suggesting that lowered Pdx-1 activity may contribute to the development of Type 2 diabetes by causing impaired expression of both GLUT-2 and insulin. Early loss of GLUT-2 expression is involved in the development of hyperglycaemia, in Pdx-1 beta-cell-specific mutants, and the combined loss of GLUT-2 together with the gradual decrease of insulin expression leads to the manifestation of diabetes. Thus lowered Pdx-1 expression, or activity resulting in impaired expression of both GLUT-2 and insulin, could cause hyperglycaemia which may progress to Type 2 diabetes. In mature beta-cells, Pdx-1 expression is required to maintain insulin production, GLUT-2 expression and glucose homeostasis (75).

Using HIT-T15 cells, a clonal beta-cell line, studies on the impact of glucose levels on Pdx-1 expression in pancreatic beta-cells suggest that while persistent hyperglycaemia (16 mmol/l glucose) extinguishes Pdx-1 mRNA and protein expression, hypoglycaemia (0.8 mmol/l glucose) actually restores Pdx-1 levels (76). Pdx-1 mRNA and protein expression were down-regulated in beta-cells from which Hnf-3β was deleted in a mouse mutant model (77). In addition, Hnf-3β was demonstrated to be an essential upstream factor that, directly or indirectly, regulates Pdx-1 levels in beta-cells *in vivo* (77).

1.5.3 Pax 4

The paired domain homeobox gene, Pax 4, is expressed in the developing pancreas at e9.5. Homozygous Pax 4 deficient mice appear normal at birth but are growth retarded by 48h and die 3-5 days after birth (18). They lack beta- and delta-cells, but have an increase in alpha-cells. Pax 4 is essential for proper beta-cell development and especially for their maturation and maintenance.

Pax 4 has been identified as a regulator of endocrine development (18). Indirect evidence from mice, containing the β-galactosidase coding sequence inserted into the Pax 4 gene, suggests that Pax 4 expression is restricted to the beta-cells at birth (18). Insulin expressing cells are detected, at e10.5, in the null mutants for Pax 4, suggesting that insulin transcription can occur in the absence of Pax 4. Pax 6, which is highly related to Pax 4, is also required for normal endocrine pancreatic development (78), and double null mutants for both Pax 4 and Pax 6 fail to produce any mature pancreatic endocrine cells (78), suggesting that these two factors together are required for endocrine cell differentiation. Using RT-PCR, Pax 4 expression was shown to peak early, at e13.5, in pancreatic development of the foetal mouse but is undetectable in adult islets (79). It appears that Pax 4 can function as a transcriptional repressor and its expression early in pancreatic development may allow it to suppress alpha-cell differentiation and permit beta-cell differentiation (79). Using a rat glucagon-producing cell line, Pax 4 was shown to act as a repressor of glucagon gene expression and was shown to inhibit the insulin promoter in the absence of Pax 6, suggesting an

active repression mechanism of Pax 4 (80). Another study showed that Pax 4 binds, with high affinity, to Pax 6 target sites of the glucagon gene promoter suggesting a competition mechanism of transcriptional inhibition (81). Pax 4 has been shown to be unable to inhibit Hnf-3α transcription, but was able to inhibit transcription of the glucagon gene, by DNA competition with Pax 6 (81). Indeed, the repression, by Pax 4 of Pax 6-mediated transcription could play a role in the restriction of glucagon gene expression to alpha-cells during pancreas development (81).

1.5.4 NeuroD1

NeuroD1 (neurogenic differentiation 1 or BETA2) is a cell-type restricted basic helix-loop-helix transcription factor expressed in all endocrine cells (82). It is expressed in the mouse pancreatic bud at e9.5. Homozygous null mutations of the NeuroD1 gene show a marked reduction in beta-, alpha- and delta-cells in newborn mice (17).

1.5.5 Hnf-1α

The hepatocyte nuclear factor, Hnf-1α, is expressed at e13.5 in the developing mouse pancreas and in beta-cells during adulthood. This transcription factor has been proposed to regulate the genetic expression of insulin and the glucose transporter, GLUT-2 (83;84). It also appears to be essential for maintaining insulin storage (83). Defects in the Hnf-1α gene, in mice, result in the

development of Type 2 diabetes two weeks after birth. Null mutant mice for Hnf-1α have impaired GSIS (85).

1.5.6 Hnf-3β

Hnf-3β (Foxa2) first appears in the early endodermal layer, from which the pancreas arises, and is later expressed in all of the islet cell types (86;87). This winged helix transcription factor is required to maintain proper circulating levels of insulin and glucagon for glucose homeostasis (88). Hnf-3β is believed to regulate transcription of the Nkx 6.1 gene (65).

1.5.7 Nkx 2.2

The Nk homeobox gene, Nkx 2.2, appears at e9.5 in the whole pancreatic bud and is expressed in all but delta islet cells (89). It may be required for terminal differentiation of beta-cells or for expression of the insulin gene (89). It is also required for maintenance of Nkx 6.1 expression. Nkx 2.2 mutant mice become diabetic after birth and die due to hyperglycaemia.

1.5.8 Nkx 6.1

Nkx 6.1 is restricted to differentiated beta-cells (90) and appears to be specific for foetal cell differentiation and function (90-92). It is expressed at e10.5 in the pancreatic bud. Targeted disruption of the Nkx 6.1 gene, in mice, results in inhibition of beta-cell formation (90) as it is required for expansion and terminal differentiation of beta-cell progenitors.

1.6 The beta-cell glucose sensing genes

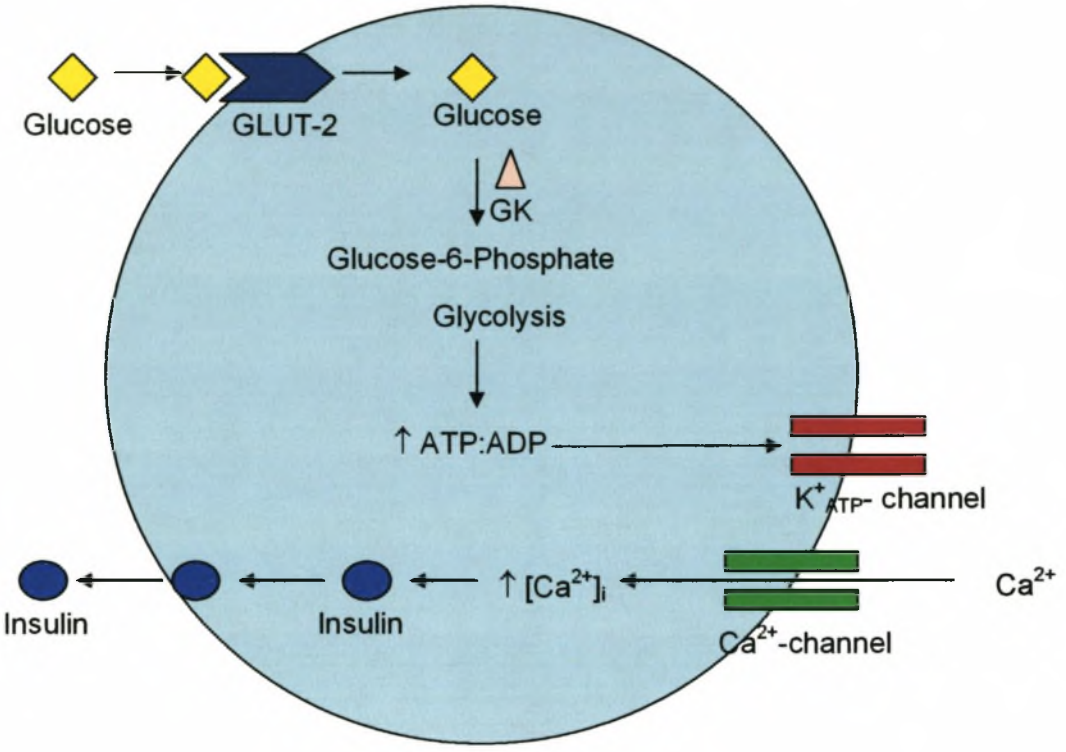
1.6.1 GLUT-2

The glucose transporter, GLUT-2, together with the glycolytic enzyme, glucokinase (GK), plays a major role in GSIS (Fig. 2). Glucose is transported into cells by facilitated diffusion, which involves binding of glucose to glucose transport proteins (93). GLUT-2 is a high-K_m facilitative glucose transporter that is expressed by beta-cells in normal animals (93). In the foetal rat pancreas, which begins to develop around e9, the duct cells transiently express GLUT-2 which is normally only expressed in mature beta-cells (94). After e16, GLUT-2 disappears from the ductal cells and appears in the newly formed beta-cells (24). In the rat, cells co-expressing GLUT-2 and insulin, at e17, form beta-cells (95).

GLUT-2 is expressed in numerous foetal and adult tissues including pancreas, liver, intestine, brain and kidney (93;96) and is most abundant in the liver, kidney and small intestines in declining order (97). GLUT-2 was shown to be localized in rat pancreatic islets, primarily to the microvesicular patterns of beta-cell membranes adjacent to neighbouring endocrine cells (98) and in the endodermal epithelial cells of the entire pancreatic primordium (94;98).

Fig. 2 A brief overview of glucose-stimulated insulin secretion

GSIS is initiated when elevated glucose levels are sensed by GLUT-2 and transported into the beta-cell. Glucose is then phosphorylated into glucose-6-phospate by GK thereby initiating glycolysis. An increase in the ATP:ADP ratio results in closure of the K^{+}_{ATP}-channels. Ca^{2+} enters the beta-cell via the voltage-gated Ca^{2+}-channels. The resultant increase in intracellular Ca^{2+} concentration triggers insulin exocytosis. In rodent models of Type 2 diabetes, GLUT-2 expression is reduced.



In normal human islets, insulin cells are strongly positive for GLUT-2, especially adjacent to the cell membranes (99). Recent studies report the expression of GLUT-2 mRNA and protein to be significantly lower in foetal and suckling rat pancreatic islets than in adult rat islets (100). Furthermore, in foetal islets, GLUT-2 mRNA and protein increased as a function of increasing glucose concentration (100). After being fed a high energy diet (2.93 Kcal/g), hyperglycaemic and hyperinsulinaemic rats (*Psammomys obesus*) showed a decrease of 71% (after 1 week) and 94% (after 3 weeks) in GLUT-2 immunostaining on the beta-cell membrane (101).

In the adult rat pancreas, GLUT-2 expression has only been described in beta-cells, where it may be essential for their glucose sensing function (96). The loss of responsive beta-cells to glucose is associated with a decrease in glucose uptake by islets and a profound reduction in immunodetectable GLUT-2 and GLUT-2 mRNA (102). Impaired expression of GLUT-2 may impair glucose sensing and result in hyperglycaemia (103). A reduction of GLUT-2 develops in concert with the onset of steady state hyperglycaemia.

Situations of increased insulin demand include a HFD, ageing and pregnancy (104). A HFD has been shown to decrease GLUT-2 and GK expression in the pancreata of adult rats fed a HFD for 10 weeks (34). More recently, mice fed a HFD for 8 weeks showed impaired glucose tolerance accompanied by compromised translocation of GLUT-2 to the plasma membrane and of Pdx-1 to

the nucleus (105). Plasma insulin concentrations were lower in rats fed a HFD and the glucose levels were higher when compared to those on a high carbohydrate diet (34). Evidence suggests, therefore, that a HFD could impair beta-cell trafficking of factors important for both beta-cell glucose recognition and insulin gene expression.

1.6.2 Glucokinase

The high-K_m glucose phosphorylating enzyme, GK, is expressed in islets where it regulates pancreatic glucose sensing (106). In the mature beta-cell, GK regulates glucose homeostasis by catalyzing the conversion of glucose to glucose-6-phosphate, after glucose is transported into the beta-cell by GLUT-2. GK appears in both beta- and alpha-cells of foetal rat islets at e16, but is predominantly expressed by beta-cells (107). The GK content of foetal islets was found to be significantly higher than that of suckling and adult rats (100).

The unique kinetics of GK underlie the ability of these cells to sense and respond to fluctuations in plasma glucose concentration (108). Modelling of changes in GK activity, as well as studies of the effects of graded increases in the enzyme, have established that glucose phosphorylation is the key point of control for glycolytic flux in the beta-cell (109;110). Consequently, even small changes in GK activity can be physiologically significant since they directly affect the threshold for GSIS (108). Overexpression of GK reduces the blood glucose concentration and appears to prevent the development of Type 2 diabetes (108).

Male GK locus transgenic mice that were fed a HFD were protected against the development of hyperinsulinaemia and hyperglycaemia, even though these animals were as obese as their non-transgenic littermates (111). This suggests that exploring ways to enhance GK activity could result in an effective strategy for treating diabetes.

Insulin secretion depends upon glucose metabolism and GK exerts nearly total control on the rate of glycolysis in beta-cells (112). Insulin is able to stimulate GK translocation and activation in the absence of glucose (113), while inhibition of insulin secretion or insulin receptor function has been found to block glucose-stimulated GK translocation and conformational changes (113). Together these results point to the regulation of GK localization and activity by insulin secreted by beta-cells in response to glucose (113). The enzymatic activity of granule-bound GK alone can support GSIS from the beta-cell (113). This is based on the observation that under glucose-starved conditions, the measured GK activity is at least 50% of the total activity measured under high glucose conditions. Under these low glucose conditions, virtually all of the endogenous GK was found to be associated with granules (113). These observations highlight the fact that GK activity in the beta-cells is modulated over a narrow range (113). Changes in the GK conformation and activation state, that correlate with GK association and dissociation from the granule, provide a novel mechanism for rapidly altering the rate of insulin secretion in response to changes in glucose concentration (113).

1.7 Insulin

Insulin first appears in the developing mouse pancreas at e12 (12). When foetal rat pancreatic cells were isolated from the pancreatic primordium, on e12-14 of pregnancy, and cultured for 48h in the presence of 5 mmol/l glucose, the cultured cells from e12 foetuses secreted 1 fmol insulin per pancreas in response to 5 or 15 mmol/l glucose (114). Insulin release from cells at e14 was 75 amol/min per pancreas when the cells were perifused for 15-20 min in 5 mmol/l glucose within 3h of isolation (114). The insulin content of rat pancreata from e13 was 0.06 pmol per pancreas and increased approximately 20-fold every second day up to 6.7 pmol on e17. Between e17 and e19 the pancreatic insulin content increased about 5-fold to 39 pmol (114). The data suggest that critical components of the insulin-secretory machinery, including K_{ATP}-channels and GK, are present in the beta-cell earlier than previously anticipated (114). The study showed that rat beta-cells, from the early foetal period, respond to an increase in glucose concentration with the release of insulin, although to a much smaller extent than adult cells (114).

The synthesis of preproinsulin from insulin mRNA occurs through the rough endoplasmic reticulum and is followed by proteolytic cleavage at the Golgi apparatus, generating mature insulin and C-peptide. Insulin and C-peptide are stored, in secretory granules, ready for release following physiological stimulation. Glucose is the main stimulus for insulin release. During prolonged fasting, however, FFA provide the body with an alternative fuel to glucose,

thereby reserving glucose for the brain and conserving body proteins that would usually be utilized as gluconeogenic substrates.

The islet beta-cell is specifically adapted for:

(i) synthesis of the insulin precursor, preproinsulin,

(ii) its processing by proteolytic cleavage to produce insulin,

(iii) storage of large quantities of insulin in membrane-limited vesicles,

(iv) secretion of insulin in response to specific stimuli such as glucose (115).

Insulin regulates numerous diverse metabolic processes through binding to a high-affinity cell-surface glycoprotein receptor. Elevated insulin levels result in down-regulation of the insulin receptor (IR) by decreasing their numbers at the cell surface. This is brought about by increased internalization and degradation and also by decreasing the IR's tyrosine kinase activity (115). The major targets of insulin, and therefore sites of glucose uptake, are liver, skeletal muscle and adipose tissue. Upon ligand binding, the IR phosphorylates the insulin receptor substrates IRS1 and IRS2, coupling IR to its downstream effector molecules, ultimately leading to intracellular changes in cell behaviour and gene expression (116).

Targeted disruption of the IR gene selectively in beta-cells results in impaired glucose tolerance in mice (117). However, disruption of the insulin-like growth

factor-1 receptor (IGF-1R) gene leads to a more complex phenotype, characterised by resting hyperglycaemia and abnormal glucose tolerance, but no significant changes in preproinsulin gene expression or islet insulin content (118). Although these mice show normal beta-cell growth and development, GSIS is inhibited *in vivo* and *in vitro* and the expression of GLUT-2 and GK are reduced (118;119).

The resultant secretion of insulin from beta-cells is multiphasic, comprising two primary phases (120), the first being transient, rapid and lasting for 2-5 minutes. In the second progressively increasing phase, glucose continually amplifies its own signal throughout the duration of the stimulus. Other nutrients, such as amino acids (121) and FFA (122), can also stimulate insulin release. FFA can potentiate GSIS by activating protein kinase C by a mechanism that appears to be mediated by Ca^{2+} mobilization (123).

Upon release from the beta-cell, the principal metabolic function of insulin is to increase the rate of glucose transport into muscle and adipose tissue. Additionally, insulin enables transmembrane transport of amino acids, glycogen formation, glucose conversion to triglycerides, nucleic acid synthesis and protein synthesis (124). It also inhibits endogenous glucose production by the liver and kidney (125).

Impaired beta-cell function is characterized by an absent first-phase response to an intravenous glucose load and a markedly reduced second-phase response (126). It is well established that the effects of exogenous FFA on insulin secretion are time-dependent both *in vivo* and *in vitro* (127). Short-term exposure to FFA stimulates insulin secretion (122), whereas long-term exposure to FFA decreases insulin secretion (53;128). Prolonged elevations in FFA, as in obesity, have been shown to induce secretory defects by selectively desensitizing the beta-cell response to glucose (52;128). Chronically elevated FFA are detrimental to beta-cell function, resulting in reduced insulin secretion.

In rats, 24h exposure to FFA transiently increased insulin mRNA levels at basal (5.6 mmol/l) levels of glucose (129). In contrast, 24h exposure of rat islets to palmitate decreased preproinsulin mRNA levels in the presence of a high glucose level (130). Thus it appeared that FFA inhibit insulin gene expression only in the presence of elevated glucose levels. This was confirmed in a study using isolated rat islets where palmitate was shown to decrease the expression of the insulin gene in the presence of 16.7 mmol/l glucose, partly through inhibition of insulin gene promoter activity (131). In support of this, another study showed that 48h exposure to palmitate decreases insulin gene expression in the presence of 30 mmol/l glucose via negative regulation of Pdx-1 (132). Recently, evidence was provided for the involvement of the fatty acid esterification pathway in mediating the inhibition of insulin gene expression by prolonged FFA exposure (133). Neutral lipid mass was increased in isolated rat islets following a 72h

exposure to palmitate, in the presence of 16.7 mmol/l glucose, that was inversely correlated to insulin mRNA levels. Depletion of insulin content may be a contributory factor to FFA-induced beta-cell dysfunction.

1.8 Hypothesis

The exposure of pups to a maternal high fat diet during gestation and/or lactation will result in a decrease in the expression of:

(i) Pdx-1 and Pax 4, important regulatory transcription factors involved in pancreatic and beta-cell development,

(ii) GLUT-2, the beta-cell specific glucose transporter, and

(iii) Glucokinase, the pancreatic glucose sensing enzyme required for glycolysis within the beta-cell,

which will adversely affect beta-cell development and function.

1.9 Aims of the study

To investigate, in the neonatal and weanling rat, the effect of exposure to a maternal high fat diet during gestation and/or lactation on:

(i) body weight

(ii) circulating glucose concentrations

(iii) circulating insulin concentrations

(iv) beta-cell development

(v) alpha-cell development

(vi) expression of the pancreatic transcription factors, Pdx-1 and Pax 4

(vii) expression of the beta-cell specific glucose sensing genes, GLUT-2 and glucokinase

CHAPTER 2

MATERIALS AND METHODS

Study design

2.1.1 Experimental groups

All animal experiments were approved by the Ethics Committee of the Medical Research Council (MRC), South Africa, rigorously adhering to the ethical NIH guidelines for the care and use of laboratory animals. Wistar rats were housed individually with free access to food and water at the MRC Primate Unit (Cape Town, South Africa). The room was maintained at a temperature of 24^0C with a 12h light cycle.

The experimental groups are summarized in Table 1. Pregnant rats were fed a HFD for the first (Group 1 dams), second (Group 2) or third (Group 3) week, or for all three (Group 4) weeks of gestation. After birth, the neonatal pancreata were collected from Groups 1-5. The individual neonatal groups were therefore exposed to a maternal HFD during the either the first (Group 1 neonates), second (Group 2) or third (Group 3) week, or all three (Group 4) weeks of gestation.

For the weanling Groups 6-10, the same range of diets were used *in utero*, as for Groups 1-5 and the dams were fed a standard laboratory diet during lactation. Additional weanling Groups 11-14 comprised dams fed a HFD throughout gestation and for specific periods of weaning, with one experimental group (Group 15) exposed to a standard laboratory diet for gestation and a HFD during lactation. The control groups comprised dams (Group 5 dams) maintained on a

standard laboratory diet and their offspring (Group 5 neonates and Group 10 weanlings).

2.1.2 Composition of diets

The standard laboratory diet comprised 10% fat, 15% protein and 75% carbohydrate as energy (2.6 kcal/g) while the HFD contained 40% fat, 14% protein and 46% carbohydrate (2.06 kcal/g).

The period of gestation in the Wistar rat is 3 weeks and lactation also lasts for 3 weeks. The weeks of gestation and/or lactation during which the dams were maintained on a HFD represent the periods of exposure of their offspring to a HFD. The experimental groups depicted in the table represent neonates (Groups 1-4) and weanlings (Groups 6-9, 11-15) that were exposed to a maternal HFD for specific periods of gestation and/or lactation. The control groups are Group 5 for the neonates and Group 10 for the weanlings. These offspring were only exposed to a standard laboratory (control) diet. When not exposed to a HFD, for example, weeks 2 and 3 of Group 1, the pups were exposed to a standard laboratory diet. Group 1 and Group 6 had identical exposure to a maternal HFD *in utero*, i.e. for the first week of gestation, with neonates from Group 1 terminated at birth and weanlings from Group 6 terminated at 3 weeks of age. This is the same for Groups 2-4 and Groups 7-9, respectively. The weanling Groups 11-14 were exposed to a maternal HFD throughout gestation and for specific weeks of lactation. Group 15 weanlings were only exposed to a maternal HFD throughout lactation.

Table1 Experimental groups

Group	Period of exposure to a maternal HFD or standard laboratory (control) diet during gestation	Period of exposure to a maternal HFD or standard laboratory (control) during weaning
1	HFD: First week Control: Second and third week	Rats terminated at birth
2	HFD: Second week Control: First and third week	Rats terminated at birth
3	HFD: Third week Control: First and second week	Rats terminated at birth
4	HFD throughout gestation	Rats terminated at birth
5	Control diet throughout gestation	Rats terminated at birth
6	HFD: First week	Control diet throughout weaning
7	HFD: Second week	Control diet throughout weaning
8	HFD: Third week	Control diet throughout weaning
9	HFD: Throughout	Control diet throughout weaning
10	Control diet throughout gestation	Control diet throughout weaning
11	HFD: Throughout	HFD: First week
12	HFD: Throughout	HFD: Second week
13	HFD: Throughout	HFD: Third week
14	HFD: Throughout	HFD: Throughout
15	Control diet throughout gestation	HFD: Throughout

Determination of circulating glucose and insulin concentrations

2.2.1 Mothers

Maternal food intake, body weight and circulating glucose and insulin concentrations were monitored during pregnancy. Blood, for measurement of the baseline circulating glucose and insulin levels, was collected from the tail veins of female virgin Wistar rats (n = 8 per group) fasted for 3h. Pregnancy was determined by the presence of a vaginal plug(s). At gestational days 7 and 14, and after delivery, dams were fasted for 3h and blood collected from the tail vein. For this procedure, dams were anaesthetized using an anaesthetic machine (Motivus Resuscitator Type AV, Crest Healthcare Technology Ltd, Johannesburg, South Africa) with fluothane (Halothane, AstraZeneca Pharmaceuticals, Johannesburg, South Africa) and 2% oxygen. Once anaesthetized, the tip of the tail was heated with a UV lamp, to facilitate blood flow, then snipped with a surgical blade and blood glucose concentrations determined using a glucometer (Precision QID, MediSense Inc., Oxfordshire, UK). Blood was collected in tubes and placed on ice before centrifugation (Biofuge 13, Heraeus Sepatech, Osterode am Harz, Germany) at 13 000 rpm for 10 min at RT and the serum used to determine the insulin concentrations with a Rat Insulin RIA Kit (Linco Research Inc., Missouri, USA).

2.2.2 Neonates and weanlings

The body weights, circulating glucose (Glucometer) and insulin (Rat Insulin RIA kit) levels in the neonates (n = 6 per group) and weanlings (n = 6 per group) were measured at postnatal days 1 and 21 respectively.

Molecular biology

2.3 RNA isolation

RNA was isolated from one-day-old neonatal (n = 10 per group) and 3-week-old weanling (n = 10 per group) pancreata using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The tissues were placed in 2 ml cryotubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and crushed (in the tubes) in liquid nitrogen. A volume of 600 µl RLT lysis buffer was added and the tissue was further disrupted and homogenized using a sterile RNase-free 1 ml syringe (Jung-Rim, Seoul, South Korea). The lysate was passed through the syringe at least 5 times to ensure complete homogenization and to increase the RNA yields. The tissue lysate was transferred to a 2 ml tube (Eppendorf AG, Hamburg, Germany) and centrifuged for 3 min at maximum speed in a microcentrifuge (Eppendorf Model 5414, Hamburg, Germany). The supernatant was carefully transferred to another 2 ml tube (Eppendorf), by pipetting, and the lysate (supernatant) used in the subsequent steps. One volume (600 µl) of 70% ethanol (Merck KGaA, Darmstadt, Germany) was added to the cleared lysate and mixed by pipetting. The sample (to a maximum of 700 µl), including any precipitate that may have formed, was applied to an RNeasy Mini column placed in a 2 ml collection tube

(Qiagen). The tube was gently closed and centrifuged for 15 s at maximum speed (≥10 000 rpm) and the flow through discarded. If the volume exceeded 700 µl, the remaining aliquots were loaded successively into the RNeasy column and centrifuged as above, discarding the flow through after each centrifugation step. A volume of 700 µl RW1 buffer was added to the RNeasy column placed in the same collection tube. The tube was gently closed and centrifuged for 15 s at maximum speed to wash the column. The flow through and collection tube were then discarded. The RNeasy column was transferred into a new 2 ml collection tube (Qiagen) and 500 µl of RPE (the buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane) was pipetted onto it. The tube was gently closed and centrifuged for 15 s at maximum speed to wash the column. The flow through was discarded and another 500 µl of RPE buffer was added onto the column, reusing the same collection tube. The sample was centrifuged for 2 min at maximum speed to dry the RNeasy silica-gel membrane to prevent residual ethanol from interfering with downstream reactions. This centrifugation ensured that no ethanol was carried over during elution. Following centrifugation, the RNeasy column was removed carefully from the collection tube to avoid contact with the flow through, which would result in ethanol being carried over. The RNeasy column was finally transferred to a new 1.5 ml tube (Qiagen) and 30 µl of RNase-free water was pipetted directly onto the RNeasy silica-gel membrane. The samples were then centrifuged for 1 min at maximum speed to elute the purified RNA.

2.4 First strand cDNA synthesis

A First Strand cDNA synthesis kit for RT-PCR (Avian Myeloblastosis Virus (AMV); Roche Diagnostics GmgH, Penzberg, Germany) was used for the first strand synthesis of single-stranded cDNA from each RNA sample for use as a template for PCR. All vessels and pipette tips that were used for cDNA synthesis were autoclaved to eliminate the risk of RNase contamination. The reagents were thawed on ice and briefly vortexed before starting. Because of the small reaction volume of the reagents required for each first strand cDNA synthesis reaction, a larger amount of master mix was prepared (Table 2), centrifuged briefly, and aliquoted into reaction tubes before adding the RNA samples. The preparation of the master mix eliminated the need to repeatedly pipette small volumes, thus yielding greater consistency between samples.

Table 2 Preparation of Master Mix for cDNA synthesis

Reagent	Volume/10 µl 1 sample	Volume/250 µl 25 samples	Final Concentration
10X Reaction Buffer	1 µl	25 µl	1X
25mM $MgCl_2$	2 µl	50 µl	5mM
dNTP mix	1 µl	25 µl	1mM
Primer: Oligo-$p(dT)_{15}$ primer or Random primer $p(dN)_6$	1 µl	25 µl	0.04 A_{260} units (1.6 µg) or 0.08 A_{260} units (3.2 µg) respectively
RNase inhibitor	0.5 µl	12.5 µl	50 units
AMV reverse transcriptase	0.4 µl	10 µl	≥20 units
RNA sample*	2-4 µl†	25-50 µl†	-

*RNA samples were omitted during preparation of the master mix and added individually after the master mix was aliquoted into separate tubes.

†A volume of either 2 µl or 4 µl of RNA was added to the mixture, depending on the concentration of the RNA samples. For groups 1-10, 4 µl of RNA was added to 5.9 µl of the master mix. For groups 11-15 only 2 µl of RNA and an additional 2 µl of sterile water (Sabax, Ladmed, Durban, South Africa) was added to 5.9 µl of the master mix as these samples had a higher RNA concentration (estimated at 10X higher).

A volume of 1 µl of control neo RNA was used as a positive control (adding 3.1 µl of sterile water to the master mix).

A volume of 1 µl of sterile water was added, instead of RNA, as the negative control.

After preparation, the mixture was briefly vortexed and centrifuged to collect the sample at the bottom of the microfuge tube. The reaction was incubated at 25^0C for 10 min, then at 42^0C for 60 min. During the first incubation, the primer anneals to the RNA template. The RNA is subsequently reverse transcribed, resulting in cDNA synthesis during the second incubation. Following the 42^0C incubation, the AMV reverse transcriptase, which synthesizes the new cDNA at the sites determined by the primer used, was denatured by incubating the reaction of 99^0C for 5 min and then cooled to 4^0C for 5 min. The cDNA was then used for RT-PCR experiments.

2.5 RT-PCR

The cDNA synthesized from RNA isolated from Groups 1-15 was then used in the subsequent RT-PCR experiments (Table 3a). All the cDNA was diluted 1:10 with sterile water before use for RT-PCR. All the primer sets (forward and reverse) for each of the target genes, viz. Pdx-1, Pax 4, GLUT-2 and GK, were diluted in sterile water to give a concentration of 100 pM/µl. Each primer was then further diluted as follows: 10 µl of 100 pM/µl primer added to 90 µl of sterile water to give a 10 pM/µl working dilution to be used for RT-PCR. A master mix was prepared for RT-PCR (Table 3b).

2.5.1 Primer design

The PCR products visualized on the agarose gel were 169 bp for Pdx-1, 132 bp for Pax 4, 100 bp for GLUT-2 and 148 bp for GK.

Primers were designed using the Primer3 website and were produced and purified by PAGE/HPLC (IDT, Coralville, IA, USA). The primer sequences were as follows:

Pdx-1 forward: 5'- GCT GGA GCT GGA GAA GGA AT -3'

Pdx-1 reverse: 5'- CGT TGT CCC GCT ACT ACG TT -3'

Pax 4 forward: 5'- CTC GAA TTG CCC AGC TAA AG -3'

Pax 4 reverse: 5'- CCC GAA GGA CTC GAT TGA TA -3'

GLUT-2 forward: 5'- CAG TGG AGC GTG AAG ACA AA -3'

GLUT-2 reverse: 5'- AGG GAA GGA GAA GGT GAA GC -3'

GK forward: 5'- CAG TGG AGC GTG AAG ACA AA -3'

GK reverse: 5'- AGG GAA GGA GAA GGT GAA GC -3'

Porphobilinogen deaminase (PBGD), the housekeeping gene used for the PCR experiments, was purified by standard desalting:

PBGD forward: 5'- GCA TAC AGA CCG ACA CTG TGG -3'

PBGD reverse: 5'- CTC TGG CAA GGT TTC CAG GG -3'

2.5.2 RT PCR programme

RT-PCR was done using the PCR Sprint (Table 3a; Thermo Hybaid, Milford, MA, USA). Briefly, the cDNA sample was denatured at 94^0C for 1 min for 1 cycle. In the next stage, the sample was denatured at 94^0C for 30 s, annealed at 52^0C (for Pdx-1, Pax 4), 57^0C and 55^0C (GLUT-2) or 57^0C (GK), then elongated at 72^0C for

1 min. This stage was repeated for 30 cycles. The final stage was an elongation step at 72^0C for 5 min comprising 1 cycle.

Table 3a RT-PCR programme

Temperature	Time	PCR step
Stage 1 Step 1		
94^0C	1 min	Denaturation (1 cycle)
Stage 2 Step 1		
94^0C	30 s	Denaturation
52, 55 or 57^0C	30 s	Annealing
72^0C	1 min	Elongation (30 cycles)
72^0C	5 min	Final elongation (1 cycle)

Table 3b Preparation of master mix for RT-PCR

Reagent	Volume/10 µl reaction	Volume/50 µl reaction	Final Concentration
10X Reaction Buffer	1 µl	5 µl	1X
Taq Polymerase	0.08 µl	0.4 µl	0.4 U
dNTP (10 mM)	0.2 µl	1 µl	200 mM
BSA (20 mg/ml)	0.125 µl	0.625 µl	50 µg/ml
Primer Forward*	0.5 µl	2.5 µl	5 pM
Primer Reverse*	0.5 µl	2.5 µl	5 pM
Water	6.265 µl	31.325 µl	-
Total	10 µl	50 µl	-

*A volume of 0.5 µl (concentration of 5 pM) was added to the master mixes for Pdx-1, Pax 4 and GK. For GLUT-2, 1 µl of primer was added (concentration 10 pM). The RT-PCR had to be further optimized for GLUT-2 and GK where 0.4 µl $MgCl_2$ was added per 10 µl reaction to give a final $MgCl_2$ concentration of 2.5 mM as opposed to the 1.5 mM concentration of the Pdx-1 and Pax 4 mixes.

A volume of 9 µl master mix was added to 1 µl cDNA sample for each reaction.

Optimization of RT-PCR

2.5.3 Annealing temperatures for primer pairs

An annealing temperature of 52^0C was used for RT-PCR for both Pdx-1 and Pax 4. The primers for these transcription factors were used under standard PCR conditions without changing any parameters. For GLUT-2, the concentration of the primer pair was increased to 10 pM, i.e. 1 µl of each primer was added per 10 µl reaction. A touchdown PCR was done where the annealing temperature was 57^0C for the first 10 cycles followed by 55^0C for the remaining 20 cycles. In addition, a $MgCl_2$ titration was done by increasing the 1.5mM $MgCl_2$ concentration present in the PCR mix to 2, 2.5 and 3 mM (Table 4). A 2.5 mM $MgCl_2$ concentration was optimum for the PCR. For GK the primer pair worked best at an annealing temperature of 57^0C. The $MgCl_2$ concentration also had to be increased to 2.5 mM (similar to GLUT-2). Primers for the housekeeping gene, PBGD, worked well at annealing temperatures of 52, 55, and 57^0C without changing any parameters under standard PCR conditions.

