EFFECTS OF GLUCOSE ON ENDOTHELIAL FUNCTION IN PREGNANCY AND THE INFLUENCE OF DIABETES

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# LIST OF CONTENTS

## ABSTRACT

17

## CHAPTER 1: INTRODUCTION

26

### 1.1 Diabetes Mellitus

27

1.1.1 Background

27

1.1.2 Rationale of study

28

1.1.3 Definition of diabetes

28

1.1.4 Classification of diabetes

29

1.1.5 Diagnosis of diabetes

29

1.1.6 Type 1 diabetes

30

1.1.7 Type 1 diabetes in pregnancy

31

1.1.8 Type 2 diabetes

31

1.1.9 Type 2 diabetes in pregnancy

32

1.1.10 Gestational diabetes

32

1.1.10.1 Definition and diagnosis

32

1.1.10.2 Features of gestational diabetes

35

1.1.11 Other specific types of diabetes

36

### 1.2 Animal models of diabetes

36

1.2.1 Animal models of type 1 diabetes

37

1.2.2 Animal models of type 2 diabetes

37

1.2.3 Animal models of diabetes in pregnancy

37

1.2.4 Advantages of animal models of diabetes

38

1.2.5 Limitations of animal models of diabetes

38

### 1.3 Pregnancy

39

1.3.1 Carbohydrate metabolism in pregnancy

39

1.3.2 Cardiovascular changes in pregnancy

40

### 1.4 Arteries

41

1.4.1 Blood flow to the fetus

41

1.4.2 Resistance arteries

41
1.5 The Endothelium

1.5.1 Endothelial function

1.5.2 Endothelium-dependent mediators of relaxation

1.5.2.1 Nitric Oxide

1.5.2.2 Prostacyclin

1.5.2.3 Endothelium Derived Hyperpolarizing Factor

1.5.3 Endothelium-derived vasoconstrictors

1.5.4 Other factors affecting endothelial function

1.5.4.1 Age

1.5.4.2 Smoking

1.5.4.3 Ethnicity

1.5.4.4 Cholesterol

1.5.5 Endothelial function in pregnancy

1.5.6 Methods of measuring endothelial function

1.5.6.1 Venous occlusion plethysmography

1.5.6.2 Brachial artery flow-mediated vasodilation

1.5.6.3 Limitations of brachial artery FMD

1.6 Endothelial dysfunction in diabetes

1.6.1 Animal studies of endothelial dysfunction in diabetes

1.6.2 Human studies of endothelial dysfunction in diabetes

1.6.3 Mechanisms of endothelial dysfunction in diabetes

1.6.4 Endothelial dysfunction and regional variations

1.6.5 Other factors influencing endothelial dysfunction in diabetes

1.6.5.1 Control of diabetes

1.6.5.2 Obesity

1.6.5.3 Lipids

1.6.5.4 Insulin resistance

1.6.5.5 Gender differences in diabetes

1.7 Endothelial dysfunction of diabetes in pregnancy

1.7.1 Type 1 diabetes

1.7.2 Gestational diabetes

1.7.3 Previous gestational diabetes

1.8 Glucose levels

1.8.1 HbA1C
1.9 Hyperglycaemia

1.9.1 Definition 62
1.9.2 Estimating hyperglycaemia 62
1.9.3 Degree of hyperglycaemia 63
1.9.4 Duration of hyperglycaemia 63
1.9.5 Pathophysiology of hyperglycaemia 64
1.9.5.1 Oxidative stress 64
1.9.5.2 Hyperglycaemia and endothelial mediators 66
1.9.6 Acute Hyperglycaemia 66
1.9.7 Hyperglycaemia and endothelium-dependent relaxation 67

1.10 Hypoglycaemia 69

1.10.1 Hypoglycaemia and pregnancy 69
1.10.2 Hypoglycaemia and endothelial function 70

1.11 Blood glucose in pregnancy 70

1.12 Summary of Introduction 72

1.13 Hypotheses 73

1.14 Aims 73

CHAPTER 2
MATERIALS AND METHODS 74

2.1 Ethics 75

2.2 Human subjects 75

2.2.1 Healthy women 75
2.2.1.1 Non-pregnant women 75
2.2.1.2 Pregnant women 75
2.2.2 Diabetic patients 75
2.2.2.1 Type 1 and type 2 diabetes in pregnancy 75
2.2.2.2 Gestational diabetes 76

2.3 Mice 76

2.4 Tissue Collection 76

2.4.1 Non-pregnant women 76
2.4.2 Pregnant women 77
2.4.3 Mice 77
2.5 Stereomicroscopic dissection
2.6 Resistance arteries
2.7 Choice of methodology
2.8 Wire myography
  2.8.1 Multi Myograph System 610M
  2.8.2 Advantages of wire myography
  2.8.3 Limitations of wire myography
2.9 Normalisation of vessel lumen
2.10 Drugs
  2.10.1 Potassium chloride
  2.10.2 Bradykinin
  2.10.3 Phenylephrine
  2.10.4 Acetylcholine
  2.10.5 U46619
  2.10.6 L-NNA
  2.10.7 Indomethacin
  2.10.8 Streptozotocin
  2.10.9 Choice of drugs
    2.10.9.1 Vasoconstriction
    2.10.9.2 Endothelium-dependent relaxation
2.11 Solutions
  2.11.1 Glucose physiological saline solutions
  2.11.2 60 mmol/L KPSS
  2.11.3 Hyper-osmolar solution
  2.11.4 Modified Krebs’ buffer
2.12 Experimental protocols
  2.12.1 Protocol 1: The effect of glucose levels on maximum constriction
  2.12.2 Protocol 2: The effect of glucose levels on endothelium-dependent relaxation
  2.12.3 Protocol 3: The effect of hyper-osmolarity
  2.12.4 Protocol 4: The effect of blockers to endothelium-dependent relaxation
2.13 Optimising experiments
   2.13.1 General measures
   2.13.2 Optimising mice experiments

2.14 Creating an animal model of diabetes
   2.14.1 Restrictions in human research
   2.14.2 Rationale for creating an animal model of diabetes
   2.14.3 Obtaining a Home Office Licence
   2.14.4 Species and strain
   2.14.5 Duration of diabetes
   2.14.6 Diabetogenic drug
   2.14.7 Procedure
   2.15.8 Impediments in creating an animal model of diabetes

2.15 Data analysis
   2.15.1 Constriction
   2.15.2 Endothelium-dependent relaxation

2.16 Statistical analysis
   2.16.1 General Principles
   2.16.2 Statistical analysis of data from patients
   2.16.3 Statistical analysis of myography data
      2.16.3.1 Constriction
      2.16.3.1 Endothelium-dependent relaxation

CHAPTER 3
THE GLUCOSE LEVELS OF WOMEN WITH DIABETES IN PREGNANCY

3.1 Introduction
3.2 Aims
3.3 Methodology
   3.3.1 Inclusion criteria
   3.3.2 Exclusion criteria
   3.3.3 Data collection
   3.3.4 Statistical analysis
3.4 Results

3.4.1 Clinical characteristics 103
3.4.2 Glucose levels 106
3.4.3 Glucose levels of type 1 diabetes in pregnancy 107
3.4.4 Glucose levels of type 2 diabetes in pregnancy 108
3.4.5 Glucose levels of gestational diabetes 108
3.4.6 HbA1C levels: comparison across types 112
3.4.7 HbA1C levels in each group 112
   3.4.7.1 Type 1 diabetes 112
   3.4.7.2 Type 2 diabetes 112
   3.4.7.3 Gestational diabetes 112
3.4.8 Glucose levels in GDM: effect of insulin 114
3.4.9 Glucose levels and outcomes in type 1 diabetes 116
3.4.10 HbA1C and outcomes in type 1 diabetes 116

3.5 Discussion 119

3.5.1 Glucose levels of women with diabetes in pregnancy 119
3.5.2 Glucose levels in type 1 diabetes 120
3.5.3 Glucose levels in type 2 diabetes 120
3.5.4 Glucose levels in gestational diabetes 120
3.5.5 Adverse outcomes 120
   3.5.5.1 Type 1 and 2 diabetes 121
   3.5.5.2 Gestational diabetes 122
3.5.6 HbA1C and glucose levels 122
3.5.7 Limitations of study 123
3.5.8 Summary 123

CHAPTER 4
THE EFFECT OF GLUCOSE ON ENDOTHELIAL FUNCTION
IN HEALTHY NON-PREGNANT AND PREGNANT WOMEN 125

4.1 Introduction 126
4.2 Aims 127
4.3 Materials and Methods 127
4.4 Results 128
4.4.1 Clinical characteristics 128
4.4.2 Arterial diameters 129
4.4.3 Constriction: effect of glucose (non-pregnant group - KCl) 131
4.4.4 Endothelium-dependent relaxation: effect of glucose (non-pregnant group – KCl) 131
4.4.5 Constriction: effect of glucose (non-pregnant group - U46619) 131
4.4.6 Endothelium-dependent relaxation: effect of glucose (non-pregnant group – U46619) 131
4.4.7 Constriction: effect of glucose (pregnant group - KCl) 136
4.4.8 Endothelium-dependent relaxation: effect of glucose (pregnant group – KCl) 136
4.4.9 Constriction: effect of glucose (pregnant group – U46619) 139
4.4.10 Endothelium-dependent relaxation: effect of glucose (pregnant group – U46619) 139
4.4.11 Comparison of endothelium-dependent relaxation in non-pregnant and pregnant groups 144
4.4.12 Effect of prolonged exposure (2 hours) of 2, 5, 8 and 12 mmol/L glucose 146

4.5 Discussion 148
4.5.1 Summary of results 148
4.5.2 Low glucose concentration and impaired relaxation 148
   4.5.2.1 The effect of vasoconstrictors 149
   4.5.2.2 The effect of pregnancy 150
4.5.3 Other findings 150
   4.5.3.1 Constriction 150
   4.5.3.2 Effect of pregnancy on endothelium-dependent relaxation 150
   4.5.3.3 Effect of prolonged incubation 151
   4.5.3.4 High glucose concentrations 152
4.5.4 Explanations for the lack of effect of high glucose levels 152
   4.5.4.1 Inadequate duration of high glucose levels 152
   4.5.4.2 Inadequate degree of high glucose levels 152
   4.5.4.3 Systemic factors 153
CHAPTER 5
THE EFFECT OF GLUCOSE ON ENDOTHELIAL FUNCTION IN DIABETES COMPLICATING PREGNANCY

5.1 Introduction
5.2 Aims
5.3 Materials and methods
5.4 Results

5.4.1 Clinical characteristics – gestational diabetes
5.4.2 Diameter of arteries – gestational diabetes
5.4.3 Constriction: effect of glucose (GDM-KCl)
5.4.4 Endothelium-dependent relaxation: effect of glucose (GDM-KCl)
5.4.5 Constriction: effect of glucose (GDM-U46619)
5.4.6 Endothelium-dependent relaxation: effect of glucose (GDM-U46619)
5.4.7 GDM: effect of vasoconstrictor on endothelium-dependent relaxation
5.4.8 Constriction: effect of glucose (type 1 and 2 diabetes)
5.4.9 Endothelium-dependent relaxation: effect of glucose (type 1 and 2 diabetes)
5.4.10 Comparison between GDM and healthy pregnant women

5.4.10.1 Using KCl
5.4.10.2 Using U46619
5.4.11 Comparison between GDM and healthy pregnant women: endothelium-dependent relaxation 175

5.4.11.1 Using KCl 175
5.4.11.2 Using U46619 175

5.5 Discussion 179

5.5.1 Summary of results 179
5.5.2 Endothelial dysfunction in GDM 180
  5.5.2.1 Explanations for impaired relaxation 180
  5.5.2.2 Implications of findings 182
  5.5.2.3 Limitations of study 183
5.5.3 Patient characteristics 183
5.5.4 The effect of glucose levels on vascular function 184
  5.5.4.1 Constriction 184
  5.5.4.2 Endothelium-dependent relaxation 184
5.5.5 Type 1 and type 2 diabetes 185
5.5.6 Summary 186

CHAPTER 6
THE EFFECT OF GLUCOSE ON ENDOTHELIAL FUNCTION IN NON-PREGNANT AND PREGNANT MICE 187

6.1 Introduction 188
6.2 Aims 189
6.3 Materials and methods 189
6.4 Results 190
  6.4.1 Arterial diameters 190
  6.4.2 Constriction: normal non-pregnant mice 191
  6.4.3 Endothelium-dependent relaxation: normal non-pregnant mice 191
  6.4.4 Constriction: normal pregnant mice 195
  6.4.5 Endothelium-dependent relaxation: normal pregnant mice 195
  6.4.6 Effect of pregnancy on constriction and endothelium-dependent relaxation 199
  6.4.7 Effect of hyper-osmolarity – pregnant mice 199
6.4.8 Normal pregnant mice: effect of endothelial blockers at 5 and 12 mmol/L glucose 202
6.4.9 Normal pregnant mice: effect of glucose levels on endothelial blockers 202
6.4.10 Blood glucose in mice 206
6.4.11 Pregnant streptozotocin-control mice 207
   6.4.11.1 Constriction: effect of glucose levels (pregnant streptozotocin-controls) 207
   6.4.11.2 Relaxation: effect of glucose levels (pregnant streptozotocin-controls) 208

6.5 Discussion 212
   6.5.1 Summary of findings 212
   6.5.2 Enhanced vasodilation at 12 mmol/L glucose 212
   6.5.3 Glucose requirements in different vascular beds 214
   6.5.4 Hyperglycaemia and hyper-osmolarity 214
   6.5.5 Hyperglycaemia and systemic factors 215
   6.5.6 Effect of pregnancy 215
   6.5.7 Effect of glucose on constriction 216
   6.5.8 Endothelial mediators of vasodilation 216
   6.5.9 Streptozotocin treated non-diabetic mice (pregnant streptozotocin-controls) 218
   6.5.10 Summary 219

CHAPTER 7
DISCUSSION 220

   7.1 Overview of study 221
   7.2 Key findings. 224
   7.3 Hypothesis 1 224
   7.4 Hypothesis 2 226
   7.5 Hypothesis 3 227
   7.6 Limitations of study 229
   7.7 Future avenues of research 231
REFERENCES

APPENDIX A: Consent form 259
APPENDIX B: Patient Information sheet 260
APPENDIX C: Pro forma for study of diabetes in pregnancy 261
APPENDIX D: Review Article: Diabetes management in pregnancy 263
APPENDIX E: Published Paper 272

FINAL WORD COUNT = 43144
LIST OF FIGURES

Figure 1.1 The Ebers Papyrus 27
Figure 1.2 Endothelium-dependent mediators of vasodilation 44
Figure 1.3 Brachial artery flow-mediated dilatation 50
Figure 1.4 Overview of glucose metabolism 60
Figure 1.5 Oxidative stress and endothelial dysfunction 65
Figure 2.1 Schematic representation of a blood vessel mounted on a wire myograph 78
Figure 2.2 Myograph with data recordings on computer 79
Figure 2.3: Multi Myograph System 610M and an individual myograph chamber 80
Figure 2.4: Schematic representation of concentration-response curve 87
Figure 2.5: Raw data trace 87
Figure 2.6: Experimental protocol 1 and 2 88
Figure 2.7: Protocol 3 89
Figure 2.8: Protocol 4: Raw data trace 90
Figure 2.9: Protocol 4 (5 mmol/L glucose) 91
Figure 2.10: Protocol 3 (12 mmol/L glucose) 91
Figure 3.1: Glucose levels: type 1 diabetes 109
Figure 3.2: Glucose levels: type 2 diabetes 110
Figure 3.3: Glucose levels: gestational diabetes 111
Figure 3.4: HbA1C: type 1 diabetes 113
Figure 3.5: HbA1C: type 2 diabetes 113
Figure 3.6: HbA1C: gestational diabetes 114
Figure 3.7: Glucose levels in diet-controlled GDM 115
Figure 3.8: Glucose levels in insulin-controlled GDM 115
Figure 3.9: Glucose levels in type 1 diabetes – normal outcome 117
Figure 3.10: Glucose levels in type 1 diabetes – adverse outcome 117
Figure 3.11: HbA1C in type 1 diabetes – normal outcome 118
Figure 3.12: HbA1C in type 1 diabetes – adverse outcome 118
Figure 4.1: Arterial diameters (non-pregnant group) 130
Figure 4.2: Arterial diameters (healthy pregnant group) 130
Figure 4.3 (A-D): Constriction: effect of glucose  
(non-pregnant group – KCl)  
132
Figure 4.4 (A-D): Relaxation: effect of glucose  
(non-pregnant group - KCl)  
133
Figure 4.5 (A-D): Constriction: effect of glucose  
(non-pregnant group – U46619)  
134
Figure 4.6 (A-D): Relaxation: effect of glucose on relaxation  
(non-pregnant group – U46619)  
135
Figure 4.7 (A-D): Constriction: effect of glucose  
(pregnant group – KCl)  
137
Figure 4.8 (A-D): Relaxation: effect of glucose  
(pregnant group – KCl)  
138
Figure 4.9 (A-D): Constriction: effect of glucose  
(pregnant group – U46619)  
140
Figure 4.10 (A-D): Relaxation: effect of glucose on relaxation  
(pregnant group – U46619)  
141
Figure 4.11: Relaxation at 5 mmol/L glucose  
(pregnant group – U46619)  
142
Figure 4.12: Relaxation at 2, 5 and 12 mmol/L glucose  
(pregnant group – U46619)  
143
Figure 4.13 (A-C): Relaxation: non-pregnant and pregnant groups  
145
Figure 4.14: Two hour incubation: aberrant responses  
147
Figure 5.1 (A & B): Arterial diameters – GDM  
163
Figure 5.2 (A-D): Constriction: effect of glucose (GDM- KCl)  
164
Figure 5.3 (A-D): Relaxation: effect of glucose levels (GDM- KCl)  
166
Figure 5.4 (A-D): Constriction: effect of glucose levels  
(GDM- U46619)  
167
Figure 5.5 (A-D): Relaxation: effect of glucose levels  
(GDM – U46619)  
168
Figure 5.6: Relaxation: effect of vasoconstrictor  
169
Figure 5.7: Constriction: effect of glucose levels, type 1 diabetes – KCl  
171
Figure 5.8: Constriction: effect of glucose levels, type 2 diabetes – KCl  
171
Figure 5.9 (A-D): Relaxation: effect of glucose (type 1 diabetes – KCl)  
172
Figure 5.10 (A-D): Relaxation: effect of glucose (type 1 diabetes – KCl)  
173
Figure 5.1: KCl Constriction: Normal vs GDM
Figure 5.12: U46619 Constriction: Normal vs GDM
Figure 5.13: Relaxation – Normal vs GDM: KCl
Figure 5.14: Relaxation – Normal vs GDM: U46619
Figure 6.1: Arterial diameters – non-pregnant and pregnant mice
Figure 6.2 (A-D): Constriction: effect of glucose (non-pregnant mice)
Figure 6.3 (A-D): Relaxation: effect of glucose (non-pregnant mice)
Figure 6.4: Relaxation: effect of glucose (non-pregnant mice)
Figure 6.5 (A-D): Constriction: effect of glucose (pregnant mice)
Figure 6.6 (A-D): Relaxation: effect of glucose (pregnant mice)
Figure 6.7: Relaxation: effect of glucose levels (pregnant group)
Figure 6.8: Constriction: non-pregnant vs pregnant mice
Figure 6.9: Relaxation: non-pregnant vs pregnant mice
Figure 6.10: The effect of hyper-osmolarity
Figure 6.11: The effect of blockers on relaxation – 5 mmol/L glucose
Figure 6.12: The effect of blockers on relaxation – 12 mmol/L glucose
Figure 6.13 (A-D): The effect of blockers at 5 and 12 mmol/L glucose
Figure 6.14: Blood glucose: mice
Fig. 6.15 (A-D): Constriction: effect of glucose levels
(pregnant STZ-controls)
Fig. 6.16 (A-D): Relaxation: the effect of glucose
(pregnant STZ-controls)
Fig. 6.17: Relaxation: effect of glucose levels
(pregnant STZ-controls)
Fig 7.1: Overview of study
# LIST OF TABLES