Table 4 Preparation of mixes for $MgCl_2$ titrations for GLUT-2 optimization RT-PCR per 10 µl reaction

Reagent	1.5 mM $MgCl_2$ concentration	2 mM $MgCl_2$ concentration	2.5 mM $MgCl_2$ concentration	3 mM $MgCl_2$ concentration
10X Reaction Buffer	1 µl	1 µl	1 µl	1 µl
Taq Polymerase	0.08 µl	0.08 µl	0.08 µl	0.08 µl
dNTP (10 mM)	0.2 µl	0.2 µl	0.2 µl	0.2 µl
BSA (20 mg/ml)	0.125 µl	0.125 µl	0.125 µl	0.125 µl
Primer Forward*	1 µl	1 µl	1 µl	1 µl
Primer Reverse*	1 µl	1 µl	1 µl	1 µl
Water	5.265 µl	4.765 µl	4.265	3.765
$MgCl_2$	0	0.5	1	1.5
Total	10 µl	10 µl	10 µl	10 µl

2.5.4 Gel electrophoresis

The PCR products for each primer pair were visualized on a 1% agarose gel (Whitehead Scientific, Cape Town, South Africa). The 1% agarose gel was prepared as follows:

1 g of agarose (Molecular grade agarose D1-LE, Whitehead Scientific, Cape Town, South Africa),

2 ml of 50X TAE (tris-acetate) buffer

[242g of Tris was dissolved in 500 ml of water. 100 ml of 0.5 M Na_2 EDTA, pH 8 and 57.1 ml glacial acetic acid was added. The volume was adjusted to 1l with dH_2O, and the solution was stored at RT], made up to 100 ml in dH_20.

The mixture was boiled for 2 min until the agarose was dissolved. Then 10 µl of EtBr was added. The beaker containing the solution was swirled under running tap water to cool down. The agarose was then poured into a gel holder and allowed to set. After reaching a gel state, the agarose was transferred to a holder containing 1X TAE buffer and electophoresed at 100 V for 60 min using a Biorad Power Pac. The DNA fragments were visualized under UV light.

2.6 LightCycler PCR

Sybr green (nucleic acid gel stain) (Stratagene, Amsterdam, The Netherlands) was used: 1 µl Sybr green with 99 µl sterile water. Then 1 µl of 1:100 Sybr green was diluted with 99 µl water to give a 1:10 000 Sybr green working stock solution. A volume of 0.33 µl of this working solution was used per 10 µl LightCycler PCR mix.

Master mixes of the 600 µl volume were prepared for Pdx-1 (Table 5), Pax 4 (Table 5), GLUT-2 (Table 6) and GK (Table 7). A volume of 1 µl cDNA of each sample was added individually to a capillary, followed by the addition of 9 µl of the respective master mixes. PBGD was prepared similarly to Pdx-1 and Pax 4, without any optimization, and worked well at the various annealing temperatures used for the different primer pairs.

2.6.1 Preparation of samples for LightCycler PCR

Capillaries were carefully placed in a LightCycler Centrifuge Adapter (Roche Diagnostics, Mannheim, Germany). A volume of 1 µl of each cDNA sample was added to a capillary, followed by 9 µl of master mix. Caps were placed and tightened on the capillaries. The mixture was then centrifuged (Eppendorf centrifuge 5414) briefly for about 1 s. The capillaries were then carefully placed in the LightCycler PCR machine, and the appropriate programme was run for each primer. LightCycler PCR was completed after 30 cycles in 40-45 min.

Table 5 Preparation of LightCycler master mixes for Pdx-1 and Pax 4 at an annealing temperature of 52^{0}C

Reagent	Volume/10µl reaction	Volume/600µl reaction
10X Reaction buffer	1 µl	60 µl
Taq Polymerase	0.08 µl	4.8 µl
dNTP (10mM)	0.2 µl	12 µl
BSA (20 mg/ml)	0.125 µl	7.5 µl
Primer Forward	0.5 µl	30 µl
Primer Reverse	0.5 µl	30 µl
Sybr Green (1:10 000)	0.33 µl	19.8 µl
cDNA	1 µl	60 µl
Water	6.265 µl	375.9 µl
Total	10 µl	600 µl

Table 6 Preparation of LightCycler master mix for GLUT-2 at annealing temperatures of 57^0C and 55^0C

Reagent	Volume/10µl reaction	Volume/600µl reaction
10X Reaction buffer	1 µl	60 µl
Taq Polymerase	0.08 µl	4.8 µl
dNTP (10mM)	0.2 µl	12 µl
BSA (20 mg/ml)	0.125 µl	7.5 µl
Primer Forward	1 µl	60 µl
Primer Reverse	1 µl	60 µl
Sybr Green (1:10 000)	0.33 µl	19.8 µl
$MgCl_2$	0.4 µl	24 µl
cDNA	1 µl	60 µl
Water	4.865 µl	291.9 µl
Total	10 µl	600 µl

Table 7 Preparation of LightCycler master mix for GK at annealing temperature of 57^{0}C

Reagent	Volume/10µl reaction	Volume/600µl reaction
10X Reaction buffer	1 µl	60 µl
Taq Polymerase	0.08 µl	4.8 µl
dNTP (10mM)	0.2 µl	12 µl
BSA (20 mg/ml)	0.125 µl	7.5 µl
Primer Forward	1 µl	60 µl
Primer Reverse	1 µl	60 µl
Sybr Green (1:10 000)	0.33 µl	19.8 µl
$MgCl_2$	0.4 µl	24 µl
cDNA	1 µl	60 µl
Water	5.865 µl	351.9 µl
Total	10 µl	600 µl

2.6.2 Generation of coefficient files

Standard curves for the house-keeping gene, PBGD, and for the target genes (Pdx-1, Pax 4, GLUT-2 and GK) were constructed using cDNA from weanling control tissue (Group 10), and the relative expression of the genes was quantified from the unknown samples using these standard curves. The ratio of target gene expression to reference PBGD gene was used to derive the relative changes in gene expression of the target genes in the different samples. Coefficient files were used to perform the ratio calculation (as the reference and target gene are amplified in the same PCR reaction).

Normal control weanling cDNA (Group 10) was used throughout as a calibrator for all of the LightCycler experiments. The concentration of the cDNA was determined using a spectrophotomer (Perkin Elmer Instruments, Lambda 25 UV/VIS, Wellesley, MA, USA), then dilutions of 1000 ng, 500 ng, 100 ng and 50 ng were done in duplicate for Pdx-1, Pax 4, GLUT-2 and GK, with PBGD also done in duplicate for each primer pair. The LightCycler PCR (Roche Diagnostics, Mannheim, Germany) was then done at the respective annealing temperatures for each primer set.

2.6.3 Analysis of gene expression (Measurement of mRNA expression)

After quantification of the genes by LightCycler PCR using RelQuant (Roche Diagnostics, Mannheim, Germany), the control measurements were subtracted and taken arbitrarily as 1. Any sample reading over 1 was taken as

overexpression, with readings under 1 representing underexpression of the particular gene.

Immunohistochemical analysis

2.7.1 Tissue preparation and sectioning

The pancreas was excised from neonate and weanling rats, placed in 4% paraformaldehyde overnight and processed in an automated tissue processor (Shandon Citadel 1000, Cheshire, UK) through ascending concentrations of ethanol from 70% - 100%, followed by xylene. The tissue was embedded in paraffin wax (Paraplast Plus, Monoject Scientific Inc., St Louis, MO, USA). Sections, 4 μm thick, were cut on a rotary microtome and mounted on slides coated with 3-aminopropyltriethoxysilane (APES). For histological examination, one slide per pup was placed in an oven at 60^0C for 30 min, dewaxed with xylene, and rehydrated through a descending series of ethanol. Sections stained with haematoxylin and eosin were examined for morphological changes.

2.7.2 Immunohistochemistry

Slides of serial sections, not stained for histology, were transfered to 50 mM Tris-buffered saline (TBS), pH 7.4, in a staining jar and were immunostained using avidin D-biotinylated horseradish H complex (Vectastain, Vector Laboratories Inc., Burlingame, CA, USA). Briefly, the sections were incubated as follows (Table 8):

(1) 0.228% periodic acid in dH_2O for 5 min,

(2) TBS for 10 minute wash,

(3) 1:20 diluted normal goat serum (NGS; MRC Delft animal facility, Cape Town, South Africa) for 20 min,

(4) primary antibody (Pdx-1, GLUT-2 or GK),

(5) TBS for 10 minute wash,

(6) rabbit biotinylated antibody (Vectastain) for 30 min,

(7) TBS for 10 minute wash,

(8) avidin D-biotinylated horseradish H complex (Vectastain) for 60 min.

The peroxidase marker was revealed by incubating the sections for 5 min in a 0.05% enzyme substrate solution of diaminobenzidine tetrachloride (DAB) (Sigma, St Louis, MO, USA) containing 0.01% hydrogen peroxide. The sections were counterstained in haematoxylin for 1 min. Method controls involved replacement of the primary antiserum with NGS.

The above protocol was standardized for GLUT-2 where 1:100 rabbit anti-rat GLUT-2 (WAK-Chemie, Bad Soden, Germany) was applied for 30 min as the primary antibody. For Pdx-1 (kindly donated by Prof C. Wright, Department of Cell Biology, Vanderbilt University Medical Center, Nashville, TN, USA) and GK (a kind gift from Dr H. Vertigan, CVGI Discovery Department, AstraZeneca, Cheshire, UK)) immunostaining, the protocol was repeated with an additional step of microwaving slides for 20 min and allowing them to cool to RT before applying NGS. Pdx-1 was diluted 1:1500, GK was diluted 1:1000 and DAB was added for only 2 min, instead of 5 min, for the GLUT-2 experiments.

Table 8 Immunohistochemistry protocol

Step	Protocol	Duration
1	Dewax slides	
2	50mM Tris buffer in staining jar	20 min
3	Microwave*	20 min
4	Cool at RT*	30 min
5	NGS 1/20 on slides in moisture chamber	20 min
6	Blot excess serum. Primary antiserum on slides in moisture chamber**	30 min or overnight**
7	Jet wash 50mM Tris: 50mM Tris buffer in staining jar	10 min
8	Make vectastain	
9	Biotinylated Ab on slides in staining jar	30 min
10	Jet wash Tris 50mM: 50mM Tris buffer in staining jar	10 min
11	Vectastain on slides in moisture chamber	60 min
12	Jet wash 50mM Tris: 50mM Tris buffer in staining jar	10 min
13	0.05% DAB + 0.01% H_2O_2 in 0.05M Tris on slides	2-5 min†
14	Jet wash tap water: tap water wash in staining jar	5 min
15	Counterstain and mount	

*Microwave and cooling down, steps 3 and 4, were only applicable for Pdx-1 and GK. These steps were omitted for GLUT-2.

**Pdx-1 and GK were incubated overnight at 4^0C; GLUT-2 was incubated for 30 min at RT.

†DAB was applied for 2 min for both Pdx-1 and GK and 5 min for GLUT-2.

2.8.1 Image analysis of pancreata immunostained for insulin (beta-cell) and glucagon (alpha-cell)

A Canon Powershot S40 digital camera (Canon, Tochigi, Japan) mounted on an Olympus BX60 light microscope (Olympus, Tokyo, Japan) attached to a personal computer was used to capture images. The camera focus, light intensity and image resolution were controlled remotely and the acquired images were transferred to the computer using Remote Capture Software from Canon. The final digitized images were all 768 X 1024 pixels. Using the X10 objective, images were acquired for alternate fields of view until the entire section was covered. For each field of view, the islets were captured using a X40 objective for nuclei counts. An islet was defined as a cluster of eight or more endocrine cells. Image Analysis was performed with the Leica Qwin Plus Software (Leica, Cambridge, UK).

Tissue parameters were measured using either the interactive option or by doing colour segmentation. Total tissue area was determined by adding the tissue measured in each field of view, using the interactive measurement option of the Leica Software. Total islet area, total alpha-cell area and total beta-cell area were determined by using colour segmentation and thresholding. The relative beta-cell volume was obtained by calculating the ratio between the area obtained by immunoreactive beta-cells and the area occupied by total islet cells. Beta-cell number was assessed by counting the number of beta-cell nuclei. Beta-cell size was calculated by dividing the measured beta-cell area by the number of beta-

cell nuclei counted and expressed in μm^2. The same procedure was used to estimate the alpha-cell volume, number and size. The ratio of beta-cell area to alpha-cell area (beta-cell:alpha-cell) was determined by dividing the total beta-cell area measured by the total alpha-cell area measured.

2.8.2 Image analysis of pancreata immunostained for Pdx-1, GLUT-2 and GK (Measurement of protein expression)

Immunolabelled sections were viewed on a Zeiss Axioskop2 light microscope (Zeiss, Jena, Germany) linked to a Zeiss Axiocam digital camera system (Zeiss, Jena, Germany). Captured micrographs of the islets of Langerhans were morphometrically analysed using the Zeiss KS 300 release 3.0 image analysis software (Carl Zeiss Vision, Hallbergmoos, Germany). Data was exported to MS Excel 97 (Microsoft Corp., Bellevue, WA, USA) and statistically analysed by a Statistica package vers 6.1 (Statsoft Inc., Tulsa, OK, USA). All of the pancreas sections were scanned for antibody activity (Pdx-1, GLUT-2 or GK). The levels of immunolabelled antibody (Pdx-1, GLUT-2 or GK) in the control (Group 5 neonates and Group 10 weanlings) were taken to equal 1, with immunoreactivity in the pups exposed to a maternal HFD expressed as a ratio of the control levels.

2.9 Statistical analysis

The data of each group were compared to the control data and reported as means ± SEM. Comparisons between the groups were analyzed using the one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons for significant tests. Significance was established at $P < 0.05$.

CHAPTER 3

RESULTS

Mothers

Pregnant dams were exposed to a HFD for either the first (Group 1), second (Group 2), or third (Group 3) week of gestation while a fourth group was fed a HFD throughout pregnancy (Group 4). The control group (Group 5) was maintained on a standard laboratory diet. Before the virgin female Wistar rats were mated, there were no differences in any of the parameters measured. This eliminated bias in the monitoring of each parameter at the various time points during gestation. Data for each of the parameters were pooled for mothers fed the same diet during the same period of gestation. This provided a larger n value for statistical purposes. For example, the two groups of dams which provided offspring for Group 1 and Group 6, respectively, were both fed a HFD for the first week of gestation only. The only difference between the two was that the offspring from Group 1 dams were euthanased at birth, and those from Group 6 dams were killed at weaning. However, this made no difference to data at birth which could therefore be pooled.

3.1 Maternal food intake

Maternal food consumption and body weight

Dams fed a HFD for any single week of gestation ingested more food in that week than in the weeks that they were maintained on a standard laboratory diet (Table 9). Group 2, Group 3 and Group 4 dams consumed significantly more food during the 21 days of gestation than the control dams.

Table 9 Maternal food consumption and body weight during pregnancy

	Control	Group 1	Group 2	Group 3	Group 4
Food intake from day 1 to day 7 of pregnancy, g	19.47 ± 0.38	28.26 ± 0.42 *	19.82 ± 0.85	17.79 ± 0.23	29.42 ± 0.91 *
Food intake from day 8 to day 14 of pregnancy, g	19.32 ± 0.69	17.54 ± 1.54	29.4 ± 1.19 *	21.16 ± 0.74	30.06 ± 0.96 *
Food intake from day 15 to day 21 of pregnancy, g	21.72 ± 0.89	21.62 ± 0.56	21.25 ± 1.07	30.91 ± 1.41 *	28.39 ± 0.99 *
Overall food intake throughout pregnancy, g	20.17 ± 0.45	22.47 ± 1.12	23.49 ± 1.10 *	23.29 ± 1.35 *	29.29 ± 0.55 *
Maternal weight before pregnancy, g	203.9 ± 3.46	210.6 ± 3.93	211.8 ± 3.61	197.7 ± 4.17	204.3 ± 4.91
Maternal weight on day 7 of pregnancy, g	218.7 ± 2.67	237.9 ± 4.49	225 ± 3.70	211.3 ± 4.69	241.4 ± 3.68 *
Maternal weight on day 14 of pregnancy, g	236.9 ± 2.42	248.2 ± 4.79	253 ± 5.82	228.1 ± 3.71	268.4 ± 4.44 *
Maternal weight on day of birth, g	238.3 ± 2.80	229.1 ± 7.81	236.6 ± 5.92	231 ± 9.69	259.9 ± 4.62 *

Data represent means ± SEM. Pregnant dams were fed a high fat (40% energy as fat) diet for either the first (Group 1), second (Group 2) or third (Group 3) week, or throughout (Group 4) gestation. Control mothers were maintained on a standard laboratory diet (10% fat). n = 8. *P < 0.05 versus control.

3.2 Maternal weight

There were no significant differences between the weights of virgin Wistar rats before they were mated (Table 9). At days 7 and 14 of gestation and after giving birth, Group 4 dams were significantly heavier than the control dams.

3.3 Maternal blood glucose concentrations

Blood glucose concentrations were significantly higher on day 7 of pregnancy in Group 1 and Group 4 dams (Table 10). Group 2 and Group 4 dams had significantly raised glycaemia on gestational day 14. After giving birth, Group 3 and Group 4 dams were hyperglycaemic.

3.4 Maternal serum insulin concentrations

No significant differences in the serum insulin concentrations were evident between experimental dams, fed a HFD during gestation, and the control dams (Table 10).

Table 10 Maternal circulating glucose and insulin concentrations

	Control	Group 1	Group 2	Group 3	Group 4
Blood glucose concentrations before pregnancy, mmol/l	5.86 ± 0.38	6.76 ± 0.60	6.16 ± 0.21	6.18 ± 0.42	6.36 ± 0.23
Blood glucose concentrations on day 7 of pregnancy, mmol/l	6.61 ± 0.41	6.86 ± 0.42 *	6.15 ± 0.18	7.1 ± 0.43	7.04 ± 0.32 *
Blood glucose concentrations on day 14 of pregnancy, mmol/l	5.51 ± 0.29	5.07 ± 0.19	5.81 ± 0.15 *	5.41 ± 0.27	5.55 ± 0.15 *
Blood glucose concentrations on day of giving birth, mmol/l	5.91 ± 0.28	6.57 ± 0.27	5.76 ± 0.212	6.9 ± 0.31 *	6.74 ± 0.21 *
Serum insulin concentrations before pregnancy, pM	126.6 ± 25.34	74.56 ± 20.26	135.9 ± 68.01	67.15 ± 9.06	176.5 ± 82.16
Serum insulin concentrations on day 7 of pregnancy, pM	173.7 ± 78.96	137.5 ± 36.5	55.05 ± 15.89	152 ± 40.84	217.7 ± 32.58
Serum insulin concentrations on day 14 of pregnancy, pM	166.4 ± 60.83	91.84 ± 21.25	229.1 ± 57.69	97.13 ± 17.42	215 ± 29.89
Serum insulin concentrations on day of giving birth, pM	152.1 ± 40.82	178.8 ± 54.41	81.78 ± 23.1	169.7 ± 42.2	155.9 ± 25.62

Data represent means ± SEM. Pregnant dams were fed a high fat (40% energy as fat) diet for either the first (Group 1), second (Group 2) or third (Group 3) week, or throughout (Group 4) gestation. Control mothers were maintained on a standard laboratory diet (10% fat). n = 8. *P < 0.05 versus control.

3.5 Optimization of GLUT-2 PCR conditions

A titration of different concentrations of $MgCl_2$ for GLUT-2 was performed and the products revealed by gel electrophoresis (Figs. 3a and b). A concentration of 1.5 mM $MgCl_2$ was in the standard PCR mix. The addition of an extra 1.0 mM $MgCl_2$, to give a final concentration of 2.5mM $MgCl_2$ was found to be optimal for RT-PCR experiments with GLUT-2.

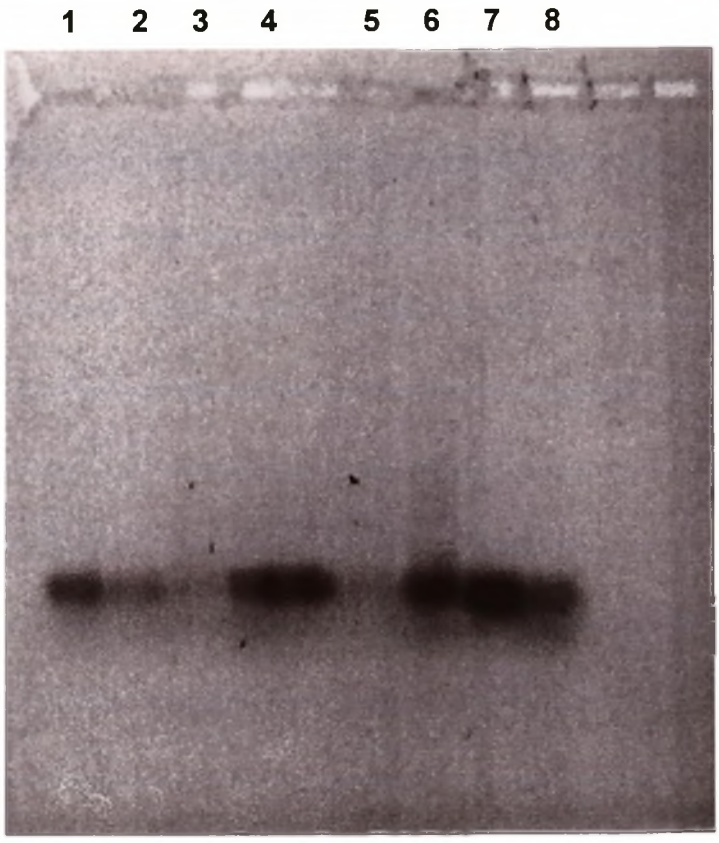


Fig. 3a Agarose (1%) gel electrophoresis for PCR optimization of GLUT-2 using 0 mM $MgCl_2$ and 0.5 mM $MgCl_2$

Lanes 1-4: Four random rat pancreata cDNA samples were loaded without $MgCl_2$.

Lanes 5-8: The same samples loaded with an additional 0.5 mM $MgCl_2$.

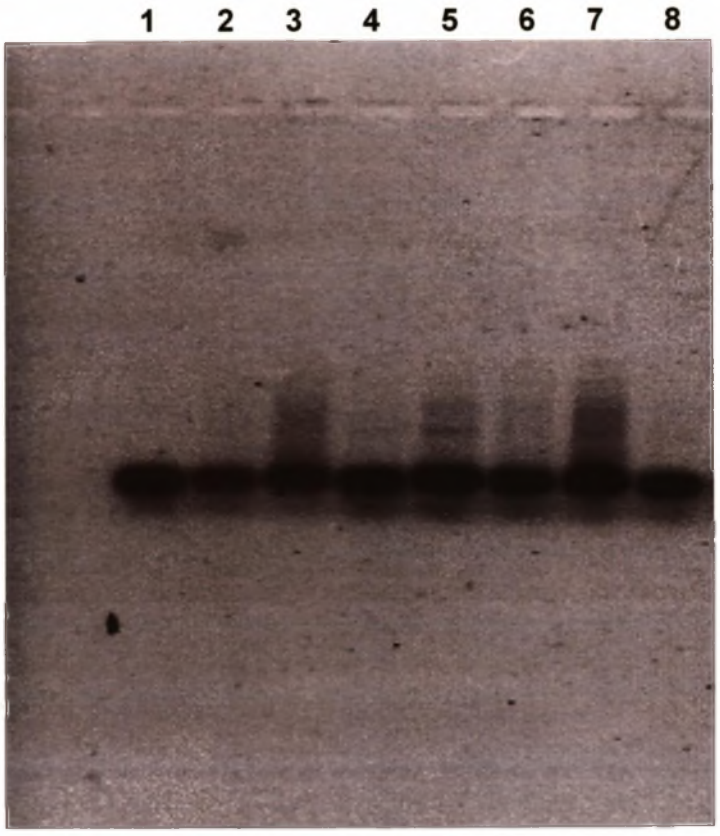


Fig. 3b Agarose (1%) gel electrophoresis for PCR optimization of GLUT-2 using 1.0 mM $MgCl_2$ and 1.5 mM $MgCl_2$

Lanes 1-4: Four random rat pancreata cDNA samples were loaded with and additional 1.0 $MgCl_2$.

Lanes 5-8: The same samples loaded with an additional 1.5 mM $MgCl_2$.

Neonates

3.6 Birth weights

Group 1 neonates had significantly decreased birth weights compared to the control (Fig. 4). The birth weights of neonates from the other experimental groups did not differ significantly from the control.

3.7 Plasma glucose concentrations in neonatal rats

Glucose concentrations were significantly lower in Group 1 and Group 2 neonates compared to the control (Fig. 5). Group 4 neonates had significantly high glucose concentrations. No significant differences in glucose concentrations were found in Group 3 neonates.

3.8 Serum insulin concentrations in neonatal rats

No significant differences were found in the insulin concentrations in any of the neonates (Fig. 6).

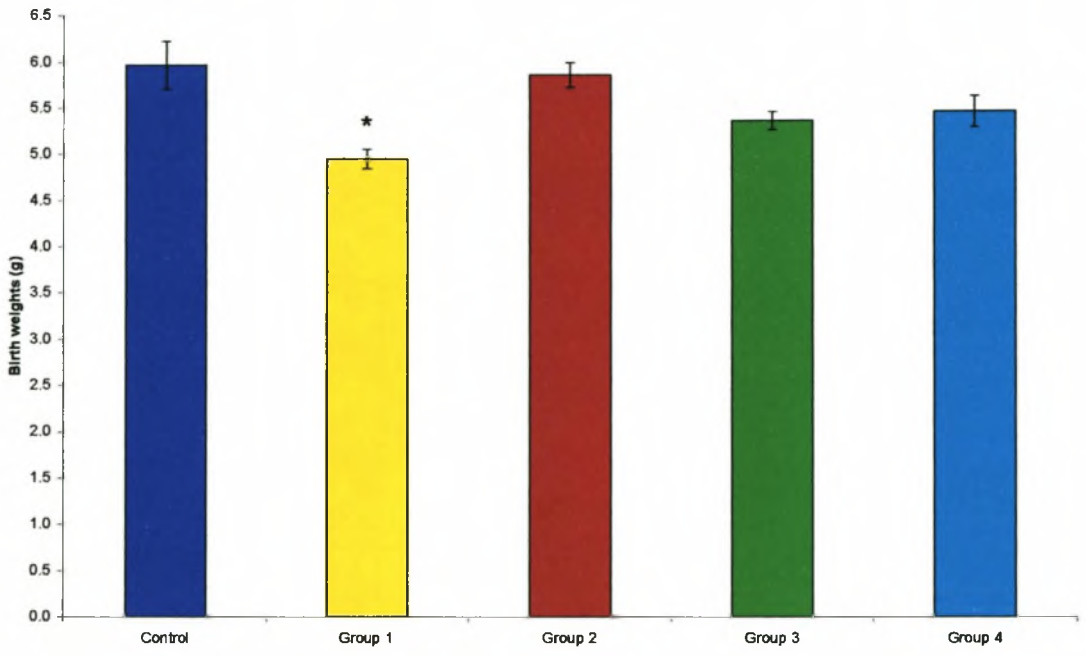


Fig. 4 Birth weights of neonatal rats

Birth weights of neonates exposed to an *in utero* HFD. Data represent mean ± SEM. n = 6. *P < 0.05.

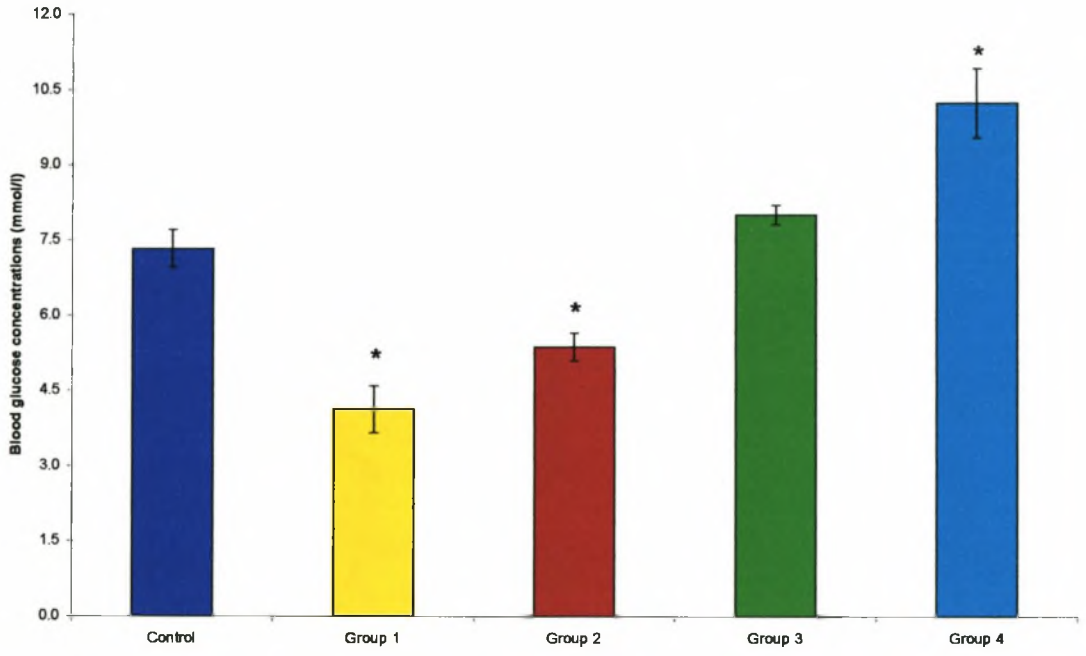


Fig. 5 Blood glucose concentrations in neonatal rats

Circulating glucose concentrations in neonates exposed to an *in utero* HFD. Data represent mean ± SEM. n = 6. *P < 0.05.

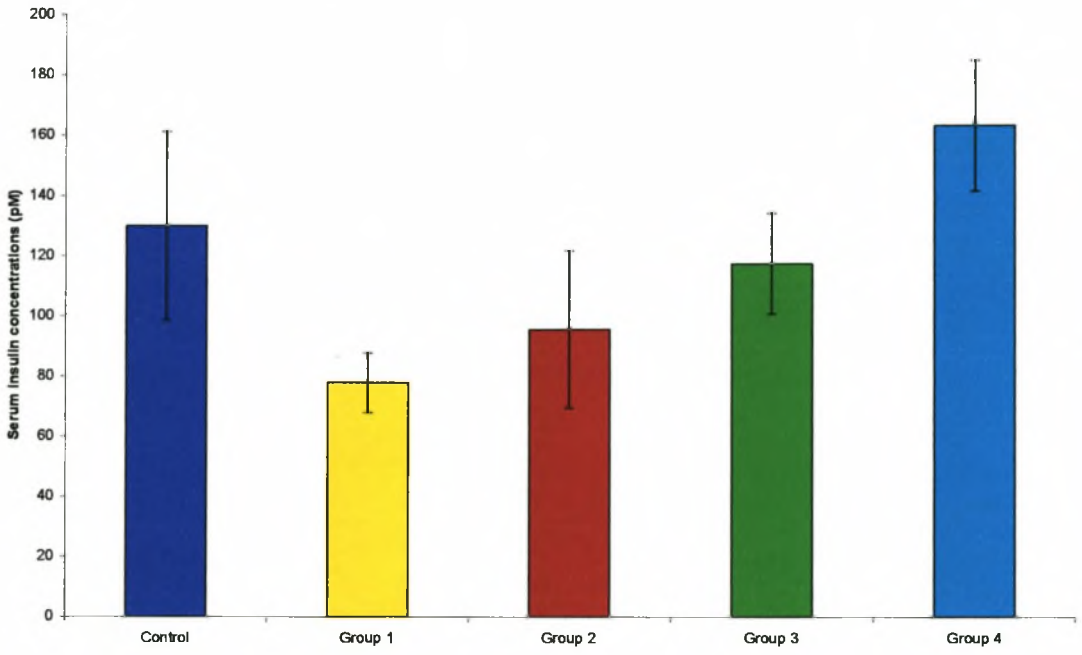


Fig. 6 Serum insulin concentrations in neonatal rats

Circulating insulin concentrations in neonates exposed to an *in utero* HFD. Data represent mean ± SEM. n = 6.

3.9 Effects of an *in utero* HFD on beta-cell development in neonatal rats

Group 4 neonates had significantly reduced beta-cell volumes (Fig. 7a). However, no significant differences were found in the beta-cell volumes of neonates exposed to a maternal HFD for any single week of gestation. Beta-cell number declined significantly in Group 1, Group 3 and Group 4 neonates (Fig. 7b). No significant differences were found in the beta-cell sizes in any of the neonates (Fig. 7c).

3.10 Effects of an *in utero* HFD on alpha-cell development in neonatal rats

Group 4 neonates had a significantly greater alpha-cell volume (Fig. 8a). There were no significant differences in the alpha-cell volume of neonates exposed to a maternal HFD for a single week of gestation. The alpha-cell number was greater in Group 4 neonates (Fig. 8b). Alpha-cell size increased in Group 1, Group 3 and Group 4 neonates (Fig. 8c).

3.11 The effect of an *in utero* HFD on the beta-cell:alpha-cell ratio in neonatal rats

No significant differences were found in the beta-cell:alpha-cell ratio (Fig. 9).

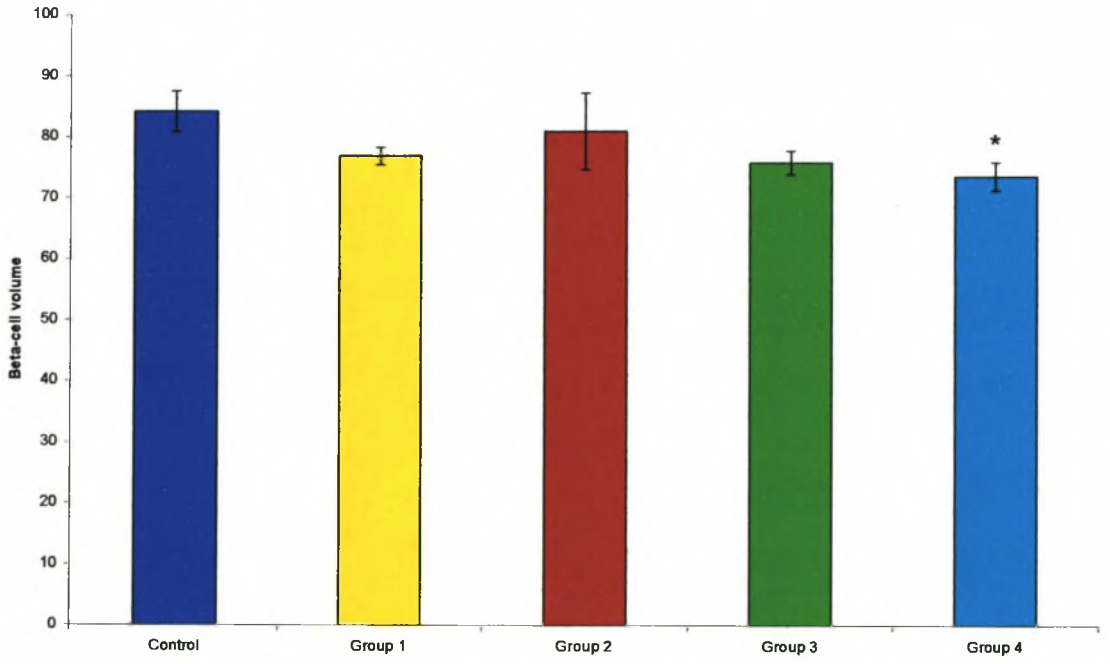


Fig. 7a The effects of exposure to an *in utero* high fat diet on beta-cell volume in neonatal rats

Beta-cell volume in neonates exposed to a maternal HFD. Data represent mean ± SEM. n = 6. *P < 0.05.

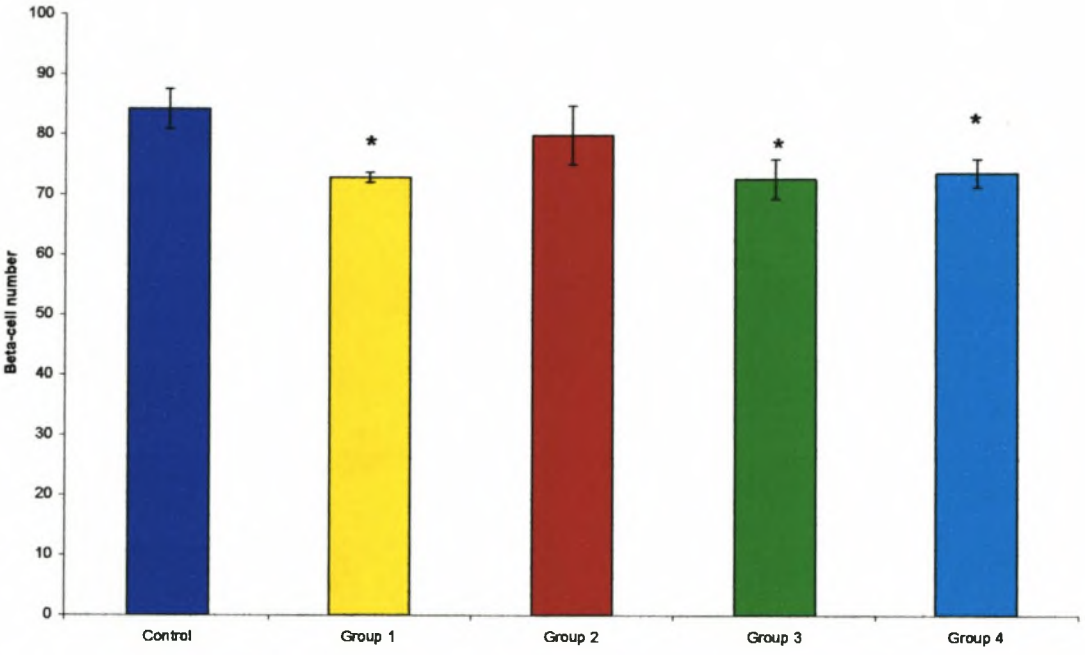


Fig. 7b The effects of exposure to an *in utero* high fat diet on beta-cell number in neonatal rats

Beta-cell number in neonates exposed to an *in utero* HFD.

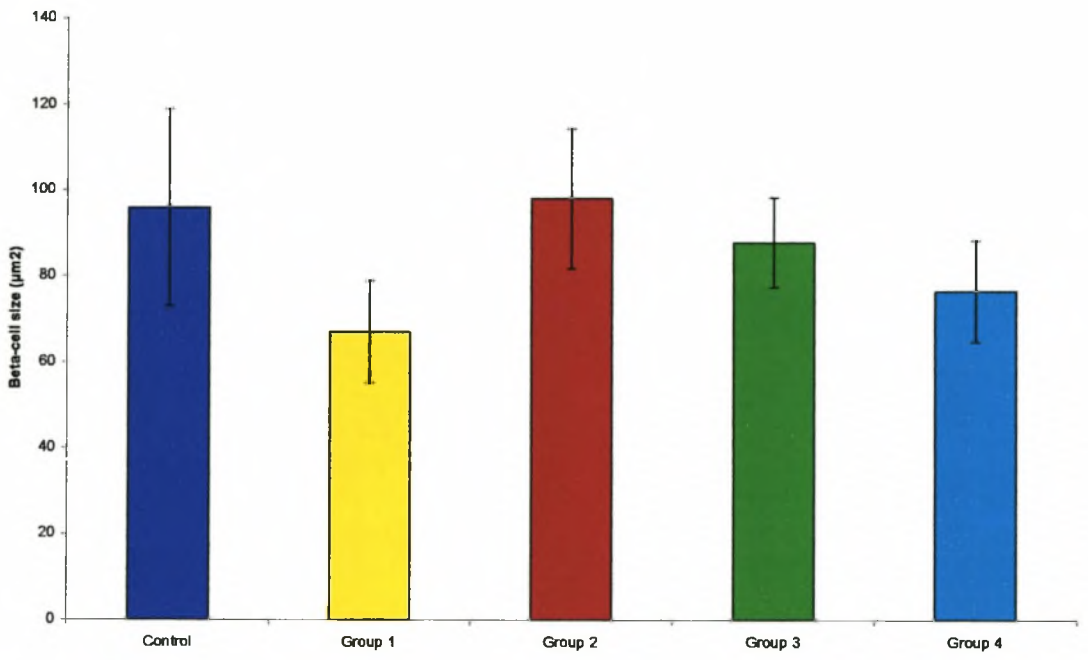


Fig. 7c The effects of exposure to an *in utero* high fat diet on beta-cell size in neonatal rats

Beta-cell size in neonates exposed to an *in utero* HFD.

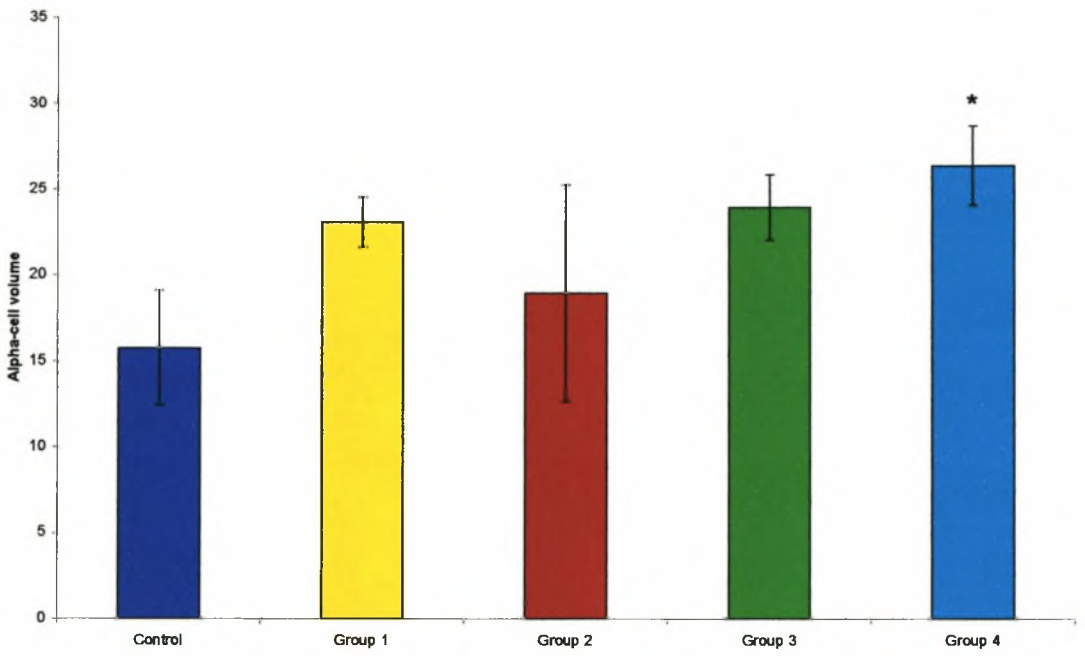


Fig. 8a The effects of exposure to an *in utero* high fat diet on alpha-cell volume in neonatal rats

Alpha-cell volume in neonates exposed to an *in utero* HFD. Data represent mean ± SEM. n = 6. *P < 0.05.

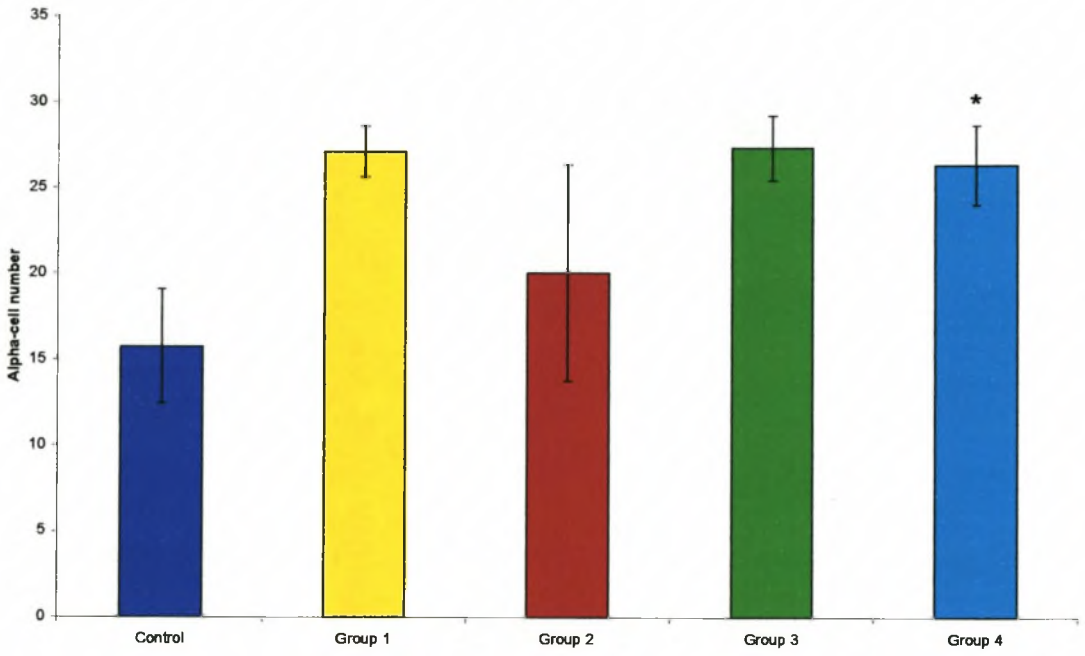


Fig. 8b The effects of exposure to an *in utero* high fat diet on alpha-cell number in neonatal rats

Alpha-cell number in neonates exposed to an *in utero* HFD.

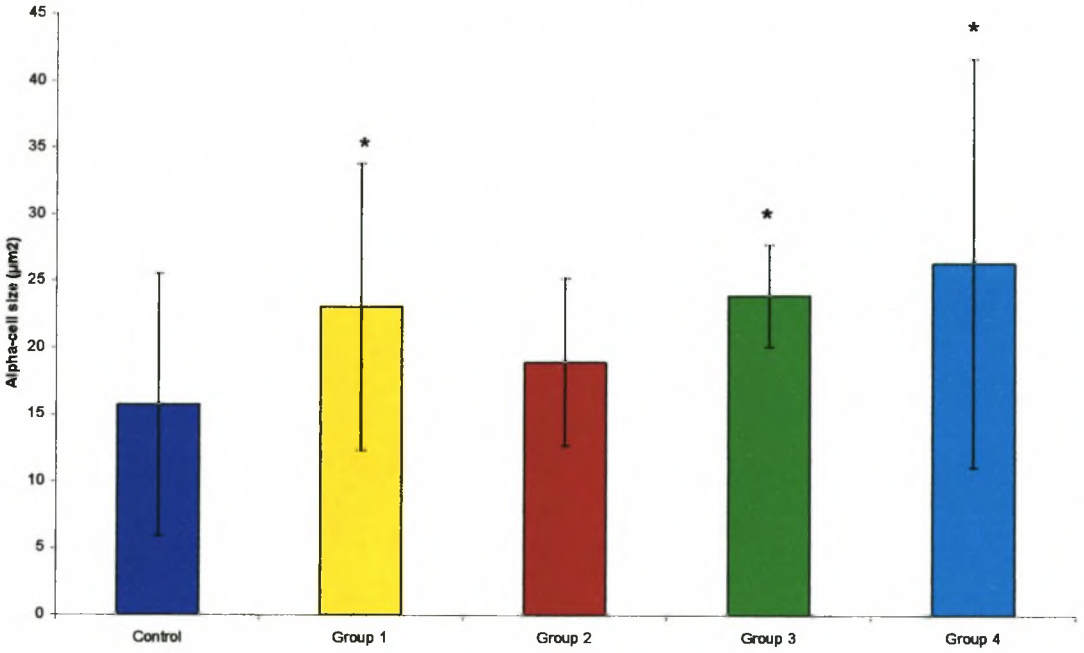


Fig. 8c The effects of exposure to an *in utero* high fat diet on alpha-cell size in neonatal rats

Alpha-cell size in neonates exposed to an *in utero* HFD.

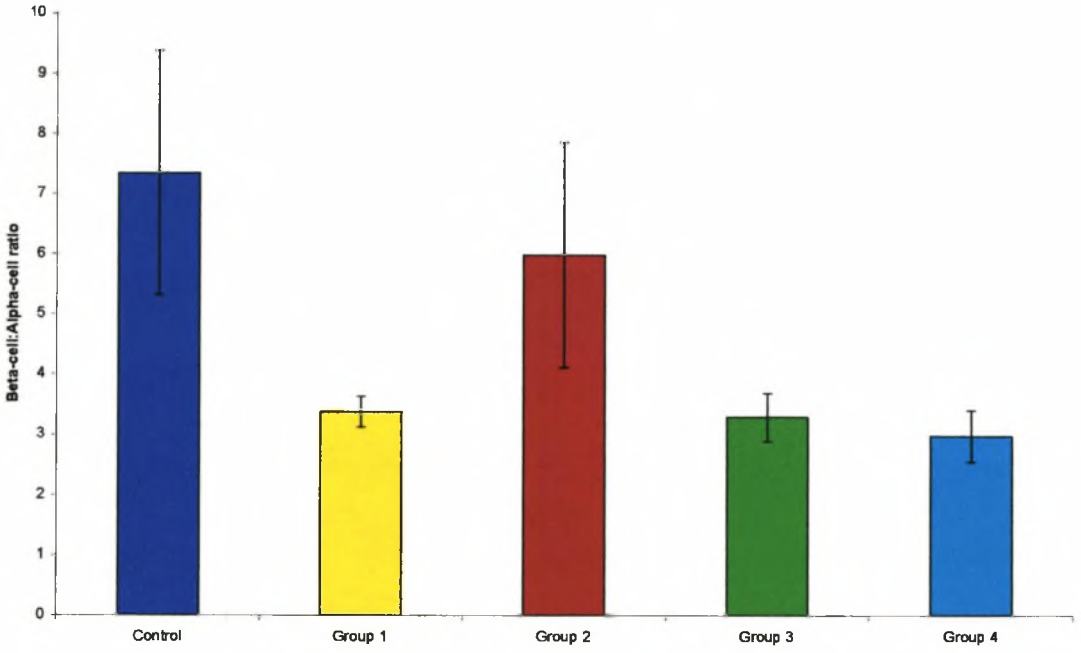


Fig. 9 The effects of exposure to an *in utero* HFD on the beta-cell:alpha-cell ratio in neonatal rats

Beta-cell:alpha-cell ratio in neonates exposed to an *in utero* HFD. Data represent mean ± SEM. n = 6.

3.12 The effect of a HFD on Pdx-1 expression in neonatal rats

3.12.1 Pdx-1 mRNA expression in neonatal rats

Relative quantitative PCR showed no significant differences in the mRNA expression profiles for Pdx-1 in any of the experimental neonatal groups (Fig. 10). There was, however, an almost significant increase ($p = 0.057$) in Pdx-1 mRNA expression levels in Group 4 neonates compared to the control.

3.12.2 Pdx-1 immunoreactivity in neonatal rats

Light microscopy revealed Pdx-1 immunostaining to be intense and nuclear in the beta-cells of all of the neonatal sections (Fig. 11). Several of these beta-cells were scattered throughout the islets. Significant reduction in immunoreactivity for Pdx-1 was only observed in Group 2 and Group 4 neonates (Fig. 12). HFD exposure for the first of final week of gestation seemed to have no effect on immunoreactivity for Pdx-1 in neonatal rats.

3.13 The effect of a HFD on Pax 4 expression in neonatal rats

3.13.1 Pax 4 mRNA expression in neonatal rats

There were no significant differences evident in the mRNA expression levels of Pax 4 in any of the neonatal groups (Fig. 13). Although levels of Pax 4 mRNA were much higher than the control levels in Group 1 neonates, there was no significance because of the high degree of variance.

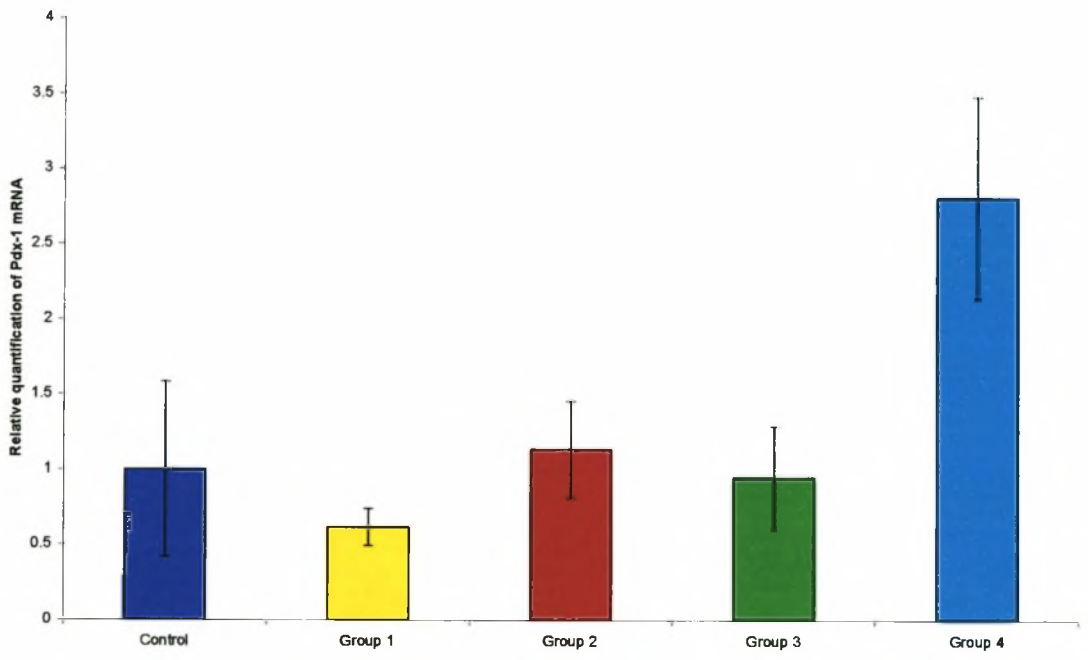
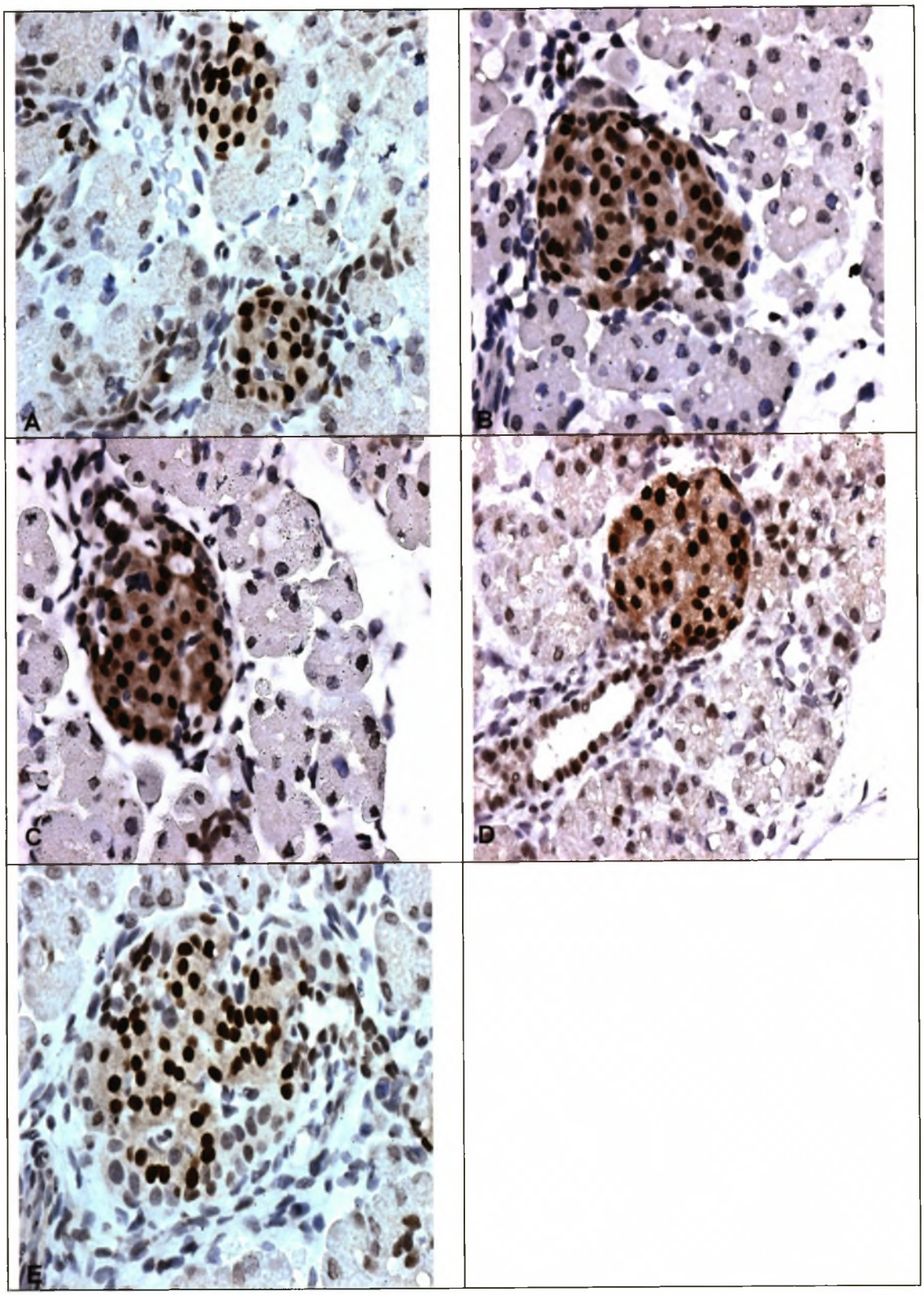


Fig. 10 Relative quantification of Pdx-1 mRNA in neonatal rats exposed to an *in utero* high fat diet

Pdx-1 mRNA expression in neonates exposed to an *in utero* HFD. Results for LightCycler PCR were normalized for Pdx-1 equalling 1. Data represent mean ± SEM. n = 10.

Fig. 11 Immunohistochemical staining for Pdx-1 in the pancreata of neonatal rats exposed to a maternal high fat diet during specific periods of gestation

Pdx-1 immunolabelling in neonates exposed to a maternal HFD for the first (A; Group 1), second (B; Group 2) or third (C; Group 3) week, or throughout gestation (D; Group 4). The control represents neonates exposed to a standard laboratory diet through gestation (E; Control). X400.



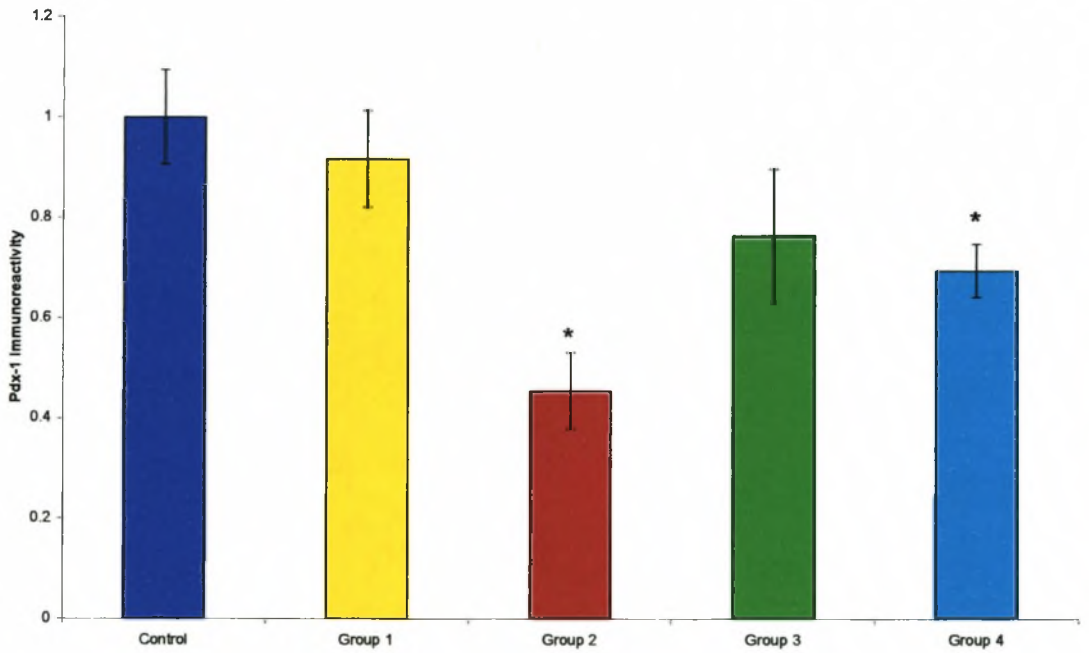


Fig. 12 Assessment of Pdx-1 immunolabelling by image analysis in neonatal rat pancreata exposed to an *in utero* high fat diet

Pdx-1 immunoreactivity in neonates exposed to an *in utero* HFD. The levels of immunolabelled Pdx-1 in the control neonates were taken to equal 1, with immunoreactivity in neonates exposed to an *in utero* HFD expressed as a ratio of the control levels. Data represent mean ± SEM. n = 6. *P < 0.05.

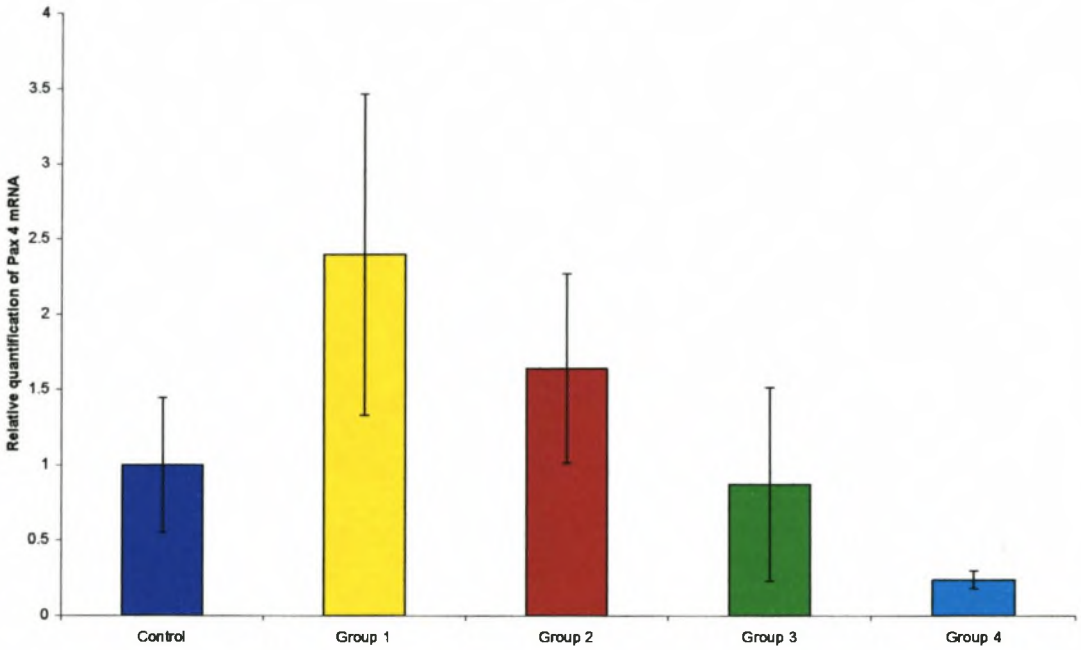


Fig. 13 Relative quantification of Pax 4 mRNA in neonatal rats exposed to an *in utero* high fat diet

Pax 4 mRNA expression in neonatal rats exposed to a maternal HFD. Results for LightCycler PCR were normalized for Pax 4 equalling 1. Data represent mean ± SEM. n = 10.

3.14 The effect of a HFD on GLUT-2 expression in neonatal rats

3.14.1 GLUT-2 mRNA expression in neonatal rats

There were no significant differences in the GLUT-2 mRNA levels in any of the experimental groups (Fig. 14), with the lowest levels detected in Group 2 neonates.

3.14.2 GLUT-2 immunoreactivity in neonatal rats

Compared with the normal dense GLUT-2 immunostaining found in the beta-cell plasma membrane of control (Group 5) neonates (Fig. 15E), there was reduced intensity of GLUT-2 immunolabelling in neonates exposed to a HFD during each week of gestation (Fig 15A-C), with the lowest intensity in those exposed to a HFD throughout gestation (Fig. 15D). There were no significant differences in the GLUT-2 immunoreactive levels in any of the neonates (Fig. 16).

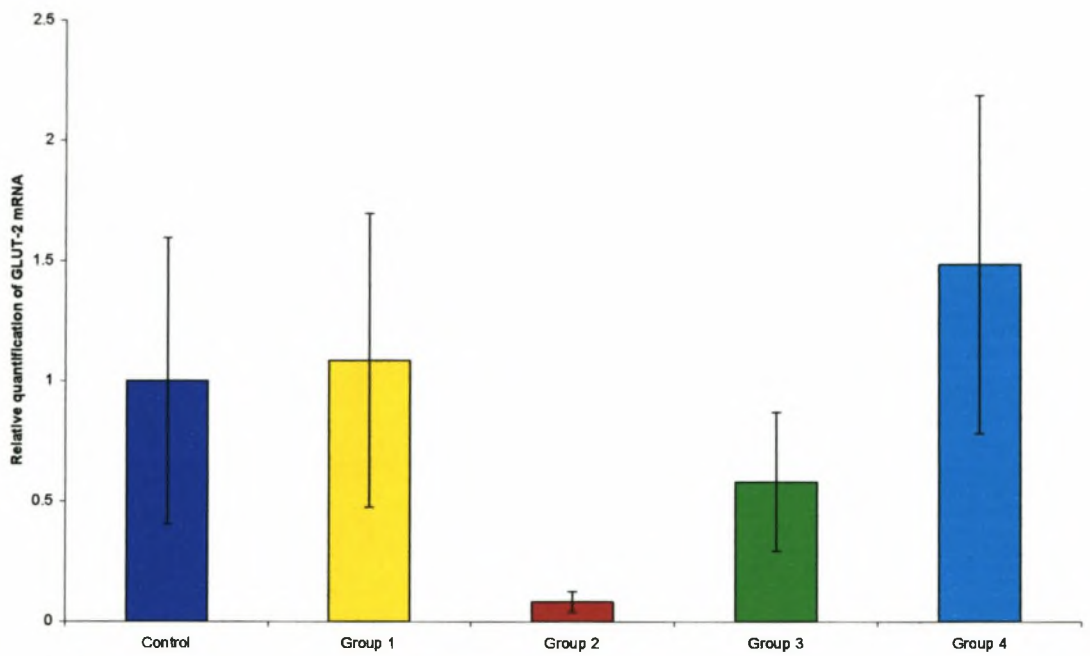
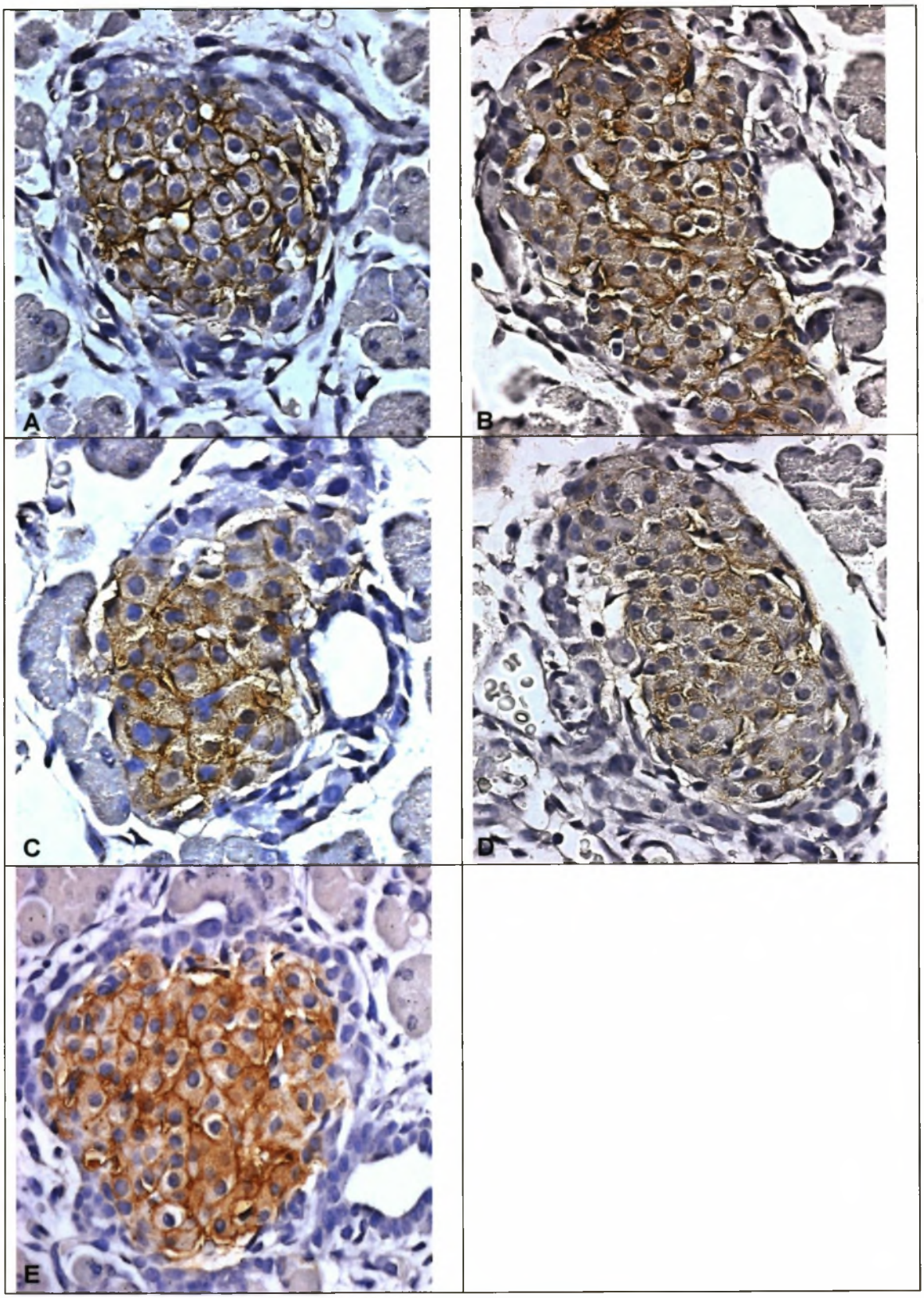


Fig. 14 Relative quantification of GLUT-2 mRNA in neonatal rats exposed to an *in utero* high fat diet

GLUT-2 mRNA expression in neonates exposed to an *in utero* HFD. Results for LightCycler PCR were normalized for GLUT-2 equalling 1. Data represent mean ± SEM. n = 10.