**Table 1.1:** WHO classification of Diabetes Mellitus  
29

**Table 1.2:** Vasoactive products synthesized by the endothelium  
43

**Table 1.3:** Studies demonstrating impaired relaxation with hyperglycaemia  
68

**Table 1.4:** Studies demonstrating vasodilation with hyperglycaemia  
68

**Table 1.5:** Studies demonstrating no effect of hyperglycaemia on vascular function  
69

**Table 2.1:** Composition of solutions used (in mmol/L)  
85

**Table 3.1:** Clinical characteristics  
104

**Table 3.2:** Range of glucose levels seen in diabetes in pregnancy  
107

**Table 4.1:** Clinical characteristics of healthy individuals  
129

**Table 4.2:** Summary of results – Chapter 4  
148

**Table 5.1:** Clinical characteristics – gestational diabetes  
162

**Table 5.2:** Summary of results - effect of change in glucose levels-DM  
178

**Table 5.3:** Summary of results – comparison between normal and GDM  
178
Diabetes in pregnancy is a potentially serious disease for both mother and fetus and its prevalence is increasing globally. Poorly controlled diabetes, with recurrent episodes of hypoglycaemia and hyperglycaemia, is associated with adverse pregnancy outcomes. As diabetes is characterised by abnormalities in vascular function, it is possible that these episodes of aberrant glucose levels may affect the arteries regulating blood flow to the fetus, thereby playing a role in the complications of this condition. The endothelium forms the innermost lining of arteries and has been shown to exert important vasoregulatory effects which can alter the haemodynamics of blood flow. Although endothelial dysfunction has been demonstrated in various vascular beds in diabetes, it is uncertain whether the crucial uterine circulation is similarly affected. The main aim of this study was to examine whether aberrant glucose levels can affect endothelial function in these arteries. Clinically relevant glucose levels were identified in this study to range from 2 to 12 mmol/L by assessing the blood glucose levels in 111 patients with diabetes in pregnancy. Patients with type 1 diabetes were noted to have wide fluctuations of blood glucose as well as a high rate of pregnancy complications. Constriction and endothelium-dependent relaxation were examined in myometrial arteries from three groups of women: healthy non-pregnant, healthy pregnant and pregnant diabetic women. The effect of changing glucose concentrations from 5 mmol/L to 2, 8 and 12 mmol/L glucose was studied using wire myography. An agonist-specific impairment of endothelium-dependent relaxation was present when arteries from healthy pregnant women were exposed to 2 mmol/L glucose for 30 minutes. Arteries from healthy non-pregnant as well as pregnant diabetic women exhibited no difference in constriction or endothelium-dependent relaxation. Myometrial arteries from women with gestational diabetes had significantly impaired endothelium-dependent relaxation compared to those from healthy pregnant women. When similar studies were performed in the uterine artery of normal pregnant mice, exposure to 12 mmol/L glucose for 30 minutes was associated with enhanced endothelium-dependent relaxation. This effect was not present in uterine arteries of non-pregnant mice, indicating a pregnancy-specific modulation of vascular function. The contribution of the endothelial mediators of vasodilation was also assessed in the uterine artery of pregnant mice. Prostacyclin did not contribute to endothelium-dependent relaxation, which was mediated by nitric oxide and to a greater extent by a non-NO/non-prostacyclin component. The contribution of these mediators was not significantly affected by changes in glucose levels. Due to the limited availability of tissue from diabetic patients, an attempt was made to create an animal model of diabetes to enable further studies. After injection of streptozotocin, only a minority of mice developed diabetes; as assessed by serial measurements of blood glucose. Mice were divided into vehicle-controls, streptozotocin-controls and diabetic mice. Uterine arteries from streptozotocin-controls did not demonstrate enhancement of endothelium-dependent relaxation with 12 mmol/L glucose, as was seen in normal mice. It is hoped that the findings of this study and the development of an animal model of diabetes in pregnancy will help to increase our understanding of the endothelial dysfunction seen in pregnant women with diabetes.
DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
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PRESENTATIONS AND PUBLICATIONS

Papers
  Submitted

Presentations
I. Annual Professional Conference (2005) - Diabetes UK
   Poster Presentation
   Acute hypoglycaemia impairs relaxation of uterine arteries in pregnant mice
II. 65th Scientific Sessions (2005) –American Diabetes Association
    Poster Presentation
    Hypoglycaemia and hyperglycaemia impair myometrial artery relaxation in pregnancies complicated by gestational diabetes
III. Annual Professional Conference (2006) - Diabetes UK
    Poster Presentation
    Hyperglycaemia enhances endothelium-dependent relaxation in uterine arteries of pregnant mice
    Oral Presentation
    Acute hyperglycemia in uterine arteries of pregnant, but not non-pregnant mice, enhances endothelium-dependent relaxation
DEDICATION

This thesis is dedicated to my loving wife and family for their constant support and also to all the patients and individuals who have helped me throughout this study.
LIST OF ABBREVIATIONS

AEP: Active Effective Pressure
ANOVA: Analysis of Variance
ATP: Adenosine Triphosphate
BH₄: Tetrahydro biopterin
BMI: Body Mass Index
cAMP: Cyclic Adenosine Monophosphate
cGMP: Cyclic Guanosine Monophosphate
CEMACH: Confidential Enquiry into Maternal and Child Health
COX: Cyclo-oxygenase
DM: Diabetes Mellitus
EDCF: Endothelium-Derived Constricting Factor
EDHF: Endothelium-Derived Hyperpolarising Factor
EDRF: Endothelium-Derived Relaxing Factor
eNOS: Endothelial Nitric Oxide Synthase
ET-1: Endothelin - 1
FMD: Flow Mediated Dilatation
GDM: Gestational Diabetes
GTP: Guanosine Triphosphate
GTT: Glucose Tolerance Test
HAPO: Hyperglycaemia and Adverse Pregnancy Outcome study
HDL: High Density Lipoprotein
ICAM: Intercellular Adhesion Molecule
IFG: Impaired Fasting Glucose
IGT: Impaired Glucose Tolerance
Ind.: Indomethacin
KCl: Potassium Chloride
KPSS: Potassium Physiological Solution
LDL: Low Density Lipoprotein
L-NNA: Nω–Nitro-L-Arginine
NO: Nitric Oxide
OGTT: Oral Glucose Tolerance Test
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGH₂</td>
<td>Prostaglandin H₂</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological Saline Solution</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>soluble Intercellular Adhesion Molecule-1</td>
</tr>
<tr>
<td>SMBG</td>
<td>Self-monitored blood glucose</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue Plasminogen Activator</td>
</tr>
<tr>
<td>TXA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
</tr>
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“Diabetic women were discouraged from becoming pregnant. They often died during the course of their pregnancy or their babies often died before birth or as infants. Any successful pregnancy was remarkable.”

Dr. Priscilla White
Pioneer in the treatment of diabetes in pregnancy;
quoted prior to the discovery of insulin
CHAPTER 1

INTRODUCTION
INTRODUCTION

1.1 Diabetes Mellitus

1.1.1 Background

Diabetes Mellitus is one of the oldest diseases known to mankind, the first record of its symptoms having been documented on papyrus scrolls by ancient Egyptians approximately 3500 years ago [King and Rubin, 2003]. The Ebers papyrus contains a description of a disease characterised by excessive urination, which is believed to be the very first description of diabetes (Fig. 1.1).

![The Ebers Papyrus: the first record of diabetes, circa 1500 BC](image)

Diabetes can be considered to be a state of reduced insulin action which occurs as a result of decreased insulin availability or diminished insulin effectiveness [Bell and Hockaday, 1996]. This leads to unduly high glucose levels and clinical symptoms such as thirst and increased urine production, which occur secondarily to the osmotic effect of raised glucose levels. The term diabetes originates from the Greek “diabainein” meaning “to go through”; a reference to the excessive urine output. A high blood glucose level is the hallmark of diabetes and plays a central role in the diagnosis of this disease and the pathophysiology of its complications. However, the deleterious effects of diabetes are a result of multiple factors such as alterations in lipid metabolism, changes in cellular metabolism, and the production of metabolites.
that are harmful to the vasculature. Diabetes Mellitus is not a single disease, but a genetically heterogeneous group of disorders that share a common factor: glucose intolerance. It is a true multi-system disorder which can affect almost all organ systems of the body, causing a variety of debilitating and potentially fatal complications. The multi-system nature of this disease, the lack of a cure, its chronicity and rising prevalence combine to pose a serious threat to global health in the 21st century.

1.1.2 Rationale of study

The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 [Wild et al., 2004]. Diabetes in pregnancy (incorporating type 1, type 2 and gestational diabetes) is a potentially serious medical disorder in pregnancy, affecting approximately 1 in 250 pregnancies in UK [CEMACH, 2005]. Patients with diabetes have a higher risk of cerebro-vascular disease, ischaemic heart disease, retinopathy, amputations, and renal failure. These myriad complications all share the common feature of abnormal vascular function, an important cause of which is the effect of glucose on vasculature. Despite its pivotal role in the pathophysiology of diabetes, the effect of aberrant glucose levels on vessels is incompletely understood. Studies in this field have produced conflicting results, which make the true effects of glucose difficult to discern. Furthermore, it is unknown if the irregular glucose levels seen in pregnancies complicated by diabetes are associated with vascular dysfunction in arteries supplying blood to the fetus. The aim of this study therefore, was to directly examine the effects of abnormal glucose levels on the function of arteries involved in the blood flow to the fetus.

1.1.3 Definition of diabetes

The WHO has defined diabetes as a metabolic disorder of multiple aetiology, characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism, resulting from defects in insulin secretion, insulin action or both [Alberti and Zimmet, 1999].
1.1.4 Classification of diabetes

The current WHO classification system is based on the aetiology of diabetes mellitus [Alberti and Zimmet, 1999]. It divides diabetes into 4 main groups: type 1, type 2, gestational diabetes and other types (Table 1).

<table>
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<tr>
<th>TYPE 1 DIABETES MELLITUS</th>
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<td>Idiopathic</td>
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<td>TYPE 2 DIABETES MELLITUS</td>
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<td>GESTATIONAL DIABETES</td>
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<td>OTHER SPECIFIC TYPES</td>
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<td>Genetic Defects of β-cell function</td>
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<td>Genetic defects of insulin action</td>
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<td>Diseases of the exocrine pancreas</td>
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<td>Infections</td>
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<td>Uncommon forms of immune-mediated diabetes</td>
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<td>Other genetic syndromes</td>
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Table 1.1: WHO classification of Diabetes Mellitus

1.1.5 Diagnosis of diabetes

The diagnosis of diabetes is made with a fasting plasma glucose level ≥ 7.0 mmol/L on two separate occasions or an oral glucose tolerance test (GTT). The GTT is more sensitive for the diagnosis of diabetes than fasting plasma glucose [Reinauer et al., 2002].

The GTT is used for diagnosis when blood glucose levels are equivocal, during pregnancy or in epidemiological studies [Alberti and Zimmet, 1999]. The test is preceded by fasting overnight until the commencement of the test, during which only water may be drunk. After the collection of the fasting sample, the subject
drinks 75 g glucose dissolved in water. Blood samples are collected 2 hours after the test load.

A diagnosis of diabetes mellitus is made if the fasting venous plasma glucose is ≥ 7.0 mmol/L or the 2-hour post glucose load value is ≥ 11.1 mmol/L. The diagnosis of gestational diabetes is made during pregnancy if a patient (with no previous diagnosis of diabetes) fulfils this criteria, or the criteria for Impaired Glucose Tolerance (IGT); which is fasting venous plasma glucose < 7.0 mmol/L and 2 hour post glucose load ≥ 7.8 mmol/L.

1.1.6 Type 1 diabetes

Type 1 diabetes is characterised by cell-mediated auto-immune destruction of islet β-cells, which leads to a complete or nearly complete absence of endogenous insulin secretion [Alberti and Zimmet, 1999]. Insulin is required for survival to prevent the development of keto-acidosis, coma and death. Type 1 diabetes is predominantly a disease of childhood and usually presents before the age of 35 [Reinauer et al., 2002]. A large multi-centre study in Europe has shown that the incidence of this disorder is much higher in the 0-14 age group than the 15-29 age group [Kyvik et al., 2004]. The incidence of type 1 diabetes in Europe has been shown to be rising at an average rate of 3.4%, with the largest rate of increase seen in children aged 0-4 years [EURODIAB Group, 2000]. There is a marked ethnic and geographic variation of type 1 diabetes; its incidence being high in Finland, Sweden and Norway and low in China and South America [Karvonen et al., 2000]. There is a low genetic predisposition to develop this type of diabetes and antibodies to β-cells are often seen at the time of diagnosis [Alberti and Zimmet, 1999].

Laboratory findings include hyperglycaemia, ketonuria, low or undetectable serum insulin and C-peptide levels, and auto-antibodies against components of the islet β-cells [Reinauer et al., 2002].
Type 1 diabetes has been subdivided into two sub-types: immune mediated and idiopathic type 1 diabetes. The immune-mediated sub-type is distinguished by the presence of auto-antibodies to glutamic acid decarboxylase, islet cell or insulin antibodies. Idiopathic type 1 diabetes has a similar clinical presentation as immune–mediated type 1 diabetes, although it is distinguished from it by a lack of demonstrable antibodies to β-cells. The idiopathic sub-type has a marked familial pattern of inheritance and is more common in Asians and Africans than Caucasians.

1.1.7 Type 1 diabetes in pregnancy

A large population study in Denmark has demonstrated a higher rate of perinatal mortality, pre-term delivery, stillbirths, congenital malformations and Caesarean delivery in patients with type 1 diabetes [Jensen et al., 2004]. Another study in Netherlands corroborated these findings, additionally reporting a higher maternal mortality rate [Evers et al., 2004]. In both studies, better glycaemic control was associated with an improved outcome, although the latter study reported complications even on attaining near-optimal control of blood glucose levels. In addition to the above complications, type 1 diabetes may also influence birth weight. A study in Scotland has reported a higher mean birth weight in 216 babies of women with type 1 diabetes [Penney et al., 2003].

1.1.8 Type 2 diabetes

Type 2 diabetes is due to insulin insensitivity combined with a failure of insulin secretion to overcome this by hyper-secretion, resulting in relative insulin deficiency [Reinauer et al., 2002]. This is the most common form of diabetes and there is a strong genetic predisposition. The specific reasons for developing these abnormalities remain unknown. Patients with this condition are usually obese and tend to be older, although the disease can occur at any age and in lean individuals. However, a recent trend for an earlier onset of type 2 diabetes has been noticed; as obesity in children and adolescents becomes more prevalent [Rosenbloom et al., 1999].
Important associations of type 2 diabetes [Reinauer et al., 2002] include:

- Family history of diabetes (in particular parents or siblings with diabetes)
- Obesity ($\geq 20\%$ over ideal body weight or BMI $\geq 25$ kg/m²)
- Ethnicity (Africans, Asians, Hispanic and Native American)
- Age $\geq 45$ years
- Previously identified impaired fasting glucose (IFG) or IGT
- Hypertension ($\geq 140/90$ mmHg in adults)
- HDL cholesterol level $<1.0$ mmol/L ($< 0.38$ g/L) and/or a triglyceride level $\geq 2.3$ mmol/L ($\geq 2.0$ g/L)
- Reduced physical activity
- Past history of gestational diabetes or delivery of babies $> 4.5$ kg

### 1.1.9 Type 2 diabetes in pregnancy

A UK study demonstrated that infants of women with type 2 diabetes in pregnancy have a 2-fold greater risk of stillbirth, a 2.5-fold greater risk of perinatal mortality and an 11-fold greater risk of congenital malformations [Dunne et al., 2003]. Another study demonstrated that the percentage of pregnancies having a serious adverse outcome was higher in type 2 patients than type 1 (16.4 vs. 6.4%), with more congenital abnormalities in the type 2 group [Roland et al., 2005]. One possible explanation cited for this was that these women had less pre-conception care. In UK, it has been estimated that only 37% of women with type 2 diabetes in pregnancy have had a measurement of glycaemic control before pregnancy [CEMACH, 2005]. As noted above, a past history of gestational diabetes is a risk factor for type 2 diabetes, emphasising the association between these two conditions.

### 1.1.10 Gestational diabetes

#### 1.1.10.1 Definition and diagnosis

Compared to type 1 and 2 diabetes, the recognition of the disorder termed gestational diabetes has been relatively recent. In the first half of the 20th century, it was perceived that women who developed diabetes years after pregnancy had
suffered from abnormally high fetal and neonatal mortality [Miller, 1946]. By the 1950s the term "gestational diabetes" was applied to what was thought to be a transient condition that affected fetal outcomes adversely, then abated after delivery [Carrington et al., 1957]. In 1964, O'Sullivan found that the degree of glucose intolerance during pregnancy was related to the risk of developing diabetes after pregnancy. He proposed statistical criteria for the interpretation of oral glucose tolerance tests during pregnancy, establishing cut-off values (approximately 2 standard deviations) for diagnosing glucose intolerance during pregnancy [O'Sullivan and Mahan, 1964]. These cut-off points were adapted to modern methods for measuring glucose and applied to the WHO definition of gestational diabetes: carbohydrate intolerance resulting in hyperglycaemia of varying severity with onset or first recognition during pregnancy [Alberti and Zimmet, 1999]. This definition does not exclude the possibility that glucose intolerance was present, but unrecognized, before pregnancy. The definition is independent of the treatment modality used during pregnancy.

The WHO definition of gestational diabetes remains controversial. The earlier definition by O’Sullivan was “a transient abnormality of glucose tolerance during pregnancy.” This definition implies that the glucose intolerance reverts back to normal after pregnancy. The objection to the WHO definition is that patients with pre-existing type 1 or type 2 diabetes may be wrongly classified as having gestational diabetes [Omori and Jovanovic, 2005]. In patients with pre-existing diabetes, abnormal carbohydrate metabolism is present for the entire duration of pregnancy, whereas it is seen mainly in the second half of pregnancy in gestational diabetes. The prevalence of maternal retinopathy and fetal congenital defects are also higher in women with pre-existing diabetes. However, in practice, it is difficult to clinically distinguish the three types of diabetes in pregnant women as there are no diagnostic markers for type 2 diabetes and the antibody tests to detect type 1 diabetes are not routinely carried out. Furthermore, post-natal GTT results are not performed in all women, which precludes the classification of their diabetes [Beischer et al., 1997].

Treatment of women with gestational diabetes (by dietary advice, glucose monitoring and insulin as needed) has been reported to improve their pregnancy
outcome [Crowther et al., 2005]. Therefore, screening of such women using a glucose tolerance test is useful in identifying those who would benefit from treatment during pregnancy. However, available data do not identify the threshold of maternal glycaemia at which the risk begins or increases during pregnancy. To resolve this issue, a large, international trial in a multi-ethnic population (the Hyperglycaemia and Adverse Pregnancy Outcome study - HAPO) is currently underway [HAPO Group, 2002]. As blood glucose levels represent a continuous variable, it is possible that a continuum of increased risk to the baby is seen rather than clear-cut distinctions between normal and abnormal groups [Scott et al., 2002].

The current lack of an evidence-based threshold for defining the glucose levels at which increased risk occurs has resulted in different guidelines worldwide for the diagnosis of gestational diabetes. Although glucose tolerance tests are used for diagnosis, the point of time in pregnancy at which it is performed, the amount of glucose taken (50, 75 and 100 grams), the duration of the test (1, 2 and 3 hours) and the cut-off values for diagnosis vary from region to region. For example, studies in Spain [Chico et al., 2005], China [Yang et al., 2005] and Mexico [Ramirez-Torres et al., 2003] have used different glucose tolerance tests to diagnose gestational diabetes. The WHO currently uses the term gestational diabetes to encompass both gestational impaired glucose tolerance (GIGT) and gestational diabetes mellitus (GDM), which were previously regarded as separate entities. Furthermore, the cut-off values for these conditions are the same as in the non-pregnant state. The normal physiological changes in pregnancy include increasing insulin resistance, resulting in raised post-prandial blood glucose levels. Therefore, the milder degrees of GIGT diagnosed later in pregnancy may actually represent normal values [Maresh, 2005]. Because of this, the European Association for the Study of Diabetes recommended in 1989 that the lower cut-off for the 2-hour value defining GIGT be raised from 7.8 to 9.0 mmol/L in the third trimester on the basis of an epidemiological study [Lind, 1989]. However, this is at variance with the current WHO guidelines.

From the above discussion, it is clear that there is no consensus of expert opinion regarding either the definition or the diagnostic criteria for gestational diabetes, although this condition has been recognised for more than 50 years. It is expected
that the results of the HAPO trial will provide the required evidence-based data from different populations around the world to clarify these issues.

1.1.10.2 Features of gestational diabetes

Risk factors for gestational diabetes [Alberti and Zimmet, 1999] include:

- Age
- Previous history of glucose intolerance
- History of large for gestational age babies
- Ethnicity (such as Asian)
- Raised fasting or random blood glucose levels

A population-based study in Sweden demonstrated that raised BMI and advanced maternal age were risk factors for impaired glucose tolerance (IGT) in pregnancy [Aberg et al., 2001]. Untreated IGT was associated with higher rates of Caesarean sections and large for gestational age infants [Ostlund et al., 2003]. It has been demonstrated that Asian women were more likely to develop gestational diabetes than Caucasian women, with GDM occurring at a lower BMI in this group [Gunton et al., 2001]. For a given population and ethnicity, the risk of diabetes in pregnancy has been demonstrated to reflect the underlying frequency of type 2 diabetes, with insulin resistance being a possible link [Ben-Haroush et al., 2004]. Post-partum studies of women with gestational diabetes have demonstrated defects in insulin secretory response and decreased insulin sensitivity, indicating typical type 2 abnormalities in glucose metabolism [Catalano et al., 1986]. Furthermore, a study reported that only 1–2% of women with previous gestational diabetes had specific islet cell antibodies, indicating a low risk of type 1 diabetes [Catalano et al., 1990]. These findings suggest a strong link between gestational diabetes and type 2 diabetes. It remains unclear, however, if these ethnic and immunological findings accurately reflect GDM, as studies have differed in methods of screening, oral and intravenous glucose loads, and diagnostic criteria.