Fig. 15 Immunohistochemical staining for GLUT-2 in the pancreata of neonatal rats exposed to a maternal high fat diet during specific periods of gestation

GLUT-2 mmunolabelling in neonates exposed to a maternal HFD for the first (A; Group 1), second (B; Group 2) or third (C; Group 3) week, or throughout gestation (D; Group 4). The control represents neonates exposed to a standard laboratory diet through gestation (E). X400.



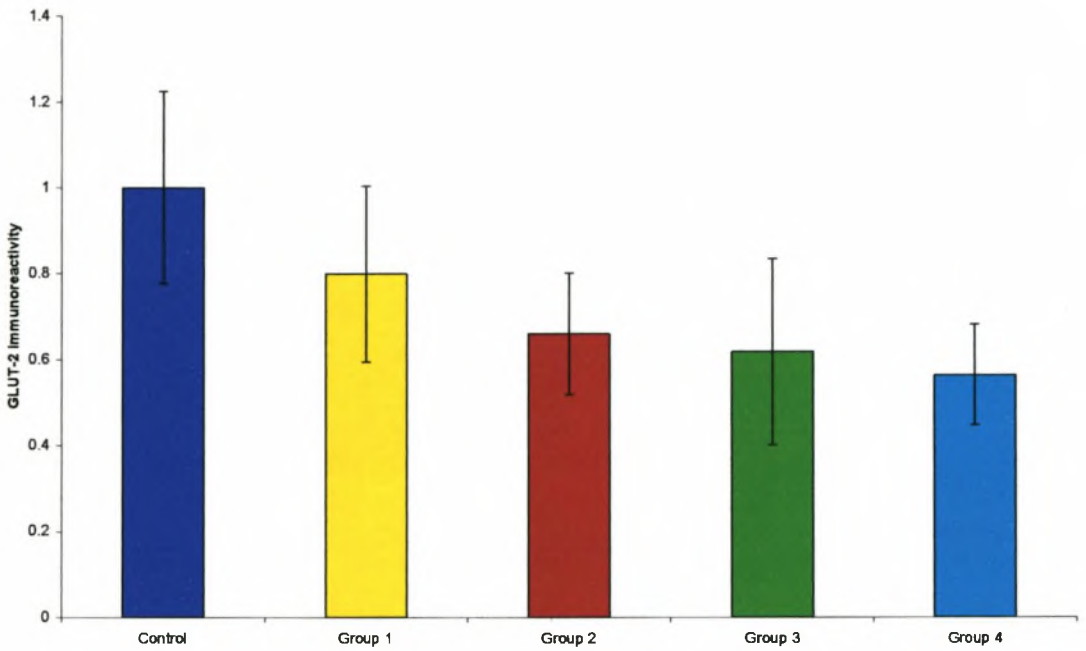


Fig. 16 Assessment of GLUT-2 immunolabelling by image analysis in neonatal rat pancreata exposed to an *in utero* high fat diet

GLUT-2 immunoreactivity in neonates exposed to an *in utero* HFD. The levels of immunolabelled GLUT-2 in the control neonates were taken to equal 1, with immunoreactivity in neonates exposed to an *in utero* HFD expressed as a ratio of the control levels. Data represent mean ± SEM. n = 6.

3.15 The effect of a HFD on GK expression in neonatal rats

3.15.1 GK mRNA expression in neonatal rats

Group 1 neonates displayed significant underexpression of GK at mRNA (Fig. 17). GK mRNA was maintained at normal expression levels in the other experimental groups.

3.15.2 GK immunoreactivity in neonatal rats

Only a few specific beta-cells showed immunoreactivity for GK (Fig. 18). These immunolabelled cells appeared to be mainly on the periphery of the islet. By means of image analysis, we found that Group 1 neonates had significantly increased immunoreactivity for GK (Fig. 19). However, Group 4 neonates had significantly reduced immunolabelling for GK. The remaining experimental groups (Group 2 and Group 3 neonates) had normal immunoreactivity for GK.

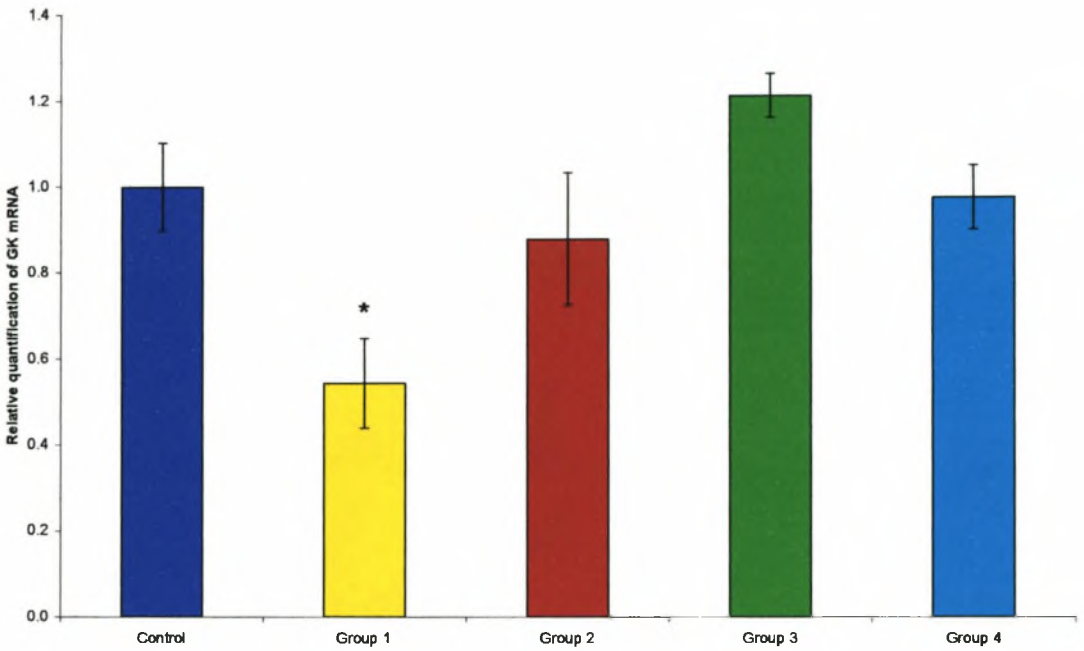
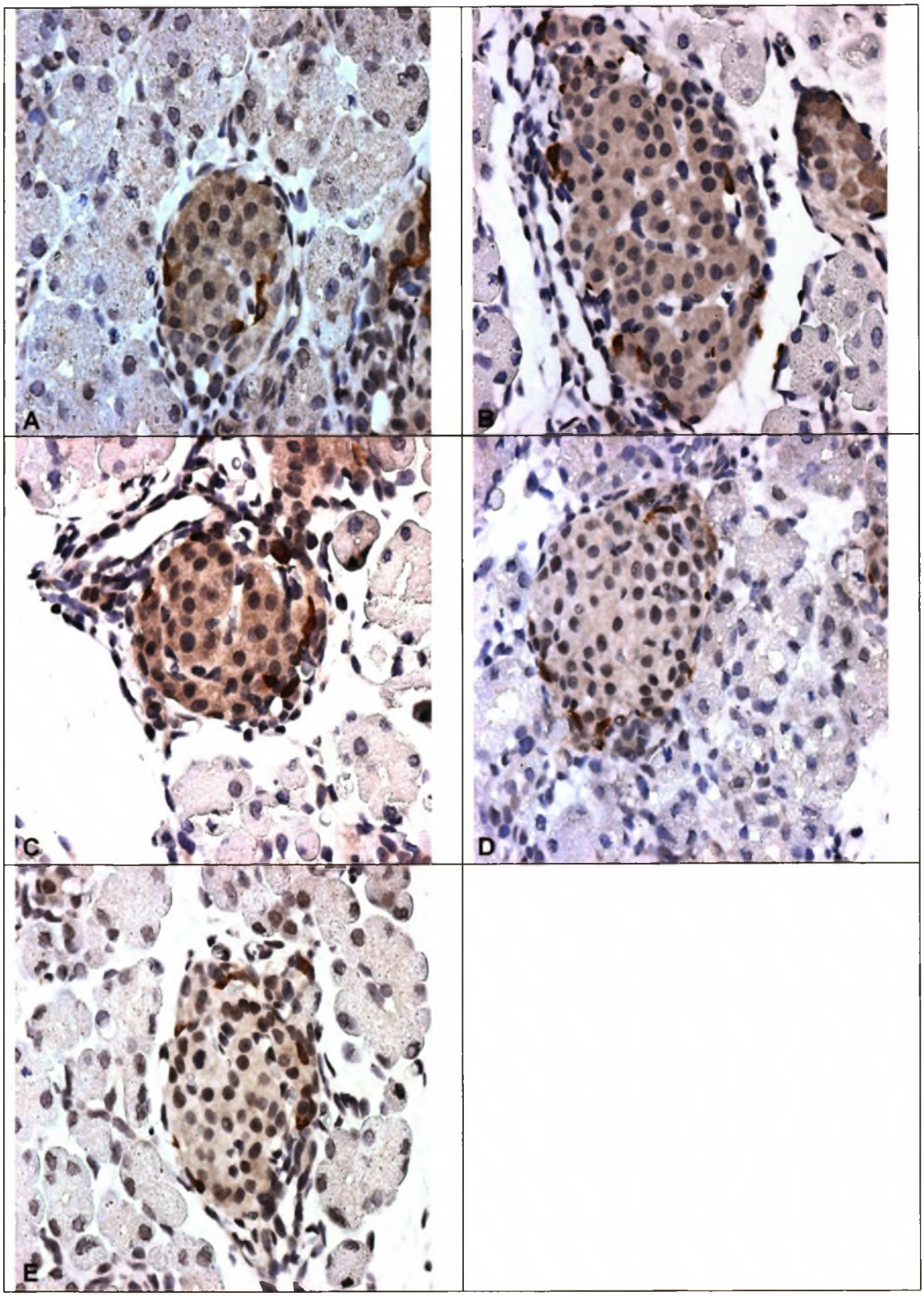


Fig. 17 Relative quantification of GK mRNA in neonatal rats exposed to an *in utero* high fat diet

GK mRNA expression in neonates exposed to a maternal HFD. Results for LightCycler PCR were normalized for GK equalling 1. Data represent mean ± SEM. n = 10. *P < 0.05.

Fig. 18 Immunohistochemical staining for GK in the pancreata of neonatal rats exposed to a maternal high fat diet during specific periods of gestation

GK immunolabelling in neonates exposed to a maternal HFD for the first (A; Group 1), second (B; Group 2) or (C; Group 3) third week or throughout gestation (D; Group 4). The control represents neonates exposed to a standard laboratory diet through gestation (E; Control). X400.



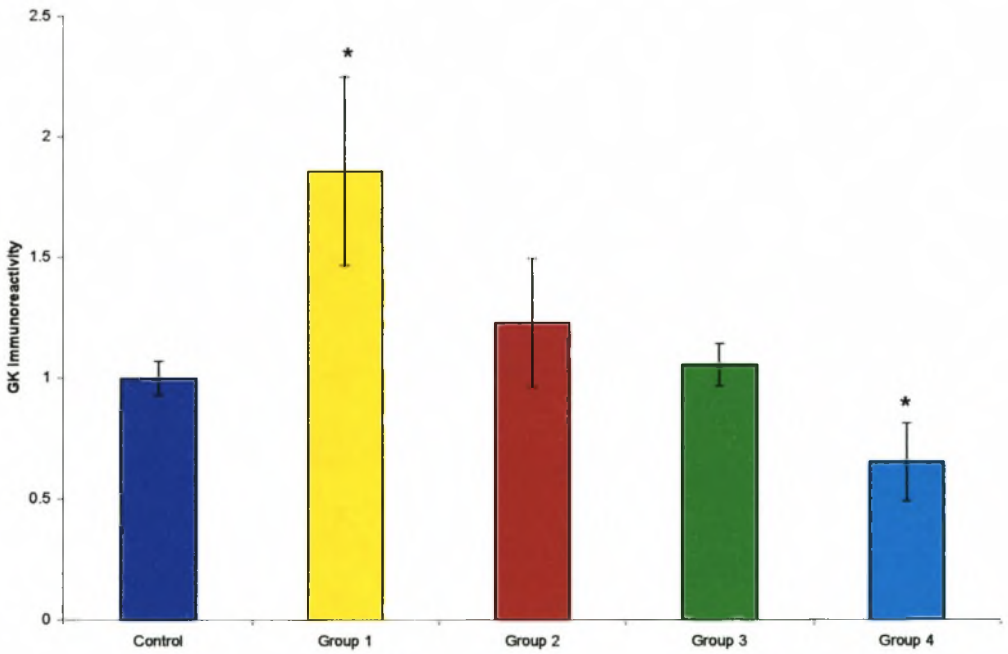


Fig. 19 Assessment of GK immunolabelling by image analysis in neonatal rat pancreata exposed to an *in utero* high fat diet

GK immunoreactivity in neonates exposed to an *in utero* HFD. The levels of immunolabelled GK in the control neonates were taken to equal 1, with immunoreactivity in neonates exposed to an *in utero* HFD expressed as a ratio of the control levels. Data represent mean ± SEM. n = 6. *P < 0.05.

Weanlings

3.16 Weights of 3-week-old weanlings

Group 6, Group 7, Group 8 and Group 9 weanlings were leaner, while Group 14 weanlings were heavier at 3 weeks of age (Fig. 20). No differences were detected in the body weights of Group 11, Group 12, Group 13 and Group 15 weanlings.

3.17 Blood glucose concentrations in weanling rats

Apart from Group 9 and Group 14 weanlings, hyperglycaemia was found in all of weanlings used in this study (Fig. 21).

3.18 Serum insulin concentrations in weanling rats

Hypoinsulinaemia was evident in weanlings exposed to a maternal HFD only during specific periods of gestation (Groups 6-9), all displaying similar circulating levels of insulin (Fig. 22). Furthermore, Group 11 and Group 14 weanlings were also hypoinsulinaemic.

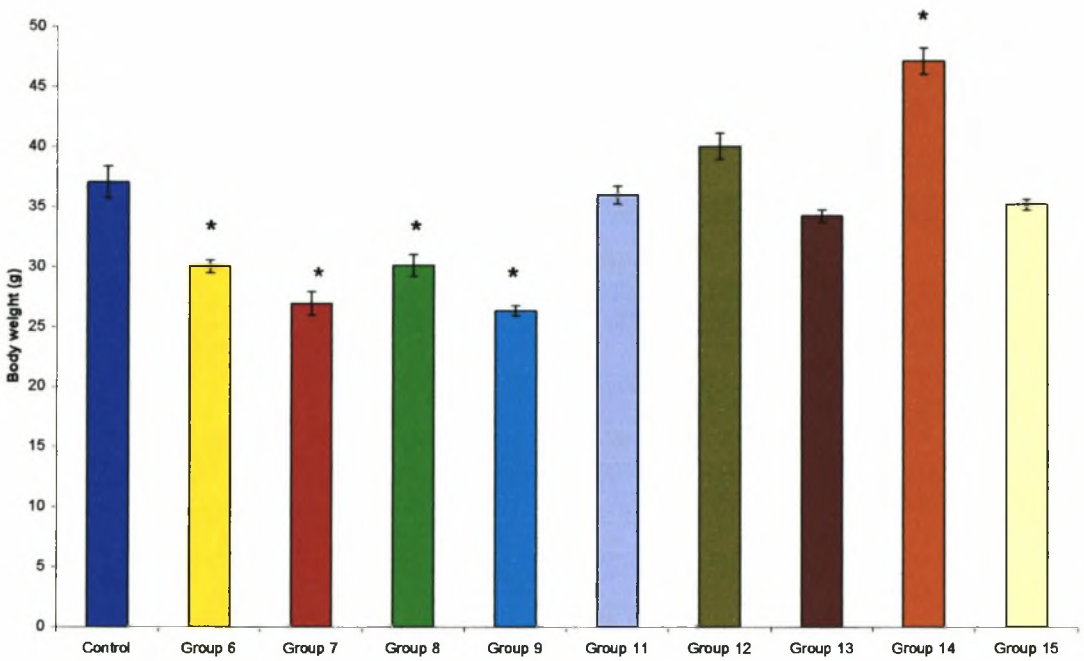


Fig. 20 Body weight at 3 weeks in weanling rats

Body weights in weanlings exposed to a maternal HFD during gestation and/or lactation. Data represent mean ± SEM. n = 6. *P < 0.05.

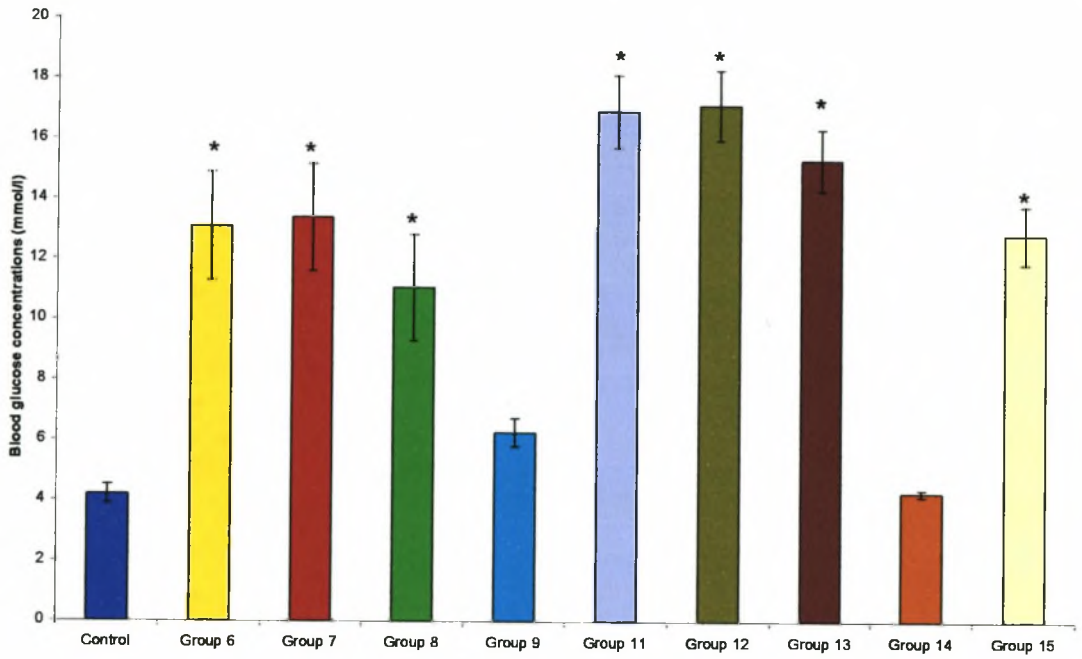


Fig. 21 Blood glucose concentrations in weanling rats

Circulating glucose concentrations in weanlings exposed to a maternal HFD during gestation and/or lactation. Data represent mean ± SEM. n = 6. *P < 0.05.

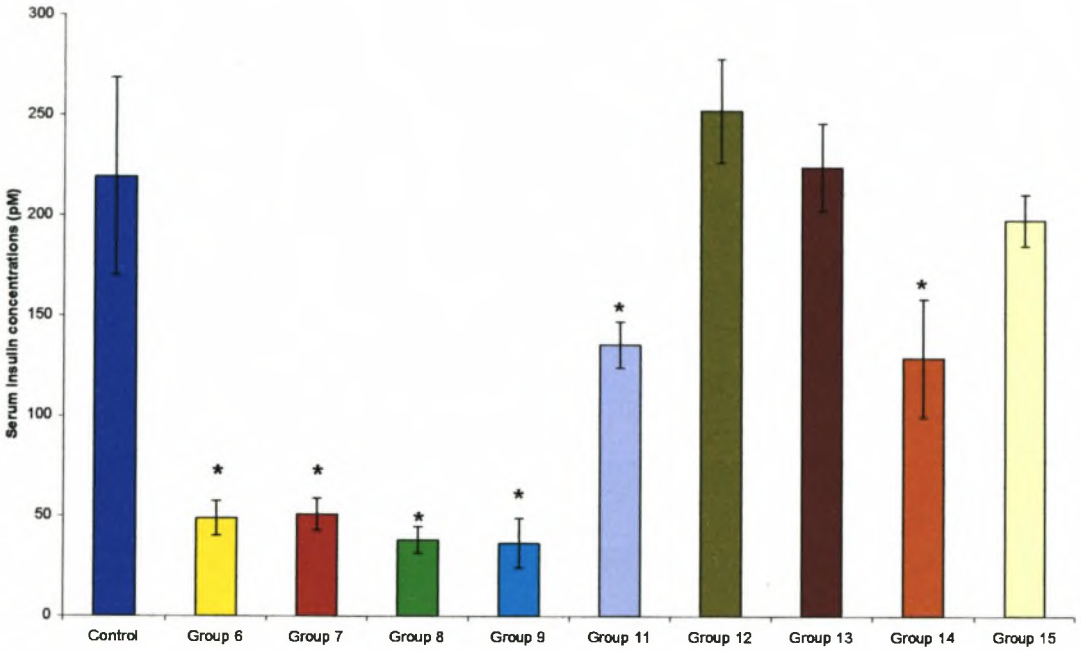


Fig. 22 Serum insulin concentrations in weanling rats

Circulating insulin concentrations in weanlings exposed to a maternal HFD during gestation and/or lactation. Data represent mean ± SEM. n = 6. *P < 0.05.

3.19 Effects of an *in utero* HFD on beta-cell volume, number and size in weanling rats

Data was only available for weanlings exposed to a maternal HFD during defined periods of gestation, followed by a standard laboratory diet for the entire duration of lactation (Groups 6-9). No significant differences were found in the beta-cell volume (Fig. 23a). However, in Group 6, Group 7 and Group 8 weanlings, both beta-cell number (Fig. 23b) and size (Fig. 23c) were reduced. No significant differences in either the beta-cell number or size were detected in Group 9 weanlings.

3.20 Effects of an *in utero* HFD on alpha-cell volume, number and size in weanling rats

No significant differences in alpha-cell volume (Fig. 24a) or number (Fig. 24b) were detected in any of the weanling groups. However, alpha-cells were significantly smaller in Group 6, Group 7 and Group 8 weanlings (Fig. 24c). No differences in alpha-cell size were detected in Group 9 weanlings.

3.21 Effects of an *in utero* HFD on the beta-cell:alpha-cell ratio in weanling rats

The beta-cell:alpha-cell ratio did not change in any of the weanlings exposed to a HFD during of gestation (Fig. 25).

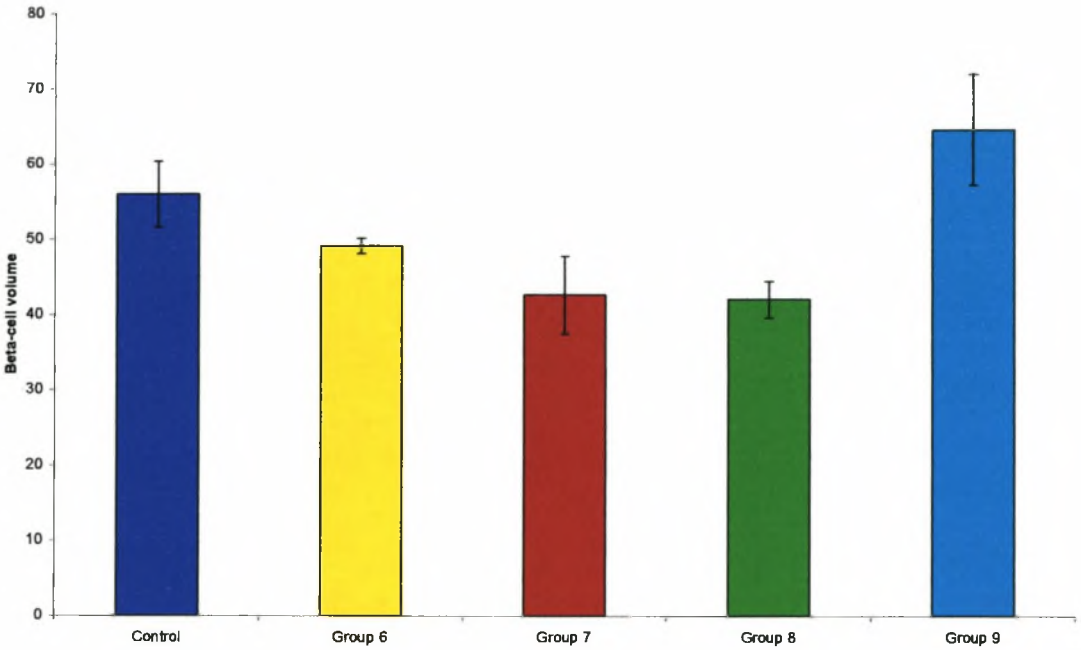


Fig. 23a The effects of exposure to a maternal HFD on beta-cell volume in weanling rats

Beta-cell volume in weanling rats exposed to *in utero* HFD. Data represent mean ± SEM. n = 6. *P < 0.05.

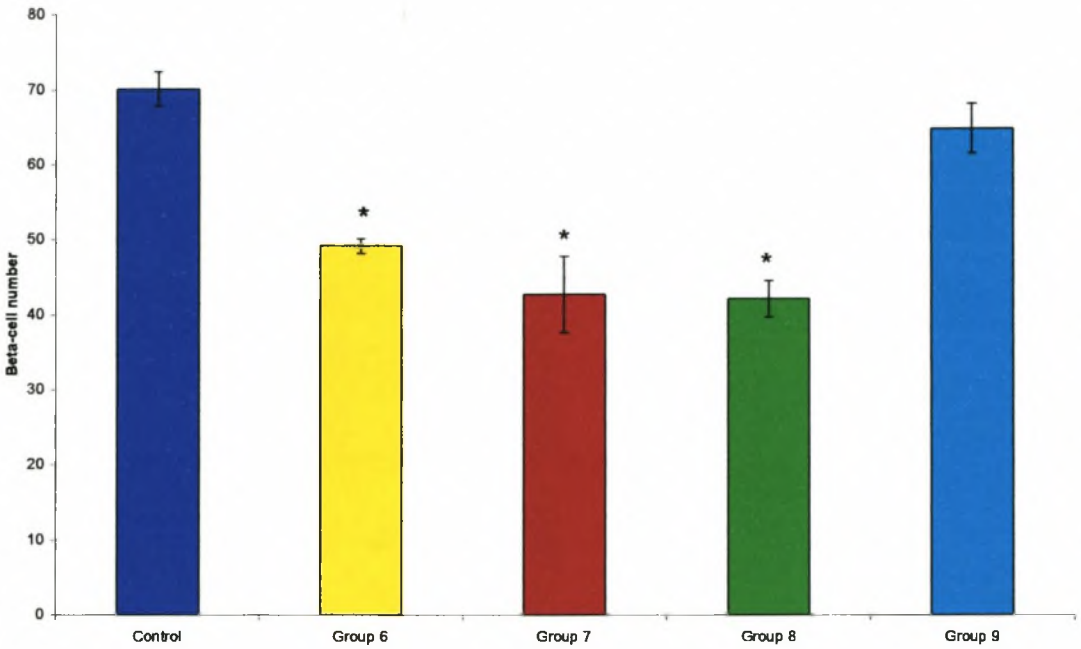


Fig. 23b The effects of exposure to a maternal HFD on beta-cell number in weanling rats

Beta-cell number in weanlings exposed to an *in utero* HFD.

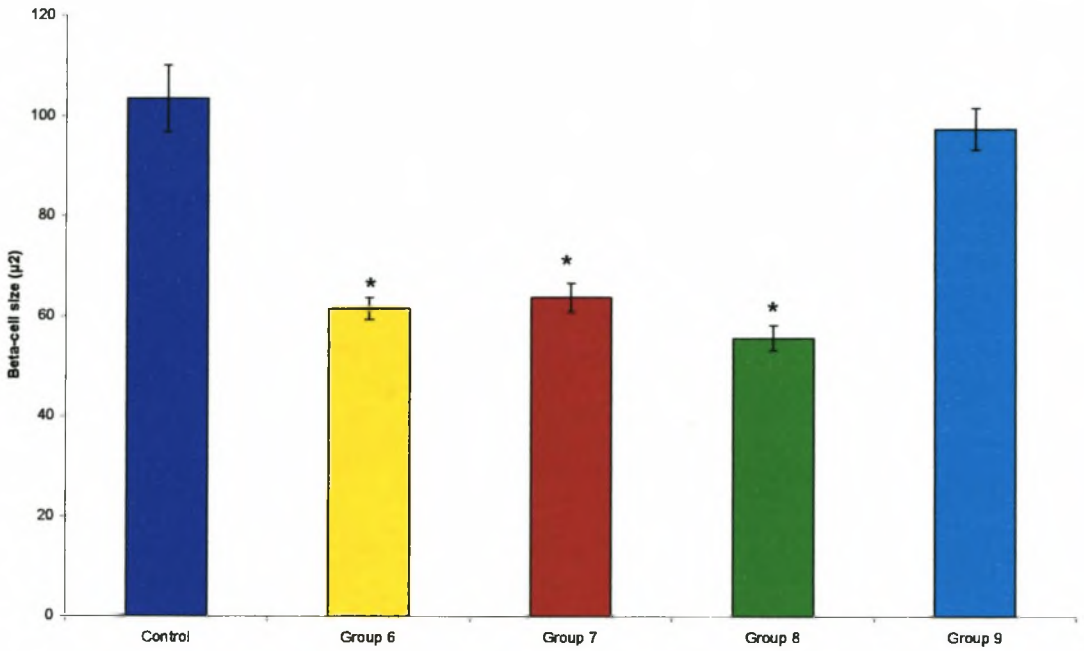


Fig. 23c The effects of exposure to a maternal HFD on beta-cell size in weanling rats

Beta-cell sizes in weanlings exposed to an *in utero* HFD.

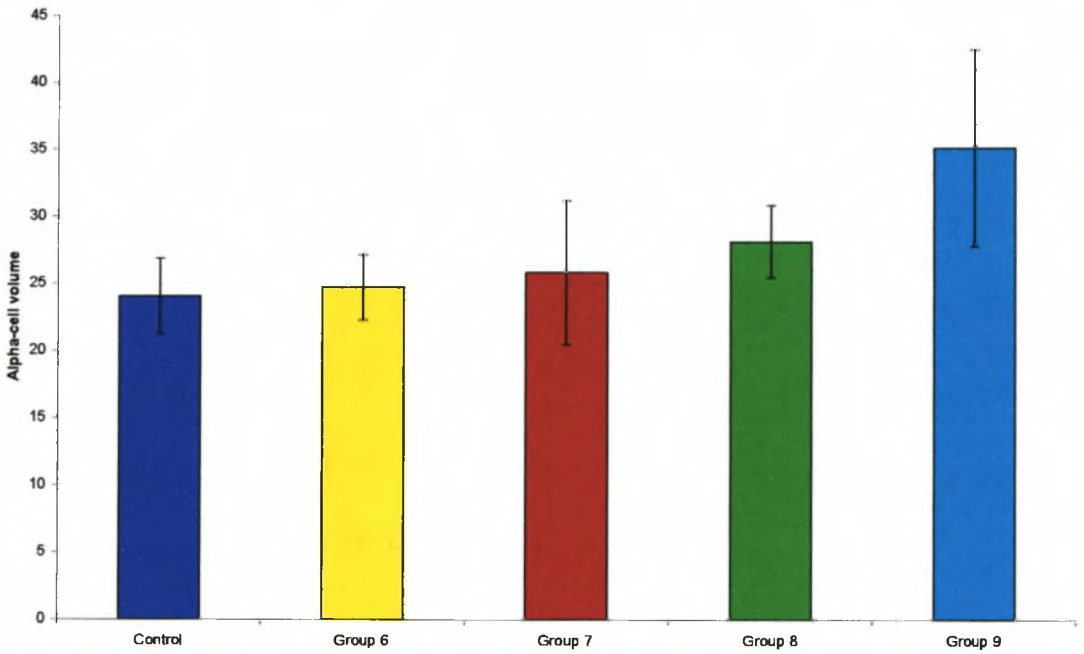


Fig. 24a The effects of exposure to a maternal HFD on alpha-cell volume in weanling rats

Alpha-cell volume in weanlings exposed to maternal HFD. Data represent mean ± SEM. n = 6. * $P < 0.05$.