Women with gestational diabetes have been reported to be older at the age of delivery than women without diabetes and more likely to have a Caesarean section [Saydah et al., 2005]. Similar findings were obtained in another study which, in
addition, found a higher rate of large for gestational age babies in GDM [Johns et al., 2006]. Thus, although the prevalence of complications in gestational diabetes is lower than that of type 1 and 2 diabetes in pregnancy, it is still higher than that seen in the normal population.

1.1.11 Other specific types of diabetes

Other types of diabetes are extremely rare in pregnancy. They include genetic defects and syndromes, drug- or chemical-induced diabetes, endocrinopathies, infections and diseases of the exocrine pancreas.

1.2 Animal models of diabetes

Animals have been used to study diabetes since the discovery in the 1880s of von Mering and Minkowski that the removal of the pancreas in dogs causes diabetes [Rees and Alcolado, 2005]. Research in dogs also led to the pioneering discovery of insulin by Banting and Best [Banting et al., 1922]. In the United Kingdom, animal research is currently controlled by the Animals (Scientific Procedures) Act 1986 which stipulates that research should involve animals with the lowest degree of neuro-physiological sensitivity. This has resulted in the vast majority of animal research in diabetes being restricted to rats and mice. Diabetes can be induced by both surgical and non-surgical methods. Surgical methods involve partial or total removal of the pancreas, whereas non-surgical methods include the injection of islet cell toxins such as streptozotocin and alloxan. Murine models of diabetes have enabled the study of hyperglycaemia, hyperlipidaemia, gestational diabetes, islet cell transplantation and the assessment of new drugs [Rees and Alcolado, 2005]. Animal models of diabetes have also been invaluable in the study of vascular function in diabetes. Streptozotocin-induced diabetes in rats has been associated with impaired endothelium-dependent relaxation of the aorta [Cameron and Cotter, 1992].
1.2.1 Animal models of type 1 diabetes

As described previously, type 1 diabetes in humans is characterised by immune-mediated destruction of the pancreatic β-cells. In animals, this destruction can be induced by the injection of toxins, of which streptozotocin is the most widely used. Streptozotocin is an N-nitroso-containing compound that acts as a nitric oxide donor in pancreatic islet cells, thereby inducing death of insulin-secreting β-cells and producing an animal model of diabetes [McEvoy et al., 1984]. A single large dose of streptozotocin or multiple small doses (e.g. 40 mg/kg on five consecutive days) can be used in rodents. In susceptible animals this induces an insulin-deficient diabetes in which immune destruction plays a role, as in human type 1 diabetes [Rees and Alcolado, 2005].

Spontaneously hyperglycaemic animal models of type 1 diabetes also exist, where animals have been in-bred for many generations. A drawback to this model is that various genes and phenotypes have been altered, which may not be relevant for either mice or humans.

1.2.2 Animal models of type 2 diabetes

Unlike type 1 diabetes, the pathogenesis of type 2 diabetes is relatively obscure and a multitude of causal factors are involved such as insulin resistance, obesity and islet cell failure. This is reflected in animal models of this disease: insulin resistance predominates in some and islet cell failure in others. Animal models of type 2 diabetes include Zucker fatty rats [Tokuyama et al., 1995], Goto-Kakizaki rats [Goto et al., 1988], Psammomys obesus sand rats [Kalderon et al., 1986] and various transgenic mice models with reduced β-cell mass [Masiello, 2006].

1.2.3 Animal models of diabetes in pregnancy

Animal models of diabetes in pregnancy have been used to investigate the fetal origins of adult disease. Using these models, it has been shown that diabetes in pregnancy predisposes to the later development of diabetes amongst offspring [Van Assche et al., 1991]. Intra-uterine malnutrition has also been shown to be associated
with a higher risk of diabetes in later life [Boloker et al., 2002]. However, studies examining vascular function in pregnant animals with diabetes are extremely rare. It is therefore unknown if aberrant vascular function is present in the uterine vascular bed of pregnant rats with diabetes.

1.2.4 Advantages of animal models of diabetes

Animal models have been responsible for many breakthroughs in the field of diabetes research, from the discovery of insulin itself to new drugs such as PPAR-γ agonists [Finegood et al., 2001]. In the development of new drugs, both drug efficacy and drug toxicity can be studied in the same model [Boelsterli, 2003]. Animal models provide unique opportunities for studying disorders such as fetal origins of disease due to their short gestation and rapid turnover. The confounding effects of concurrent diseases such as hypertension and hyperlipidaemia, which are often present in human studies of diabetes, can be reduced by using animal models. Knockout mice have provided valuable insights into the pathophysiology of diabetes [LeRoith and Gavrilova, 2006]. Mouse and human genomes bear striking similarities to each other. Approximately 99% of mouse genes have a homologue in the human genome, making it a leading mammalian system for studying human physiology and disease [Chinwalla et al., 2002]. When studying pregnancy, the easier access to tissue from animals compared to humans enables a broader range of experiments; an advantage which has been availed of in the present study.

1.2.5 Limitations of animal models of diabetes

The genetic homogeneity of inbred animals and the lack of environmental triggers in a pathogen-free setting have raised concerns about the validity of animal models. Some of the commonly used animal models have specific genetic defects that are not seen in humans [Lohmann, 1998]. A lack of reproducible animal models of certain human diabetic complications and the disappointing results of studies aimed at preventing type 1 diabetes, based on strategies that were successful in rodents, have also been noted [Rees and Alcolado, 2005].
1.3 Pregnancy

1.3.1 Carbohydrate metabolism in pregnancy

During pregnancy, a progressive rise in insulin resistance occurs that begins near mid-pregnancy and progresses through to the third trimester [Buchanan and Xiang, 2005]. The insulin resistance appears to result from a combination of increased maternal adiposity and the rise in hormones secreted by the growing fetoplacental unit, which are antagonistic to the actions of insulin. These include human chorionic somatomammtropin, progesterone, cortisol, and prolactin [Butte, 2000]. The fact that placental hormones are responsible for insulin resistance is also suggested by the observation that following delivery, this resistance is diminished [Sivan et al., 1997].

Pancreatic β–cells normally increase their insulin secretion to compensate for the insulin resistance of pregnancy. A progressive increase in basal and postprandial insulin concentrations is seen with advancing pregnancy. By the third trimester, basal and 24-hour mean insulin concentrations may double [Lesser and Carpenter, 1994]. This ensures that maternal glucose levels do not increase to a large degree compared with the large changes in insulin sensitivity. Thus glucose regulation during pregnancy requires adequate β–cell function to overcome progressive insulin resistance.

By the third trimester, postprandial glucose concentrations are significantly elevated and the glucose peak is prolonged [Cousins et al., 1980]. This may be due to impaired insulin-mediated glucose utilization, suppression of endogenous glucose production, and inadequate increase in first-phase insulin secretion [Di Cianni et al., 2003]. Women with gestational diabetes have decreased insulin sensitivity in comparison with weight-matched control groups [Ryan et al., 1985; Xiang et al., 1999]. Furthermore, decreased insulin secretion rates have also been noted in women with GDM, making them unable to compensate for the increased insulin resistance [Homko et al., 2001]. The ultimate effect of these numerous metabolic changes in pregnancy is a pre-disposition for glucose intolerance; which in individuals with risk factors for diabetes, can lead to GDM.
1.3.2 Cardiovascular changes in pregnancy

Pregnancy is associated with numerous cardio-vascular adaptations, which promote healthy fetal development without compromising maternal circulation in other organ systems. This includes a rise in blood volume; which reaches a plateau at approximately 34 weeks and can exceed the non-pregnant value by up to 45-55% at term [Rovinsky and Jaffin, 1965]. This is associated with an increase in the cardiac volume by about 40%, mainly due to an increase in the stroke volume, but also due to an increase in heart rate [Walters et al., 1966].

Despite this marked increase in blood volume, the blood pressure in normal pregnancies does not rise correspondingly, due to a reduction in the peripheral arterial resistance. Although studies have investigated whether this reduced resistance is a result of vaso-relaxants such as prostacyclin or nitric oxide (NO), its precise cause remains unknown. Prostacyclin has been associated with attenuation in angiotensin 2-induced uterine vasoconstriction in sheep [Magness et al., 1992]. Increased generation of NO has been demonstrated in hand blood flow of pregnant women [Williams et al., 1997]. Increased urinary excretion of nitrate, which was inhibited by chronic infusion of the nitric oxide synthase inhibitor L-N arginine methyl ester (L-NAME), has been demonstrated during pregnancy in rats [Conrad et al., 1993]. However, other studies in rats have suggested that increased NO production is not responsible for this decrease in vascular resistance [Ahokas et al., 1991; Umans et al., 1990].

It has been hypothesized that the elevated levels of urinary nitrate excretion may reflect local increases in NO production in specific vascular beds [Poston et al., 1995]. For example; in the sheep uterine circulation, oestrogen-induced vasodilation has been demonstrated to be mediated by NO [Rosenfeld et al., 1996]. The above studies indicate that the state of pregnancy is associated with important alterations to vascular function in the uterine circulation. Although teleologically these changes are aimed at improving blood flow to the fetus, they may also lead to altered effects of glucose on vascular function. This possibility has been investigated further in this study.
1.4 Arteries

1.4.1 Blood flow to the fetus

A substantial portion of cardiac output (approximately 10% at term) is directed to the uterine circulation to meet the needs of the growing fetus [Assali et al., 1978]. To accommodate this increase in uterine blood flow, the uterine vasculature undergoes marked morphologic changes: the diameters of the blood vessels (both veins and arteries) increase resulting in a fall in resistance of the smaller vessels [Moll, 2003]. Aberrant vascular function in the uterine arteries has been demonstrated to have deleterious effects on the fetus. Reduction in uterine blood flow was associated with fetal hypoxemia, acidemia and changes in fetal cardiovascular function [Stevens and Lumbers, 1990]. An association between increased uterine artery resistance and recurrent pregnancy loss has also been demonstrated [Habara et al., 2002]. In pregnant streptozotocin-induced diabetic rats, decreased basal prostacyclin production with reduced myometrial blood flow has been reported [Takeda and Kitagawa, 1992]. However, little is known about whether the vascular changes associated with diabetes in pregnant women affect blood flow to the fetus.

1.4.2 Resistance arteries

Poiseuille’s equation states that in tubes of uniform diameter carrying a homogeneous liquid (such as water) the flow is proportional to the fourth power of radius [Badeer, 2001]. Although this equation cannot be directly applied to biological systems (as arteries are not rigid tubes and blood is heterogeneous) it still highlights the fact that even small changes in vessel diameter will result in disproportionately large changes to blood flow. This concept forms the basis of resistance arteries, which have been defined as pre-capillary vessels that contribute both passively to the resting resistance and actively to the blood flow control during altered demands [Duling, 1991]. Although most small pre-capillary arteries satisfy the first part of this definition by contributing to resistance, it is less certain which among these can control blood flow during altered demands. The limitations of the
various methodologies used to study vessels have resulted in heterogeneous data and uncertainty regarding the location of true resistance arteries.

In one of the earliest studies, Sugiura et al. showed that in the dog mesentery the average fall in blood pressure was 7.5 mm Hg between the aorta to 1mm-diameter arteries, and 17 mm Hg between aorta to 200 µm-diameter arteries [Sugiura and Freis, 1962]. This study concluded that in dogs, arterioles less than 200 µm in diameter fulfilled the criteria for resistance vessels. Most of the available evidence originates from measurements conducted in small animals during anaesthesia, which in itself can alter the vascular resistance [DeLano et al., 1991]. It has been reported that 100- to 300-µm mesenteric arteries from conscious rats also comply with the criteria for resistance arteries [Christensen and Mulvany, 1993]. In the human uterine circulation, myometrial arteries are likely to represent the prime site of resistance because the pregnancy-associated increase in diameter and loss of contractile function in the more distal spiral arteries eliminate their functional role in vascular control [Kublickien et al., 1997]. These myometrial arteries are important in regulating uterine blood flow, as they are densely innervated and exhibit a more pronounced effect to vasoconstrictors than the main branches of the uterine artery [Akerlund, 1994]. Due to these reasons, the effects of glucose on myometrial arteries have been examined in this study.

1.5 The Endothelium

1.5.1 Endothelial function

The endothelium is a single layer of cells which constitutes the inner—most surface of the vessel wall. It forms the interface between blood flow and the blood vessel wall. Previously thought to be an inert layer of cells, it is now known to regulate vasomotor tone, by secreting various vasoactive factors which modulate both relaxation and constriction (Table 1.2). Our understanding of its function has improved dramatically over the past decades since Furchgott’s pioneering discovery of the vasoregulatory role of the endothelium [Furchgott and Zawadzki, 1980]. The interest in endothelial function is based on its pivotal role in various diseases such as diabetes, coronary artery disease, hypertension and pre-eclampsia. Ischaemic heart disease, cerebro-vascular disease and renal failure are leading causes of death in the
developed world. These disorders are often a result of atherosclerosis, hypertension and diabetes. Research into endothelial function may pave the way for novel therapies aimed at alleviating the morbidity and mortality from these conditions. Furthermore, as endothelial dysfunction is a common feature of these inter-related disorders, knowledge gained by studying one disease may be applicable to another.

| Vasoregulation | Relaxation          | Nitric Oxide                  |
|                |                     | Prostacyclin                  |
|                |                     | EDHF                          |
|                | Contraction         | Endothelin - 1                |
|                |                     | PGH$_2$                       |
|                |                     | Thromboxane A$_2$             |
| Permeability   | VCAM-1, ICAM-1, PCAM-1, P and E selectin |
| Vasculogenesis | VEGF, TGF β         |
| Haemostasis    | Coagulation         | Prostacyclin                  |
|                |                     | Thromboxane A$_2$             |
|                |                     | vWF                           |
|                | Fibrinolysis        | tPA                           |
|                |                     | PAI-1                          |

Table 1.2: Vasoactive products synthesized by the endothelium
Abbreviations listed on page 23.

1.5.2 Endothelium-dependent mediators of relaxation

The three mediators responsible for endothelium-dependent relaxation are nitric oxide (NO), prostacyclin (PGI$_2$) and the endothelium derived hyperpolarizing factor (EDHF). However, not all endothelial cells release all of the mediators. Variation with species is seen: endothelium-dependent relaxation in fish is mediated mainly by prostanoids, whereas that in frogs by NO [Vanhoutte and Scott-Burden, 1994]. In mammalian species, all three mediators are involved in endothelium-dependent relaxation, although there are variations between vascular beds.
1.5.2.1 Nitric Oxide

Nitric oxide (NO) is an important endothelium derived relaxing agent that diffuses easily across cell membranes, as it is lipophilic and has a small molecular weight. NO is synthesized from L-arginine by the enzyme endothelial Nitric Oxide Synthase (eNOS), which is stimulated by shear stress [Ayajiki et al., 1996]. The NO crosses the endothelium and reaches the smooth muscular tissue of the arterial wall, releasing cyclic Guanosine Monophosphate (cGMP) from GTP [Rapoport and Murad, 1983]. This in turn regulates the cytosolic Ca$^{2+}$ and causes smooth muscle relaxation leading to vasodilation (Figure 1.2).

![Endothelium-dependent mediators of vasodilation](image)

**Figure 1.2:** Endothelium-dependent mediators of vasodilation

Abbreviations listed on page 23.

In vessels stripped of endothelium, the response to stimulation by NO releasers such as acetylcholine or shear stress is lost, despite the persistent response to exogenous nitrates and sodium nitroprusside [Furchgott and Zawadzki, 1980]. Endothelium-dependent vasodilation has become synonymous with intact or normal endothelial function and preserved NO bioavailability.
NO production has been demonstrated to vary depending on gender. Hayashi et al. demonstrated that basal NO release from endothelium-intact aortic rings in rabbits depends on circulating estradiol concentration, as this basal release was greater in females than males, but returned to the level of males after oophorectomy [Hayashi et al., 1992]. In addition to the effects of oestrogen, another explanation for the gender differences is that the main endothelial mediator of vasodilation may vary in males and females. EDHF has been demonstrated to be the predominant endothelium-derived relaxing factor in female mice, whereas NO and PGI$_2$ were more important in male mice [Scotland et al., 2005].

Despite reduced bioavailability of NO, vascular disease is not necessarily associated with a complete loss of either flow-mediated or agonist-induced vasodilation, because mediators which are thought to play a minor role in the regulation of tone in healthy vessels may compensate (at least partially) for the lack of NO. For example, depending on the vascular bed, either PGI$_2$ [Sun et al., 1999] or EDHF [Huang et al., 2001] can mediate flow-induced dilation in eNOS knockout mice. Exogenously applied NO decreases EDHF-mediated responses in isolated arteries [Bauersachs et al., 1996; Nishikawa et al., 2000]. After angioplasty, the regenerated endothelium in the porcine coronary artery generates less NO; but relaxation to bradykinin is maintained via an EDHF-dependent mechanism [Thollon et al., 2002]. These studies reveal a more complicated inter-relationship between the endothelial mediators than previously thought. They also suggest that the blocking of one mediator, rather than isolating the remaining mediators, may actually increase their role in inducing endothelium-dependent vasodilation.

### 1.5.2.2 Prostacyclin

Prostacyclin is a prostaglandin derivative produced by vascular endothelial cells from arachidonic acid by the enzymes cyclo-oxygenase (COX) and prostacyclin synthase [Parkington et al., 2004]. Its release is stimulated by bradykinin and adenine nucleotides [Guerci et al., 2001]. Prostacyclin acts by stimulating adenylate cyclase and by increasing intracellular cyclic adenosine monophosphate (cAMP) in vascular smooth muscle [Kukovetz et al., 1979]. This in turn stimulates ATP.
sensitive $K^+$ channels to cause hyperpolarization of the cell membrane and inhibit the development of contraction [Parkington et al., 2004]. cAMP also increases the extrusion of $Ca^{2+}$ from the cytosol in vascular smooth muscle and inhibits the contractile machinery [Abe and Karaki, 1992; Bukoski et al., 1989]. Unlike NO, the vasodilatory effect of prostacyclin depends on the expression of certain receptors in vascular smooth muscle [Halushka et al., 1989]. Hence in arterial beds that do not express such receptors, prostacyclin does not contribute to endothelium-dependent relaxation. Prostacyclin facilitates the release of NO by endothelial cells [Shimokawa et al., 1988]. Furthermore, the action of prostacyclin in vascular smooth muscle is potentiated by NO, as the increase in cGMP in target cells inhibits a phosphodiesterase that breaks down cAMP [Delpy et al., 1996]. Therefore, NO indirectly prolongs the half-life of the second messenger of prostacyclin.

### 1.5.2.3 Endothelium Derived Hyperpolarising Factor (EDHF)

EDHF is defined as a mediator of vascular relaxation via a non-NO, non-prostanoid mechanism [McGuire et al., 2001]. This mediator was so-named as these cyclo-oxygenase and nitric oxide inhibitor-independent dilator responses are associated with vascular smooth muscle hyperpolarisation. EDHF-mediated responses involve an increase in the intracellular calcium concentration, the opening of calcium-activated potassium channels of small and intermediate conductance and the hyperpolarization of the endothelial cells [Feletou and Vanhoutte, 2006]. An increase in potassium ion efflux from a cell results in a more negative resting membrane potential, leading to hyperpolarisation. The amplitude of the hyperpolarisation is inversely proportional to the extracellular concentration of $K^+$ ions and the hyperpolarisation disappears totally in concentrations higher than 25 mmol/L [Chen and Suzuki, 1989]. The identity of this so-called EDHF is controversial, probably because more than one type of EDHF exists with substantial species and regional heterogeneity.

EDHF appears to play a more important role in mediating relaxation in smaller arteries than larger arteries. A study in rats has demonstrated that the contribution of NO was most prominent in the aorta, whereas that of EDHF was most prominent in the distal mesenteric arteries [Shimokawa et al., 1996]. One explanation for this may be the association between EDHF and gap junctions. Electrical conductance
can occur directly from endothelium to smooth muscle via gap junctions, formed between the cells by connexin proteins. Gap junctions are conspicuously present in small vessels, where the apposition of 2 cells is the closest. This may explain the greater role for EDHF in small arteries [Pepper et al., 1992]. In murine resistance vessels the predominant agonist-induced endothelium-dependent vasodilation in vivo and in vitro is mediated by an EDHF-like principle that requires functional gap junctions [Brandes et al., 2000]. This has been corroborated by Luksha et al. who demonstrated that gap junctions are involved in the EDHF-mediated responses to bradykinin in small subcutaneous arteries in normal pregnancy [Luksha et al., 2004]. Non-NO/non-prostanoid endothelium dependent hyperpolarization has been postulated to be the pre-dominant mechanism for endothelium-dependent relaxation in the human sub-cutaneous vascular bed [Buus et al., 2000].

There also appears to be a gender variation in the effect of EDHF, as male mice exhibit markedly reduced EDHF activity compared to females [Scotland et al., 2005]. This effect may be mediated by the female sex hormone oestrogen. There is evidence that oestrogen deficiency in ovariectomized rats specifically impairs EDHF; possibly by reduced expression of connexin-43, a component of gap junctions in resistance arteries [Liu et al., 2002]. Further evidence for a link between oestrogen and EDHF comes from a study demonstrating variations in the contribution of EDHF to relaxation depending on the day of the oestrus cycle in perfused rat uterine vasculature [Lucca et al., 2000].

1.5.3 Endothelium-derived vasoconstrictors

The endothelium also secretes vasoconstrictors, which play a role in regulating vascular tone (Table 1.2). Endothelin is present in healthy individuals in low concentrations and is involved in vascular counter-regulation for preserving peripheral resistance [Guerci et al., 2001]. Under certain conditions, the endothelium also produces endothelium derived contracting factors such as prostaglandin H2 and thromboxane A2, which activate specific receptors on the vascular smooth muscle.
1.5.4 Other factors affecting endothelial function

1.5.4.1 Age

Endothelial function can deteriorate with age [Gerhard et al., 1996; Lyons et al., 1997]. eNOS expression has been demonstrated to be up-regulated with age [Briones et al., 2005]. However, the production of reactive oxygen species from NADPH oxidase also increases with age, which may counter-act increased NO production [Briones et al., 2005; van der Loo et al., 2004]. Endothelium-dependent relaxation to acetylcholine has been demonstrated to be reduced with advancing age in humans [Taddei et al., 1995].

1.5.4.2 Smoking

Both short-term smoking [Lekakis et al., 1997] and passive smoking [Celermajer et al., 1996] have been reported to impair endothelium-dependent dilatation. The latter study in healthy young adults demonstrated that the impairment was related to the degree of exposure.

1.5.4.3 Ethnicity

Compared to Caucasians, endothelial cells from Afro-Caribbeans have been described to have reduced release of bioactive NO, with an accompanying increase in the release of superoxide anions [Kalinowski et al., 2004].

1.5.4.4 Cholesterol

Hypercholesterolaemia has been associated with impaired endothelium-dependent relaxation in human resistance vessels [Shiode et al., 1996]. This impairment has been demonstrated to be reversed by reducing serum cholesterol [Leung et al., 1993].

1.5.5 Endothelial function in pregnancy

Enhanced sensitivity to acetylcholine-mediated relaxation in pregnancy, compared to the non-pregnant state has been demonstrated in uterine arteries [Lucca et al., 2000] and mesenteric arteries [Gerber et al., 1998] of rats. Another study has reported enhanced NO-mediated relaxation in the uterine artery of pregnant rats [Ni
et al., 1997]. In healthy pregnant women, endothelium-dependent relaxation (measured by flow mediated dilatation) has been found to be enhanced in the third trimester of pregnancy, compared to the first and second trimester [Faber-Swensson et al., 2004]. In contrast to these findings, McCarthy et al. demonstrated similar endothelium-dependent relaxation in small subcutaneous arteries of both pregnant and non-pregnant women [McCarthy et al., 1994]. This suggests that endothelial function may vary in different vascular beds in pregnancy, emphasising the importance of studying the relevant vascular bed directly.

The differences in endothelial function between vascular beds in pregnancy are further illustrated by human studies examining subcutaneous and myometrial arteries. Luksha et al. found that EDHF and NO contributed equally to endothelium-dependent relaxation in subcutaneous arteries from healthy pregnant women [Luksha et al., 2004]. However, in myometrial arteries from healthy pregnant women, neither NO inhibition alone nor EDHF inhibition alone affected endothelium-dependent relaxation [Kenny et al., 2002]. When both NO and EDHF inhibition were combined, a significant impairment of relaxation was noted. This implies a compensatory mechanism among the endothelial mediators which can rectify the absence of one particular mediator in the myometrial vascular bed. This feature was reported by Kenny et al. to be absent in arteries isolated from healthy non-pregnant women. These studies indicate that the role of NO and EDHF varies in subcutaneous and myometrial vascular beds during normal pregnancy. The inter-relationship between these mediators may also be altered in pregnancy, with changes in the amount of relaxation contributed by each mediator.

1.5.6 Methods for measuring endothelial function

The evaluation of endothelial function can theoretically be made by evaluating any of its specific functions such as vasoregulation, haemostasis, vasculogenesis or permeability. Of these, the most important is the vasoregulatory role of endothelium, which has resulted in the measurement of endothelium-dependent vasodilation becoming the main parameter in both basic science and clinical research for assessing endothelial function. Early studies examined endothelial
function by catheterising coronary arteries [Ludmer et al., 1986]. More recent studies have utilised forearm plethysmography and ultrasonography.

1.5.6.1 Venous occlusion plethysmography

The underlying principle of venous occlusion plethysmography involves the arrest of venous outflow from the forearm such that it begins to swell. The rate and degree of swelling reflects forearm vascular resistance, which is a function of normal vascular endothelial function [Alam et al., 2004]. The increase in forearm volume is taken to represent blood flow in the resistance vessels of the forearm (muscle, soft tissues and skin). Venous occlusion plethysmography has been used to investigate endothelial dysfunction in diabetes using intra-arterial infusions of drugs such as acetylcholine and glyceryl trinitrate [McVeigh et al., 1992].

1.5.6.2 Brachial artery flow-mediated vasodilation

Most clinical studies have utilised this technique, making it the most common method for assessing endothelial function. Ischaemia is induced by the inflation of an arterial occlusion cuff, positioned on the proximal or mid-forearm. After cuff deflation, brachial artery flow increases because of downstream vessel dilation, and this augmented flow increases brachial artery shear stress, resulting in vasodilation [Anderson and Mark, 1989]. The precise mechanism of flow-mediated vasodilation is unknown, but the release of NO through shear stress-mediated stimulation of the endothelium is thought to be involved [Faulx et al., 2003]. In patients with impaired NO bioavailability (and therefore impaired endothelial function), the dilator response is diminished, and in some patients a constrictor response is seen.

Although the use of this technique is widespread, it is subject to important limitations, which throw into doubt the validity of some of the previous conclusions reached about endothelial function. As these limitations are important in critically evaluating such studies, they have been examined in detail.
1.5.6.3 Limitations of brachial artery FMD

Although most reported inter-observer variability rates are low (< 5%), they are usually expressed as a percentage of the baseline diameter. However, when expressed as the percent increase above baseline diameter, variability is more marked, with coefficients of variation up to 33% [Woodman et al., 2001]. Another potential problem relates to image timing. Because of the wide baseline variability in the normal response to increased flow, present techniques may fail to detect the true peak of FMD [Bressler et al., 2000]. The location of the occlusion cuff may also affect measurements, as FMD was more pronounced when the occlusion cuff was placed on the proximal upper arm, as opposed to the forearm [Berry et al., 2000].

Visualization of the vascular intima is often difficult in patients who are obese, because the distance between the ultrasound probe and the vessel increases and this attenuates the ultrasound signal. This is of particular relevance to diabetes, as a large number of patients are either overweight or obese. Raised BMI has been associated with diminished flow-mediated dilation [Olson et al., 2006].

Age, sex and ethnicity can also influence results. Although the compliance of elastic arteries generally decreases with increasing age, brachial artery compliance in women may actually increase [van der Heijden-Spek et al., 2000]. Young, healthy
African-American men and women have significantly reduced post-ischemic brachial artery dilation compared with similar Caucasian subjects [Perregaux et al., 2000].

Measurements of endothelium-dependent relaxation are based on shear stress in large conduit arteries, which triggers the release of NO. It is uncertain how this stimulus affects the other mediators of endothelium-dependent relaxation. The degree to which shear stress influences endothelium-dependent relaxation in resistance arteries is also unknown.

From the above discussion, it can be inferred that the results obtained from FMD studies often cannot be extrapolated to other systemic vessels or resistance arteries. It is therefore necessary to examine the relevant vascular bed directly in order to achieve a proper understanding of its endothelial function.

### 1.6 Endothelial dysfunction in diabetes

Impaired endothelium-dependent vasodilation has been demonstrated in various vascular beds such as forearm, cerebral, coronary and subcutaneous arteries in patients with diabetes as well as various animal models of this disease. However, few studies have examined the uterine circulation in pregnancies complicated by diabetes, either in animals or humans. It is therefore uncertain if abnormal endothelial function in pregnancies complicated by diabetes is responsible for the higher rate of complications seen in this condition.

#### 1.6.1 Animal studies of endothelial function in diabetes

Studies investigating endothelium-dependent vasodilation in animal models of type 1 diabetes have yielded conflicting results. Early studies showed normal [Head et al., 1987; Mulhern and Docherty, 1989] and even enhanced [Bhardwaj and Moore, 1988] endothelium-dependent relaxation in rat models of diabetes. However, more recent studies have demonstrated impaired endothelium-dependent relaxation in streptozotocin-induced diabetic rats [Otter and Chess-Williams, 1994; Pieper and Peltier, 1995]. Endothelial dysfunction has been reported in large conduit arteries.
such as the aorta in diabetic rabbits [Tesfamariam et al., 1989] as well as in smaller vessels such as mesenteric arteries in diabetic rats [Palmer et al., 1998]. Some of the discrepancies noted may be due to differences in the vascular bed, animal model and agonists used. Damage to the endothelium during vessel preparation can also make interpretation of results difficult. Nevertheless, the majority of studies have demonstrated impaired endothelium-dependent relaxation with diabetes.

1.6.2 Human studies of endothelial function in diabetes

Human research on endothelial function in diabetes has similarly led to divergent results. Patients with type 1 diabetes and normoalbuminuria have been demonstrated to have both impaired [Lekakis et al., 1997] and normal [Lambert et al., 1996; Smits et al., 1993] endothelium-dependent relaxation. Type 1 patients with a long duration of disease, but good glycaemic control were reported to have normal endothelial function [Enderle et al., 1998], whereas patients with uncomplicated type 2 diabetes demonstrated endothelial dysfunction [Gazis et al., 1999].

The reasons for the above discrepancies may involve differences in the type of diabetes, duration and glycaemic control; as well as the presence of clinical characteristics affecting endothelial function such as hypertension, hyperlipidaemia and obesity. These factors are of particular relevance in type 2 diabetes and may confound results. Most of these studies were performed using brachial artery flow-mediated dilation, the limitations of which have been previously discussed. Another reason for the discordant results may be small study sizes. For example, the findings of Smits et al. were based on 11 diabetic patients in the study group.

1.6.3 Mechanisms of endothelial dysfunction in diabetes

A) Decreased production of vasorelaxants
Elevated glucose levels have been demonstrated to inhibit insulin-stimulated NO release in human endothelial cells [Salt et al., 2003]. Hyperglycaemia has been associated with depletion of L-arginine, a precursor of NO, in mesangial cells of rats [Trachtman et al., 1997]. Reduced serum arginine levels have also been
observed in diabetic rats [Pieper and Peltier, 1995]. However, another study demonstrated that although serum arginine was diminished in diabetic rats, NO synthase was increased [Rosen et al., 1996]. This increased NO generation may reflect a compensatory mechanism to balance the enhanced inactivation of NO [Angulo et al., 1998]. Exogenous L-arginine improved the endothelium-dependent vasodilation in diabetes in some studies [Matsunaga et al., 1996; Pieper and Peltier, 1995], whereas this effect was not seen in others [Mayhan et al., 1997; Zsofia Koltai et al., 1997]. The different results may be due to certain systemic effects of arginine that have been noticed in patients with diabetes: high doses of arginine were associated with increased plasma insulin [MacAllister et al., 1995]. Insulin itself is associated with vasodilation that is mediated by NO [Steinberg et al., 1994].

B) Enhanced inactivation of vasorelaxants
Endothelial dysfunction has been prevented by anti-oxidants, suggesting that impaired vasodilation may be due to an accelerated inactivation of NO by superoxide anions [Rosen et al., 1996]. Reduced NO availability through the interaction between NO and superoxide anion has been demonstrated to be a causal factor for the impaired vasodilation seen in a rat model of type 2 diabetes [Kim et al., 2002].

C) Decreased responsiveness of smooth muscle to vasorelaxants
An impaired response to nitrovasodilators has been noted in patients with diabetes, suggesting a generalized reduction in the sensitivity of the smooth muscle to NO [Calver et al., 1992; McVeigh et al., 1992].

D) Enhanced generation of vasoconstrictors
Endothelium-derived constricting factors (Table 1.2) may be released along with the mediators of relaxation, opposing their effect on smooth muscle cells. The impaired relaxation with acetylcholine in diabetic rats has been associated with stimulation of the thromboxane A2 - prostaglandin H2 receptor [Mayhan et al., 1991]. In the aorta of diabetic animals, the impaired relaxations have been demonstrated to be restored by non-specific cyclo-oxygenase blockade, but not by thromboxane A2 synthase blockers; suggesting that the culprit is a prostaglandin endoperoxide [Shimizu et al., 1993; Tesfamariam et al., 1989]. In contrast to these findings, cyclo-oxygenase
inhibition did not restore impaired endothelium-dependent relaxations in mesenteric arteries of diabetic rats [Taylor et al., 1992]. These studies suggest that the role of constrictor prostanoids in diabetes varies depending on the vascular bed. The relative potency of the vasoconstrictor effect may also differ at individual sites.

1.6.4 Endothelial dysfunction and regional variations

Regional variations in endothelial dysfunction exist, which may be an important reason for the conflicting reports in diabetes vascular research. A study in diabetic rats has demonstrated impaired endothelium-dependent relaxation in the mesenteric circulation which was absent in the aorta of the same animals [Taylor et al., 1994]. Similarly, loss of bradykinin-mediated vasodilation in the hindquarters vascular bed of diabetic rats has been reported with normal renal and mesenteric vasodilatations [Kiff et al., 1991]. A possible explanation for these findings could be that endothelial cells from individual vascular beds exhibit metabolic differences, and may be affected differentially by hyperglycaemia [Sobrevia and Mann, 1997]. A recent study looking at the femoral, mesenteric and carotid vascular beds in diabetic rats has demonstrated heterogeneity in endothelial responses in these arteries [Shi et al., 2006]. This study also illustrated that under physiological conditions, the production of EDHF can be curtailed by the production of NO. As NO synthesis is impaired in diabetes, this inhibition is alleviated; resulting in improved EDHF-mediated vasodilation.

The above studies indicate that endothelium-dependent relaxation varies in different vascular beds in diabetes. As mentioned previously, studies have demonstrated a gradient in the release of endothelial mediators, with a progressively increasing contribution of EDHF in the more distal vessels (Section 1.5.2.3). The overall picture is further complicated by the fact that in diabetes, reduced bioavailability of NO can affect other mediators such as EDHF. Most studies have been performed in animals, which restricts conclusions from being drawn regarding these changes in humans. This emphasizes the importance of studying relevant vascular beds directly to assess the effect of diabetes in humans.
1.6.5 Other factors influencing endothelial dysfunction in diabetes

1.6.5.1 Control of diabetes

Endothelial dysfunction associated with diabetes is closely related to metabolic control of the disease; endothelial function improving with better control. This has been demonstrated in both animal and human studies of diabetes. Streptozotocin-induced diabetic rats, with good metabolic control (HbA1C 5.5 – 7.4%) had similar endothelium-dependent relaxation as non-diabetic rats, whereas rats with HbA1C levels greater than 7.5% showed a significantly impaired response [Angulo et al., 1998]. In contrast to type 2 diabetic patients with cardiovascular complications, type 1 patients with a long duration of diabetes and good long-term metabolic control have been demonstrated to have similar endothelial function as control subjects [Enderle et al., 1998]. In a study of ten patients with type 1 diabetes, poorly controlled diabetes over 48 hours resulted in reduced flow-mediated dilation [Sorensen et al., 2005]. However, a concurrent impairment of endothelium-independent vasodilation with nitroglycerin was also seen with hyperglycaemia. This restricts the assessment of endothelial function in this study and raises the possibility of aberrant smooth muscle function. Von Willebrand factor, a marker of endothelial function, was found to be elevated during poor glycaemic control in this study; although other markers such as soluble intercellular adhesion molecule (sICAM) were found to be normal. In contrast, both of these endothelial markers were found to be unaffected by improved glycaemic control in patients with type 2 diabetes [Yudkin et al., 2000]. Another study in patients with type 2 diabetes demonstrated improved flow-mediated dilation with improved glycaemic control [Gaenzer et al., 2002]. These studies indicate that glucose levels can influence endothelial function, although the precise association between the two remains poorly characterised.

1.6.5.2 Obesity

Obesity is commonly seen in patients with type 2 and gestational diabetes. However, conflicting reports exist regarding the relationship between overweight/obesity and endothelial dysfunction. Obesity has been independently associated with endothelial dysfunction which was related to insulin resistance
Various studies have reported that weight reduction in obese individuals improved endothelium-dependent vasodilation [Sasaki et al., 2002; Sciacqua et al., 2003]. This improvement has also been linked to a reduction in plasma glucose levels [Raitakari et al., 2004]. However, it has also been demonstrated that basal release of endothelial nitric oxide is maintained in overweight and obese adults [DeSouza et al., 2005]. Furthermore, a study examining monozygotic twins reported that endothelial dysfunction was not correlated with obesity, but with the accompanying metabolic abnormality of adiponectin deficiency [Pietilainen et al., 2006]. From the above, it is evident that the link between obesity and endothelial dysfunction is complex; with involvement of factors such as insulin resistance, hyperglycaemia and hypo-adiponectinemia.

**1.6.5.3 Lipids**

Diabetes is associated with alterations in lipid profile, which may have an effect on endothelial function. In type 1 diabetes, the cholesterol lowering agent pravastatin has been demonstrated to improve endothelial function [Joyce et al., 2004]. Lowering LDL cholesterol has been associated with improvement in endothelial function in obese women with previous gestational diabetes [Bergholm et al., 2003]. Fatty acids have been demonstrated to impair endothelium-dependent relaxation in animal conduit arteries [Edirisinghe et al., 2006] and resistance arteries [Sainsbury et al., 2004].

Another pathway for endothelial dysfunction in diabetes involves LDL oxidation. In a study where patients with type 2 diabetes were given two different meals, (resulting in two different levels of post-prandial hyperglycaemia), LDL was more susceptible to oxidation after the meal that produced greater hyperglycaemia [Ceriello et al., 1999]. Oxidized LDL is degraded through lectin-like oxidized LDL receptor-1 (LOX-1), which is expressed in vascular endothelium [Sawamura et al., 1997]. Oxidized LDL uptake by LOX-1 reduces eNOS expression [Mehta et al., 2001], leading to diminished NO bioavailability. This mechanism may represent a link between hyperglycaemia and endothelial dysfunction.
1.6.5.4 Insulin resistance

Endothelial dysfunction has been associated with insulin resistance [Kim et al., 2006]. An enhancement in endothelial function was noticed when insulin resistance was reduced with rosiglitazone [Pistrosch et al., 2004]. Responses of angiographically normal coronary arteries to acetylcholine were impaired in subjects with insulin resistance, which was linked to oxidative stress [Shinozaki et al., 2001].

These studies indicate the multi-factorial aetiology of endothelial dysfunction in diabetes. Obesity and insulin resistance are closely associated with type 2 diabetes but not with type 1 diabetes, suggesting a distinct difference in the pathogenesis of endothelial dysfunction in these two disorders.

1.6.5.5 Gender differences in diabetes

There is evidence to suggest that gender differences exist in the effects of diabetes on vascular function. An early study demonstrated that diabetic women had a higher relative mortality from coronary heart disease than men [Garcia et al., 1974]. Relaxation to acetylcholine in the aorta of diabetic rats was described to be impaired in females, but not in males [Pinna et al., 2001]. In this study the vascular tissue from female rats was more susceptible to oxidative damage, but also more responsive to antioxidant treatment. Gender differences in vascular function, although subtle, may be of importance in the pathophysiology of diabetes in pregnancy.