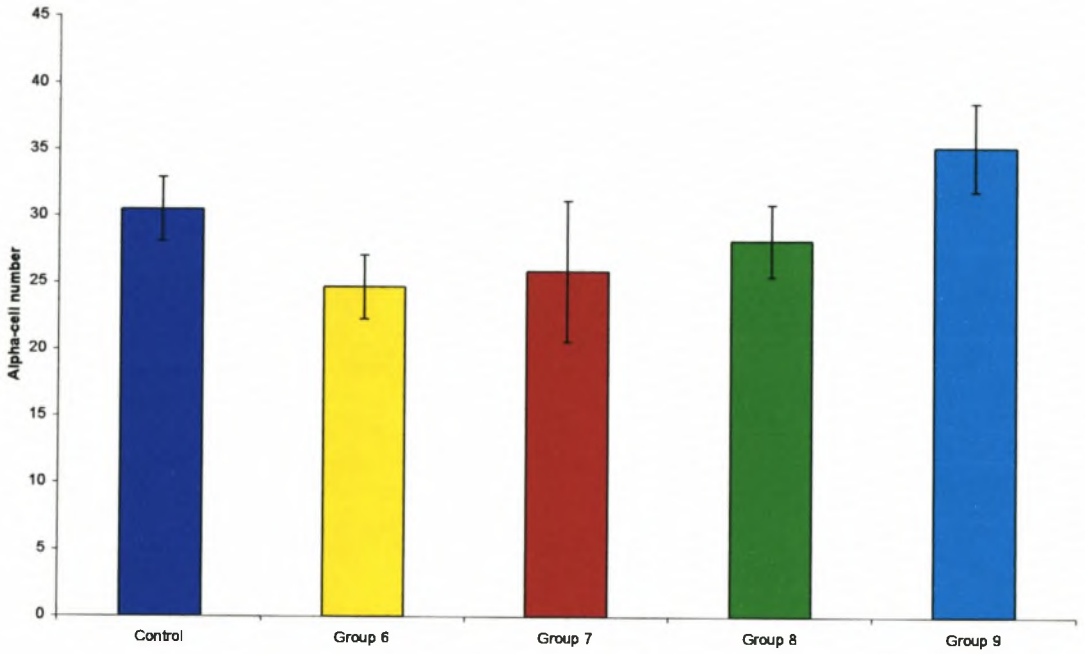


Fig. 24b The effects of exposure to a maternal HFD on alpha-cell number in weanling rats

Alpha-cell numbers of weanlings exposed to an *in utero* HFD.

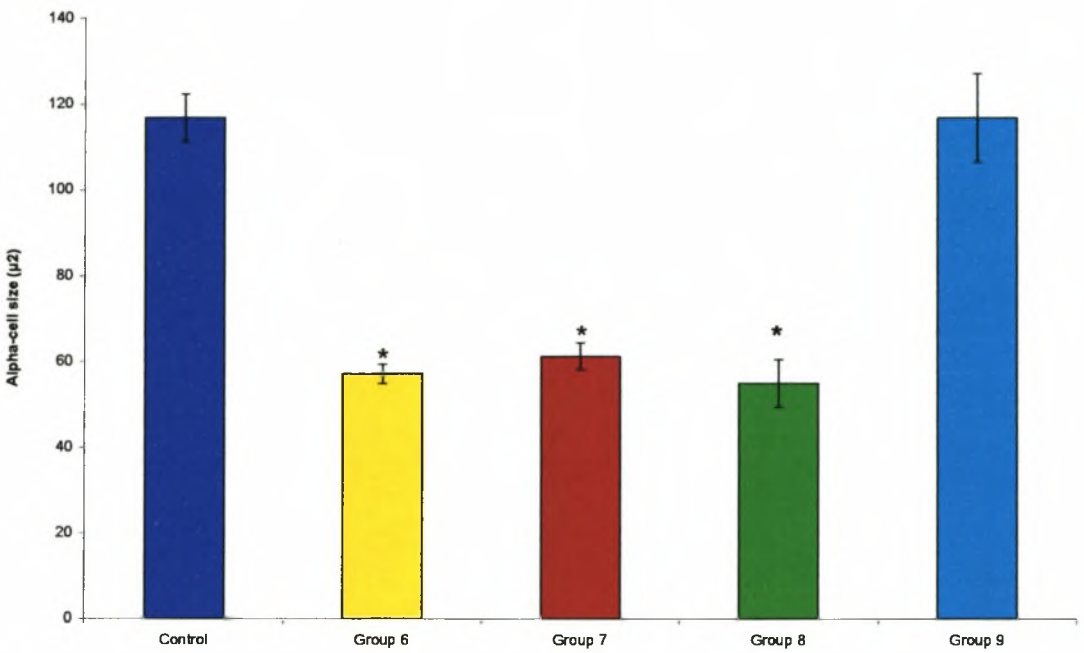


Fig. 24c The effects of exposure to a maternal HFD on alpha-ceil size in weanling rats

Alpha-cell sizes of weanlings exposed to an *in utero* HFD.

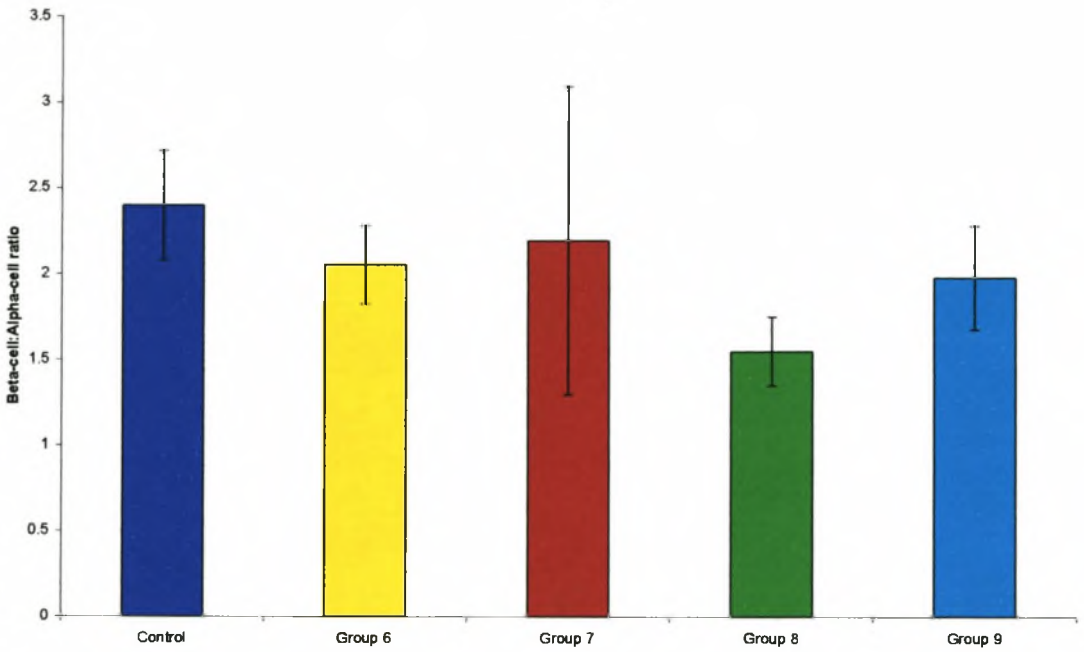


Fig. 25 The effects of exposure to a maternal HFD on the beta-cell:alpha-cell ratio in weanling rats

The beta-cell:alpha-cell ratio in weanlings exposed to an *in utero* HFD. Data represent mean ± SEM. n = 6.

3.22 The effect of a maternal HFD on Pdx-1 expression in weanling rats

3.22.1 Pdx-1 mRNA expression in weanling rats

Pdx-1 mRNA was overexpressed in Group 8 and Group 14 weanlings (Fig. 26). Pdx-1 mRNA was underexpressed in Group 15 weanlings. The expression profiles for mRNA in the remaining weanling groups, viz. Groups 6, 7, 9, 11-13, were all similar to the control.

3.22.2 Pdx-1 immunoreactivity in weanling rats

Nuclear staining of Pdx-1 was demonstrated in beta-cells throughout the islets (Figs. 27a and b). HFD exposure throughout any period of gestation and/or lactation did not seem to affect immunoreactivity for Pdx-1, as no significant differences were detected in the weanlings (Fig. 28).

3.23 The effect of a maternal HFD on Pax 4 expression

3.23.1 Pax 4 mRNA weanling rats

Significantly reduced Pax 4 mRNA levels were found in Group 6, Group 8, Group 9 and Group 15 weanlings (Fig. 29). No differences were detected in the other weanling groups.

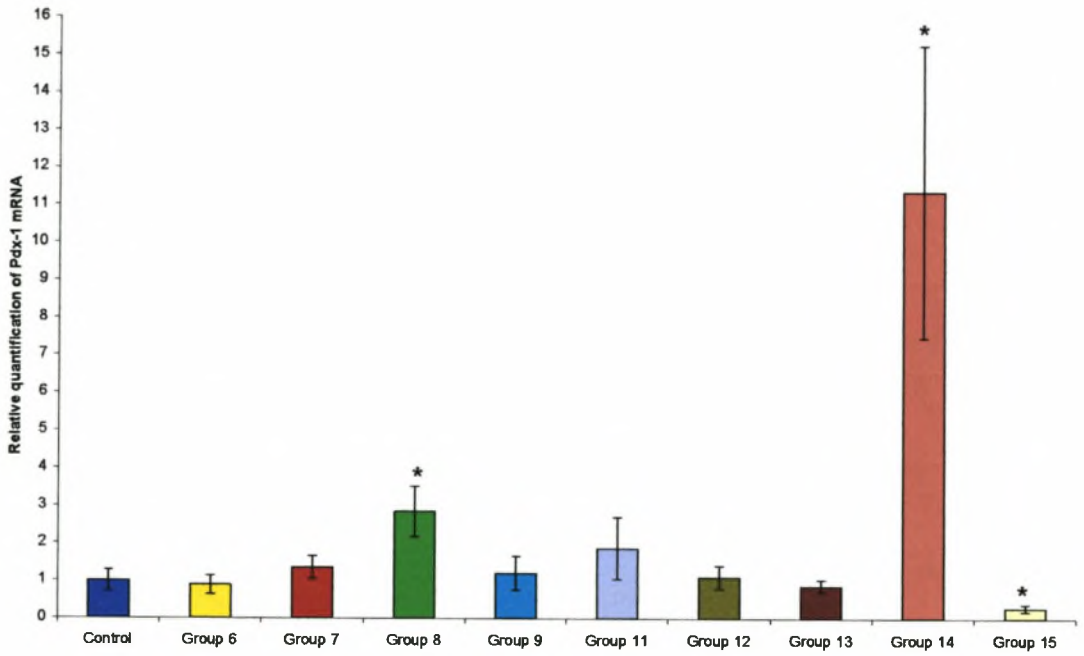
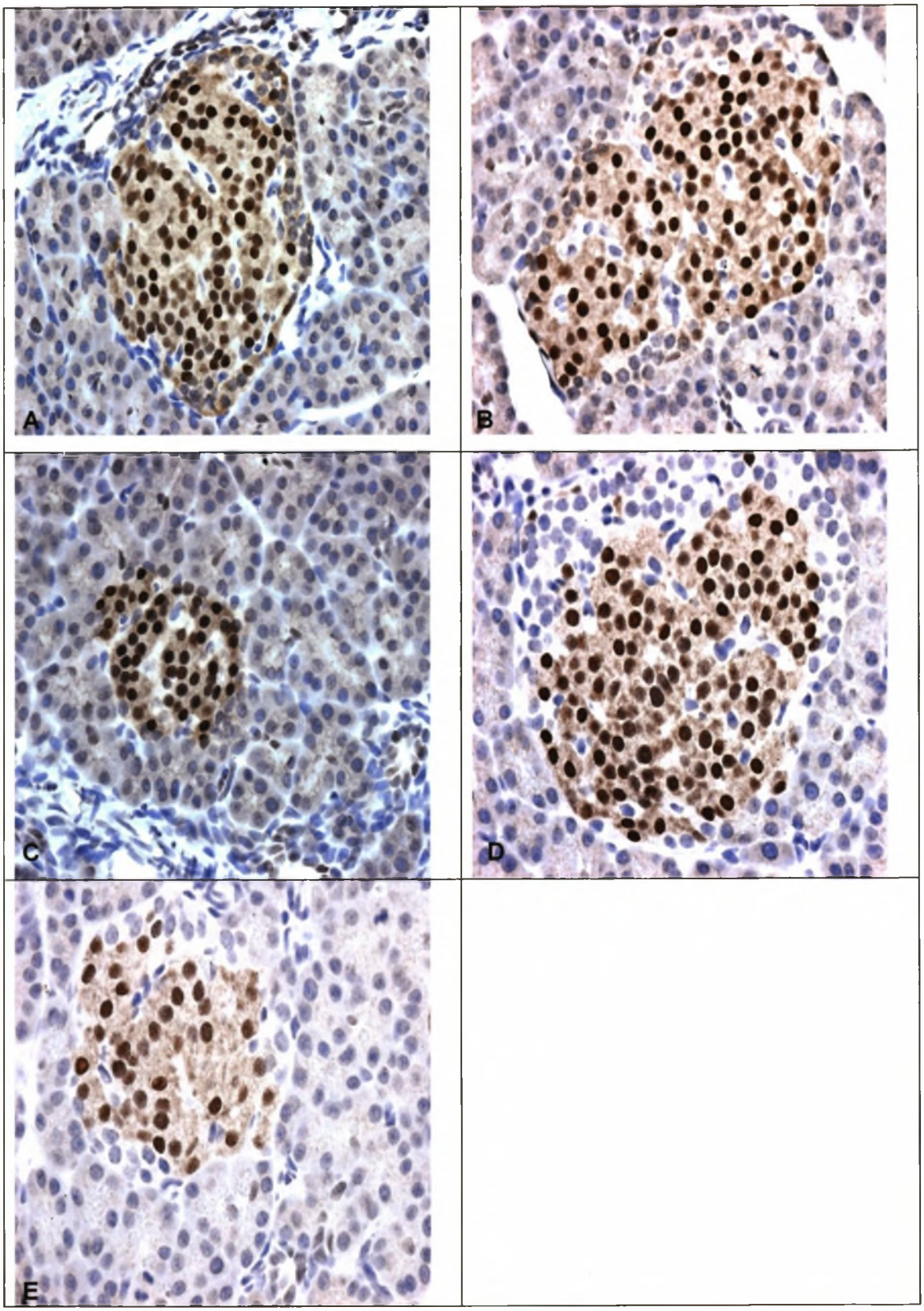


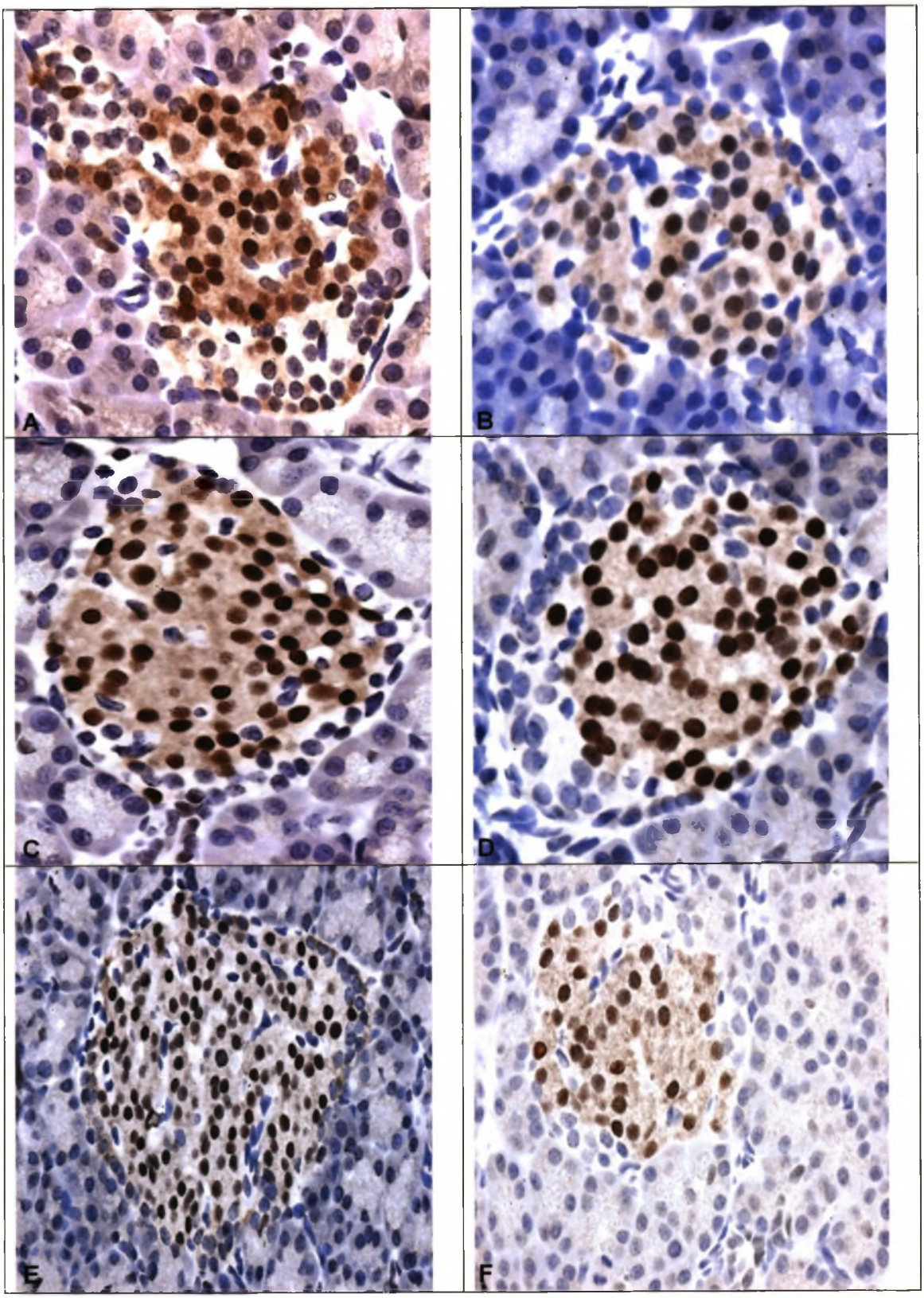
Fig. 26 Relative quantification of Pdx-1 mRNA in weanling rats exposed to a maternal high fat diet

Pdx-1 mRNA expression in weanlings exposed to a maternal HFD during gestation and/or lactation. Results for LightCycler PCR were normalized for Pdx-1 equalling 1. Data represent mean ± SEM. n = 10. *P < 0.05.

Fig. 27a Immunohistochemical staining for Pdx-1 in the pancreata of weanling rats exposed to a maternal high fat diet during specific periods of gestation

Pdx-1 immunolabelling in weanlings exposed to a maternal HFD for the first (A; Group 6), second (B; Group 7) or third (C; Group 8) week of gestation, or throughout gestation (D; Group 9) followed by exposure to a standard laboratory diet during lactation. The control represents weanlings exposed to a standard laboratory diet throughout both gestation and lactation (E; Control). X400.





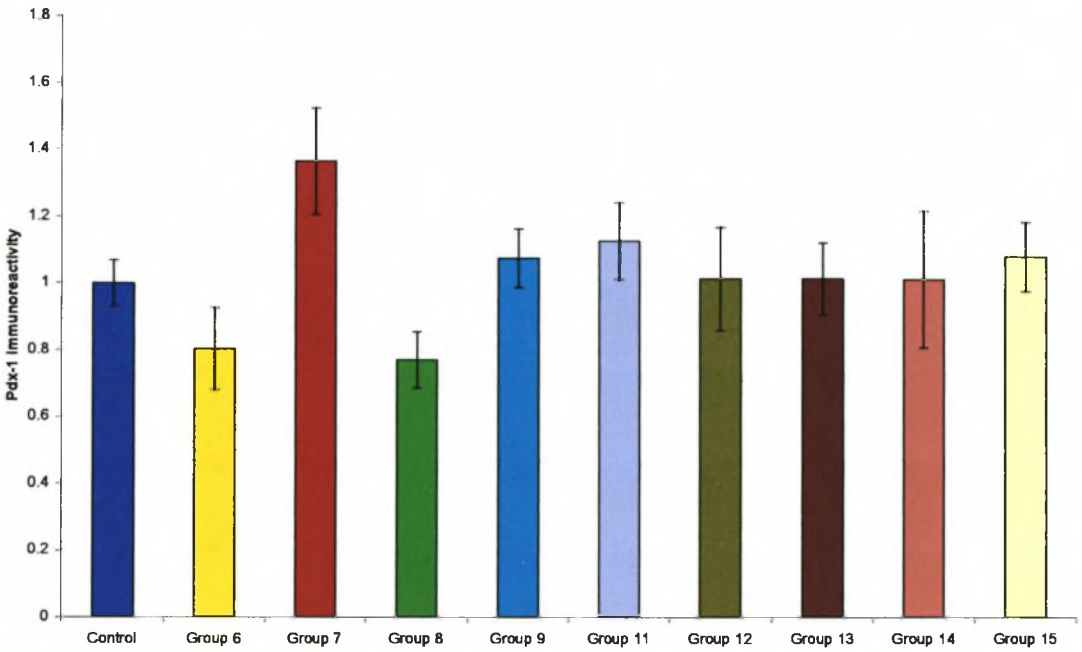


Fig. 28 Assessment of Pdx-1 immunolabelling by image analysis in weanling rat pancreata exposed to a maternal high fat diet

Pdx-1 immunoreactivity in weanlings exposed to a maternal HFD during gestation and/or lactation. The levels of immunolabelled Pdx-1 in the control weanlings were taken to equal 1, with immunoreactivity in weanlings exposed to an *in utero* HFD expressed as a ratio of the control levels. Data represent mean ± SEM. n = 6.

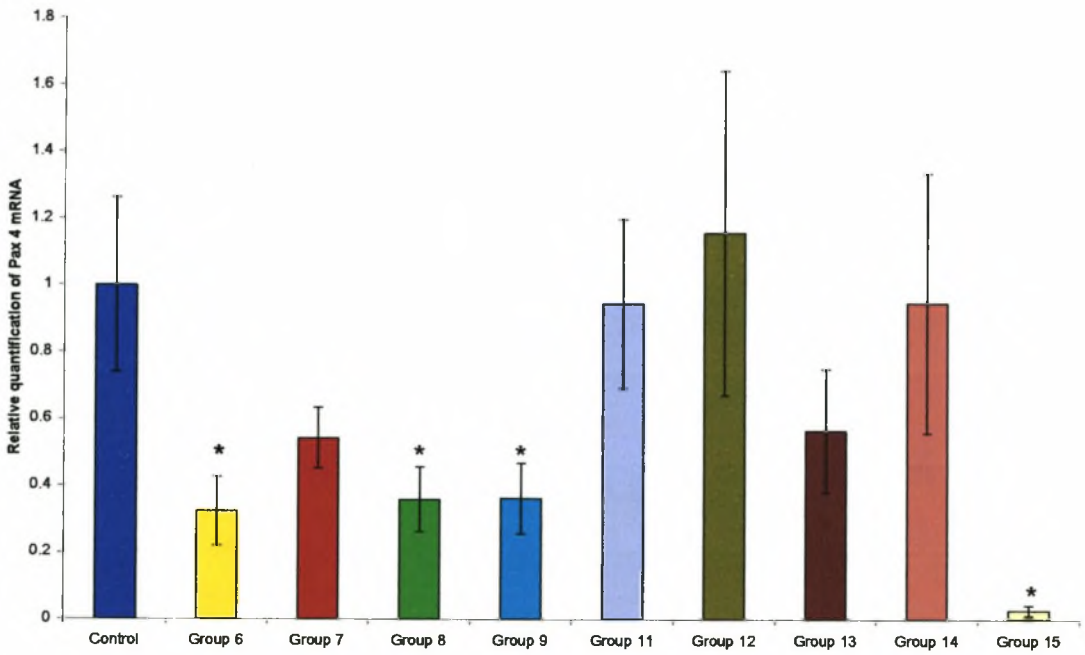


Fig. 29 Relative quantification of Pax 4 mRNA in weanling rats exposed to a maternal high fat diet

Pax 4 mRNA expression in weanlings exposed to a maternal HFD during gestation and/or lactation. Results for LightCycler PCR were normalized for Pax 4 equalling 1. Data represent mean ± SEM. n = 10. *P < 0.05.

3.24 The effect of a maternal HFD on GLUT-2 expression in weanling rats

3.24.1 GLUT-2 mRNA expression in weanling rats

GLUT-2 mRNA was significantly underexpressed in Group 6, Group 11, Group 12, Group 13, Group 14 and Group 15 weanlings (Fig. 30). Extremely low, but non-significant, levels of GLUT-2 mRNA were also detected in Group 7 and Group 8 weanlings. Although still lower than the control, a much higher expression level of GLUT-2 mRNA was found in Group 9 weanlings relative to the other experimental groups, but the differences were not significant.

3.24.2 GLUT-2 immunoreactivity in weanling rats

Intense immunostaining for GLUT-2, around the beta-cell membrane, was evident in weanlings exposed to a maternal HFD during any period of gestation and/or lactation (Figs. 31a and b). This immunolabelling was found throughout the islet in most of the beta-cells. Significantly raised GLUT-2 immunostaining was evident in Group 8, Group 9, Group 11, Group 12, Group 13, Group 14 and Group 15 weanlings (Fig. 32).

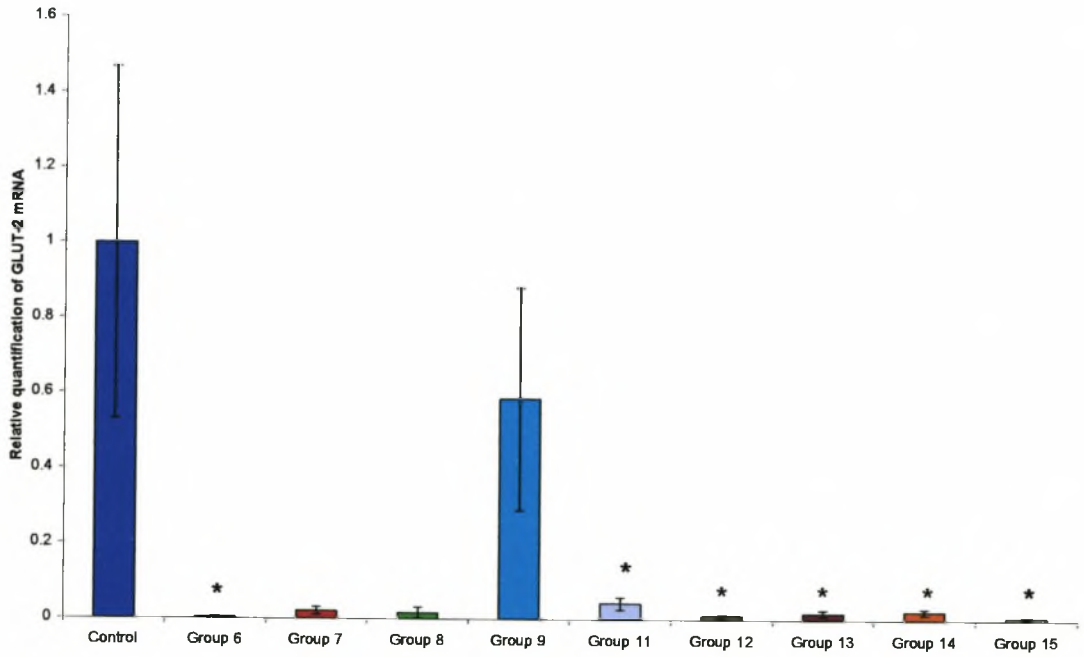


Fig. 30 Relative quantification of GLUT-2 mRNA in weanling rats exposed to a maternal high fat diet

GLUT-2 mRNA expression in weanlings exposed to a maternal HFD during gestation and/or lactation. Results for LightCycler PCR were normalized for GLUT-2 equalling 1. Data represent mean ± SEM. n = 10. *P < 0.05.

Fig. 31a Immunohistochemical staining for GLUT-2 in the pancreata of weanling rats exposed to a maternal high fat diet during specific periods of gestation

GLUT-2 immunolabelling in weanlings exposed to a maternal HFD for the first (A; Group 6), second (B; Group 7) or third (C; Group 8) week of gestation, or throughout gestation (D; Group 9) followed by exposure to a standard laboratory diet for the duration of weaning. The control represents weanlings exposed to a standard laboratory diet throughout both gestation and lactation (E; Control). X400.

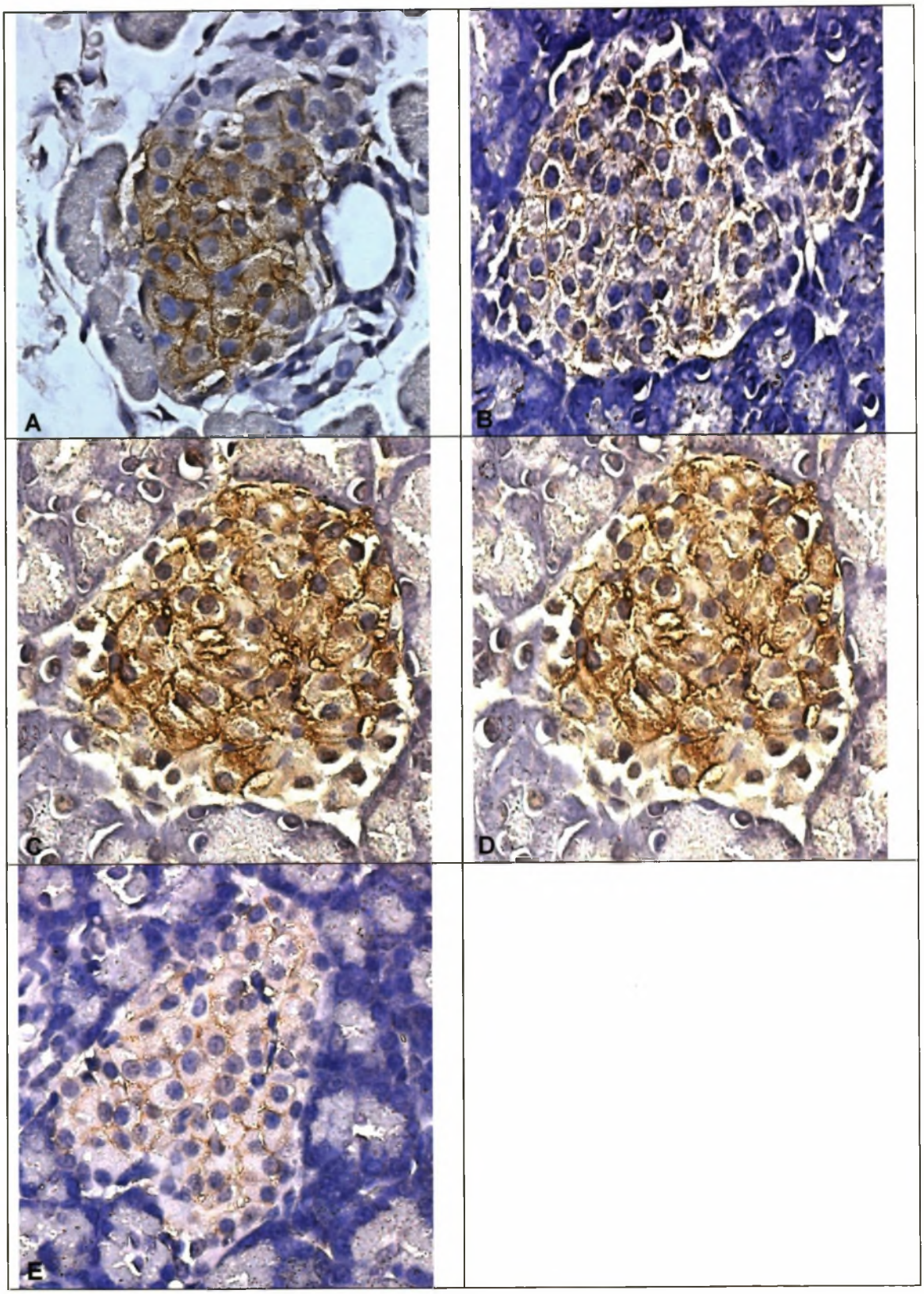
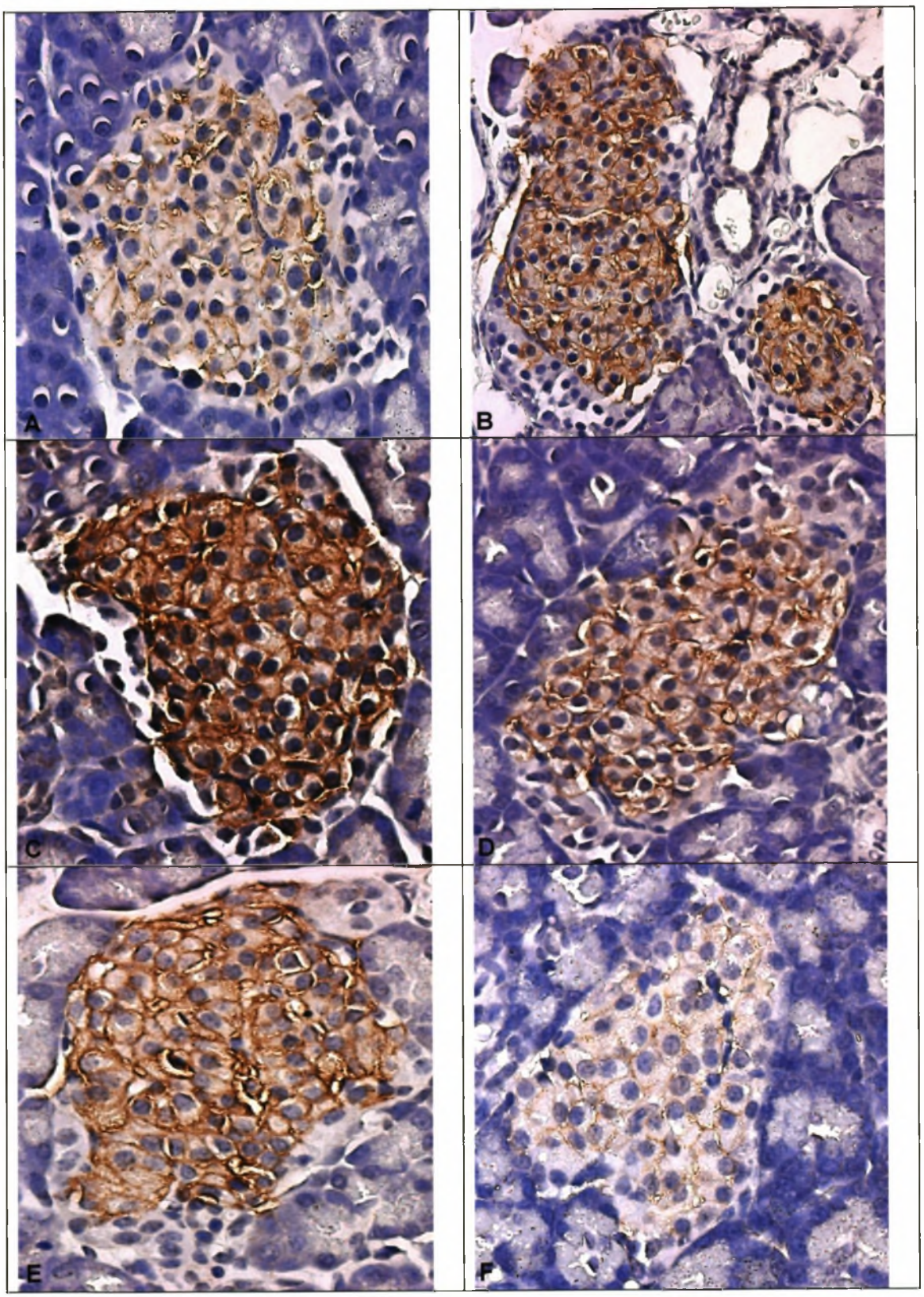


Fig. 31b Immunohistochemical staining for GLUT-2 in the pancreata of weanling rats exposed to a maternal high fat diet throughout gestation and/or for specific periods of lactation

GLUT-2 immunolabelling in weanling rats exposed to a maternal HFD throughout gestation and for either first (A; Group 11), the second (B; Group 12), or the third week of lactation (C; Group 13); or throughout gestation and lactation (D; Group 14); or throughout lactation (E; Group 15). The control represents weanlings exposed to a standard laboratory diet throughout both gestation and lactation (F; Control). X400.