1.7 Endothelial dysfunction of diabetes in pregnancy

1.7.1 Type 1 diabetes

In pregnant rats with streptozotocin-induced diabetes, enhanced endothelium-dependent relaxation to acetylcholine has been reported [Omer et al., 1999]. In this study, the activity of NOS was raised in renal, cardiac, aortic and uterine vascular beds, but not in the placenta. Using a microvascular skin perfusion technique, Ramsay et al. demonstrated improved endothelial function during pregnancy compared to the post-natal period in both healthy women and women with type 1 diabetes [Ramsay et al., 2003]. However, women with diabetes had impaired
responses compared to healthy women, both during pregnancy and in the postnatal period. Another study in pregnant women with type 1 diabetes using flow-mediated dilation of the brachial artery described impaired vasodilation, which correlated with the duration of diabetes [Savvidou et al., 2002]. In contrast, a small study investigating subcutaneous arteries from 9 pregnant women with type 1 diabetes demonstrated normal endothelial function in this vascular bed [Ang et al., 2002]. Apart from this study, type 1 diabetes in pregnancy has generally been associated with endothelial dysfunction.

1.7.2 Gestational diabetes

Subcutaneous arteries from women with gestational diabetes displayed impaired relaxation to acetylcholine compared to healthy pregnant women [Knock et al., 1997]. This study also suggested that in this vascular bed, prostacyclin may be of more importance to relaxation than NO. Using the novel technique of transcranial Doppler during visual stimulation, altered endothelial function has also been demonstrated in the cerebral circulation of women with GDM [Rosengarten et al., 2004]. Gestational diabetes has been associated with pre-eclampsia [Bryson et al., 2003]: both disorders share endothelial dysfunction as a common factor [Savvidou et al., 2003]. Furthermore, as in diabetes, the endothelial dysfunction of pre-eclampsia has been reported to be reversed by anti-oxidants [Chambers et al., 2001].

1.7.3 Previous gestational diabetes

Endothelial dysfunction has been demonstrated in patients with impaired glucose tolerance in pregnancy [Paradisi et al., 2002] and in women with previous gestational diabetes [Anastasiou et al., 1998]. Hannemann et al. used both laser Doppler fluximetry of maximum skin microvascular hyperaemia and brachial artery flow-mediated dilatation to assess endothelial function in seventeen patients with previous gestational diabetes [Hannemann et al., 2002]. Although microvascular hyperaemia was impaired compared to healthy controls, flow-mediated dilation was similar in both groups. This suggests aberrant function in the microvascular circulation that was not evident in the larger arteries. A similar study using the laser Doppler technique demonstrated that acetylcholine induced vasodilation was reduced in seventeen women with previous gestational diabetes compared with controls [Hu et al., 1998]. In contrast to the findings of Hannemann et al., both
Aberrant homeostasis and microvascular function were found to be abnormal in this study. Oral vitamin C has been demonstrated to improve endothelial function in women with previous GDM [Lekakis et al., 2000]. The small number of studies makes interpretation of vascular function in women with previous gestational diabetes challenging. Studies investigating GDM are also often constrained by relatively low number of study participants. Nevertheless, a general trend of impaired endothelium-dependent relaxation in these women has been demonstrated.

**Figure 1.4:** Overview of glucose metabolism
1.8 Glucose levels

Glucose is the key molecule for energy production in the biological world. The oxidation of glucose represents the culmination of a series of steps by which energy from the sun is directly or indirectly harnessed by organisms as diverse as anaerobic bacteria, plants and human beings (Fig. 1.4). The levels of this molecule are therefore closely regulated with varying degrees of complexity in all life forms. The breakdown of the physiological mechanisms controlling the homeostasis of glucose results in diabetes.

Considerable controversy exists over the precise levels of glucose which represent normoglycaemia, hypoglycaemia and hyperglycaemia. Data for these levels are based on population studies and the risk for developing complications of diabetes at each glycaemic level. Furthermore, numerous methods are currently in use for measuring glucose levels. The term “blood glucose” can refer to capillary blood, arterial blood, venous whole blood or plasma, all of which can vary in their values from the same individual, albeit to slight degrees. The overall uncertainty of glucose measurement has been reported to be about 5% during serial measurement [Reinauer et al., 2002].

The WHO has defined a fasting venous plasma glucose concentration less than 6.1 mmol/L as normal [Alberti and Zimmet, 1999]. Although this choice is arbitrary, such levels have been observed in people with normal glucose tolerance. Values above this are associated with a progressively greater risk of developing micro- and macro-vascular complications [Alberti, 1996; Engelgau et al., 1997]. In healthy pregnant women, random blood glucose levels at various stages in gestation have been reported to vary from 4.3 (median) to 7.3 (95th percentile) mmol/L [Ostlund and Hanson, 2004]. Another study of healthy pregnant women demonstrated a range of blood glucose (mean ± SD) from 3.0 ± 0.3 to 5.8 ± 0.3 mmol/L during pregnancy [Parretti et al., 2001]. Furthermore, daily mean glucose values increased slightly between 28 (4 ± 0.3 mmol/L) and 38 (4.3 ± 0.3 mmol/L) weeks of pregnancy, with the mean post-prandial glucose levels reaching a maximum of 5.8 mmol/L. The small rise in blood glucose values normally seen in the third trimester has been attributed to increasing insulin resistance, as discussed previously. A third study of healthy non-obese pregnant women reported a blood glucose level (mean ± SD) of
4.6 ± 1.0 mmol/L with a peak post-prandial level of 5.9 ± 0.9 mmol/L; this peak being reached in 71.4 ± 30 minutes [Yoge et al., 2004]. These three studies provide evidence that in healthy pregnant women, the blood glucose level varies approximately between 3 to 6.8 mmol/L.

1.8.1 HbA1c

HbA1c (also referred to as glycated or glycosylated haemoglobin) is the sub-fraction of haemoglobin formed as a result of irreversible, non-enzymatic glycation of the haemoglobin β-chain [Snedl et al., 2001]. The measurement of HbA1c is expressed as a percentage of total haemoglobin. The formation of glycosylated haemoglobin occurs over the average life span of erythrocytes (approximately 120 days) [Bunn et al., 1976]. Therefore, HbA1c can serve as a retrospective indicator of the average glucose concentration over the previous 8 to 10 weeks [Reinauer et al., 2002]. HbA1c concentration has been demonstrated to fall when diabetic control is improved by treatment [Koenig et al., 1976]. Although HbA1c levels have been extensively used in epidemiological studies looking at the chronic effects of diabetes, its value in pregnancy is less certain. This is because adverse outcomes have been demonstrated in pregnancy despite achieving a near optimal HbA1c concentration [Evers et al., 2004].

1.9 Hyperglycaemia

1.9.1 Definition

For the purpose of diagnosing diabetes, a fasting venous plasma glucose of ≥ 7.0 mmol/L has been considered to be hyperglycaemic and in the diabetic range [Alberti and Zimmet, 1999].

1.9.2 Estimating hyperglycaemia

Currently, self-monitored blood glucose (SMBG) is the most prevalent method for measuring blood glucose levels during pregnancy, where a drop of capillary blood from the fingertip is analysed using a glucometer. However, the timing of this test varies; with both pre-and postprandial values being measured. In the UK, preprandial levels taken three times a day and an additional fourth reading before bed
are usually advised for pregnant women with diabetes who are on insulin. Limitations of this method include poor patient compliance and errors due to improper techniques. Levels are normally measured before meals; however these values may also be affected by snacks between meals, leading to errors in interpretation. SMBG may not detect diurnal fluctuations of glucose levels, as a study has estimated that in pregnant women with poorly controlled type 1 diabetes, ten daily SMBG determinations may be required to detect these fluctuations [Kerssen et al., 2006]. Concern has also been raised about the reliability of self-generated data, as falsification has been reported [Kendrick et al., 2005].

The recent advent of a new technique, called the continuous glucose monitoring system (CGMS) has simplified the estimation of glucose levels. The blood glucose is monitored continuously by a subcutaneous implant, allowing precise estimation of glucose fluctuations. This new method has revealed that standard measures for glycaemic control may markedly underestimate the frequency of both hyperglycaemia [Praet et al., 2006] and hypoglycaemia [Cheyne and Kerr, 2002] in patients with diabetes. CGMS is of particular value in estimating the duration of each episode of hyperglycaemia and hypoglycaemia; a measurement which is difficult to make using SMBG. However, CGMS is primarily used as a research tool at present due to its cost and limited availability.

1.9.3 Degree of hyperglycaemia
A study using CGMS in women with diabetes in pregnancy demonstrated a mean peak glucose value (± SD) of 10.1 ± 3.2 mmol/L in type 1 diabetes patients, 7.2 ± 1.6 mmol/L in patients with diet-controlled GDM and 8.2 ± 2.5 mmol/L in patients with insulin-treated GDM. [Ben-Haroush et al., 2004].

1.9.4 Duration of hyperglycaemia
Buhling et al. examined the duration of hyperglycaemia present in women with diabetes in pregnancy using CGMS. The mean duration (± SD) of hyperglycaemia above 8.9 mmol/L varied from 7.5 ± 14 minutes in healthy pregnant women to 14 ± 21 minutes in women with gestational diabetes [Buhling et al., 2004]. The peak of postprandial hyperglycaemia in women with GDM and type 1 diabetes has been estimated to occur at approximately 90 minutes [Ben-Haroush et al., 2004].
### 1.9.5 Pathophysiology of hyperglycaemia

#### 1.9.5.1 Oxidative stress

Reactive oxygen species are atoms or molecules capable of independent existence that contain one or more unpaired electrons [Mehta et al., 2006]. These include various derivatives of oxygen and nitrogen such as superoxide (\(\text{O}_2^-\)), hydroxyl (\(\text{OH}^-\)), peroxide (\(\text{H}_2\text{O}_2\)) and peroxynitrite (\(\text{ONOO}^-\)). These molecules can damage protein, RNA and DNA structure, but are normally neutralized by innate antioxidant defence mechanisms, thus maintaining a stable intracellular environment. Oxidative stress implies a disturbance of this delicate balance, resulting in an excess of free radicals and its sequelae.

Hyperglycaemia exerts part of its deleterious effects by promoting oxidative stress [Monnier et al., 2006]. Hyperglycaemia has been reported to cause an overproduction of superoxide by the mitochondrial electron-transport chain [Nishikawa et al., 2000]. In normal pregnant women, it has been demonstrated that hyperglycaemia is associated with increased production of reactive oxygen species in neutrophils [Petty et al., 2005].

The link between reactive oxygen species and endothelial dysfunction involves, in part, NO metabolism (Fig. 1.5). The enzyme endothelial nitric oxide synthase (eNOS) regulates vascular tone through production of NO. Upon electron transfer from various sources, eNOS oxidises L-arginine to L-citrulline with the simultaneous release of a single molecule of NO. (6\(R\))-5,6,7,8-tetrahydrobiopterin (\(\text{BH}_4\)) is required as a co-factor for these electron transfer reactions [Vasquez-Vivar et al., 1998]. When L-arginine and \(\text{BH}_4\) are available in adequate quantities, these reactions generate NO. However, if either or both are deficient, eNOS switches from a coupled state (generating NO) to an uncoupled state, producing superoxide (\(\text{O}_2^-\)) [Alp and Channon, 2004]. The resultant \(\text{O}_2^-\) can react further with existing NO and generate peroxynitrite (\(\text{ONOO}^-\)). Diabetes is linked to this process by causing depletion of L-arginine and \(\text{BH}_4\) [Cai et al., 2005].
Anti-oxidants have been shown to attenuate some of the deleterious effects of hyperglycaemia. In intestinal arterioles of normal rats, hyperglycaemia of 16.6 mmol/L (200 mg/dl) suppressed acetylcholine mediated vasodilation; this was prevented by superoxide dismutase [Bohlen and Lash, 1993]. Treatment with anti-oxidants has been demonstrated to protect against impaired endothelium-dependent relaxation caused by elevated glucose in the aorta of diabetic rabbits [Tesfamariam and Cohen, 1992]. Vitamin C (which is an anti-oxidant) has been reported to reverse diabetes-induced impairment in endothelium-dependent relaxation in mesenteric arteries of diabetic rats [Sridulyakul et al., 2006].
1.9.5.2 Hyperglycaemia and endothelial mediators

Hyperglycaemia has been associated with the generation of vasoconstrictor prostanoids in normal rabbit aorta [Tesfamariam et al., 1990]. Ozkan et al. demonstrated that during hyperglycaemia, reactive oxygen species such as superoxide play a role in the impairment of EDHF-mediated relaxations [Ozkan and Uma, 2005]. NO availability has been reported to be reduced when the blood glucose levels were elevated above normal levels in diabetic women [Konukoglu et al., 2003]. However, the plasma nitrite and nitrate measured in this study are not accurate markers of vascular NO. In the rat spinotrapezius muscle, acute hyperglycaemia has been shown to suppress arteriolar NO production [Lash et al., 1999]. The association between hyperglycaemia and the endothelial mediators of vasodilation is unresolved, as it is unknown which of the three endothelial mediators is affected the most by alterations in glucose concentration.

1.9.6 Acute hyperglycaemia

Short periods of hyperglycaemia, referred to as “glucose spikes”, usually seen after a meal, are becoming increasingly recognised as an important factor in the pathogenesis of complications in diabetes. Flow-mediated vasodilation in the post-prandial state was found to be diminished in patients with type 2 diabetes; the reduction being inversely correlated to the magnitude of post-prandial hyperglycaemia [Shige et al., 1999].

Indirect evidence for the deleterious effects of glucose spikes also comes from studies which have demonstrated improvement in endothelial function when such spikes were suppressed. Acarbose, an α-glucosidase inhibitor that limits glucose spikes, improves endothelial dysfunction in rats exhibiting repetitive blood glucose fluctuation [Azuma et al., 2006]. As acarbose also improves the lipid profile [Ogawa et al., 2004], some of the benefits may be due to indirect effects on the endothelium. Another study has demonstrated that the avoidance of spikes in type 2 diabetes patients by intensive hospital treatment of hyperglycaemia improved endothelial function [Yasuda et al., 2006].
The mechanism of how glucose spikes mediate endothelial dysfunction is believed to be related to free radical production. Glucose fluctuations during post-prandial periods have been demonstrated to be a more effective trigger for oxidative stress than chronic sustained hyperglycaemia [Monnier et al., 2006]. Constant as well as intermittent high glucose levels have also been shown to enhance endothelial cell apoptosis through mitochondrial superoxide overproduction [Piconi et al., 2006].

1.9.7 Hyperglycaemia and endothelium-dependent relaxation

Studies examining the association between hyperglycaemia and endothelium-dependent relaxation have produced contradictory results. Impaired, unaltered and enhanced endothelium-dependent relaxation has been demonstrated in both animal and human vascular beds. These studies have been summarised in Table 1.3 (impaired), Table 1.4 (enhanced) and Table 1.5 (unaltered). These conflicting results may be attributable to differences in the species, vascular bed, methodology and both the degree and duration of hyperglycaemia. However, different results were obtained even in the same vascular bed (upper limb circulation) using similar methods. The conflicting results of these studies suggest that the actions of glucose on endothelial function are complex. They also indicate that the glucose concentration, its duration of exposure, the vascular bed studied and the methodology used are four distinct variables that require standardisation in order to understand the intricate relationship between glucose and endothelial function.
<table>
<thead>
<tr>
<th>Author</th>
<th>Vascular Bed</th>
<th>Method</th>
<th>Duration</th>
<th>Level of Hyperglycaemia</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bohlen and Lash, 1993</td>
<td>Intestinal arterioles of normal rats</td>
<td>Direct microscopic visualization</td>
<td>1 hour</td>
<td>16.6 mmol/L</td>
<td>Suppression of vasodilation</td>
</tr>
<tr>
<td>Williams et al., 1998</td>
<td>Brachial artery of healthy humans</td>
<td>Plethysmography</td>
<td>6 hours</td>
<td>16.6 mmol/L</td>
<td>Impaired endothelium-dependent relaxation</td>
</tr>
<tr>
<td>Renaudin et al., 1998</td>
<td>Trapezius muscle of normal rats</td>
<td>Intravital microscopy</td>
<td>45 min</td>
<td>16.9 ± 0.5 mmol/L</td>
<td>Vasoconstriction</td>
</tr>
<tr>
<td>Kawano et al., 1999</td>
<td>Brachial artery in normal, IGT and type 2 DM patients</td>
<td>FMD</td>
<td>1-2 hours</td>
<td>OGTT 75 g Norm 8.5 mmol/L IGT 12.2 mmol/L DM 13.3 mmol/L</td>
<td>Impaired dilatation at 1 hour in all groups</td>
</tr>
<tr>
<td>Title et al., 2000</td>
<td>Brachial artery of healthy humans</td>
<td>FMD</td>
<td>Up to 4 hours</td>
<td>75 g OGTT</td>
<td>Reduced FMD which was restored with Vitamin C and E</td>
</tr>
<tr>
<td>Beckman et al., 2001</td>
<td>Brachial artery of healthy humans</td>
<td>Plethysmography</td>
<td>6 hours</td>
<td>16.6 mmol/L</td>
<td>Reduced endothelium-dependent vasodilation, which was restored by Vitamin C</td>
</tr>
<tr>
<td>Affonso Fde et al., 2003</td>
<td>Renal artery of normal rabbits</td>
<td>Tissue perfusion</td>
<td>3 hours</td>
<td>15 mmol/L</td>
<td>Impaired endothelium-dependent relaxation</td>
</tr>
</tbody>
</table>

Table 1.3: Studies demonstrating impaired relaxation with hyperglycaemia

<table>
<thead>
<tr>
<th>Author</th>
<th>Vascular Bed</th>
<th>Method</th>
<th>Duration</th>
<th>Level of Hyperglycaemia</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cipolla et al., 1997</td>
<td>Posterior cerebral artery of rats</td>
<td>Pressure Myography</td>
<td>1-2 hours</td>
<td>44 mmol/L</td>
<td>Endothelium-dependent vasodilation</td>
</tr>
<tr>
<td>van Veen et al., 1999</td>
<td>Forearm blood flow of healthy humans</td>
<td>Forearm blood Flow</td>
<td>5 minutes</td>
<td>20% glucose infusion</td>
<td>Vasodilation not modified by hyperinsulinemia</td>
</tr>
<tr>
<td>Hoffman et al., 1999</td>
<td>Forearm blood flow of healthy humans</td>
<td>Plethysmography</td>
<td>1 hour</td>
<td>20% glucose infusion</td>
<td>Sympathoexcitation and peripheral vasodilation</td>
</tr>
<tr>
<td>Oomen et al., 2002</td>
<td>Skin of type 1 DM patients</td>
<td>Skin microvascular flow</td>
<td>1 hour</td>
<td>12 mmol/L</td>
<td>Increased blood flow</td>
</tr>
</tbody>
</table>

Table 1.4: Studies demonstrating vasodilation with hyperglycaemia
<table>
<thead>
<tr>
<th>Author</th>
<th>Vascular Bed</th>
<th>Method</th>
<th>Duration</th>
<th>Level of Hyperglycaemia</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Houben et al., 1993</td>
<td>Brachial artery</td>
<td>Laser Doppler flowmetry</td>
<td>24 hours</td>
<td>15 mmol/L</td>
<td>No effect on basal blood flow or endothelium-dependent or independent relaxation</td>
</tr>
<tr>
<td>Brands and Fitzgerald, 1998</td>
<td>Hind limb blood flow of streptozotocin treated diabetic rats</td>
<td>Arterial catheter</td>
<td>6 days</td>
<td>22 mmol/L</td>
<td>No effect on endothelium-dependent relaxation</td>
</tr>
<tr>
<td>Charkoudian et al., 2002</td>
<td>Leg skin blood flow in healthy individuals</td>
<td>Laser Doppler Flowmetry</td>
<td>6 hours</td>
<td>11.1 mmol/L</td>
<td>No effect on vasodilation</td>
</tr>
<tr>
<td>Reed et al., 2004</td>
<td>Forearm of healthy individuals</td>
<td>Forearm blood flow</td>
<td>6 hours</td>
<td>11.1 mmol/L</td>
<td>No effect on endothelial function</td>
</tr>
<tr>
<td>Oomen et al., 2004</td>
<td>Skin of type 2 DM patients</td>
<td>Laser Doppler flowmetry</td>
<td>3.5 hours</td>
<td>12 mmol/L</td>
<td>No change in micro-circulation</td>
</tr>
</tbody>
</table>

Table 1.5: Studies demonstrating no effect of hyperglycaemia on vascular function

1.10 Hypoglycaemia

1.10.1 Hypoglycaemia and pregnancy

Hypoglycaemia has been defined as a clinical and biological syndrome, caused by an abnormal decrease in plasma glucose levels to below 3 mmol/L [Virally and Guillausseau, 1999]. However, the definition of hypoglycaemia is controversial, as it has both a quantitative as well as a clinical aspect. In contrast to hyperglycaemia, hypoglycaemia can be associated with more pronounced symptoms such as tremors, headaches and behavioural changes. The threshold for developing these features can vary from patient to patient. As a result, clinically relevant hypoglycaemia may not correlate with its quantitative measurement and vice versa. A study in ten pregnant women with type 1 diabetes demonstrated an association between hypoglycaemia and an increase in frequency and amplitude of fetal heart rate accelerations, as well as a rise in maternal catecholamine levels [Bjorklund et al., 1996]. This is in contrast to a similarly sized study (also in type 1 diabetic patients) which reported...
that hypoglycaemia had no significant effect on fetal movements, heart rate or breathing [Reece et al., 1995]. Safety and ethical concerns limit the study of hypoglycaemia in women during pregnancy, making its precise effects uncertain. Nevertheless, hypoglycaemia is a common occurrence in poorly controlled diabetes, its true frequency now becoming evident in pregnancy with new techniques such as continuous glucose monitoring (CGMS) [Yoge et al., 2003]. Asymptomatic hypoglycaemic events in women with gestational diabetes have been reported to be particularly common overnight in insulin-treated women [Yoge et al., 2004]. Hypoglycaemia approximately 160 minutes after a meal has also been noted in women with diabetes in pregnancy [Ben-Haroush et al., 2004].

1.10.2 Hypoglycaemia and endothelial function

A study investigating the effect of hypoglycaemia on rat middle cerebral artery demonstrated that reducing the glucose concentration from 5.5 mmol/L to 1.0 or 0.5 mmol/L for 1.5 hours each had no significant effect on arterial diameter [Swafford et al., 1998]. The production of prostacyclin has been found to be increased in cultured human endothelial cells exposed to a low concentration of glucose [Watanabe and Jaffe, 1995]. Little is known about the effect of hypoglycaemia on endothelial function, as research in diabetes has concentrated on the vascular effects of hyperglycaemia. The surprising paucity of published studies examining endothelial function during hypoglycaemia prevents any meaningful analysis of its effects.

1.11 Blood glucose in pregnancy

Karlsson and Kjellmer were among the first to demonstrate a relationship between fetal outcome and glycaemic control; reporting a linear correlation between the third trimester mean maternal blood glucose and the perinatal mortality rate [Karlsson and Kjellmer, 1972]. Since then, various studies have shown the beneficial aspects of glucose control on pregnancy outcome in diabetes [Crowther et al., 2005; Drexel et al., 1988]. However, the criteria for satisfactory metabolic control vary widely, and studies showing a low perinatal morbidity and mortality differ in their details. For example, in the study by Crowther et al., although outcome improved with treatment in GDM patients, the actual glucose levels these women attained were not assessed. Karlsson and Kjellmer reported a low perinatal morbidity at a mean third
trimester blood glucose value of less than 5.5 mmol/l. These mean values were obtained by averaging daily measurements that were taken at three unspecified times during the day, without considering the effect of meals on these levels. When estimating blood glucose levels, the lack of taking into account the time since the last meal is a recurring problem in many studies. This makes interpreting the true degree of glycaemic control difficult.

Most studies currently use HbA1C as a marker for glycaemic control. HbA1C measures long-term glycaemic control, reflecting a time-weighted mean over the previous 3 to 4 months. Improvement of HbA1C is associated with a reduction of complications associated with type 2 diabetes [Stratton et al., 2000]. However, there is uncertainty regarding the precise correlation between HbA1C and the various phases of glycaemia such as basal, post-prandial and fasting. Other limitations of HbA1C include differences in predictive value between type 1 and type 2 diabetes, a considerable degree of biological variability between subjects and analytical variability [Jeffcoate, 2004].

Pregnancy complications have been demonstrated to be reduced in diabetic women with good glycaemic control, as evaluated using HbA1C [Boulot et al., 2003]. However, a large study in Netherlands surveying women with type 1 diabetes in pregnancy reported increased maternal and perinatal complications, despite achieving satisfactory HbA1C levels of less than 7.0% [Evers et al., 2004]. Rebound hyperglycaemia elicited by hypoglycaemia has been put forward as a possible explanation for this outcome, as hypoglycaemia is more common in well controlled type 1 patients [ter Braak et al., 2002]. In women with type 1 diabetes, HbA1C levels during pregnancy were found to have no correlation with neonatal hypoglycaemia or macrosomia [Taylor et al., 2002]. These studies signify that using HbA1C to assess glycaemic control may not be useful in predicting the complications of diabetes in pregnancy.

Numerous studies have examined Doppler flow parameters during hyperglycaemia. In healthy pregnant women, acute hyperglycaemia did not affect Doppler flow in the umbilical or uterine arteries at any stage of oral glucose tolerance testing [Yogevo et al., 2003]. Three studies have demonstrated no correlation between uterine and
umbilical artery Doppler waveforms and the degree of glycaemic control [Dicker et al., 1990; Ishimatsu et al., 1991; Kofinas et al., 1991]. Although these studies indicate that Doppler flow is unaffected by maternal blood glucose levels, it is uncertain whether blood flow in smaller resistance arteries are affected by glucose levels during pregnancy.

The above discussion outlines the limitations of using HbA1C and absolute glucose levels in assessing control during diabetes in pregnancy. It can be surmised that other parameters, such as fluctuation in the levels of glucose, may be of importance in diabetes complicating pregnancy. As previously described, glucose fluctuations during post-prandial periods may be a more effective trigger for oxidative stress than chronic sustained hyperglycaemia. However, the effects of acute hypoglycaemia and hyperglycaemia on resistance artery function in pregnancy are unknown.

1.12 Summary of Introduction

Poorly controlled diabetes in pregnancy, with recurrent hypo- and hyperglycaemia, is associated with an increased incidence of complications. Hyperglycaemia has been linked to aberrant endothelial function, which may have an important role in the pathophysiology of these complications, as controlling hyperglycaemia improves pregnancy outcome. However, studies investigating the effects of glucose on endothelial function have produced conflicting results, raising the possibility of heterogeneity of these effects depending on the vascular site and degree of hyperglycaemia. To discern the true effects of glucose concentrations on endothelial function, it is therefore necessary to examine the relevant vascular bed directly. In diabetes complicating pregnancy, the myometrial arterial bed is important in determining blood supply to the fetus. However, the effects of hypo- and hyperglycaemia in this vascular bed are not known, either in healthy individuals or diabetic patients. It has been demonstrated that fluctuations in blood glucose may be more important than absolute values in the pathogenesis of aberrant vascular function. However, the value of certain studies has been limited by the high glucose levels examined, which are not seen clinically. Defining clinically relevant glucose concentrations and characterising their effect on endothelial function in myometrial
arteries of healthy women and pregnant women with diabetes is therefore important in advancing our limited knowledge of this disorder.

1.13 Hypotheses

I. The effects of glucose levels on endothelial function vary in different vascular beds
II. Acute change in glucose level from the normal range is associated with alterations in endothelial function
III. Diabetes in pregnancy is associated with endothelial dysfunction in myometrial arteries

1.14 Aims

1. Identify clinically relevant glucose levels in diabetes complicating pregnancy
2. Characterise the effect of glucose levels in myometrial arteries of healthy pregnant and non-pregnant individuals
3. Examine the effect of acute changes in glucose levels in myometrial arteries in pregnancies complicated by diabetes
4. Examine the effect of acute changes in glucose levels in the uterine artery of pregnant and non-pregnant mice
5. Discern the contributions of the endothelial mediators of vasodilation in the uterine artery of pregnant mice
6. Enable further characterisation of the vascular effects of diabetes in pregnancy by creating an animal model of diabetes
CHAPTER 2

MATERIALS AND METHODS
MATERIALS AND METHODS

2.1 Ethics

The study conformed to the Declaration of Helsinki (2000) and was approved by the Central Manchester Local Research Ethics Committee under the title “Endothelial vascular behaviour in pregnancies complicated by diabetes”. Informed written consent was obtained from all participating patients (sample information sheet and consent form is given in the Appendix). Diabetic patients were recruited from the three hospitals in Greater Manchester having the largest number of patients with diabetes in pregnancy (St. Mary’s Hospital, Hope Hospital and North Manchester Hospital). All animal studies were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.2 Human Subjects

2.2.1 Healthy women

2.2.1.1 Non-pregnant women

Healthy women under the age of 60 with no co-existing disease and not on medication were selected. All patients had elective hysterectomies, the most common indication being menorrhagia.

2.2.1.2 Pregnant women

Healthy women with no documented disease and not on medication, who had uncomplicated singleton pregnancies were selected. All patients had elective Caesarean section deliveries at term, the most common indications being previous Caesarean section or breech presentation.

2.2.2 Diabetic Patients

2.2.2.1 Type 1 and type 2 diabetes in pregnancy

Patients with type 1 or type 2 diabetes diagnosed before pregnancy and not on medication during pregnancy (apart from insulin) were selected. Type 2 diabetes
patients with co-morbidities such as hypertension were excluded. Type 1 patients in the ongoing Diabetes And Pre-eclampsia Intervention Trial (DAPIT) were also excluded as they were taking vitamins A and E or placebo.

2.2.2 Gestational diabetes

All the patients with GDM fulfilled the WHO criteria for having gestational diabetes i.e.; the glucose intolerance was first recognized during pregnancy with a fasting blood glucose ≥ 7.0 mmol/L and/or 2 hour ≥ 7.8 mmol/L. Patients with GDM who had no other co-existing disease and were not on medication during pregnancy (except insulin) were selected. GDM was diagnosed either in the second or third trimester of pregnancy, with a glucose tolerance test (GTT) usually being performed between 24 to 28 weeks. Each hospital had different policies regarding post-natal glucose tolerance tests. Of the 16 patients, 8 had a normal post-natal GTT and 8 patients had normal post-natal blood glucose readings, but no formal GTT.

2.3 Mice

Female C57BL/6JOlac mice (Harlan, UK) were used as they have been reported to have similar blood glucose levels as healthy humans. Fasting glucose levels of 4.4 ± 0.2 mmol/L [Schreyer et al., 1998] and mean levels of 5.3 ± 1.3 mmol/L [Keren et al., 2000] have been reported in these mice. Furthermore, this strain has been extensively used over the years in studies dealing with vascular research examining normal mice [Laufs et al., 2005], knockout mice [Waldron et al., 1999] and steptozocin-induced animal models of diabetes [Cai et al., 2005]. Non-pregnant mice, pregnant mice at term (day 19) and their offspring were humanely killed by cervical dislocation, as per the Animals (Scientific Procedures) Act 1986.

2.4 Tissue collection

All tissue was collected in cold Modified Krebs’ buffer (Table 2.1) and immersed in this solution until dissection of the arteries was completed.

2.4.1 Non-pregnant women

Tissue samples were taken from the lower segment of the hysterectomy specimens, in order to correspond to the anatomically equivalent site of Caesarean section biopsies.
2.4.2 Pregnant women
After the fetus was delivered by Caesarean section, myometrial biopsies were obtained from the upper lip of the transverse lower segment incision of the uterus.

2.4.3 Mice
After the mice had been humanely killed, the main uterine arteries were dissected bilaterally.

2.5 Stereomicroscopic dissection
Samples obtained were examined under a light microscope and small arteries were dissected out using fine forceps and small dissecting scissors, taking care not to damage the vessel wall. The length of arteries was measured using a calibrated microscope eyepiece. Vessels were cut into lengths of about 2 mm and mounted in a wire myograph chamber, where their diameter was assessed.

2.6 Resistance arteries
As discussed in Section 1.4.2, mesenteric arteries in rats with a diameter of 100 to 300 µm have been demonstrated to be resistance arteries [Christensen and Mulvany, 1993]. The diameter of the uterine arteries of both pregnant and non-pregnant mice studied fell in this range. The diameter of resistance arteries in humans is uncertain; however because of the reasons discussed in Section 1.4.2, myometrial arteries with diameters in the approximate range of 200 to 500 µm were used in this study.

2.7 Choice of methodology
The decision regarding which technique to use for studying vascular function is of considerable importance. This decision should be made by weighing the relative benefits and drawbacks of each methodology in the context of the particular experiments envisaged. In this study, vessel function at four different glucose levels was examined in a vascular region inaccessible by techniques such as plethysmography and flow-mediated dilation. As four glucose levels were investigated, single-vessel techniques such as pressure myography were unsuitable.
It was therefore decided that investigating vascular function using wire myography would be the most appropriate way to achieve the aims of the study.

### 2.8 Wire myography

Wire myography is an *in vitro* technique for studying the mechanical, morphological and pharmacological properties of small vessels. It was first proposed by Bevan *et al.* [Bevan and Osher, 1972] and subsequently developed by Halpern and Mulvany [Halpern *et al.*, 1978]. In this technique, segments of vessels are mounted as ring preparations, with two fine wires through their lumen which are then secured at both ends. During the experiment, the circumference of the vessel is kept constant; therefore the vessels are examined under isometric conditions (Fig. 2.1).

![Fig. 2.1: Schematic representation of a blood vessel mounted on a wire myograph](image)

This is necessary as the force production is dependent on the extent of stretch, according to the active tension-length relation of all muscles. Also, the sensitivity of vessels to different agonists is dependent on stretch [Aalkjaer and Mulvany, 1983]. The force exerted on the wire during constriction and relaxation is measured by a sensitive strain gauge, transmitted to a computer and graphically represented as a curve (Fig. 2.2). Mounted vessels have been demonstrated to maintain their functional characteristics *ex-vivo* [Mulvany and Aalkjaer, 1990]. Refinement of the
mechanistic aspects of this technique has occurred over the decades and a considerable body of work has developed around this methodology. It has proven to be a robust and reproducible method in the study of vascular function.

2.8.1 Multi Myograph System 610M

All experiments described were performed on a four-chamber Multi Myograph System 610M (Danish Myo Technology A/S, Aarhus, Denmark: Fig. 2.3). The artery preparations in all four chambers were kept under physiological conditions at 37° C, and aerated with a gas mixture containing air / 5% CO₂ (British Oxygen Company, Surrey, UK).

![Myograph with data recordings on computer](image)

Fig. 2.2: Myograph with data recordings on computer
2.8.2 Advantages of wire myography

1. Four arteries can be studied in parallel, enabling a more comprehensive study of vessel function from a single subject. This is of particular relevance to this study as it enables the study of 4 different glucose levels.

2. Vessels are studied *ex-vivo*, maintaining their functional integrity

3. By studying isolated vessels, greater control of experimental conditions (such as glucose levels, solutions and oxygenation) is possible than studying perfusion of a tissue region *in vivo*.

4. It allows resistance arteries from any vascular bed to be studied, irrespective of whether they were obtained from humans or animals.

---

**Fig 2.3:** Multi Myograph System 610M (above) and an individual myograph chamber (left)
2.8.3 Limitations of wire myography

Mounted vessels in wire myography have a flattened, rather than the customary cylindrical shape. Other techniques such as plethysmography or pressure myography are therefore considered to approximate physiological function more closely than wire myography, as vessels maintain their normal shape. The delicate endothelial cell layer is prone to damage if the two wires are not carefully inserted. The wires exert pressure on the ends of the vessel wall, rather than distributing it evenly along the circumference of the artery. Furthermore, vessels tend to retract as they are stretched, due to elasticity. By studying isolated arteries, tissue-specific systemic factors (such as neuronal and hormonal input) which may influence vessel function are abolished. Additionally, vessels are exposed to solutions bereft of blood cells and plasma. Shear stress, an important stimulus for NO release in vivo [Kublickiene et al., 1997], cannot be reproduced using wire myography. However, these limitations are outweighed by the benefit wire myography confers in enabling four arteries to be studied concurrently in the present study.

2.9 Normalisation of vessel lumen

Normalisation is a process whereby the vessel circumference is calculated and set to a standardized level, enabling comparison of responses across different vessels. The rationale of this procedure is three-fold. The behaviour of elastic structures like vessels can only be compared if the conditions are standardised and uniform. Furthermore, the sensitivity of preparations to agonists is dependent of the degree of stretch, so it is important that these conditions be clearly defined. Finally, because the active response of a vessel is dependent on the degree of stretch, it is useful to set vessels to an internal circumference which gives the maximum response. Normalisation was performed using the Myodaq 2.01 Multi + software program (Myonic Software, National Instruments Corporation, USA).

In practice, the vessel is stretched in a series of small steps at two minute intervals, and the resting tension determined for each stretch. The wall tension is the measured force divided by the wall length and is expressed in mN/mm (milli-Newton per
millimetre). From the wall tension, the Laplace relation can be used to determine the Active Effective Pressure ($P_i$):

$$P_i = \frac{\text{Wall Tension}}{2 \pi \text{ Internal Circumference}}$$

Active effective pressure is thus an estimate of the pressure which would be necessary to extend the vessel to the measured internal circumference.

The active effective pressure is calculated at each step until a pressure of 13.3 kPa (100 mm Hg) is reached. An exponential curve is then fitted to the internal circumference pressure data and the point on the curve corresponding to 100 mm Hg is determined and denoted IC$_{100}$. Having found this, the internal circumference is set to 90% of this, as previous studies have shown that a maximal force is generated at this setting [Mulvany and Aalkjaer, 1990].

### 2.10 Drugs

All drugs and solutes were obtained from Sigma-Aldrich (UK). All drugs were newly re-constituted from powder form on each day of experimentation apart from bradykinin and U46619 which were made from the frozen stock solution.

#### 2.10.1 Potassium chloride

At concentrations of 60 mmol/L, potassium chloride constricts vessels by depolarization of smooth muscle cells. This property has been utilised in previous studies to induce arterial constriction [Lagaud et al., 2002; Vial and Evans, 2005]. An advantage of using potassium to constrict vessels is that it produces a regular and sustained constriction, enabling greater reproducibility when analysing constriction and relaxation.

#### 2.10.2 Bradykinin

Bradykinin has been demonstrated to cause relaxation of blood vessels that is endothelium-dependent, as denudation of the endothelium abolished this response [Cherry et al., 1982; Whalley et al., 1987]. Bradykinin is a stimulator of eNOS and NO production through Ca$^{2+}$-mediated mechanisms in endothelial cells via the B$_2$ receptor [Leeb-Lundberg et al., 2005]. It has also been demonstrated to be
associated with prostacyclin release [Lamontagne et al., 1992] as well as relaxation mediated by EDHF [Ohlmann et al., 1997].

2.10.3 Phenylephrine

Phenylephrine is structurally related to epinephrine and induces constriction of vessels by its action as an \( \alpha_1 \) agonist [Sofowora et al., 2004].

2.10.4 Acetylcholine

Acetylcholine mediates endothelium-dependent relaxation by the activation of muscarinic receptors located on endothelial cells [Walch et al., 2001].

2.10.5 U46619

U46619 (9, 11-dideoxy-11\( \alpha \), 9\( \alpha \)-epoxymethanoprostaglandin F\( _{2\alpha} \)) is a prostanoid which acts as a specific thromboxane receptor agonist. This biologically relevant vasoconstrictor has previously been demonstrated to provide a sustained and reproducible constriction in myometrial arteries [Ong et al., 2003].

2.10.6 L-NNA

L-NNA (N\( \omega \)-Nitro-L-arginine) is an inhibitor of the enzyme Nitric Oxide Synthase (NOS) [Lamontagne et al., 1991; Ding and Triggle, 2000]. Exposure to a concentration of 100 \( \mu \)M L-NNA has been previously used in the aorta of C57BL/6 mice to block the action of NO [Rabelo et al., 2003].

2.10.7 Indomethacin

Indomethacin is a cyclo-oxygenase inhibitor that inhibits both COX-1 and COX-2 isoforms [Mitchell and Warner, 2006]. In the carotid artery of C57BL/6 mice, concentrations of 10 \( \mu \)M indomethacin have been used to block cyclo-oxygenase activity [Quan et al., 2006].

2.10.8 Streptozotocin

Streptozotocin is a nitrosourea derivative isolated from Streptomyces achromogenes with broad-spectrum antibiotic and anti-neoplastic activity [Bono, 1976]. It is a powerful alkylationing agent that has been shown to interfere with glucose transport [Wang and Gleichmann, 1998], glucokinase function [Zahner and Malaisse, 1990]
and induce multiple DNA strand breaks [Bolzan and Bianchi, 2002]. A single large
dose of streptozotocin or multiple small doses can induce an insulinopaenic diabetes
due to its toxic effects on pancreatic β–cells [Rees and Alcolado, 2005].

2.10.9 Choice of drugs

2.10.9.1 Vasoconstriction

60 mmol/L potassium was used to constrict myometrial arteries as it produced a
sustained and reproducible constriction in these vessels. However, at this
concentration, the effect of EDHF on subsequent endothelium-dependent relaxation
was blocked, as discussed in Section 1.5.2.3. Therefore, experiments were also
performed using U46619 as a vasoconstrictor in myometrial arteries. Initial
experiments in mice uterine arteries demonstrated diminished constriction in this
vascular bed with 60 mmol/L potassium, but greater constriction with
phenylephrine. Similar enhanced constriction with phenylephrine compared with
potassium has been previously noted in mouse arteries [Nobe et al., 2006].
Preliminary experiments using U46619 in these arteries resulted in less reproducible
constriction compared with phenylephrine. It was therefore decided to use
phenylephrine as the vasoconstrictor in experiments investigating mice uterine
arteries.