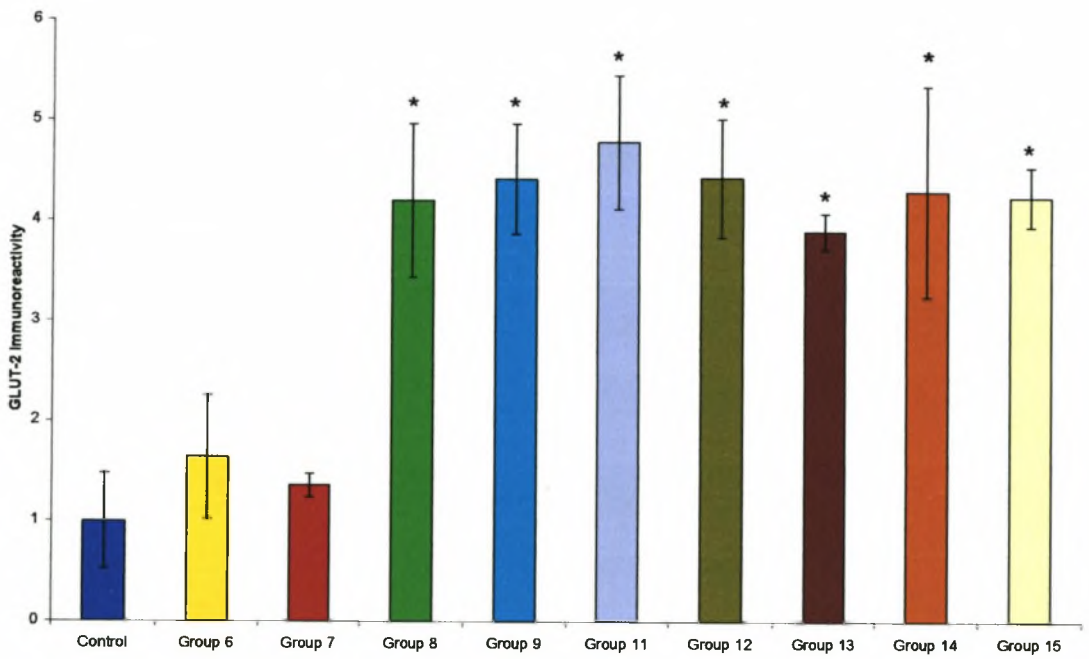


Fig. 32 Assessment of GLUT-2 immunolabelling by image analysis in weanling rat pancreata exposed to a maternal high fat diet

GLUT-2 immunoreactivity in weanlings exposed to a maternal HFD during gestation and/or lactation. The levels of immunolabelled GLUT-2 in the control neonates were taken to equal 1, with immunoreactivity in neonates exposed to an *in utero* HFD expressed as a ratio of the control levels. Data represent mean ± SEM. n = 6. *P < 0.05.

3.25 The effect of a maternal HFD on GK expression in weanling rats

3.25.1 GK mRNA expression in weanling rats

The mRNA levels of GK were significantly reduced in Group 6, Group 7, Group 8, Group 12, Group 13, Group 14 and Group 15 weanlings (Fig. 33). GK mRNA levels were comparable to the control in Group 9 and Group 11 weanlings.

3.25.2 GK immunoreactivity in weanling rats

Similar to GK immunoreactivity in the neonatal rats, immunolabelling for GK was found in a few beta-cells on the periphery of the islet (Figs. 34a and b). In some of the islets, immunostained GK cells formed a circular border. The immunostaining for GK was intense in the sections. Significantly reduced immunoreactivity for GK was found in Group 6, Group 7, Group 8, Group 11, Group 12, Group 13, Group 14 and Group 15 weanlings (Fig. 35). GK immunoreactivity was normal, showing similar levels to the control in Group 9 and Group 15 weanlings.

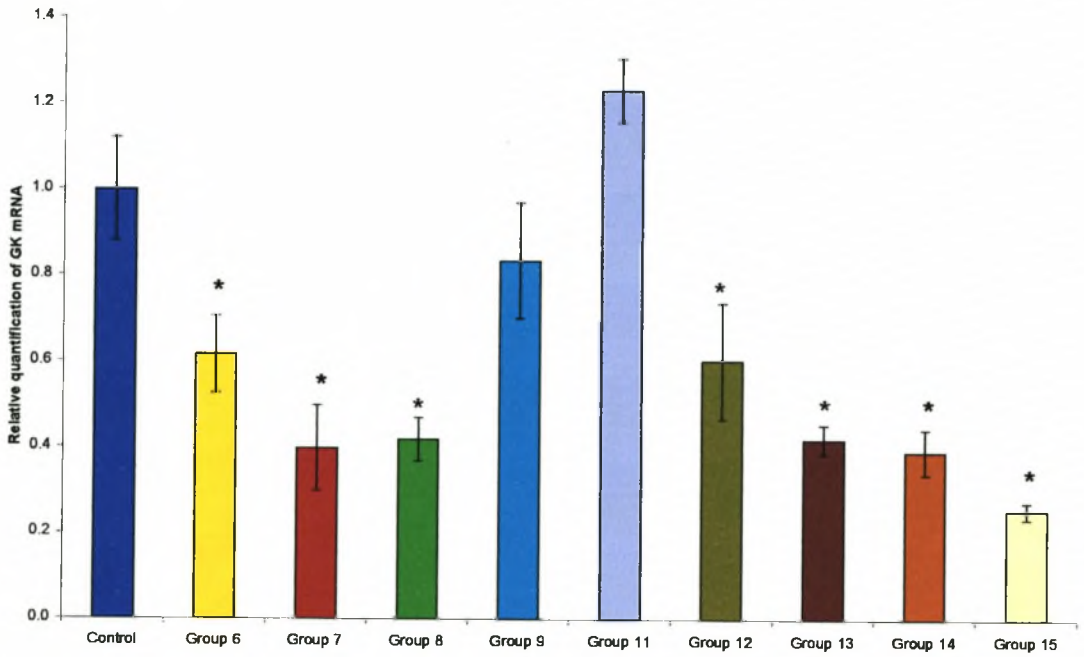


Fig. 33 Relative quantification of GK mRNA in weanling rats exposed to a maternal high fat diet

GK mRNA expression in weanlings exposed to a maternal HFD during gestation and/or lactation. Results for LightCycler PCR were normalized for GK equalling 1. Data represent mean ± SEM. n = 10. *P < 0.05.

Fig. 34a Immunohistochemical staining for GK in the pancreata of weanling rats exposed to a maternal high fat diet during specific periods of gestation

GK immunolabelling in weanlings exposed to a maternal HFD for the first (A; Group 6), second (B; Group 7) or third (C; Group 8) week, or throughout gestation (D; Group 9) followed by exposure to a standard laboratory diet for the duration of weaning. The control represents weanlings exposed to a standard laboratory diet throughout both gestation and lactation (E; Control). X400.

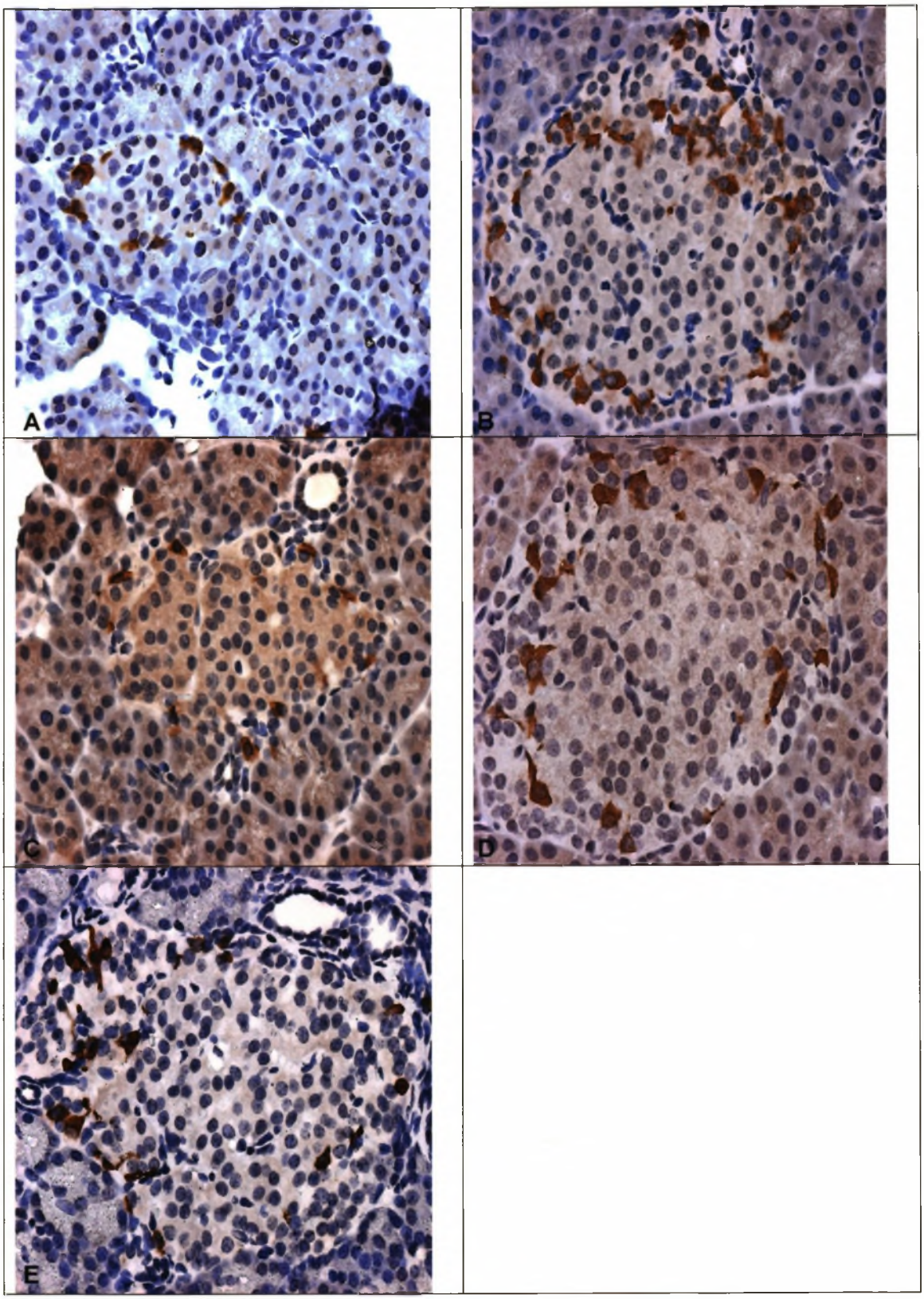
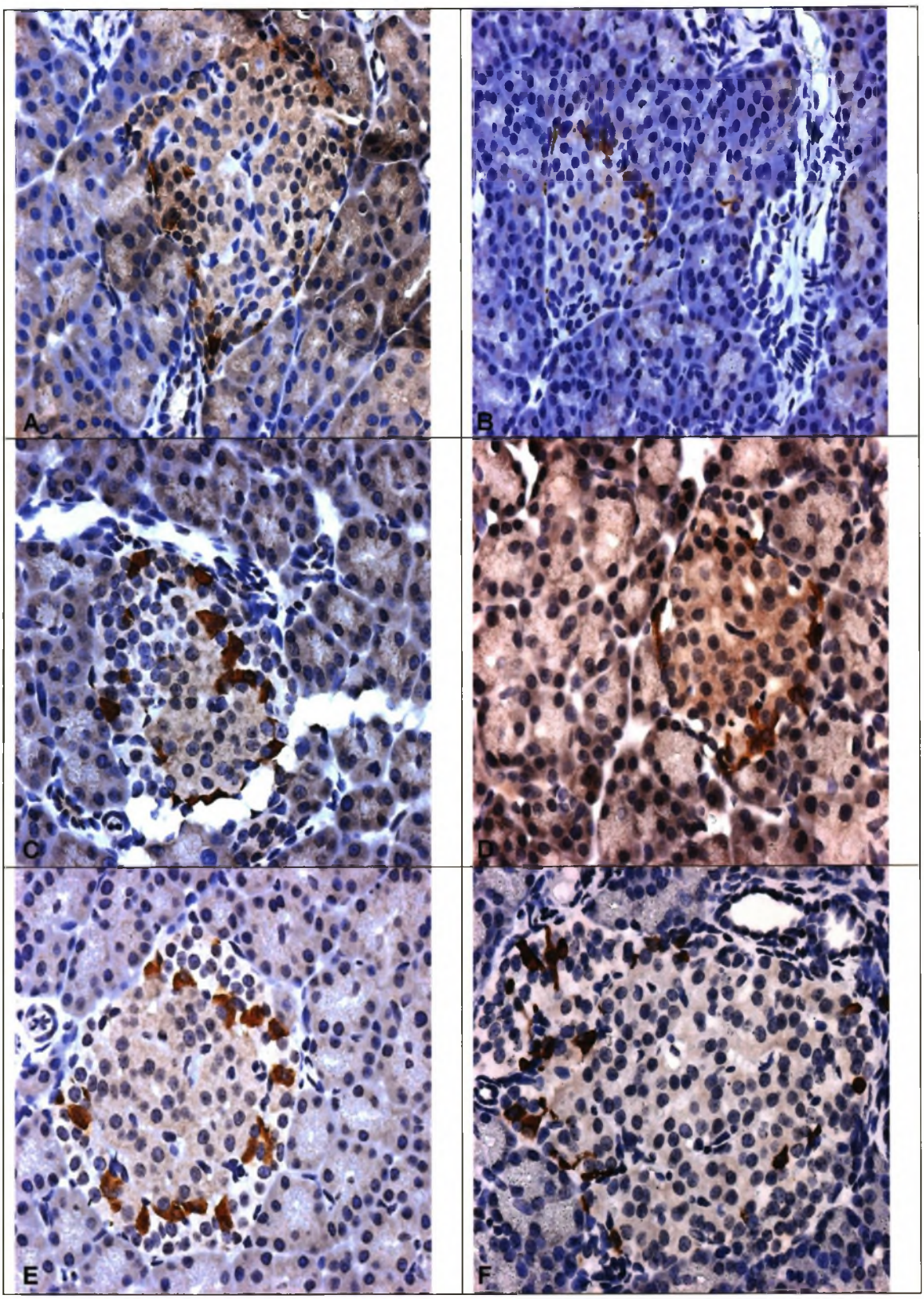


Fig. 34b Immunohistochemical staining for GK in the pancreata of weanling rats exposed to a maternal high fat diet throughout gestation and/or for specific periods of lactation

GK immunolabelling in weanling rats exposed to a maternal HFD throughout gestation and for either first (A; Group 11), the second (B; Group 12), or the third week of lactation (C; Group 13); or throughout gestation and lactation (D; Group 14); or throughout lactation (E; Group 15). The control represents weanlings exposed to a standard laboratory diet throughout both gestation and lactation (F; Control). X400.



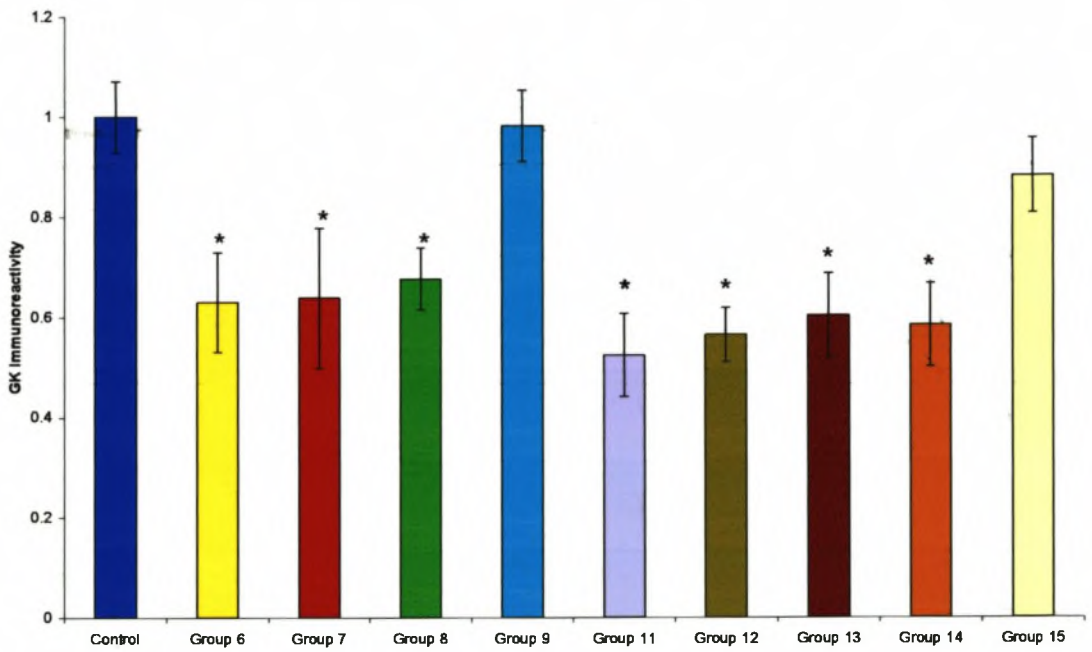


Fig. 35 Assessment of GK immunolabelling by image analysis in weanling rat pancreata exposed to a maternal high fat diet

GK immunoreactivity in weanlings exposed to a maternal HFD during gestation and/or lactation. The levels of immunolabelled GK in the control weanlings were taken to equal 1, with immunoreactivity in weanlings exposed to a maternal HFD expressed as a ratio of the control levels. Data represent mean ± SEM. n = 6. *P < 0.05.

Fig. 27b Immunohistochemical staining for Pdx-1 in the pancreata of weanling rats exposed to a maternal high fat diet throughout gestation and/or for specific periods of lactation

Pdx-1 immunolabelling in weanling rats exposed to a maternal HFD throughout gestation and for either first (A; Group 11), the second (B; Group 12), or the third week of lactation (C; Group 13); or throughout gestation and lactation (D; Group 14); or throughout lactation (E; Group 15). The control represents weanlings exposed to a standard laboratory diet throughout both gestation and lactation (F; Control). X400.

CHAPTER 4

DISCUSSION

Mothers

4.1 Maternal food intake and weight

During early gestation, the mother develops hyperphagia which, together with endocrine changes, allows her to increase her net body weight, which is mainly due to the accumulation of fat depots in the first two thirds of gestation (134). This occurs in both in women and rats (134). In a study where 20-22 week old female Wistar rats were fed a moderately high fat (36% energy as fat) diet for 14 weeks, the body weight and total energy intake, notably the dietary fat intake, increased significantly compared to those on the control (3% fat) diet (135). Earlier studies (136) also reported that a HFD can induce overconsumption and weight gain and the increased caloric intake due to the HFD would contribute to excess body weight. Similarly, our data indicate that the HFD was more palatable than the standard laboratory diet as pregnant dams consumed more food when maintained on a HFD. Dams fed a HFD for the second or third week of pregnancy or throughout gestation had higher overall food intakes after all 21 days of gestation suggesting that, apart from the increase in food intake to ensure that their progeny grow and develop normally, a HFD given during the latter two weeks of gestation or throughout gestation induces greater overall food consumption in these pregnant dams. Dams used in our study, maintained on a HFD throughout pregnancy, were significantly heavier than those maintained on a standard laboratory diet at days 7 and 14 of pregnancy and on the day of delivery. This corresponded to the high food consumption of these dams at those time intervals of pregnancy which would have contributed to their gaining weight.

It has been reported that fat-rich diets may contribute to early weight gain not only because fat provides the substrate for triacylglycerol accumulation, but also because food intake increases as a result of the reduction in leptin secretion by adipose tissue (135).

Dietary fat induces overconsumption (137) and weight gain through its low satiety properties and high caloric density (136). A HFD promotes hyperphagia possibly related to palatability (138) and an attenuated satiety response to fat compared to carbohydrates (139-141) which may be related to postabsorptive factors such as stomach distension, nutrient absorption, hormonal release or oxidation of nutrients (138). Protein has been reported to be more satiating than carbohydrate (142-144) since high protein intake correlates with reduced overall food intake (144). Therefore, in terms of satiety potential, it appears that proteins are most effective, followed by carbohydrates, with the least satiation occurring with fat consumption.

Weight gain and feeding are mainly modulated by neural and hormonal inputs to the hypothalamus (145). In the study presented here, food consumption increased in the pregnant dams fed the HFD. On the day of delivery, the increase in body weight in Group 4 dams could be attributed to the increase in their overall food intake during pregnancy. While a high protein diet has a greater satiety effect than a HFD and turns off the feeding response, dams fed a HFD consumed more food ingesting higher dietary fat and protein than dams

maintained on a standard laboratory diet. However the changes induced in the offspring (which will be discussed later) appear to be due to the high fat content of the diet. In addition, the energy derived from carbohydrate was reduced in the HFD. By increasing the fat component of the diet, the levels of the other macronutrients would be affected. The protein derived from calories was kept similar in both the HFD and standard laboratory diet as protein deficiency has a deleterious effect on islet cell development (29). Therefore the carbohydrate levels were reduced in the HFD which may be beneficial as high carbohydrate feeding has been shown to cause the immediate onset of hyperinsulinaemia in rats (146).

4.2 The effect of HFD feeding on maternal circulating glucose and insulin concentrations

In the study reported here, high fat feeding, in the week of pregnancy prior to the measurement of the circulating glucose concentrations in dams, induced acute hyperglycaemia which occurred only at that particular time point. This was evident in all dams fed a HFD for only a single week of gestation. In addition, dams fed a HFD throughout pregnancy were found to be hyperglycaemic at the two days (7 and 14) of pregnancy when blood glucose was measured. Similarly, on the day of delivery, both these dams and those fed a HFD for the final week of gestation were hyperglycaemic. Since dams that were fed a HFD throughout gestation remained hyperglycaemic at all the time intervals measured, and particularly on the day of giving birth it could be suggested that prolonged high fat

feeding, in addition to the metabolic demands of pregnancy, induce hyperglycaemia.

Pregnancy induces profound changes in maternal metabolism and insulin secretion, both of which allow optimum nutrient supply to the foetus (147). Maternal insulin resistance normally develops in the last trimester in both women and rats (148;149). In the study for this thesis, circulating insulin levels were measured on day 20 of pregnancy in some of the mothers. Although the insulin levels of dams fed a HFD during gestation did not differ significantly from the control, all of the insulin levels were higher than those detected in non-pregnant dams. Significantly higher insulin concentrations were found in dams fed a HFD for the first or second week of gestation or throughout gestation (data not shown) and this can be attributed to the reported hyperinsulinaemia associated with pregnancy in the final trimester. In addition, glucose concentrations in these dams on day 20 of pregnancy in these dams were normal, confirming that there was at least sufficient insulin in the circulation to maintain glucose homeostasis. The circulating insulin levels did not differ significantly in the dams during pregnancy or on the day of delivery, but were higher relative to those on a standard laboratory diet, when measured after the dams were fed a HFD.

Gestational diabetes is defined as any form of diabetes mellitus or impaired glucose tolerance with first onset during pregnancy (150). A rat model for gestational diabetes was developed in a recent study (151). The female rats

were fed a cafeteria style diet, 4 weeks prior to mating, and this resulted in increased body weight that persisted throughout pregnancy (151). While pregnant, these rats exhibited impaired glucose tolerance with insulin resistance, as determined by hyperinsulinaemic clamp studies on day 22 of pregnancy (151). In another model of gestational diabetes, using uteroplacental insufficiency, pregnancy resulted in rats developing progressive hyperglycaemia and hyperinsulinaemia accompanied by impaired glucose tolerance and insulin resistance (152). These rats were also significantly heavier after gestation (152). Although hyperglycaemia was detected, after birth, in dams fed a HFD throughout gestation in the present study, the circulating insulin levels were comparable to the control. Unlike the previously described models of gestational diabetes, the insulin status appears to be normal which indicates that dams used in this study did not fully develop gestational diabetes. We conclude, therefore, that the changes induced in the offspring were mainly attributed to the pups being exposed to maternal HFD during critical periods of pancreatic development and growth possibly exacerbated by the maternal hyperglycaemia. The metabolic status of the mothers during pregnancy and lactation, however, was not the focal point of this study. The effects of high fat feeding on pregnant dams may however be determined in further studies taking into consideration their glucose and insulin responses.

Neonates

4.3 The effect of an *in utero* HFD on birth weight

Nutrition during the early phases of life is of major importance for proper tissue development and functional maturation (153). Programming is the process by which the physiology or metabolism of a foetus and/or neonate may be altered by a stimulus or insult during a critical period of development (154). During gestation, we exposed pups to a HFD, indirectly, through the mother. It is known that the diet, *in utero*, is critical to the development of the offspring. An *in utero* protein deficient diet has been shown to decrease birth weights in rats (31;155). Women, undernourished in the first trimester of pregnancy, were found to produce offspring who were small at birth (154). Using a HFD instead of a low protein diet, in this thesis study, we have now found that dams fed a HFD for only the first week of gestation gave birth to pups with significantly reduced birth weights. It has been hypothesized that low birth weight predisposes the offspring to the development of Type 2 diabetes and insulin resistance syndrome in adult life (25). By extrapolation, pups exposed to a maternal HFD for the first week of gestation may therefore be susceptible to developing diabetes later in life. Foetal growth and development are determined primarily by the genetic potential of the foetus, but this can be influenced by environmental factors, such as the nutritional status of the mother and/or the capacity of the placenta to transport these nutrients to the foetus (26). Interestingly, pups that were exposed to a HFD for any other week of gestation, other than the first, or throughout gestation had normal birth weights.

Pregnancy results in profound changes in both maternal metabolism and insulin secretion to allow optimal nutrient supply to the foetus (147). Furthermore, the insulin demands on the mother, during pregnancy, dramatically increase due to enhanced insulin resistance of maternal tissues and increased food intake, and this occurs especially during the latter half of pregnancy (156). Our data showed that pregnant dams consumed more food when maintained on a HFD. It has been reported that foetal metabolism, and consequently foetal growth, directly depend on the nutrients crossing the placenta (134). The mother, therefore, must adapt her metabolism in order to support this continuous draining of substrates (134). The body weights and food consumption of dams, in this thesis study, fed a HFD for only the first week of gestation did not differ significantly from the control.

At critical periods of foetal and early life, certain metabolic processes can be programmed, for an organism to accommodate a particular level of nutrition throughout life, thus conferring a survival advantage (25). However, a subsequent improvement in nutrition, for example, can lead to obesity, diabetes and cardiovascular disease in adult life (157). Studies in a rat model fed a high carbohydrate diet have shown that, by switching the nature of calories from high fat to high carbohydrate, extensive adaptations at the molecular, cellular and biochemical levels occur in islets isolated from 12-day old rats (158;159). Our study suggests that Group 1 neonates expect to be fed the same diet (i.e. HFD) for life. A change to a standard laboratory diet after the first week of gestation

and continued for the remainder of gestation, concomitant with reduced caloric intake from the dams, may contribute to the growth retardation in these offspring. Apart from being leaner, Group 1 neonates were hypoglycaemic with the lowest circulating insulin concentrations. A reduction in body weight has been associated with reduced glucose (160) and insulin (31) levels in rats fed a low protein diet. Furthermore, specific foetal hypoinsulinaemia is associated with intrauterine growth retardation in humans (161). We have now shown that a HFD fed to pregnant dams for the first week of gestation results in offspring with low birth weights and reduced glucose and insulin levels, similar to findings in low protein fed rats.

Interestingly, despite being heavier at term, Group 4 dams gave birth to Group 4 neonates of normal weight. This appears to have set a specific growth trajectory in these offspring. The Group 4 neonates, similar to the control neonates, were only exposed to one type of diet without any dietary switch. Therefore the Group 4 neonates expect to consume a HFD throughout life and adapt promptly which may account for their normal growth.

4.4 The effect of exposure to an *in utero* HFD on circulating glucose and insulin levels, the expression of transcription factors, glucose sensing genes and islet cell development in neonatal rats

The pancreatic beta-cell is designed to synthesize, package and release insulin on demand in order to control blood glucose homeostasis (162). Unlike other cell types it is constantly monitoring the nutrient status of the organism (162). Insulin secretion is stimulated by intracellular signals derived from the metabolism of a variety of nutrients, the most important being glucose (162). In addition to signalling the beta-cell to secrete insulin, nutrient secretagogues, like glucose, also generate important signals required to regulate gene expression, protein biosynthesis and cell proliferation (162). Chronic exposure to FFA has been shown to decrease Pdx-1 transactivation of GLUT-2, GK and insulin genes in islets (132). Glucose stimulates proinsulin biosynthesis at both the translational and transcriptional level (163-165). In the study presented here, neonates were exposed to a maternal HFD during defined periods of gestation. We therefore, related the circulating glucose levels to expression levels of the glucose transporter, GLUT-2, and the glycolytic enzyme, GK, which are both important for glucose metabolism within the beta-cell, ultimately resulting in insulin secretion. Pdx-1 is reported to regulate the transcription of the GLUT-2 (63), GK (64) and insulin (62) genes, and therefore, indirectly, plays a role in GSIS. We therefore monitored the changes in circulating glucose and insulin levels associated with exposure to an *in utero* HFD and related this to the expression of the glucose sensing genes involved in GSIS, GLUT-2 and GK, and the transcription factor,

Pdx-1. Pdx-1 is also important for endocrine cell differentiation and in maintaining the beta-cell phenotype. Another, transcription factor, Pax 4, is important for ensuring proper beta- and alpha-cell development. The expression levels of these transcription factors, therefore, were discussed in relation to islet cell development.

(i) Neonates exposed to an *in utero* HFD for the first week of gestation (Group 1)

Neonates that were exposed to a maternal HFD for the first week of gestation were hypoglycaemic, with the lowest, though not significant, serum insulin levels. Both Pdx-1 and GLUT-2 mRNA levels and immunoreactivity were normal. However, GK mRNA expression was significantly reduced, while GK immunoreactivity was significantly higher. This suggests that transcription of GK may have been compromised by the HFD but, in contrast, translation of GK into protein has been increased. Insulin secretion depends upon glucose metabolism and GK exerts nearly total control on the rate of glycolysis in the beta-cells (112). The unique kinetics of GK underlie the ability of these cells to sense and respond to fluctuations in plasma glucose concentration (108). Modelling of changes in GK activity, as well as studies of the effects of graded increases in the enzyme, have established that glucose phosphorylation is the key point of control for glycolytic flux in the beta-cell (109;110). Consequently, even small changes in GK activity can be physiologically significant, since they directly affect the threshold for GSIS (108). In addition to reducing blood glucose concentration,

overexpression of GK appears to prevent the development of Type 2 diabetes (108). Our results would support this claim since we found that increased immunoreactivity for GK reduced blood glucose concentrations in one-day-old neonates that were exposed to an *in utero* HFD for the first week of pregnancy. The increase in GK protein expression should therefore increase the rate of glycolysis within the beta-cell which, dependent on downstream components of GSIS, could result in an increase in insulin secretion. In another study, male GK locus transgenic mice, that were fed a HFD, were protected against the development of hyperinsulinaemia and hyperglycaemia, even though these animals were as obese as their nontransgenic littermates (111). This suggests that if GK activity could somehow be enhanced, it might be an effective strategy for treating diabetes.

This thesis research showed that beta-cell volume and size was unaltered in these offspring, but there were significantly fewer beta-cells. The Pdx-1 expression levels were relatively normal. It is possible that the reduction in beta-cell number may be due to underexpression of other transcription factors important for beta-cell development, and also, in part, to the exposure to the HFD for the first week of pregnancy. Although not significant, Pax 4 mRNA was overexpressed, and was indeed the highest of all the groups. This may account for the significantly larger alpha-cells, since Pax 4 is required for normal alpha-cell development. Measuring the expression levels of Pax 4 immunoreativity in these neonates could endorse this finding.

(ii) Neonates exposed to an *in utero* HFD for the second week of gestation (Group 2)

Neonatal rats exposed to a maternal HFD for the second week of gestation were hypoglycaemic. Glucose activates Pdx-1 through an insulin-dependent cell signalling pathway involving phosphotidylinositol 3-kinase, and the stimulation of this pathway leads to phosphorylation and activation of a cytoplasmic form of Pdx-1 that translocates to the nucleus (73). In a recent study, a decrease in circulating glucose, in rats exposed to a low protein diet, was suggested to contribute to the reduced Pdx-1 expression, indicating that a low protein diet during the foetal (and lactation periods) could lead to alterations in islet size and functionality (31). These effects were linked to a reduction in Pdx-1 protein, but not Pdx-1 mRNA expression (31). Similarly, in our study, hypoglycaemia may have induced the reduction of Pdx-1 protein expression when foetuses were exposed to an *in utero* HFD for the second week of gestation. The serum insulin levels of these offspring were also reduced, though not significantly. The expression of Pdx-1 mRNA in these offspring was normal but there was a significant reduction in Pdx-1 immunoreactivity, concomitant with lowest, though not significant, expression of GLUT-2 mRNA detected. Pdx-1 is known to regulate transcription of the GLUT-2 gene, and the reduced Pdx-1 protein expression appears to impair the expression of GLUT-2 mRNA. However, the GK expression, at both mRNA and protein levels, was normal and therefore appeared to be unaffected by the reduced levels of Pdx-1 protein.

The reduction in Pdx-1 immunoreactivity does not seem to affect beta-cell development as the beta-cell volume, number and size appeared to be normal. Pax 4 mRNA was overexpressed in these offspring, though not significantly, but this may have been sufficient to ensure normal beta-cell development, despite the reduced Pdx-1 immunoreactivity. In addition, normal alpha-cell development has also been maintained in these offspring.

(iii) Neonates exposed to an *in utero* HFD for the third week of gestation (Group 3)

Normal circulating glucose and insulin levels, similar to the control levels, were found in neonates that were exposed to a maternal HFD for the third week of gestation. This appeared to be attributed to normal expression profiles of the glucose sensing genes, GLUT-2 and GK, both in terms of mRNA levels and immunoreactivity. In addition, Pdx-1 mRNA expression and immunoreactivity was normal. GSIS seemed to be intact, possibly be due to similarity in GK expression profiles to the control levels (both mRNA expression and immunoreactivity), to ensure normal glycolysis. The normal Pdx-1 expression may also have played a role in ensuring the normal regulation of the GK and insulin genes.

These neonates were exposed to a HFD during the final week of gestation, which is reportedly the most quantitatively important phase of islet histogenesis (24). The rapid increase in islet cell mass that occurs in the rat in late foetal and early neonatal life may explain why pancreatic morphology is so sensitive to nutritional

insult at this time (158). Since the expression of Pdx-1 (mRNA and immunoreactivity) and Pax 4 mRNA were normal, the reduced beta-cell number in these offspring appeared to be dietary-induced. It is interesting that alpha-cells were significantly larger, and, since Pax 4 mRNA levels were normal, this effect on alpha-cells may be attributed either to changes in Pax 4 protein expression or to other transcription factors involved in alpha-cell development. An important transcription factor involved in alpha-cell development is Pax 6. Pax 6, which is strongly related to Pax 4, is also required for normal endocrine pancreatic development and both of these transcription factors are required for endocrine cell differentiation (78).

(iv) Neonates exposed to an *in utero* HFD throughout pregnancy (Group 4)

An earlier study demonstrated that when adult rats were fed a HFD (40% of energy as fat) for 4-21 weeks, plasma glucose concentrations in both fasting and postprandial conditions were higher than in rats fed a low fat diet (5% energy as fat) (166). In our study, we found that exposure of foetuses to a high fat (40%) diet throughout pregnancy resulted in raised blood glucose levels compared to animals on a control (10%) fat diet. This overt hyperglycaemia may have resulted from the exposure of these offspring to a maternal HFD for the entire duration of pregnancy. These Group 4 neonates also had the highest circulating insulin levels relative to neonates that were exposed to an *in utero* HFD for a single week of gestation. Immunoreactivity for both Pdx-1 and GLUT-2 was reduced, which was significant in Pdx-1 and lowest for GLUT-2 relative to the other

experimental groups. With less Pdx-1 protein available to transactivate GLUT-2 gene expression, the resultant decreased availability of GLUT-2 on the beta-cell membrane may impair glucose uptake into the beta-cell for metabolism. In addition, the GK immunolabelling was reduced, and appears to have contributed to the hyperglycaemia evident in these neonatal rats by reducing the rate of glycolysis. Although, the Pdx-1 mRNA levels were almost significantly increased ($p = 0.057$), the Pdx-1 protein levels were, however, significantly reduced. These neonates may be trying to compensate for their hyperglycaemia by increasing their Pdx-1 mRNA output, but appear to be unable to translate mRNA into higher levels of Pdx-1 protein expression that would be required to maintain normal expression of the glucose sensing genes, GLUT-2 and GK, and insulin. Therefore, the reduced immunoreactivity for Pdx-1 may also have contributed to the hyperglycaemia.

Morphometric analysis of islets showed that beta-cell volume was significantly reduced in neonates that were exposed to an *in utero* HFD for the entire duration of pregnancy. Furthermore, counting the actual number of beta-cells revealed a significant decrease. It is interesting that, despite the compromised beta-cell development, these neonates displayed normal circulating insulin levels. Hyperglycaemia can result in decreased beta-cell mass by inducing apoptosis (167;168). This may have played a role in the reduction of beta-cell volume in the case of neonates exposed to a HFD throughout pregnancy. In contrast, the alpha-cell volume, number and size appear to be stimulated by a HFD *in utero*

and were all significantly increased in Group 4 neonates. Since these glucagon-secreting alpha-cells are functionally antagonistic to insulin, they could further stimulate glucose release into the circulation and therefore account for the hyperglycaemia in these neonates.

The significant reduction in Pdx-1 immunoreactivity as a result of exposure to a HFD appears, not surprisingly, to have compromised beta-cell development. In these offspring, alpha-cell development appears to be accelerated at the expense of the beta-cell. Apart from Pdx-1, Pax 4 is also required for normal beta-cell development. Interestingly, the Pax 4 mRNA levels were also reduced in these offspring (the lowest levels detected in all of the groups, but not significantly different from the control mRNA levels). Therefore reduced expression of the transcription factors, Pdx-1 (at the protein level) and Pax 4 (at mRNA level) appear to result in retarded beta-cell development. Unfortunately, Pax 4 protein levels were not measured as no commercial antibody was available at the start of this study. This would provide further insight into the role of Pax 4 in beta-cell development in offspring that were exposed to an *in utero* HFD. Furthermore, the expression levels of other transcription factors that are important for beta-cell maturation, such as Nkx 2.2 and Nkx 6.1, as well as Pax 6, which is involved in alpha-cell development, should be determined in future studies to further our knowledge of the effects of an *in utero* HFD on islet cell development. Immunohistochemical studies using antibodies directed against

somatostatin and pancreatic polypeptide would be useful to show us how this *in utero* HFD also affects the delta and pancreatic polypeptide cells respectively.

4.5 Summary of the effects of exposure to an *in utero* HFD on GSIS in neonatal rats

It has been reported that after being fed a high energy diet (2.93 Kcal/g), hyperglycaemic and hyperinsulinaemic rats (*Psammomys obesus*) showed a 71% (after 1 week) and 94% (after 3 weeks) decrease in GLUT-2 immunostaining on the beta-cell membrane (101). It is interesting that a reduction in GLUT-2 immunoreactivity was found in the hyperglycaemic Group 4 neonates, which develops from exposure to an *in utero* HFD throughout gestation.

Another study using a clonal beta-cell line to investigate the impact of glucose levels on Pdx-1 expression in pancreatic beta-cells, showed that chronic hyperglycaemia inhibited Pdx-1 mRNA and protein expression (76). In the study presented here, only neonates that were exposed to a HFD throughout gestation were hyperglycaemic at birth. Although hyperglycaemia may have played a role in the reduced Pdx-1 protein expression, at mRNA level, these offspring had higher expression levels of Pdx-1. The differences modulation in Pdx-1 mRNA levels can be attributed to differences in *in vivo* and *in vitro* models. In our study, the pups were not exposed to chronic hyperglycaemia, but hyperglycaemia appears to be a consequence of the *in utero* HFD exposure.

It has been reported that normal Pdx-1 expression is required in mature beta-cells to maintain insulin production, GLUT-2 expression and glucose homeostasis (75). Furthermore, Pdx-1 has been reported to regulate the expression of GLUT-2 in a dosage dependent manner suggesting that lowered Pdx-1 activity may contribute to the development of Type 2 diabetes by causing impaired expression of both GLUT-2 and insulin (75). In another study, early loss of GLUT-2 expression was shown to be involved in the development of hyperglycaemia in Pdx-1 beta-cell-specific mutants and that the combined loss of GLUT-2 and gradual decrease of insulin expression together was found to lead to the manifestation of diabetes (75). Thus lowered Pdx-1 expression or activity resulting in impaired expression of both GLUT-2 and insulin could cause hyperglycaemia which may progress to Type 2 diabetes (75). In this thesis study, the neonates exposed to an *in utero* HFD throughout pregnancy, had lowered immunoreactivity for Pdx-1 and GK which therefore appears to have contributed to the development of hyperglycaemia. A recent study in MIN6 beta-cells showed that inhibition of GSIS through the inactivation of IGF-1R, resulted in elevated glucose concentrations which regulate preproinsulin, Pdx-1 and GK gene expression in beta-cells (74). These studies showed that released insulin, acting via IR, is likely to be an important mediator of the effects of glucose on Pdx-1 gene expression in normal MIN6 beta-cells (74). However, insulin-independent mechanisms were sufficient to allow the control by glucose of GK gene expression in wild type cells and regulation of the Pdx-1 gene in the absence of IGF-1R (74). Although the insulin levels in the offspring of our study were normal

(only slightly raised) at this stage, the reduced Pdx-1 and GK immunoreactivity, concomitant with the hyperglycaemia, suggests that the Group 4 neonates (that have been exposed to a maternal HFD throughout gestation) would be most susceptible (relative to the neonates exposed to a maternal HFD for only a single week of gestation) to develop Type 2 diabetes later in life if they are unable to maintain glucose homeostasis.

A HFD has been shown to decrease both GLUT-2 mRNA expression (34) and translocation to the plasma membrane (105). Reduced gene expression of GLUT-2 in beta-cells was also observed in neonatal rats treated with streptozotocin (96) and in Goto-Kakizaki diabetic rats (98). Other genetic models of diabetes such as Wistar Kyoto and Zucker diabetic rats (98;102), which are hyperinsulinaemic and hyperglycaemic, express less GLUT-2. Now we have shown that GLUT-2 (although not significant), GK and Pdx-1 immunoreactivity is already reduced at birth in normal Wistar rats exposed to a maternal HFD throughout pregnancy.

4.6 Summary of the effects of exposure to an *in utero* HFD on islet cell development in neonatal rats

An *in utero* protein deficient diet in rats has been shown to reduce pancreatic islet size, islet vascularization and the number of beta-cells (27;28) We have previously shown, in adult Vervet monkeys, a reduction in beta-cell volume after maintenance on a HFD for 18 months (37). Mice, maintained on a high fat/high

protein (HF/HP) diet or a HFD, showed a reduction in beta-cell mass, with increased apoptosis relative to proliferating beta-cells at 20 and 30 weeks of age (169). In rodent models of obesity without diabetes, there is an adaptive increase in beta-cell mass to meet the insulin demands (170). Studies suggest that beta-cell mass is also adaptively increased in non-diabetic obese humans (171). Beta-cell mass is regulated by a balance of beta-cell replication and apoptosis as well as development of new islets from exocrine pancreatic ducts (neogenesis) (172;173). Disruption of any of the pathways of beta-cell formation or increased rates of beta-cell death would result in decreased beta-cell mass and this would reduce the capacity to produce insulin (174). Our data demonstrate compromised beta-cell development in neonates that were exposed to a HFD *in utero* and this suggests that either beta-cell replication pathways are being inhibited by a HFD during gestation, or that apoptosis in the beta-cells is being induced by the HFD, or both. In a study where adult CD-1 mice were injected with streptozotocin to induce hyperglycaemia, islets were shown to gradually lose their regenerative potential when exposed to high circulating glucose concentrations for an extended period of time (175). It may therefore be possible that the hyperglycaemia, evident in Group 4 neonates, may have inhibited neogenesis. Furthermore, increased duration of exposure to a HFD, as in Group 4 neonates, resulted in the most profound inhibition of beta-cell development relative to those neonates only exposed to a maternal HFD for a single week of gestation.

This study demonstrated that *in utero* HFD exposure, for the first or final week of gestation, reduces beta-cell number in neonatal rats. It appears that the longer the period of HFD exposure *in utero*, the greater the inhibitory effect of the HFD on beta-cell development. This was evident in Group 4 neonates that were exposed to a HFD throughout pregnancy, resulting in a significant decrease in beta-cell volume and data suggesting it is due to there being significantly fewer beta-cells. A decrease in beta-cell number would result in an increase in the functional load for each individual beta-cell, thereby making the maintenance of glucose homeostasis harder to achieve. A decrease in beta-cell mass alone, except if it is severe, cannot cause diabetes because of the great reserve for insulin production of the endocrine pancreas (176). A reduced beta-cell mass would however be subjected to a persistently increased functional load, which, in the long term, may exhaust insulin release (176). Providing that beta-cell development is not normalized later in life, we speculate that beta-cell failure could develop in these animals.

Larger alpha-cells were found in neonates exposed to a maternal HFD for the first or final week of gestation. Furthermore, exposure to a maternal HFD throughout pregnancy not only resulted in larger alpha-cells, but also increased alpha-cell volume and number. The HFD, directly or indirectly, appeared to have a stimulatory effect on alpha-cell development. Earlier research reports that volume density of alpha-cells was significantly elevated, with both elevated pancreatic and plasma glucagon concentrations, in weanling female mice

maintained on a high protein diet (177). The increase in alpha-cell volume density and pancreatic glucagon concentration appeared to be due initially to alpha-cell hypertrophy. The authors speculated that these changes were compensatory responses to the increased functional demand on alpha-cells (i.e. glucagon biosynthesis and secretion) imposed by chronic high-protein feeding (177). In our study, it appears that exposure to a maternal HFD throughout pregnancy has induced alpha-cell hyperplasia and hypertrophy resulting in an increase in alpha-cell volume. Pdx-1 immunoreactivity, which was reduced in offspring exposed to a maternal HFD throughout gestation, appears to have resulted in compromised beta-cell development. We therefore speculate the HFD *in utero* may modulate the expression of key genes involved in alpha-cell development thereby resulting in accelerated alpha-cell growth. This concept needs to be investigated in further studies.

Weanlings

4.7 The effect of a HFD *in utero*, and during lactation, on the body weight of weanling rats

In our study, Wistar weanling rats exposed to a HFD during defined periods of gestation followed by a standard laboratory diet after birth were leaner. This may be due to a decrease in the maternal food intake since pregnant dams consumed less food when fed a standard laboratory diet as opposed to the HFD. Foetal metabolism and foetal growth directly depend on nutrients crossing the placenta (134). Since foetal growth is largely determined by maternal nutrition, a lower food intake by the mother would be expected to retard growth in the offspring, particularly during pregnancy and lactation, as these are critical periods of growth in the offspring and in beta-cell development. A recent study reported that weight was significantly decreased in offspring exposed to a maternal low protein diet throughout gestation and lactation in 3-week-old weanling Wistar rats which persisted into adulthood even after the pups were fed a standard laboratory diet (178). The lower body weights in the weanlings of our study correlates well with a study done in mice where body weight increased after high fat feeding, but decreased after low fat intervention (179). The marked reduction in body weight in low fat fed mice was attributed to the lower energy intake (179). During such critical periods, either stimulus or insult may have long-lasting consequences on tissue or organ function postnatally (180).

Exposure to a HFD is known to increase circulating levels of FFA. A HFD may therefore be detrimental to pancreatic development due to an excess, in the diet, of FFA, which apart from increasing body weight by the storage of accumulated fat, also compete with glucose for metabolism (44). A HFD can induce overconsumption and weight gain and the increased caloric intake due to high fat feeding would contribute to excess body weight (136). In a study where 20-22 week old female Wistar rats were fed a moderately high fat (36% energy as fat) diet for 14 weeks, the body weight and total energy intake, notably the dietary fat intake, increased significantly compared to the control (3% fat) diet (135). Fat-rich diets may contribute to early weight gain not only because fat provides the substrate for triacylglycerol accumulation, but also because food intake increases as a result of the reduction in leptin secretion by adipose tissue (135). Indeed, those progeny exposed to a HFD throughout gestation and weaning were significantly heavier and if these rats became obese they could be at risk for the development of Type 2 diabetes mellitus. In contrast, weanlings that were exposed to a maternal HFD throughout gestation and only for a single week of lactation had normal body weights. Offspring exposed to a maternal HFD only throughout lactation also had normal body weights at 3 weeks. This shows that continuous exposure to a maternal HFD throughout both gestation and weaning results in heavier weanlings at 3 weeks.

4.8 The effect of a HFD *in utero* and during lactation on circulating glucose and insulin levels, the expression of transcription factors, glucose sensing genes and islet cell development in weanling rats

(i) Weanlings exposed to an *in utero* HFD for the first week of gestation (Group 6)

Weanling rats, exposed to a maternal HFD for only the first week of gestation, were hyperglycaemic and hypoinsulinaemic. Although immunoreactivity for GLUT-2 was normal, the expression of GLUT-2 mRNA was significantly reduced. Furthermore, both GK mRNA and immunoreactivity were significantly reduced. GLUT-2 transports glucose into the beta-cell, whereby GK phosphorylates glucose ultimately resulting in insulin secretion. The reduction in these glucose sensing genes, GLUT-2 (at mRNA level) and GK (both mRNA expression and immunoreactivity), may therefore have contributed to both the hyperglycaemia and hypoinsulinaemia that was evident in these offspring. We speculate that less glucose would be transported into the beta-cell for metabolism by GLUT-2 and, with the reduced GK levels, the rate of glucose metabolism would decrease thereby resulting in reduced insulin secretion. Pdx-1 mRNA expression and immunoreactivity was normal in Group 6 weanlings. This suggests that Pdx-1 may not be responsible for the reduced expression of the glucose sensing genes, and the ensuing hyperglycaemia and hypoinsulinaemia.

The beta-cell number and size was significantly reduced in weanlings exposed to a maternal HFD for the first week of gestation. The significantly reduced Pax 4

mRNA levels detected in these offspring may, in part, explain the compromised beta-cell development and the smaller alpha-cell sizes, since Pax 4 is an important transcription factor that regulates endocrine cell development. In the Group 6 weanlings, Pdx-1 mRNA expression and immunoreactivity was normal, suggesting that it may not play a role in the compromised islet cell development.

(ii) Weanlings exposed to an *in utero* HFD for the second week of gestation (Group 7)

Hyperglycaemia and hypoinsulinaemia was evident in those offspring exposed to an *in utero* HFD only for the second week of gestation. Low levels of GLUT-2 mRNA were detected (not significant) with immunoreactivity for GLUT-2 appearing to be normal. However, GK mRNA was underexpressed and immunoreactivity for GK was reduced, both significantly. Pdx-1 mRNA expression and immunoreactivity was normal. We speculate that although glucose transport may be adequate (since GLUT-2 immunoreactivity was unaffected by the HFD intervention), the reduced GK expression, both mRNA expression and immunoreactive levels, may be responsible for reduced insulin secretion from the beta-cell. Furthermore, these low serum insulin levels are insufficient to stimulate glucose uptake into the peripheral tissues, therefore hyperglycaemia persists.

Significantly fewer beta-cells, that were also smaller when compared to the control, were found in Group 7 weanlings. Pdx-1 mRNA and immunoreactivity did

not differ significantly from the control levels. However, the highest levels of immunolabelling for Pdx-1 were found in these offspring relative to all the experimental groups. This suggests that the pups may be attempting to improve beta-cell development.

(iii) Weanlings exposed to an *in utero* HFD for the third week of gestation (Group 8)

In weanlings exposed to an *in utero* HFD for only the third week of gestation, hyperglycaemia and hypoinsulinaemia was evident. GLUT-2 mRNA levels were reduced, though not significantly. However, the immunoreactivity for GLUT-2 was significantly increased. Both the GK mRNA expression and protein (determined by image analysis) levels were significantly reduced in these offspring. We postulate that GLUT-2 protein levels have been increased in response to hyperglycaemia in order to restore the plasma glucose levels to within the normal range. However, this compensatory mechanism is not yet effective as some defect in the glucose metabolism pathway seems to exist, resulting in low circulating insulin levels despite the hyperglycaemia. The reduced expression of GK (both at mRNA and protein level) appears to be the defect contributing to the reduced circulating insulin levels. With less GK available within the beta-cell, the rate of glycolysis would decrease, resulting in reduced metabolism of glucose. This may cause reduced GSIS from the beta-cell. It would be interesting to study each step of the GSIS pathway in order to determine whether any other components are affected by the HFD intervention. Another explanation may be

that insulin may not be fully processed resulting in a high proinsulin/insulin ratio in the circulation. Further studies determining the insulin content and insulin secretion would provide further insight into changes that may occur metabolically in the beta-cell after exposure to a HFD. An approximate increase of 70% GLUT-2 protein content was found in islets from 12-day rats fed a high carbohydrate diet (146). A similar increase in GLUT-2 protein was reported in another study in islets of normoglycaemic and hyperinsulinaemic pregnant rats (181). We have now shown that, in weanling rats exposed to a maternal HFD for the final week of gestation, GLUT-2 protein levels were also significantly higher.

Pdx-1 mRNA was significantly overexpressed, with normal immunoreactivity for Pdx-1. It appears that the HFD may, in fact, have a stimulatory effect by increasing the mRNA levels of Pdx-1, but somehow this mRNA is not effectively translated into protein, perhaps due to some post-translational defect. Furthermore, Pdx-1 does not appear to have a role in the reduced expression of the glucose sensing genes. Therefore the reduction in both GK mRNA expression and immunoreactivity, and the hyperglycaemia and hypoinsulinaemia, may be largely due to the HFD.

Pax 4 mRNA was significantly reduced. This, along with the dietary exposure during the final week of gestation, may have caused the reduction in beta-cell number and resulted in the smaller beta- and alpha-cells, which may be via reduced differentiation of ductal cells into islet cells. The Pdx-1 mRNA was

significantly increased and may be an indication that the animal is attempting to correct the compromised islet cell development by increasing the expression of this transcription factor which is not only important for beta-cell development from ductal cells, but also in the maturation of these insulin-producing cells.

(iv) Weanlings exposed to an *in utero* HFD throughout gestation (Group 9)

Circulating glucose levels were normal in weanling rats that were exposed to a maternal HFD throughout gestation. However, these pups were hypoinsulinaemic. Although the GLUT-2 mRNA levels in weanlings exposed to a HFD for the duration of pregnancy were reduced relative to the control, they were much higher compared to the other experimental groups (though not significant). GLUT-2 immunoreactivity, however, was significantly higher. It appears that glucose transport, via GLUT-2 compensatory overexpression at protein level, has succeeded in maintaining glucose homeostasis. However, the circulating insulin levels were significantly reduced. Both GK and Pdx-1 mRNA expression and immunoreactivity remained intact. It appears that the hypoinsulinaemia may be due mainly to the HFD because expression of GK and Pdx-1 was normal (both mRNA and immunoreactivity) in these weanlings.

Although the Pax 4 mRNA was significantly reduced in Group 9 weanlings, both beta-cell and alpha-cell development were normal. This may be explained by the normal expression of Pdx-1, which is considered the master regulator of beta-cell development and maintenance.

(v) Weanlings exposed to a maternal HFD throughout gestation and for the first week of lactation (Group 11)

Weanlings exposed to a maternal HFD throughout pregnancy and continued into the first week of lactation, were hyperglycaemic and hypoinsulinaemic at 3 weeks of age. Although GK mRNA expression was highest amongst the experimental groups, it did not differ significantly from the control levels. However, immunoreactivity for GK was significantly lower. GLUT-2 overexpression at protein level (i.e. immunoreactivity), which would increase glucose transportation into the beta-cell, appears not to be adequate to reduce the circulating glucose levels, as hyperglycaemia persists in these offspring. This overexpression may be an adaptive response to reduced GK immunoreactivity, which would definitely play a role in inhibiting insulin secretion from the beta-cell, hence the hypoinsulinaemia. Pdx-1 mRNA expression and immunoreactivity profiles were similar to those of the control. The HFD intervention therefore appeared to have little effect on the gene expression of Pdx-1 or on the Pax 4 mRNA levels which were also normal.

(vi) Weanlings exposed to a maternal HFD throughout gestation and for the second week of lactation (Group 12)

Weanlings that were exposed to a maternal HFD for the entire duration of pregnancy, and for the second week of lactation, were hyperglycaemic, but had normal circulating insulin levels. GLUT-2 mRNA was reduced, but immunoreactivity for GLUT-2 was increased, both significantly. Both GK mRNA

expression and immunoreactivity were significantly reduced. It appears that GLUT-2 overexpression at protein level is an adaptive response to reduce the hyperglycaemia. Despite the reduced levels of GK, insulinaemia remained intact. Pdx-1 mRNA expression and immunoreactivity, and Pax 4 mRNA expression were all normal. Since expression of Pdx-1 was normal, the regulation of the insulin gene should be normal, and is at this stage, sufficient to ensure normal circulating insulin levels. However, hyperglycaemia was evident, which may indicate that insulin may not be fully functional or perhaps not fully processed by the beta-cell. Measurement of proinsulin or C-peptide in these offspring would provide further insight.

(vii) Weanlings exposed to a maternal HFD throughout gestation and for the third week of lactation (Group 13)

Hyperglycaemia had developed in weanling rats exposed to a maternal HFD throughout gestation, and for the final week of lactation, but the circulating insulin levels remained normal. Expression for GLUT-2 was significantly reduced at mRNA level, but significantly upregulated at protein level. GK mRNA expression and immunoreactivity were both significantly reduced. The HFD appears to have induced hyperglycaemia and reduced gene expression of GK. GLUT-2 overexpression at protein level appears to be an adaptive response, which is unsuccessful for effective glucose clearance and transport into the beta-cell. No significant differences were found in Pdx-1 mRNA expression or immunoreactivity, or in Pax 4 mRNA expression.

(viii) Weanlings exposed to a maternal HFD throughout both gestation and lactation (Group 14)

Exposure to a maternal HFD throughout both gestation and weaning seemed to have little effect on the blood glucose concentration, but did reduce the serum insulin levels significantly in weanling rats. GLUT-2 mRNA levels were significantly reduced, but there was a significant increase in GLUT-2 immunoreactivity. It therefore appears that these pups are trying to increase insulin secretion by producing more GLUT-2 protein, thereby not only clearing glucose from the circulation, but also transporting more glucose into the beta-cell for metabolism. GK mRNA expression and immunoreactivity, however, were both significantly reduced. Therefore the rate of glycolysis would be reduced which may contribute to the hypoinsulinaemia. Although Pdx-1 mRNA was significantly overexpressed (highest amongst all of the groups), Pdx-1 immunoreactivity remained unaltered.

Pax 4 mRNA maintained normal expression levels. The effects of the HFD on islet cell development, as well as on Pax 4 protein expression, needs to be elucidated in future studies. It appears that the animal has managed to adapt to a HFD i.e. it has been programmed *in utero* to respond to a HFD. Hypoinsulinaemia is already evident in these offspring and the HFD has promoted a weight gain which would contribute to the development of obesity and insulin resistance. We postulate that if animals were to be maintained on a

HFD until and during adulthood, they may eventually reach a point of saturation where hyperglycaemia becomes manifested.

(ix) Weanlings exposed to a maternal HFD throughout lactation (Group 15)

Pups that were exposed to a standard laboratory diet throughout pregnancy, then to a HFD throughout lactation were hyperglycaemic with normal circulating insulin concentrations at 3 weeks of age. GLUT-2 mRNA expression was reduced, with higher immunoreactivity, both significant changes. Although GK mRNA was significantly underexpressed, immunoreactivity for GK was normal. HFD exposure only for the duration of lactation was sufficient to induce hyperglycaemia in these offspring. Again, as in the previous groups that were exposed to a maternal HFD during pregnancy and for defined periods of weaning, the GLUT-2 overexpression at protein level does not seem to be adequate and cannot restore glucose homeostasis. Similar to GK, Pdx-1 mRNA expression was significantly lower, but immunoreactivity was normal. This suggests that reduced Pdx-1 transcription may have resulted in both the reduction of GLUT-2 and GK mRNA, as Pdx-1 is a key regulator of expression of these genes. Pax 4 mRNA was also significantly underexpressed and was lowest amongst the groups. It would be interesting to determine whether islet cell development is compromised in these offspring, as two of their key transcription factors were downregulated at mRNA level.

4.9 Summary of the effects of exposure to a HFD *in utero* and during lactation on GSIS in weanling rats

Hyperglycaemia, hypoinsulinaemia, reduced expression of GK mRNA and immunoreactivity all occurred in weanling rats that were exposed to a maternal HFD for either the first (Group 6), or second (Group 7) or third (Group 8) week of gestation. Exposure to the HFD during these critical periods of pancreatic development resulted in low circulating insulin levels that appear to be unable to counteract the hyperglycaemia.

Glucose utilization results in insulin release from the beta-cell which stimulates the uptake of glucose into the peripheral tissues thereby maintaining glucose homeostasis. These raised glucose levels were accompanied by relatively normal GLUT-2 immunoreactivity but low insulin levels in Group 6, Group 7 and Group 8 weanlings. This suggests that, although glucose transport may be adequate, some component of GSIS is compromised. This is supported by another study where the plasma insulin concentrations were lower, in adult rats fed a HFD, and the glucose levels were higher when compared to those on a high carbohydrate diet (34). Impaired expression of GLUT-2 may impair glucose sensing and result in hyperglycaemia (103). However, in these groups, the GLUT-2 immunoreactivity was normal, but hyperglycaemia persisted, suggesting that the plasma insulin levels are insufficient to stimulate glucose uptake into the peripheral tissues.

In female Sprague-Dawley rats, fed a HFD (58% energy as fat) for 8 weeks, the body weight, insulin levels and triglycerides increased significantly (182). A longer term HFD induces a progressive impairment of insulin secretion *in vivo* (182). Furthermore, FFA are known to stimulate insulin secretion on a short-term basis (128) or inhibit insulin secretion on a long-term basis (183). The impairment of both insulin gene expression and insulin secretion suggests that a HFD is strongly diabetogenic in rodents (182). In our study, weanlings that had maximum exposure to a maternal HFD (Group 14) had significantly reduced circulating insulin concentrations, which may be due to a reduction in insulin gene expression. This appears to occur via a Pdx-1 independent mechanism as Pdx-1 immunoreactivity was normal in these offspring. It is also possible that FFA derived from the metabolism of the HFD may have inhibited insulin secretion. The mechanism appears to be via reduced expression of the glycolytic enzyme, GK.

An extensive study on yeast, which examined the relationship between yeast protein and message levels, revealed that the transcript levels provide little predictive value with respect to the extent of protein expression (184). The authors speculated that there was no predictive correlation between steady-state levels of mRNA and those of protein in mammalian cells (184). Similarly, we do not find a correlation in GLUT-2 mRNA and protein expression (immunoreactivity) in most of the weanling rats used in this study. GLUT-2 mRNA was significantly underexpressed, but in contrast, immunoreactivity for GLUT-2

was significantly increased in some of the weanling groups. It therefore appears that the HFD exposure inhibits transcription of the GLUT-2 gene but stimulates the translation of GLUT-2 into protein.

A HFD *in utero* and/or during lactation resulted in significant differences in the circulating glucose and insulin concentrations and in the expression of the glucose sensing genes, GLUT-2 and GK. This suggests that metabolic changes are taking place, enabling the animal to physiologically adapt in order to restore glucose homeostasis in response to the HFD.

Pdx-1 mRNA expression and immunoreactivity were relatively normal in most of the weanlings, the only significance detected at mRNA level, in those exposed to the HFD for the third week of gestation (increased in Group 8), throughout both gestation and lactation (increased in Group 14) or throughout lactation (reduced in Group 15). Therefore Pdx-1 expression in most of these weanlings does not appear to be markedly changed by the HFD. The changes in the expression levels of GLUT-2 and GK therefore seem to be altered by the HFD in a Pdx-1-independent manner. Pax 4 mRNA was significantly reduced in weaned rats exposed to a HFD for the first (Group 6) or third (Group 8) week of gestation, throughout gestation only (Group 9) or throughout lactation only (Group 15). The reduction in Pax 4 mRNA appears to have contributed to the smaller alpha-cells in Group 6 and Group 8 weanlings. The reduced mRNA expression levels of the

Pdx-1 and Pax 4 genes, in Group 15 weanlings, may inhibit the development and maturation of the islet cells. This needs to be investigated further.

Hyperglycaemia is the clinical hallmark of Type 2 diabetes. Furthermore, reduced expression of the glucose sensing genes, GLUT-2 and GK, would inhibit glucose transport and glycolysis, respectively, in the beta-cell. This, in turn, would limit insulin secretion from the beta-cell. Therefore, in offspring characterized by these conditions, beta-cell failure may eventually develop. Therefore it is important to monitor these changes in transcription factor and glucose sensing gene expression, as the animal ages. A HFD is known to decrease expression of Pdx-1, GLUT-2 and GK and promote weight gain in adult rats (34;105;135). This has been shown in some of the weanlings used in this study, and together with aging, will make these offspring prime candidates for the development of Type 2 diabetes. However, the pancreas has the ability to adapt to increased metabolic needs in terms of increased insulin demand that occurs during pregnancy, aging, high fat feeding and diabetes (or hyperglycaemia). Furthermore, a significant reduction in beta-cell mass is not sufficient to induce diabetes due to the great reserve of the pancreas. But with time, the pancreas may be exhausted by the great demand for insulin, which could occur by continuous high fat feeding and this would subsequently lead to Type 2 diabetes.

4.10 Summary of the effects of exposure to a HFD *in utero* and during lactation on islet cell development in weanling rats

Weanling rats exposed to a maternal HFD for either the first (Group 6), or second (Group 7) or third (Group 8) week of gestation had significantly fewer beta-cells which were also smaller. This compromisation in beta-cell development would inhibit beta-cell function, the main one being the maintenance of glucose homeostasis. The hypoinsulinaemia which occurred in these offspring appeared to play a role in the hyperglycaemia that was evident. In addition, these offspring exhibited reduced expression for GK (both mRNA expression and immunoreactivity), suggesting a reduced rate of glycolysis in the beta-cell. This would contribute to reduced insulin secretion and hypoinsulinaemia. Furthermore, significantly smaller alpha-cells were found in these offspring. Apart from *in utero* HFD exposure, Pax 4 also appears to have played a role in the small alpha-cell sizes, as the mRNA expression of this transcription factor was reduced significantly in Group 6 and Group 8 weanlings.

In weanlings exposed to a maternal HFD throughout gestation (Group 9 weanlings), both beta-cell and alpha-cell development appeared to be normal. Although Pax 4 mRNA was significantly underexpressed, it did not affect islet cell development. Pdx-1 expression, at both mRNA and immunoreactive levels, were similar to the control levels, and appeared to be adequate to ensure normal development of both the beta- and alpha-cells.

No data is available yet on the effects of a HFD on islet cell development in the offspring that had dietary insult *in utero* and/or during specific periods of lactation (Groups 11-15). This will be explored in further studies. Since the corresponding pancreatic weights are available, we shall be able to determine the beta-cell and alpha-mass in these offspring, in addition to the other parameters of islet cell development that were studied in the other groups.

Neonates and weanlings

4.11 Weights of offspring at birth and 3 weeks of age

Groups were done in parallel, with some offspring euthanased at birth (postnatal day 1) after exposure to a HFD *in utero*, and other groups, that had the same exposure to a HFD *in utero*, monitored after weaning (3 weeks of age). This allowed us to detect any changes or adaptations that may have occurred in these offspring.

In neonatal rats, we showed that exposure to an *in utero* HFD for only the first week of gestation resulted in low birth weight (Group 1 neonates). Group 6 weanlings had identical HFD exposure to Group 1 neonates, and were exposed to standard laboratory diet for the duration of weaning. At weaning, Group 6 weanlings were also leaner. The other weanlings that were exposed to a maternal HFD during gestation, then to a standard laboratory diet during weaning, had normal birth weights (Group 2, Group 3 and Group 4 neonates), but had reduced body weights at 3 weeks of age (Group 7, Group 8 and Group 9 weanlings). In contrast, offspring that were exposed to a maternal HFD throughout both gestation and weaning (Group 14 weanlings), which normal birth weights (Group 4 neonates), were significantly heavier at 3 weeks of age. Our data indicate that dams consumed more food when fed a HFD. This suggests that exposure to the maternal HFD during lactation resulted in a significant weight gain in Group 14 weanlings. The exposure of these weanling rats to the increased amount of fat from the HFD would contribute to the body weight gain.