2.10.9.2 Endothelium-dependent relaxation

Bradykinin was used in myometrial arteries, as it has been noted to produce a more
reproducible relaxation in these vessels than acetylcholine [Kenny et al., 2002].
Preliminary experiments with bradykinin in mice uterine arteries demonstrated
minimal relaxation with bradykinin. A possible explanation for this result is that
there are diminished amounts of endothelial cells responsive to bradykinin in this
vascular bed. Using confocal microscopy, it has been demonstrated that individual
endothelial cells in the aorta of mice respond in different ways to endothelium-
dependent vasodilators such as acetylcholine and bradykinin [Marie and Beny,
2002]. Endothelial cells which responded to acetylcholine were found to be different
from those activated by bradykinin. Furthermore, these endothelial cells were not
homogeneously distributed, with a significantly higher percentage of cells
responding to acetylcholine compared with bradykinin. This may explain why acetylcholine produced more reproducible relaxations than bradykinin in mice uterine arteries. Subsequent mice experiments were therefore performed using acetylcholine.

2.11 Solutions

All solutions were freshly made up each day, titrated to a pH of 7.4 and stored in a fridge. The composition of the various solutions in mmol/L is given in Table 2.1.

<table>
<thead>
<tr>
<th>Solute</th>
<th>Glucose Solutions</th>
<th>KPSS</th>
<th>Hyper-osmolar Solution</th>
<th>Modified Krebs' Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>128</td>
<td>72.45</td>
<td>128</td>
<td>136.9</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>11.9</td>
</tr>
<tr>
<td>KCl</td>
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<td>60</td>
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<td>2.7</td>
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<tr>
<td>MgSO₄</td>
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<td>2.4</td>
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<tr>
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<td>1.6</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>KH₂PO₄</td>
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<td>1.18</td>
<td>1.18</td>
<td>0.5</td>
</tr>
<tr>
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<td>0.034</td>
<td>0.034</td>
<td>0.034</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>2, 5, 8 or 12</td>
<td>2, 5, 8 or 12</td>
<td>5</td>
<td>11.5</td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.1: Composition of solutions used (in mmol/L)

2.11.1 Glucose physiological saline solutions

The glucose solutions used consisted of Physiological Saline Solution (PSS) to which 2, 5, 8 or 12 mmol/L of glucose was added.

2.11.2 60 mmol/L KPSS

Potassium Physiological Saline Solution (KPSS) consisted of the previously described PSS with an equi-osmolar substitution of NaCl with KCl. 2, 5, 8 or 12 mmol/L of glucose was added to the solution as before.
2.11.3 Hyper-osmolar solution

This solution consisted of PSS to which 5 mmol/L glucose and 7 mmol/L mannitol were added. This resulted in a solution equi-osmolar to PSS containing 12 mmol/L glucose (equi-osmolar substitution of glucose with mannitol).

2.11.4 Modified Krebs’ buffer

This solution was used to temporarily preserve tissue while arteries were dissected out, because of its ability to maintain its pH at room temperature without having to be aerated. Although this solution contained a relatively high concentration of glucose (11.5 mmol/L), the vessels were only exposed to this for less than 30 minutes.

2.12 Experimental protocols

2.12.1 Protocol 1: The effect of glucose levels on maximum constriction

For each experiment, arteries were all initially placed inside individual chambers in PSS with a glucose concentration of 5 mmol/L (representing normoglycaemia). Arteries were constricted with a vasoconstrictor (KCl, U46619 or phenylephrine) and allowed to plateau. The maximum active effective pressure in the first two curves at 5 mmol/L glucose for each artery was taken and the data averaged. After relaxation (described in Protocol 2), vessels were subsequently exposed to PSS containing 2, 8 or 12 mmol/L glucose for 30 minutes, leaving a control vessel at 5 mmol/L glucose. To avoid methodological error, vessels were randomly allocated to a particular glucose level. Arteries were washed with fresh glucose solutions three times in these 30 minutes. The maximum active effective pressure after change in glucose concentration in two subsequent curves was similarly taken and the data averaged (Fig. 2.4 and 2.5).
Fig. 2.4: Schematic representation of concentration-response curve
Fixed dose of vasoconstrictor added (green arrow). Endothelium-dependent vasodilator added at maximum constriction of artery in incremental concentrations (red arrows) at two minute intervals. After five doses of vasodilator, drugs were washed off (blue arrow).

Protocol 1, 2 and 3

Fig. 2.5: Raw data trace
Actual raw data trace of an experiment, illustrating the scheme of concentration-response curves in Protocol 1, 2 and 3. The red arrow represents the change of solution to one of different glucose concentration or osmolarity. Similar traces were obtained simultaneously from four myograph chambers for each experiment.
2.12.2 Protocol 2: The effect of glucose levels on endothelium-dependent relaxation

As described in Protocol 1, arteries were all initially placed inside individual chambers in PSS with a glucose concentration of 5 mmol/L. Vessels were constricted and subsequently an endothelium-dependent vasodilator (bradykinin or acetylcholine) was added in incremental concentrations from $10^{-10}$ to $10^{-6}$ Mol/L at two-minute intervals. The drugs were then washed away with fresh PSS. Two such constriction-relaxation curves were performed at 5 mmol/L glucose and the data was averaged. Vessels were subsequently exposed to PSS containing 2, 5, 8 or 12 mmol/L glucose for 30 minutes. Vessels were washed with fresh PSS glucose solutions three times in these 30 minutes. Two further constriction-relaxation curves were performed as before and the data averaged. This has been schematically represented in Fig. 2.6. A paired comparison was made of the endothelium-dependent relaxation before and after the change in glucose levels.
2.12.3 Protocol 3: The effect of hyper-osmolarity

This was similar to Protocol 2, except an artery kept was kept as control at 5 mmol/L glucose and another artery incubated in a hyper-osmolar solution with mannitol (Fig 2.8). The hyper-osmolar solution contained 5 mmol/L glucose to which 7 mmol/L mannitol had been added to make it equi-osmolar to a 12 mmol/L glucose solution. Endothelium-dependent relaxation was assessed in the same manner as in Protocol 2.

![Diagram of normal and hyper-osmolar solutions](image)

**Fig. 2.7: Protocol 3**

Normal Solution (red): glucose solution (with 5 mmol/L glucose)
Hyper-osmolar solution (light blue): glucose solution (with 5 mmol/L glucose) + 7 mmol/L mannitol
2.12.4 Protocol 4: The effect of blockers to endothelium-dependent relaxation

Arteries were initially placed in a glucose solution containing PSS with 5 mmol/L glucose and a constriction-relaxation curve performed as normal. Vessels were then incubated in either a 5 (Fig. 2.6) or 12 (Fig. 2.7) mmol/L glucose solution for 30 minutes and a second curve performed. Subsequently, the four arteries were treated as follows:
1) Control
2) $10^{-4}$ M L-NNA added
3) $10^{-5}$ M Indomethacin added
4) Both $10^{-5}$ M L-NNA and $10^{-5}$ M Indomethacin added

After a 20 minute exposure to these drugs, a final concentration-response curve was performed in the previous manner.

![Protocol 4 Graph](image)

**Fig. 2.8:** Protocol 4: Actual raw data trace illustrating Protocol 4. Red arrow represents change of solution to 12 mmol/L glucose (or 5 mmol/L in control). Green arrow represents addition of one of the following in each vessel (no drug in control): L-NNA ($10^{-4}$ M), indomethacin ($10^{-5}$ M), L-NNA + indomethacin.
Fig. 2.9: Protocol 4 (5 mmol/L glucose)
A schematic representation of Protocol 3 at 5 mmol/L glucose. Control= no drugs added, L-NNA=10^{-4} M L-NNA added, Ind=10^{-5} M Indomethacin added added for 20 minutes

Fig. 2.10: Protocol 4 (12 mmol/L glucose)
A schematic representation of Protocol 3 at 5 (red) and 12 mmol/L glucose (orange) Control= no drugs added, L-NNA=10^{-4} M L-NNA added, Ind=10^{-5} M Indomethacin added added for 20 minutes
2.13 Optimising experiments

2.13.1 General measures

As the main aim was to investigate the effect of acute changes in glucose levels, all experiments were immediately performed as soon as tissue was obtained. This helped to avoid storage for prolonged periods at a fixed glucose level: overnight storage of tissue for up to 19 hours has been reported [Ang et al., 2002]. All solutions were freshly made on each day of experimentation to prevent deterioration in glucose levels of solutions from storage over time. Reduction in the potency of drugs over time was minimised by freshly re-constituting drugs immediately prior to the experiments.

When dissecting the arteries, care was taken not to damage the vessel wall by undue stretching, with handling restricted to the ends of the artery. When inserting wires inside the vessel, particular care was taken not to damage the endothelial layer: vessels where such damage occurred were discarded. Damage to vessels was also offset by ensuring wires had blunt edges, which prevented endothelial damage due to scraping of the inner wall. The Multi Myograph Systems were calibrated at regular intervals to ensure accurate responses. The average of two concentration-response curves was taken to avoid the effect of any drug sensitization.

2.13.2 Optimising mice experiments

Uterine arteries from mice were found to have a limited ability to maintain constriction, with occasional spontaneous relaxation to the baseline. This was prevented by the following measures:

1. **Smaller incremental stretches when normalising**
   
   As mouse uterine arteries were more delicate than human myometrial arteries, performing smaller increments while stretching the vessel during normalisation was found to be beneficial. This was found to prevent damage to the vessel wall from excessive tension.
2. Extra washing of vessels after each constriction/relaxation curve
   It was surmised that spontaneous relaxation could be due to acetylcholine
   remaining in the vessel or in the vessel chamber despite a single wash; as
   such relaxation was not present during the first curve. By washing the vessel
   three times after each run, this problem was alleviated.

3. Longer interval between curves
   It was observed that constriction was not maintained if the artery was
   subjected to multiple constriction and relaxation curves without an adequate
   time interval in between. Spontaneous relaxation to baseline was minimised
   by allowing at least 10 minutes between each curve.

Vessels that continued to relax spontaneously to the baseline despite the above
measures were excluded from analysis.

2.14 Creating an animal model of diabetes

2.14.1 Restrictions in human research
The availability of tissue samples from both healthy individuals and diabetic
patients was found to be limited. Only a minority of patients underwent Caesarean
section, restricting myometrial biopsies from both healthy and diabetic pregnant
women. A large number of patients had co-existing disease or were on regular
medication and hence could not be included in the study. This was particularly true
of type 2 diabetes patients who often had co-existing hypertension. Most type 1
diabetes patients were recruited to the Diabetes And Pre-eclampsia Intervention
Trial (DAPIT) and were taking vitamins A and E or placebo, excluding them from
the present study. Of the women who fulfilled the inclusion criteria, consent was
often declined as the biopsies were perceived by the patients to be obtained by an
invasive procedure. As a result, only a small group of type 1 and 2 patients could be
enrolled in the study, despite recruiting from the hospital with the highest number of
pregnant diabetic patients in the Greater Manchester region (St. Mary’s Hospital).
To increase the number of patients in the study, approval was obtained to recruit
diabetic patients from two further hospitals (Hope Hospital, Salford and North
Manchester Hospital, Crumpsall) which had the second and third highest number of
patients with diabetes complicating pregnancy in the region. Despite this, the overall number of patients recruited was still less than anticipated. The difficulties in attempting to study diabetes in pregnancy using clinical research protocols have been acknowledged in the past [Farrell et al., 1982]. It was therefore decided to create an animal model of diabetes to enable progress of the study.

2.14.2 Rationale for creating an animal model of diabetes

The advantages of an animal model of diabetes have been previously discussed (Section 1.2.4). Research in the field of pregnant diabetic animals has focused on fetal defects and the fetal origins of adult disease. Little is known about endothelial function in uterine arteries of diabetic rodents. Characterisation of uterine artery endothelial function in diabetic rodents would therefore be valuable in discerning the role of vascular responses in the pathogenesis of these conditions. If endothelial function in diabetic animals was comparable to that seen in human pregnancies complicated by diabetes, it would provide a basis for future work in this field. It would also enable a broader range of studies to be undertaken, which would not have been possible if research had been confined to human subjects.

2.14.3 Obtaining a Home Office Licence

In collaboration with Dr. Nicholas Ashton, a Home Office Project Licence was obtained to create an animal model of diabetes (Project licence no: PPL 40/2916). The proposal for a project licence was written by the author, with assistance from Dr. Ashton. The abstract describing the purpose of the study has been posted by the Home Office on the Internet at:


2.14.4 Species and strain

Both rat and mouse models of diabetes can be produced by injection of streptozotocin (see Section 1.2). C57BL/6JOla mice (Harlan, UK) were chosen mainly because this was the same strain used in earlier work, thus enabling comparison with previous results. Other advantages of this strain have been discussed in Section 2.3. Furthermore, the use of mice to establish baseline
characteristics of endothelial function enables the future utilization of genetic manipulation technologies, such as knock-out models.

2.14.5 Duration of diabetes

Endothelium-dependent relaxation in the aorta of rats injected with streptozotocin is affected by the duration of diabetes [Pieper, 1999]. Relaxation to acetylcholine was increased at 24 hours following injection with streptozotocin compared with controls, normal after 1 and 2 weeks of disease and impaired at 8 weeks of disease. Therefore all experiments in the present study were performed 8 weeks after injection, with regular testing of blood glucose in the intervening period.

2.14.6 Diabetogenic drug

Streptozotocin was used to induce diabetes as it is the most commonly used drug for diabetogenesis. A single intra-peritoneal injection of streptozotocin was used as described in other studies [Alp et al., 2003; Soriano et al., 2001]. A dose of 200 mg/kg body weight has been demonstrated to induce diabetes [Sango et al., 2002]. Other research groups in Manchester have successfully utilised murine models of diabetes, which has facilitated local experience with this technique [Burnand et al., 2004].

2.14.7 Procedure

Normal female C57 BL/6JOla mice of approximately 8 weeks of age were given a single intra-peritoneal injection of 200mg/kg body weight of streptozotocin. Blood glucose was measured from a superficial vein by the tail-prick method, using an Accu-chek advantage glucose meter (Roche Diagnostics, UK).

All mice were provided with food and water ad libitum and their general condition monitored on a daily basis over 8 weeks. Body weight and blood glucose were measured periodically. The initial batch of 18 mice did not develop diabetes, as random blood glucose levels measured over 8 weeks were persistently lower than the accepted threshold for diabetes of 11.1 mmol/L (200mg/dl) [Soriano et al., 2001]. Only 5 out of 10 mice developed diabetes in the second batch, of which one subsequently died.
Mice were divided into 3 categories:

- **Category 1**: vehicle controls: injected with vehicle alone
- **Category 2**: streptozotocin-controls: injected with streptozotocin, but blood glucose levels remained in the normal range
- **Category 3**: diabetic mice: injected with streptozotocin, with glucose levels in the diabetic range.

After an interval of 8 weeks from the date of injection, mice were allowed to become pregnant. At term (day 19) mice were humanely killed and the main uterine artery carefully dissected out. Subsequent steps were similar to that described previously for normal pregnant mice.

### 2.14.8 Impediments in creating an animal model of diabetes

The poor success rate for inducing diabetes in mice, the stipulation of waiting 8 weeks prior to experimentation and the lack of any diabetic mice in the initial batch limited the range of studies possible during the allotted time. Furthermore, diabetic mice were noted to have an impaired ability to conceive. This effect has been previously noted in mice with uncontrolled streptozotocin-induced diabetes [Diamond *et al.*, 1989]. Deterioration in the health of diabetic mice during pregnancy was also noticed. Therefore, only results from Category 2 mice (streptozotocin injected, but not diabetic) have been presented here. However, these results can be used in future comparisons of endothelial function in the three categories of mice, as this work is being continued by the post-graduate student Joanna Stanley. Despite these impediments, the initiation of the above steps in creating an animal model of diabetes will enable future investigations into vascular function in diabetes complicating pregnancy.
2.15 Data analysis

Data obtained as above was analysed using Myodata 2.02 software (Myonic Software, National Instruments Corporation, USA).

2.15.1 Constriction

The maximum active effective pressures obtained in the two constriction curves at 5 mmol/L glucose were averaged. Maximum active effective pressures in the two curves after change of glucose concentration were similarly averaged.

2.15.2 Endothelium-dependent relaxation

The baseline for calculating the amount of relaxation was taken as the resting level before constriction. The average force per unit length between markers of drug addition on the concentration response curve was taken. This ensured reproducibility of results and enabled comparison between experiments. The active tension produced by the vessels was calculated in mN/mm to eliminate variations arising due to different vessel lengths. The maximum active tension obtained when the vessels constricted and reached a plateau was taken as 100%, and the reduction in tension on adding vasodilators was calculated as a percentage of this. This eliminated variation in the numerical value of tension developed in different vessels, thereby allowing comparison between arteries.

2.16 Statistical analysis

2.16.1 General Principles

All results were analysed using GraphPad Prism version 4.00 for Windows, (GraphPad Software, San Diego USA) and plotted graphically using this software. Data was tested for normality using the Kolmogorov-Smirnov Test. Normally distributed data were analysed using the appropriate parametric test. At 95% confidence intervals, a p value of less than 0.05 was taken to be statistically significant in all analyses. Bonferroni post-hoc testing was performed on all statistically significant results. 95% confidence intervals have been stated for major comparisons. All data were expressed as mean ± standard error (SEM = standard deviation / \sqrt{n} ) except where stated.
2.16.2 Statistical analysis of data from patients

To compare normally distributed data between groups of women, results were analysed using a one-way ANOVA. Bonferroni post tests were used to discern differences between groups.

Serial measurements of blood glucose and HbA\textsubscript{1C} in women were also compared during pregnancy. For each woman, blood glucose and HbA\textsubscript{1C} levels at a fixed point in pregnancy were matched with their corresponding levels at a subsequent time point. Missing values were excluded and paired t-tests were performed on normally distributed data.

2.16.3 Statistical analysis of myography data

In the myography studies, “N” refers to the number of subjects or animals studied and “n” indicates the number of arteries analysed.

2.16.3.1 Constriction

Paired t-tests (two-tailed) were used to assess constriction before and after changes in glucose concentration.

2.16.3.2 Endothelium-dependent relaxation

Repeated measures ANOVA was used to compare relaxation in the same artery after changing the glucose concentration. Ordinary two-way ANOVA was used to calculate differences in endothelium-dependent relaxation when comparing relaxation between groups.
CHAPTER 3

The glucose levels of women with diabetes in pregnancy
3.1 Introduction

The glucose levels of women with diabetes in pregnancy play an important role in determining the outcome of pregnancy. Karlsson and Kjellmer were among the first to demonstrate the relationship between fetal outcome and glucose control, showing that the third-trimester mean maternal blood glucose was linearly correlated with the perinatal mortality rate [Karlsson and Kjellmer, 1972]. Improved control of diabetes has been associated with a reduction in the perinatal complication rate [DCCT Group, 1996; Drexel et al., 1988].

Studies examining the control of diabetes in pregnancy are often subject to important limitations. Instead of examining glucose levels, most of these studies have taken HbA\textsubscript{1C} as the marker for glycaemic control [DCCT Group, 1996], the limitations of which have been discussed in Section 1.11. Furthermore, HbA\textsubscript{1C} has been demonstrated to be a poor predictor of adverse outcome in pregnant women with diabetes [Brustman et al., 1987; Evers et al., 2004]. Studies on diabetes in pregnancy have often focused on a particular class: type 1, type 2 or gestational diabetes. As a result, variations in glucose levels between the three types of diabetes have not been adequately characterised. The effect of hypoglycaemia during diabetes in pregnancy is not as well defined as that of hyperglycaemia, as research in this field has concentrated on high levels of glucose.

As discussed in Section 1.9.7, conflicting results have been obtained in studies examining the effect of hyperglycaemia on endothelial function. Some of these studies were based on theoretical rather than empirical values of hyperglycaemia. This has led to investigations at levels as high as 44 mmol/L glucose [Cipolla et al., 1997], which is not seen clinically. Furthermore, although hypoglycaemia is frequently seen in patients on insulin during pregnancy (see Section 1.10.1), its frequency has not been adequately characterised. It is therefore important to define clinically relevant glucose levels in pregnant women with diabetes before examining its effects on endothelial function.
3.2 Aims

The main aim of this section was to define a clinically relevant range for blood glucose levels in women undergoing treatment at a tertiary centre for diabetes in pregnancy.