These rats may be prone to become obese later in life, which would make them prime candidates to develop Type 2 diabetes.

4.12 The effect of a maternal HFD on the circulating glucose and insulin concentrations in neonatal and weanling rats in relation to the expression of the transcription factor, Pdx-1, and glucose sensing genes, GLUT-2 and GK

Although changes in the serum insulin concentrations in pups that were exposed to an *in utero* HFD were detected at birth, these were not significant. However, hypoinsulinaemia was evident in these pups at 3 weeks of age. This reduction in the circulating insulin levels appeared to be responsible for the hyperglycaemia that was evident in weanlings that were exposed to a maternal HFD for the first, or second or third week of gestation. In order to understand why the circulating insulin levels had been reduced, we needed to examine the expression levels of the genes involved in GSIS, GLUT-2 and GK, and also Pdx-1, which is the transcription factor that not only regulates insulin gene transcription, but is also reported to regulate GLUT-2 and GK gene expression.

(i) Group 1 neonates and Group 6 weanlings

Hypoglycaemia was evident at birth in offspring that were exposed to a maternal HFD for only the first week of gestation but, between birth and weaning, these pups had already developed hyperglycaemia. Their reduced (though not significant) insulin levels at birth had become significantly reduced at 3 weeks of

age. The expression of GLUT-2 mRNA, which was normal at birth, was significantly reduced once they reached weaning. Furthermore, the GK mRNA levels were significantly reduced both at birth and weaning, but the Pdx-1 mRNA levels remained normal at both birth and weaning. Both Pdx-1 and GLUT-2 immunoreactivity were normal at birth and weaning, but GK immunoreactivity, which was significantly overexpressed at birth, was significantly underexpressed at weaning.

The change in the circulating glucose levels, that were significantly low at birth then significantly high at weaning, can be attributed to the GLUT-2 mRNA levels which were normal at birth but reduced significantly by the time the pups were weaned. Meanwhile, GK mRNA expression was also significantly reduced at birth and remained underexpressed at weaning, while GK immunoreactivity was significantly overexpressed at birth but significantly reduced at weaning. Underexpression of the glucose sensing genes, GLUT-2 (at mRNA level) and GK (both mRNA expression and immunoreactivity) seem therefore to have been the cause of these offspring developing hyperglycaemia at weaning. The reduced circulating insulin levels may be due to the significant underexpression of GLUT-2 and GK mRNA and the significant reduction of GK immunoreactivity, limiting the rate of glycolysis within the beta-cell. We speculate that all of these changes occur because GSIS is compromised. Pdx-1 mRNA expression and immunoreactivity was normal, and therefore does not seem to play a role in the hypoinsulinaemia evident in Group 6 weanlings.

(ii) Group 2 neonates and Group 7 weanlings

In offspring that were exposed to an *in utero* HFD for the second week of gestation, the hypoglycaemia at birth changed to hyperglycaemia at weaning while low circulating insulin levels deteriorated even further. In these offspring, GLUT-2 mRNA levels were reduced at birth and weaning, though not significantly; GK mRNA expression was normal at birth but significantly reduced at 3 weeks of age; Pdx-1 mRNA was normal both at birth and weaning while Pdx-1 immunoreactivity was significantly reduced at birth but increased to normal levels at weaning. GLUT-2 immunoreactivity was normal at birth and weaning and GK immunoreactivity was normal at birth but significantly reduced at weaning. From this we speculate that the development of hyperglycaemia and the deteriorating insulin levels in these offspring at weaning may be attributed to the significantly reduced GK mRNA expression and immunoreactivity which would impair glycolysis and GSIS. The hypoinsulinaemia would lead to hyperglycaemia because of the reduced availability of insulin to stimulate glucose uptake into peripheral tissues. It is possible that the very low, though not significant, GLUT-2 mRNA levels that were evident both at birth and weaning may contribute to the hyperglycaemia by reduced transport of glucose into the beta-cell for metabolism. The effect of the reduced GK immunoreactivity would be to limit the metabolism of glucose within the beta-cell. However, Pdx-1 immunoreactivity, which was reduced at birth, had increased to normal levels in these pups at weaning possibly to increase transcription of the GK and insulin

genes in order to restore glucose homeostasis and the insulin levels to within the normal range.

(iii) Group 3 neonates and Group 8 weanlings

In offspring that were exposed to a maternal HFD for the third week of gestation, the normal circulating glucose and insulin levels measured at birth had deteriorated at weaning into hyperglycaemia and hypoinsulinaemia respectively. GLUT-2 mRNA expression was normal at birth and weaning. Pdx-1 mRNA expression was normal at birth, but was significantly higher at weaning, while GLUT-2 immunoreactivity appeared normal at birth but was significantly higher at weaning. Both GK mRNA expression and immunoreactivity were normal at birth but reduced significantly at weaning, while Pdx-1 immunoreactivity was normal at both birth and weaning.

It appears that GLUT-2 protein (as determined by image analysis) was overexpressed in these weanlings in an attempt to increase the rate of glucose transport into the beta-cell for metabolism. Pdx-1 mRNA was also overexpressed, suggesting that these offspring may be trying to increase protein expression of Pdx-1 as this important transcription factor has been reported to regulate the GLUT-2, GK and insulin gene expression. Although Pdx-1 mRNA levels were overexpressed, the increase in functional Pdx-1 protein, that would be required to increase transcription of the GK gene, did not occur. The levels of immunoreactive GLUT-2 protein had already been increased in response to the

hyperglycaemia and hypoinsulinaemia, for improved glucose clearance from the circulation for metabolism within the beta-cell. However, the reduced GK immunoreactivity appears to compromise glycolysis and GSIS.

(iv) Group 4 neonates and Group 9 weanlings

In offspring that were exposed to an *in utero* HFD for the entire duration of pregnancy, hyperglycaemia, which was evident at birth, had been normalized at weaning. The normal insulin levels at birth, however, were significantly reduced in these pups at weaning. The restoration of hyperglycaemia to normal in these offspring at weaning can be attributed to the normal GK and Pdx-1 immunoreactivity, which were both reduced at birth. This may be aided by the low GLUT-2 immunoreactivity (lowest amongst all of the groups) at birth being significantly increased at weaning, resulting in increased glucose transport into the beta-cell, and the restoration of glucose homeostasis. Because the glucose levels had been normalized at weaning, the mRNA expression and immunoreactivity for Pdx-1 and GK were normal despite the low circulating insulin levels. With hyperglycaemia at birth, both GK and Pdx-1 immunoreacitvity were significantly reduced, while normal glucose levels at weaning were accompanied by normal GK and Pdx-1 immunoreactivity. This could suggest that high blood glucose levels play a role in the modulation of both Pdx-1 and GK gene expression and it would be interesting to see whether expression of these genes is modulated as the animal matures.

(v) Group 4 neonates and Group 14 weanlings

In offspring that were exposed to a maternal HFD throughout both gestation and weaning, hyperglycaemia at birth had normalized at weaning. The normal insulin levels at birth had decreased to hypoinsulinaemia at weaning. In these offspring, GLUT-2 immunoreactivity had been increased significantly between birth and weaning and this seems to be a sufficient compensatory mechanism at this stage, to restore glucose homeostasis. However, GLUT-2 mRNA had been significantly reduced, and the effect of this may soon impede the translation of GLUT-2 into protein. Additionally, the significant reduction in GK mRNA levels and immunoreactivity could result in a decrease in glycolysis in the beta-cell. With less glucose being metabolized in the beta-cell, less insulin will be secreted, and this may explain, in part, why the hypoinsulinaemia was evident in these weanling rats. Significantly higher Pdx-1 mRNA expression was detected in these offspring, with Pdx-1 immunoreactivity normal at weaning. Although the HFD may have a stimulatory effect on Pdx-1 mRNA expression (as these weanlings were exposed to a maternal HFD for their entire lives), we speculate that these animals may be attempting to increase protein synthesis of Pdx-1 in order to increase the expression levels of the GLUT-2, GK and insulin genes, therefore facilitating the maintenance of glucose homeostasis. The underexpression of GLUT-2 and GK mRNA may be associated with the hypoinsulinaemic status evident in these weanling rats.

4.13 The effect of a maternal HFD on islet cell development in neonatal and weanling rats in relation to expression of Pdx-1 and Pax 4, transcription factors that are involved in islet cell development

(i) Group 1 neonates and Group 6 weanlings

In pups that were exposed to a maternal HFD for the first week of gestation, beta-cell volume was not significantly reduced, but the beta-cell number and size was significantly reduced, both at the neonatal and weanling stages of life. Pdx-1 immunoreactivity was normal both at birth and weaning. However, Pax 4 mRNA, that was normal at birth, was significantly underexpressed at weaning. This may be responsible for inhibiting the development of the beta-cells, as Pax 4 is required for beta-cell maturation. These smaller beta-cells may be immature and therefore unable to secrete sufficient insulin, contributing to the hyperglycaemia in these pups at weaning. Pax 6 requires the expression of Pax 4 in order for alpha-cells to develop normally. The reduced Pax 4 mRNA expression may therefore account for the smaller alpha-cells. It is only by comprehensive recording of Pax 4 protein expression, and transcription factors associated with alpha-cell development, plus the circulating levels of glucagon secreted by the alpha-cells to increase glucose levels, that can one start to elucidate the mechanisms of all of the changes observed.

(ii) Group 2 neonates and Group 7 weanlings

In offspring exposed to a maternal HFD for the second week of gestation, beta-cell number and size, which appeared to be normal at birth, had significantly been reduced at weaning. The beta-cell volume, normal at birth, was also reduced at weaning, though not significantly. Alpha-cell size, which was larger at birth, had also been reduced significantly at weaning. The data indicate that these changes may be independent of Pdx-1 and Pax 4 expression as the profiles of these transcription factors were not significantly altered in weanling rats. The expression of other transcription factors involved in beta-cell maturation, such as Nkx 2.2 and Nkx 6.1 may have been altered by the HFD exposure during the second week of gestation. This needs to be investigated in further studies.

(iii) Group 3 neonates and Group 8 weanlings

In offspring exposed to a maternal HFD for the third week of gestation, there were significantly fewer beta-cells, at both birth and weaning, and normal beta-cell sizes measured at birth were significantly reduced at weaning. In the weanlings, Pdx-1 mRNA expression, which was normal at birth, was significantly increased at weaning. This suggests that the offspring may be attempting to increase the low protein levels for Pdx-1 so that the beta-cells may develop and mature normally. However, the significant underexpression of Pax 4 mRNA may be the cause of both the compromised beta-cell development, and the reduced alpha-cell sizes in these weanlings. The reduced beta-cell size and number in

these weanlings may be associated with the hyperglycaemia and hypoinsulinaemia in the weanlings as there would be reduced capacity for the beta-cells to secrete insulin in order to maintain glucose homeostasis.

(iv) Group 4 neonates and Group 9 weanlings

In pups that were exposed to a maternal HFD throughout gestation, the significantly reduced beta-cell volume and number at birth were all normalized at weaning. Elevated FFA have already been reported to contribute to the reduction in beta-cell mass that accompanies diabetes (54) and may well have played a role during gestation. After birth, the mothers were maintained on a standard laboratory diet and therefore ingested less food, and this appears to have contributed to the normalization of beta-cell development at weaning, which shows that the effect may be reversed by ingestion of a diet with a lower fat component. In order to understand how these changes occurred, we need to examine the data on Pdx-1 and Pax 4 expression. At birth, Pdx-1 immunoreactivity was reduced and could be the cause of the compromised beta-cell development, although other transcription factors involved in beta-cell development cannot be excluded. However, the almost significant increase in Pdx-1 mRNA levels in these neonates appears to have been an attempt to increase expression of Pdx-1 protein in order to correct and maintain normal beta-cell development. It is interesting that, once these pups have been weaned, normal expression of both Pdx-1 mRNA and protein occurred and the resultant normalization of beta-cell development in terms of volume, number and size was

evident. This highlights the retained ability, at this stage, to initiate an adaptive response to correct the compromised beta-cell development that was evident at birth. Although hyperglycaemia existed in these offspring at birth, their blood glucose levels were restored to normal, at weaning, probably associated with the improved beta-cell development. However, hypoinsulinaemia, had developed by the time these pups were weaned, suggesting that, although beta-cell development may be adequate in these weanlings, the beta-cells may not be fully mature. This is supported by the significant underexpression of Pax 4 mRNA which is required for normal beta-cell maturation. As previously discussed, GLUT-2 immunoreactivity (protein expression levels) had been increased for increased glucose transport. The glucose- or lipid-infused rat model is used to study beta-cell mass and alterations in islet-specific gene expression, and an increase in insulin secretion at the renormalized glucose level was thought to be due to an increase in the catalytic rate of GK, GK being the rate limiting factor for glucose entry into the beta-cell (185;186). However, no significant changes in GK mRNA expression and immunoreactivity were evident in Group 9 weanlings, and this may contribute to the hypoinsulinaemia. An increase in GK gene expression would increase the rate of glycolysis and therefore induce more GSIS which may return the circulating insulin levels to normal.

4.14 The effects of maternal HFD exposure during lactation on glucose sensing genes in 3-week-old weanling rats (Group 9 and Group 14)

A comparison of groups of weanling rats, exposed to a maternal HFD throughout pregnancy, followed by a standard laboratory diet (Group 9) or a HFD (Group 14) for the duration of lactation, showed that hyperglycaemia at birth had been normalized in both groups of weanlings, with hypoinsulinaemia, however, evident in both. Expression of GLUT-2 and GK mRNA were further reduced in Group 14 weanlings, after continued exposure to a HFD during lactation, while the mRNA profiles of these glucose sensing genes remained normal in Group 9 weanlings that were exposed to a standard laboratory diet during weaning. Several studies have shown that HFD-induced obesity and insulin resistance in rodents can be improved by reducing dietary fat content (187-189). In rats, a reduction in dietary fat from 40% to 30% of total energy was reported to rapidly reverse insulin insensitivity, as determined by glucose tolerance (188). Another study done in rats showed an almost linear relationship between the amount of dietary fat and glucose, with a threshold between 30% and 40% energy provided as fat (189). In a recent study, insulin resistance was induced by high fat feeding for 20 weeks in male C57BL/6J mice (179). The mice were then fed a low fat diet or maintained on a HFD for a further 12 weeks (179). Glucose tolerance and insulin sensitivity improved more efficiently with low fat feeding (179). Low fat feeding resulted in a decrease in both plasma glucose and insulin levels (179). Plasma glucose levels were reduced to severely low levels in low fat-fed mice, resulting in severe hypoglycaemia (179). Our Group 9 weanlings were first exposed to a HFD (40%

as energy) throughout gestation then to a low fat diet (10% as energy) for the duration of lactation. Within a 3 week period, glucose homeostasis was restored, which emphasizes the importance of low fat feeding particularly in early developmental stages. Moreover, it shows that the animals have the ability to restore glucose homeostasis once the HFD insult is eliminated, which may have practical applications for patients with Type 2 diabetes. The data show that exposure to a HFD during weaning has a deleterious effect on the glucose sensing genes, GLUT-2 and GK, at the level of mRNA transcription. This was demonstrated in Group 14 weanlings that were permanently exposed to a maternal HFD.

CHAPTER 5

CONCLUSIONS, PERSPECTIVES AND FUTURE STUDIES

Conclusions

5.1.1 Mothers

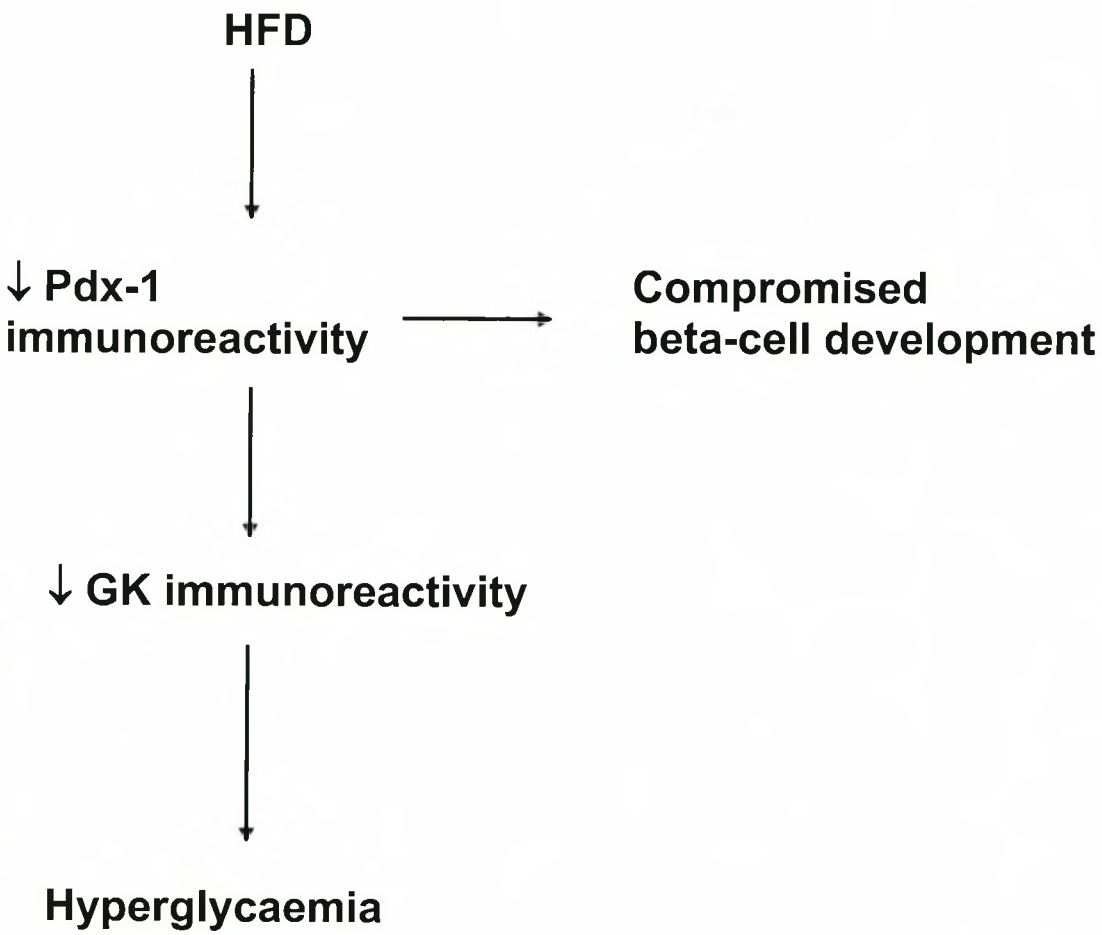
A HFD appeared more palatable and induced hyperphagia in pregnant dams. Dams fed a HFD throughout pregnancy were heavier after giving birth. In addition, high fat feeding in pregnant dams induced hyperglycaemia. High fat feeding therefore had a diabetogenic effect in pregnant dams.

5.1.2 Neonates

The leaner neonates, exposed to a HFD for the first week of gestation, may be susceptible to develop diabetes later in life. Exposure to a maternal HFD throughout gestation had the most adverse effect on beta-cell development in neonates which may be explained, in part, by the reduced Pdx-1 immunoreactivity (Fig. 36). Furthermore, these neonates were hyperglycaemic contributed by impaired glycolysis as GK immunoreactivity was reduced. Of all the neonates studied these were the most susceptible to beta-cell failure in adulthood.

Fig. 36 The effects of an *in utero* HFD on beta-cell development and function in neonatal rats

Neonates that were exposed to an *in utero* HFD throughout gestation (Group 4) were hyperglycaemic. The HFD exposure throughout gestation resulted in reduced immunoreactivity for Pdx-1, a transcription factor required for beta-cell development and maintenance. This reduction in Pdx-1 expression appears to have contributed to the compromised beta-cell development, evident by the reduction in both beta-cell volume and number in these neonates. Furthermore, Pdx-1 is reported to regulate the expression of the GK gene, therefore the reduced Pdx-1 levels may, concomitant with the HFD exposure *in utero*, be responsible for the reduced GK immunoreactivity. Hyperglycaemia ensues due to a decrease in the rate of glycolysis thereby causing low GSIS inadequate to maintain glucose homeostasis. Since Pdx-1 also regulates the expression of the insulin gene, it may be possible that the reduction of Pdx-1 results in reduced insulin gene expression. A HFD may, also, directly inhibit beta-cell development and reduce GK expression, thereby inducing hyperglycaemia.



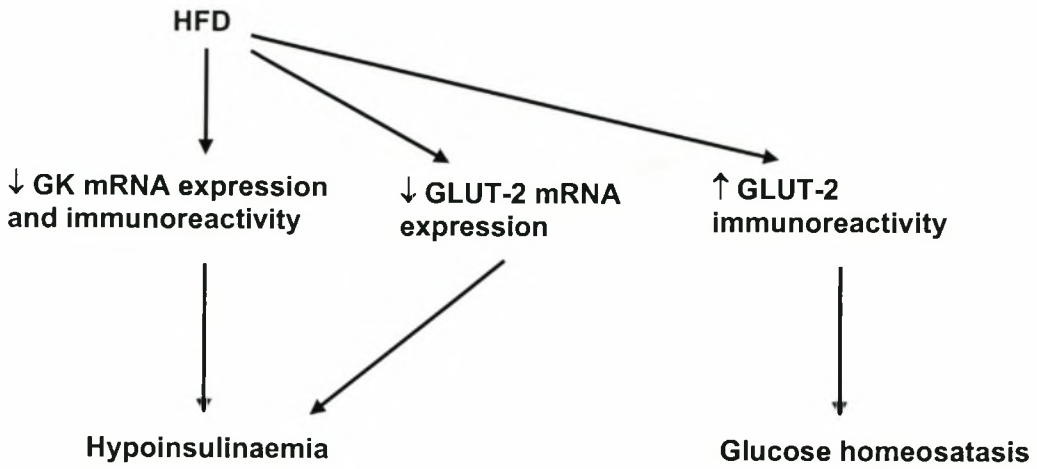
5.1.3 Weanlings

Weanlings that were only exposed to a HFD during defined periods of gestation were leaner, suggesting that the reduced maternal food intake may play a role as the dams were maintained on a standard laboratory diet for the duration of lactation. These weanlings were all hypoinsulinaemic, associated with hyperglycaemia in those exposed for only a single week of gestation. A reduction in both GK mRNA expression and immunoreactivity was evident in these weanlings therefore impairing glycolysis and, ultimately, insulin secretion. The low circulating insulin levels appear unable to stimulate glucose uptake in peripheral tissues therefore hyperglycaemia persisted. In addition, beta-cell number and size were both reduced, increasing the functional load for each beta-cell and thus contributing to hyperglycaemia.

Weanlings that were exposed to a HFD throughout gestation and continued for a single week of lactation were hyperglycaemic which was also associated with reduced GK expression. Exposure to a maternal HFD, from conception until weaning, induced an increase in the weight of progeny at 3 weeks of age and was associated with adverse effects on the mRNA expression of glucose sensing genes (Fig. 37). In contrast, those offspring weaned on a standard laboratory diet were able to normalize the expression of the glucose sensing genes. This demonstrates the adverse effects of high fat feeding during lactation and its ability to modulate gene expression.

Fig. 37 The effects of a maternal HFD on beta-cell function in weanling rats

Weanlings exposed to a maternal HFD throughout gestation and lactation (Group 14) had reduced mRNA expression of both glucose sensing genes, GLUT-2 and GK. It appears that the rate of glycolysis within the beta-cell is adversely affected by the HFD resulting in reduced GSIS, and subsequently, hypoinsulinaemia ensues. Interestingly, GLUT-2 was overexpressed at protein level (as determined by image analysis) suggesting an adaptative response by which glucose homeostasis is being maintained in these weanlings.



This highlights the importance of maternal nutrition during the early developing stages of life in progeny. Exposure to a maternal HFD *in utero* (and/or lactation) has adverse effects on the expression of beta-cell transcription factors, glucose sensing genes and islet cell development in some neonatal and weanling offspring which may predispose them to beta-cell failure and the development of Type 2 diabetes later in life.

5.2 Perspectives

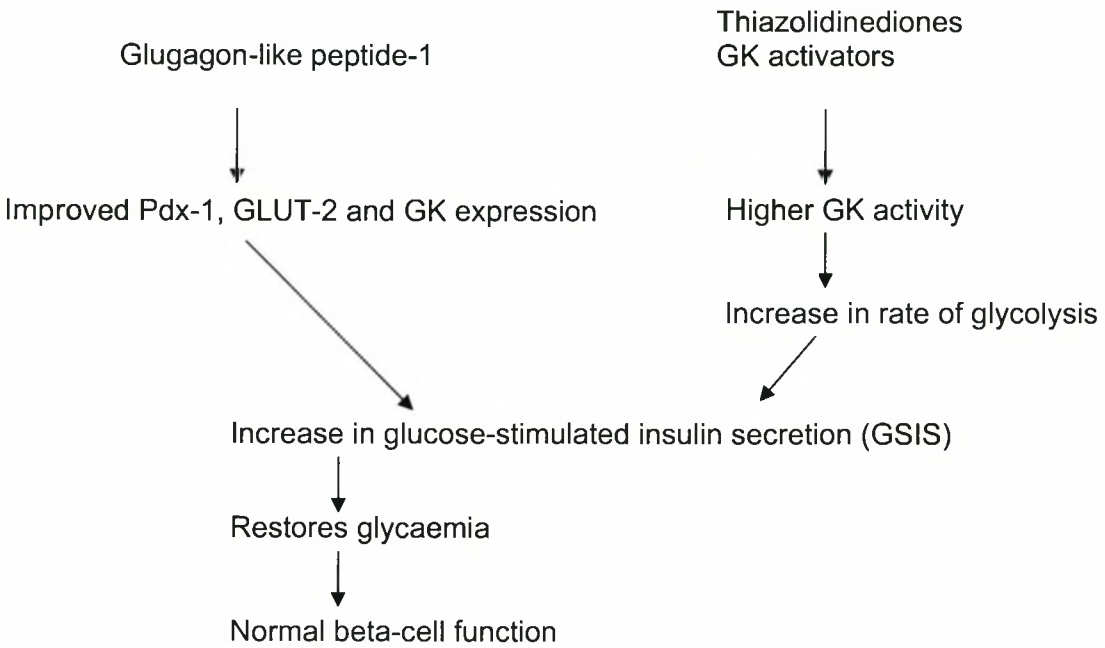
This study has demonstrated the importance of maternal nutrition for normal development of the endocrine pancreas and for beta-cell function in young offspring. A HFD *in utero* was shown to have adverse effects inducing hyperglycaemia and hypoinsulinaemia in some of the offspring. Furthermore GK mRNA and immunoreactivity was reduced in most of the weanlings coupling the hypoinsulinaemia to the hyperglycaemia. Glucose sensing is the initial event of GSIS and it is therefore important that adequate expression of GLUT-2 and GK is maintained for ensuring optimal insulin secretion in response to glucose. GK is believed to play a more important in glucose sensing than GLUT-2, although both are required for normal beta-cell function. Therapeutic agents directed towards increasing beta-cell GK expression and activity, and therefore glycolysis, will improve insulin secretion thereby restoring glucose homeostasis patients with Type 2 diabetes.

Since transcription factors play major roles in pancreatic development, the regulation of their expression is important for maintaining normal pancreatic development and beta-cell function. Studies that further our overall knowledge of the basic mechanisms involved in the generation of functional insulin-producing beta-cells, and the factors that adversely affect the process, could reveal the appropriate target sites for the development of pharmaceutical agents to ensure normal beta-cell development.

It is therefore important to maintain optimal expression profiles for Pdx-1, GLUT-2 and GK as these are key genes involved in beta-cell function (Fig. 38). Pdx-1, in particular, is an attractive target as it is involved in the maintenance of both beta-cell development and function, and is also reported to regulate the key beta-cell genes, insulin, GLUT-2 and GK. Fortunately, some therapeutic agents that modulate expression of these beta-cell genes are already available or in development. The incretin, glucagon-like peptide-1 (GLP-1), which is in the final phases of pharmaceutical development, has been shown to upregulate Pdx-1 expression (190;191). In addition, improved GLUT-2, GK and insulin expression occurred after administration of GLP-1, in aged rats (192). Thiazolidinediones (TZDs) increase GLUT-2 gene expression (193), endogenous GK expression and GK enzymatic activity in beta-cell lines (194). Peroxisomal proliferator-activated receptor-γ (PPAR-γ) directly increases GK expression in beta-cells resulting in an increase in GK activity (194).

Fig. 38 Therapeutic maintenance of beta-cell function by modulation of Pdx-1, GLUT-2 and GK expression

The reduced expression of the key beta-cell genes Pdx-1, GLUT-2 and GK (induced by the HFD) can be reversed by the modulation of their expression by therapeutic agents. Glucagon-like peptide-1 improves Pdx-1, GLUT-2 and GK expression. Thiazolidinediones (which can also improve GLUT-2 and GK expression) and GK activators increase GK activity and thereby glycolysis. These agents contribute to augmenting GSIS thereby restoring glucose homeostasis and maintaining normal beta-cell function. The discovery of novel agents that can regulate expression the Pdx-1 gene, and increase the glucose sensing ability of the beta-cell by modulating GLUT-2, and particularly, GK expression, are important for improving insulin secretion and beta-cell function in patients with Type 2 diabetes.



PPAR-γ regulates gene expression of glucose sensing apparatus thereby improving glucose sensing ability of beta-cells, contributing to the restoration of beta-cell function (193;194). The recently discovered anti-diabetic agents, glucokinase activators (GKA) enhance insulin release by activating glucose metabolism (195).

A HFD has adverse effects on beta-cell genes and promotes obesity, both of which may ultimately lead to the development of Type 2 diabetes. Anti-obesity drugs, currently in development, are important as obesity is a major risk factor for insulin resistance and Type 2 diabetes. Early detection of pathological changes by monitoring beta-cell function in people with obesity can prevent the onset of Type 2 diabetes. Following a healthy lifestyle by reducing dietary fat intake (particularly saturated fat) would be an important step towards preventing the development of Type 2 diabetes in people displaying abnormal beta-cell function. Ways to ameliorate the HFD-induced attenuation of glucose sensing and insulin secretion are important for future research in Type 2 diabetes.

5.3 Future studies

Several of the findings in this thesis study can lead to further studies. These include:

- The effect of HFD *in utero* on other transcription factors involved in beta-cell maintenance (e.g. Nkx 2.2, Hnf-1α) and in alpha-cell maintenance (e.g. Pax 6)

- Determining the rates of insulin secretion in isolated islets from these offspring in response to glucose
- Measuring C-peptide to reflect beta-cell function
- Determining the effect of a HFD *in utero* on different components of GSIS (e.g. ATP production, the K_{ATP}- and Ca^{2+}-channels and intracellular Ca^{2+} concentration)
- The use of various therapeutic agents that modulate the expression of these key beta-cell genes thereby maintaining glucose homeostasis and restoring beta-cell function
- Repeating the study in near-term rat foetuses, adult rats, non-pregnant and pregnant dams and in Vervet monkeys

CHAPTER 6

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ANNEXURE

Abbreviations

ANOVA one-way analysis of variance

AMV Avian Myeloblastosis Virus

APES aminopropyltriethoxysilane

bp base pair

BSA bovine serum albumin

cDNA complementary deoxyribonucleic acid

DAB dimethylaminobenzaldehdye

dH_20 distilled water

DNA deoxyribonucleic acid

dNTP deoxynucleotide triphosphate

EDTA ethylenediaminetetraacetic

e embryonic day (or day of gestation)

EtBr ethidium bromide

Foxa2 forkhead box A2

FFA free fatty acids

GK glucokinase

GLP-1 glucagon-like peptide-1

GSIS glucose-stimulated insulin secretion

GLUT-2 glucose transporter-2

Hnf hepatocyte nuclear factor

HFD high fat diet

HF/HP high fat/high protein

IGF-1R insulin-like growth factor-1 receptor

IL-6 interleukin-6

IR insulin receptor

IRS insulin receptor substrate

K_m Michalis Menten constant

LPL lipoprotein lipase

$MgCl_2$ magnesium chloride

mRNA messenger RNA

MODY maturity-onset diabetes of the young

n sample number

Na_2 sodium

NeuroD1 neurogenic differentiation 1

NGS normal goat serum

Nkx Nk homeobox protein

PAGE/HPLC Polyacrylamide gel electrophoresis/High performance liquid chromatography

Pax paired domain homeobox

PBGD porphobilinogen deaminase

PCR polymerase chain reaction

Pdx-1 pancreatic duodenal homeobox-1

PPAR-γ peroxisomal proliferator-activated receptor-gamma

RIA radio immunoassay

RNA ribonucleic acid

RT room temperature

RT-PCR reverse transcriptase PCR

SEM standard error of the mean

TAE tris-acetate

Taq Taq polymerase

TNF-α tumor necrosis factor-alpha

TBS tris-buffered saline

TZDs thiazolidinediones

UV ultra-violet

WHO World Health Organization

The period of gestation in the Wistar rat is 3 weeks and lactation also lasts for 3 weeks. The weeks of gestation and/or lactation during which the dams were maintained on a HFD represent the periods of exposure of their offspring to a HFD. The experimental groups depicted in the table represent neonates (Groups 1-4) and weanlings (Groups 6-9, 11-15) that were exposed to a maternal HFD for specific periods of gestation and/or lactation. The control groups are Group 5 for the neonates and Group 10 for the weanlings. These offspring were only exposed to a standard laboratory (control) diet. When not exposed to a HFD, for example, weeks 2 and 3 of Group 1, the pups were exposed to a standard laboratory diet. Group 1 and Group 6 had identical exposure to a maternal HFD *in utero*, i.e. for the first week of gestation, with neonates from Group 1 terminated at birth and weanlings from Group 6 terminated at 3 weeks of age. This is the same for Groups 2-4 and Groups 7-9, respectively. The weanling Groups 11-14 were exposed to a maternal HFD throughout gestation and for specific weeks of lactation. Group 15 weanlings were only exposed to a maternal HFD throughout lactation.

Table1 Experimental groups

Group	Period of exposure to a maternal HFD or standard laboratory (control) diet during gestation	Period of exposure to a maternal HFD or standard laboratory (control) during weaning
1	HFD: First week Control: Second and third week	Rats terminated at birth
2	HFD: Second week Control: First and third week	Rats terminated at birth
3	HFD: Third week Control: First and second week	Rats terminated at birth
4	HFD throughout gestation	Rats terminated at birth
5	Control diet throughout gestation	Rats terminated at birth
6	HFD: First week	Control diet throughout weaning
7	HFD: Second week	Control diet throughout weaning
8	HFD: Third week	Control diet throughout weaning
9	HFD: Throughout	Control diet throughout weaning
10	Control diet throughout gestation	Control diet throughout weaning
11	HFD: Throughout	HFD: First week
12	HFD: Throughout	HFD: Second week
13	HFD: Throughout	HFD: Third week
14	HFD: Throughout	HFD: Throughout
15	Control diet throughout gestation	HFD: Throughout