Secondary aims include:

a) Retrospectively examine glucose levels and HbA\textsubscript{1C} values at fixed points through pregnancy in women with type 1, type 2 and gestational diabetes.

b) Examine diabetes control in women with normal and adverse outcome in pregnancy.

3.3 Methodology

Ethical approval was obtained from the audit department of the Central Manchester NHS Trust. The study was instigated in discussion with Dr. Moulinath Banerjee (Department of Medicine, Manchester Royal Infirmary). Pregnancy records of patients with type 1, type 2 and gestational diabetes who had undergone treatment at the diabetes ante-natal clinic of St.Mary’s Hospital, Manchester during the period 2001-2006 were retrospectively analysed. It was envisaged to include a time-matched group of healthy pregnant women for comparison. However, glucose levels in healthy pregnant women were found to be measured infrequently, which precluded further study of this group. All case note evaluation and data collection were performed by the author. The results were collated by the audit department of the Central Manchester NHS Trust. Statistical advice was obtained from Professor Graham Dunn (Professor of Biomedical Statistics, University of Manchester) on analysis of the data, which was performed by the author.
3.3.1 Inclusion criteria:

1) Patients with a proven diagnosis of diabetes
   a) Documented glucose intolerance:
      - Positive GTT or
      - Fasting blood glucose ≥ 7.0 mmol/L on at least two separate occasions or
      - Random blood glucose readings ≥ 11.1 mmol/L on at least two separate occasions.

   b) Patients with type 1 or 2 diabetes diagnosed before pregnancy.
   c) Patients with no previous diabetes but found to have gestational diabetes with documented glucose intolerance (as above) during pregnancy.

2) Patients with a clearly classified type of diabetes
   a) Patients classified as either type 1 or type 2 before pregnancy
   b) Patients with no diabetes prior to pregnancy, but classified as having gestational diabetes during pregnancy with documented glucose intolerance.

3.3.2 Exclusion criteria:

a) Patients being monitored for marginally elevated glucose levels but not having diabetes.

b) Patients with risk factors for diabetes (such as previous large for gestational age baby) but not having blood glucose levels in the diabetic range.

c) Patients considered to have gestational diabetes, but without documented glucose intolerance.

d) Patients who were diagnosed to have gestational diabetes in a previous pregnancy, but without documentation of normal glucose tolerance test in the subsequent post-natal period.

e) Patients where there was uncertainty regarding the type of diabetes.

f) Patients on medication during pregnancy, such as heparin. Unfractioned heparin has been demonstrated to have vasoactive properties such as causing endothelium-dependent vasodilation [Tasatargil et al., 2005].
Patients diagnosed to have gestational diabetes, but subsequently found to have a positive glucose tolerance test in the post-natal period were re-classified as having type 2 diabetes.

3.3.3 Data collection
Pregnant women whose expected date of delivery fell between January 2001 and March 2006 were included in the study. 131 case notes of patients were randomly selected, of which 111 fulfilled the inclusion criteria. Blood glucose readings recorded during each clinic appointment were analysed. Readings obtained at the following time points were recorded: pre-conception, first documented value at the start of pregnancy, 10 ± 1 week, 20 ± 1 week, 34 ± 1 week and the last recorded reading during pregnancy. The blood glucose readings represented the maximum and minimum values obtained by the patient (from self-monitoring of blood glucose) in the week preceding the clinic appointment. In patients with gestational diabetes, the first blood glucose reading represented the initial value after the diagnosis of GDM had been confirmed. The diagnosis of GDM was made between 22 and 32 weeks of gestation in the patients studied.

3.3.4 Statistical analysis
Data was analysed as discussed previously in Section 2.16.1 and 2.16.2.

3.4 Results

3.4.1 Clinical characteristics
A summary of clinical characteristics is given in Table 3.1. Parameters where a significant difference was noted between groups have been described below. Data has been expressed as mean ± SEM.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Type 1 diabetes</th>
<th>Type 2 diabetes</th>
<th>Gestational diabetes</th>
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<td>Number of Patients</td>
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<td>16</td>
<td>51</td>
</tr>
<tr>
<td>Age at delivery (years)</td>
<td>30.5 ± 0.8</td>
<td>34.5 ± 1.1</td>
<td>33.6 ± 0.7</td>
</tr>
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<td><strong>Ethnicity</strong></td>
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<td>31</td>
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<td>9</td>
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<td>Unspecified</td>
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<td>2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
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<td>30.9 ± 1.9</td>
<td>30.3 ± 0.9</td>
</tr>
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<td><strong>Treatment</strong></td>
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<td>44</td>
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<tr>
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<td>0</td>
<td>21</td>
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<td><strong>Vascular Complications</strong></td>
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<td>0</td>
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<td>Neuropathy</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Ischaemic Heart Disease</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mean Gestation (weeks)</td>
<td>37.4 ± 0.2</td>
<td>38.3 ± 0.3</td>
<td>38.7 ± 0.2</td>
</tr>
<tr>
<td><strong>Mode of Delivery</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Vaginal</td>
<td>16 (38%)</td>
<td>4 (33.3%)</td>
<td>29 (56.8%)</td>
</tr>
<tr>
<td>Assisted Vaginal</td>
<td>6 (14.2 %)</td>
<td>1 (8.3%)</td>
<td>7 (13.7%)</td>
</tr>
<tr>
<td>Elective Caesarean</td>
<td>7 (16.6 %)</td>
<td>3 (25%)</td>
<td>6 (11.7%)</td>
</tr>
<tr>
<td>Emergency Caesarean</td>
<td>11 (26.1 %)</td>
<td>4 (33.3%)</td>
<td>8 (15.6%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (4.7 %)</td>
<td>0</td>
<td>1 (1.9%)</td>
</tr>
<tr>
<td>Termination of Pregnancy</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mean birth weight (g)</td>
<td>3511 ± 94</td>
<td>3499 ± 250</td>
<td>3575 ± 86</td>
</tr>
<tr>
<td>Birth weight &gt; 4000 g</td>
<td>9 (20%)</td>
<td>3 (19%)</td>
<td>14 (27%)</td>
</tr>
<tr>
<td><strong>Adverse Outcome</strong></td>
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<td>Stillbirth</td>
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<td>0</td>
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<td>1</td>
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</tr>
<tr>
<td>Early Pregnancy Loss</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Congenital Malformations</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 3.1: Clinical characteristics**

Data represented as mean ± SEM. BMI was based on weight at initial ante-natal clinic booking.
Patient Numbers:
The numbers of patients studied in the type 1, type 2 and gestational diabetes group were 44, 16 and 51 respectively.

Age:
The age at delivery of patients with type 1 diabetes was significantly lower (30.5 ± 0.8 years) compared to both type 2 and gestational diabetes (one-way ANOVA, Bonferroni post test: p < 0.05).

Ethnicity:
Most type 1 patients were Caucasian, whereas most patients with gestational diabetes were Asian. Ethnicity was unspecified in five patients.

Body Mass Index (BMI):
The BMI was based on the weight at the initial ante-natal clinic booking. BMI was significantly lower in type 1 diabetes (26.6 ± 0.8) compared to gestational diabetes (30.3 ± 0.9) (one-way ANOVA, Bonferroni post test: p < 0.05).

Treatment:
Most patients with gestational diabetes (30 out of 51) and all women with type 1 and 2 diabetes were treated with insulin during pregnancy.

Vascular complications:
Vascular complications were noticed only in patients with type 1 diabetes. Three patients had pre-existing retinopathy, of which one worsened during pregnancy. Two had new onset retinopathy diagnosed during pregnancy. Proteinuria was present in one patient with type 1 diabetes and microalbuminuria in a further two; all of which had preceded pregnancy and did not worsen subsequently. One patient with type 1 diabetes had pre-existing ischaemic heart disease.

Gestational Age:
The mean gestation period was significantly lower in women with type 1 diabetes (37.4 ± 0.2 weeks) compared to gestational diabetes (38.7 ± 0.2 weeks) (one-way ANOVA, Bonferroni post test: p < 0.001).

Mode of delivery:
The pre-dominant mode of delivery was normal vaginal in type 1 (38%) and gestational diabetes (56.8%). In type 2 diabetes, emergency Caesarean section was as common as normal vaginal delivery (33.3% each). In all groups, emergency Caesarean deliveries were higher than elective Caesarean deliveries. The mode of
delivery was unknown in 3 pregnancies due to a change in residence or transfer of care with loss of follow-up.

**Termination of pregnancy:**
Termination of pregnancy was performed in one woman with type 2 diabetes due to severe congenital defects (cerebral ventricle defects). The reason for termination was not specified in two patients with type 1 diabetes.

**Birthweight:**
No significant difference was noticed in birth weight between patients with type 1 (3511 ± 94 g), type 2 (3499 ± 250 g) or gestational diabetes (3575 ± 86 g) (one-way ANOVA, Bonferroni post test: p > 0.05). However, women with GDM were noted to have the highest rate of babies born with a birth weight greater than 4000 g.

**Congenital Malformations:**
These included two cases of ventricular septal defect, one case of ventricular septal hypertrophy and one case of Down’s syndrome in patients with type 1 diabetes. A case of cerebral ventricle defect in type 2 diabetes and a case of ventricular hypertrophy was noted in gestational diabetes.

**3.4.2 Glucose levels**
The analysis of 792 glucose readings recorded from women with type 1, type 2 and gestational diabetes is summarised in Table 2. As expected, more variability was seen among maximum values than minimum values. The overall range of values was 1 to 28 mmol/L glucose. The lower quartile for minimum glucose levels was 3 mmol/L glucose whereas the upper quartile for maximum glucose levels was 11 mmol/L glucose. Mean ± SD values for minimum glucose levels were 4.0 ± 1.5 and for maximum glucose levels 9.7 ± 3.4 mmol/L. These results indicate that most glucose levels were in the range of 2 to 12 mmol/L glucose.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Minimum Glucose (mmol/L)</th>
<th>Maximum Glucose (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of values</td>
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<td>396</td>
</tr>
<tr>
<td>Minimum</td>
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<td>&lt; 7.2</td>
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<tr>
<td>Median</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Upper Quartile</td>
<td>&gt; 4.7</td>
<td>&gt; 11</td>
</tr>
<tr>
<td>Maximum</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>4.0 ± 1.5</td>
<td>9.7 ± 3.4</td>
</tr>
</tbody>
</table>

Table 3.2: Range of glucose levels seen in diabetes in pregnancy
Collective glucose levels of women with type 1, type 2 and gestational diabetes.

3.4.3 Glucose levels of type 1 diabetes in pregnancy

Glucose readings were plotted from the pre-conception period to the end of pregnancy in 44 women with type 1 diabetes (Fig. 3.1). Narrower ranges of minimum and maximum glucose levels with less hyperglycaemia were seen as pregnancy progressed; indicating improved glycaemic control. The mean minimum and maximum pre-conception values were 4.3 and 13.1 mmol/L respectively. These values improved to 3.1 and 8.8 mmol/L respectively for the last recorded value in pregnancy. The very high levels of hyperglycaemia seen at the beginning of pregnancy (23, 26 and 28 mmol/L glucose) were absent in the last recorded readings, where the highest reading was 14 mmol/L. Paired maximum glucose levels from women at week 10 were significantly higher than corresponding values at week 34 (paired t-test, mean difference = 2.88, 95% confidence interval = 1.32 to 4.44, p < 0.001).
3.4.4 Glucose levels of type 2 diabetes in pregnancy

In 16 women with type 2 diabetes, glucose readings were plotted from the pre-conception period to the end of pregnancy (Fig. 3.2). The mean minimum and maximum pre-conception values were 5.1 and 11.3 mmol/L, although these readings could be obtained from only 3 patients in the group. The mean first recorded lowest and highest readings in pregnancy were 6.0 and 9.6 mmol/L whereas the corresponding values at the end of pregnancy were 4.5 and 9.0 respectively. The very high glucose readings noticed in type 1 patients were absent in type 2 patients during pregnancy. In contrast to type 1 diabetes, no significant difference was seen between paired glucose values in women at week 10 and week 34, (paired t-test, p > 0.05).

3.4.5 Glucose levels of gestational diabetes

Glucose readings were examined from the time of diagnosis to the end of pregnancy in 51 women with gestational diabetes (Fig. 3.3). The diagnosis of gestational diabetes was made at a mean ± SEM of 29.4 ± 1.4 weeks with an interquartile range of 28 to 30.5 weeks. Therefore, the first recorded readings in this group were taken later in pregnancy compared to the first trimester readings in type 1 and 2 diabetes. The mean first-recorded minimum and maximum blood glucose values were 4.6 and 8.9 mmol/L, measured at a mean of 29 ± 1 weeks. Mean minimum and maximum glucose levels at the end of pregnancy were 3.8 and 8.2 mmol/L. Paired maximum glucose levels at the time of diagnosis were not significantly different from those recorded at the end of pregnancy (paired t-test, p > 0.05).
Fig 3.1: Glucose levels: type 1 diabetes

Maximum and minimum glucose levels in patients with type 1 diabetes (N = 44) at different time points of pregnancy. Maximum values indicated by green dots, minimum values by blue dots and mean values by horizontal red bars.

Pre-Con: Pre-conception values

First: First recorded values in pregnancy

Last: Last recorded values before delivery
**Fig 3.2: Glucose levels: type 2 diabetes**

Maximum and minimum glucose levels in patients with type 2 diabetes (N = 16) at different time points of pregnancy. Maximum values indicated by green dots, minimum values by blue dots and mean values by horizontal red bars.

**Pre-Con:** Pre-conception values

**First:** First recorded values in pregnancy

**Last:** Last recorded values before delivery
Fig 3.3: Glucose levels: gestational diabetes

Maximum and minimum glucose levels in patients with gestational diabetes (N = 51) at different time points in pregnancy. Maximum values indicated by green dots, minimum values by blue dots and mean values by horizontal red bars.

**First:** First recorded values in pregnancy (28 to 30.5 weeks interquartile range)

**Last:** Last recorded values before delivery
3.4.6 HbA<sub>1C</sub> levels: comparison across types

The first recorded HbA<sub>1C</sub> levels represent first trimester levels in type 1 and 2 diabetes, but second or third trimester levels in gestational diabetes, depending on the time of diagnosis. Therefore only the 34th week values can be considered to be time-matched across all three groups. There was no significant difference in HbA<sub>1C</sub> values between the three groups at week 34 (one-way ANOVA, Bonferroni post test: p > 0.05).

3.4.7 HbA<sub>1C</sub> levels in each group

3.4.7.1 Type 1 diabetes
Paired data from women at week 10 and week 34 were compared. HbA<sub>1C</sub> levels were significantly higher at week 10 compared to week 34 (Fig. 3.4, paired t-test, mean difference = 1.56, 95% confidence interval = 0.79 to 2.34, p < 0.001).

3.4.7.2 Type 2 diabetes
Paired results from women at week 10 and week 34 showed no significant improvement in HbA<sub>1C</sub> levels (Fig. 3.5, paired t-test, p > 0.05).

3.4.7.3 Gestational diabetes
In women with gestational diabetes, no significant difference was noted on comparing their first recorded HbA<sub>1C</sub> with the corresponding final values (Fig. 3.6, paired t-test, p > 0.05).
Fig 3.4: HbA1c: type 1 diabetes

HbA1c levels in type 1 diabetes at different time points, from pre-conception to end of pregnancy. Horizontal lines represent median values.

**Pre-Con**: Pre-conception values
**First**: First recorded values in pregnancy
**Last**: Last recorded values before delivery

---

Fig 3.5: HbA1c: type 2 diabetes

HbA1c levels at different time points during pregnancy in type 2 diabetes. Horizontal lines represent median values.

**First**: First recorded values in pregnancy
**Last**: Last recorded values before delivery
Fig 3.6: HbA1c: gestational diabetes
HbA1c levels at different time points during pregnancy in gestational diabetes. Horizontal lines represent median values.
First: First recorded values in pregnancy
Last: Last recorded values before delivery

3.4.8 Glucose levels in GDM: effect of insulin

Of a total of 51 patients, 21 were diet-controlled and 30 were treated with insulin.

Diet-controlled: Paired maximum blood glucose levels measured at the time of diagnosis of GDM (first maximum) were compared with that at the end of pregnancy (last maximum). No significant difference was seen between the two glucose levels. (Fig. 3.7, paired t-test, \( p > 0.05 \)).

Insulin-controlled: In this group, the first glucose levels measured at the time of diagnosis of GDM represented values before the start of insulin. As expected, this first set of maximum glucose values was significantly higher than the last set, where patients were on insulin (paired t-test, mean difference = 1.45, 95% confidence interval = 0.32 to 2.59, \( p < 0.05 \)). Mean ± SD of first and last maximum glucose levels were 10.0 ± 2.1 and 8.5 ± 1.9 mmol/L.
Fig 3.7 & 3.8: Treatment and glucose levels in GDM

Glucose levels in patients with diet-controlled (N=21, fig 3.7) and insulin-controlled (N=30, fig 3.8) gestational diabetes at different time points in pregnancy. Red horizontal bars represent mean values.

**First:** First recorded values in pregnancy

**Last:** Last recorded values before delivery
3.4.9 Glucose levels and outcomes in type 1 diabetes:

Adverse outcome was defined as stillbirth or special care baby unit (SCBU) admission for greater than 24 hours. In both normal outcome (N=32) and adverse outcome (N=12) groups, paired maximum glucose values were significantly higher at week 10 compared to week 34 (paired t-test, p < 0.05).

3.4.10 HbA₁C and outcomes in type 1 diabetes:

In patients with a normal outcome, as well as those with an adverse outcome, a significant reduction in mean HbA₁C levels was seen between 10 and 34 weeks (paired t-test, p < 0.05).
Fig 3.9 Type 1 Diabetes: Normal Outcome

Fig 3.10 Type 1 Diabetes: Adverse Outcome

Fig 3.9 & 3.10: Adverse outcome and glucose levels in type 1 diabetes

Blood glucose levels in patients with type 1 diabetes who had a normal (N=32, fig 3.9) and an adverse (N=12, Fig 3.10) pregnancy outcome. Maximum values indicated by green dots, minimum values by blue dots and mean values by horizontal red bars.

First: First recorded values in pregnancy

Last: Last recorded values before delivery
**Fig 3.11** Type 1 Diabetes: Normal Outcome

Figures showing HbA1C levels over time for patients with type 1 diabetes with normal outcomes. Values are compared at different time points (First, Week 10, Week 20, Week 34) with a significant difference marked by *p < 0.01.

**Fig 3.12** Type 1 Diabetes: Adverse Outcome

Figures showing HbA1C levels over time for patients with type 1 diabetes with adverse outcomes. Values are compared at different time points (First, Week 10, Week 20, Week 34) with a significant difference marked by *p < 0.01.

**Fig 3.11 & 3.12:** Outcomes and HbA1C in type 1 diabetes

HbA1C levels in patients with type 1 diabetes who had a normal (N=32, Fig 3.11) and an adverse (N=12, Fig 3.12) pregnancy outcome. **First:** First recorded values in pregnancy.
3.5 Discussion

3.5.1 Glucose levels of women with diabetes in pregnancy

The main aim of this section was to identify a clinically relevant range for glucose values in women with diabetes complicating pregnancy. These results indicate that such a range would be between 2 and 12 mmol/L glucose. This range is markedly lower than the glucose levels that have been used to study vascular function in the past.

This section has demonstrated that women with type 1 diabetes in pregnancy have wide fluctuations in their blood glucose level (between 1 and 28 mmol/L) even when treated at a tertiary care centre. In comparison, a narrower range of glucose levels was seen in women with type 2 diabetes (2 to 18 mmol/L) and gestational diabetes (2 to 17 mmol/L). The lack of ante-natal blood glucose measurements in healthy pregnant women prevented comparison with a non-diabetic group. However, as described in Section 1.8, previous studies have reported a range of 3 to 6.8 mmol/L glucose in healthy pregnant women. A small rise in mean blood glucose values in the third trimester due to increasing insulin resistance has been observed in healthy pregnant women [Parretti et al., 2001]. However, in the present study, a similar rise in glucose levels towards the end of pregnancy was not seen in the diabetic patients. This was probably due to insulin treatment and also because only the range of glucose levels was recorded.

The results of this section demonstrate heterogeneity in glucose levels between the three types of diabetes in pregnancy. In the group studied, type 1 diabetes patients had a glycaemic profile distinct from both type 2 and gestational diabetes. Type 1 diabetes was characterised by extremes of hyperglycaemia and hypoglycaemia, which were absent in type 2 and gestational diabetes. This can be attributed to the relative lack of endogenous insulin production in type 1 diabetes and its inappropriate replacement with insulin therapy; although other mechanisms such as a deficient glucagon and epinephrine response may also play a role [Cryer, 1991].
3.5.2 Glucose levels in type 1 diabetes complicating pregnancy

Patients with type 1 diabetes had the poorest glycaemic control, with recurrent episodes of hypo- and hyperglycaemia. The hypoglycaemic episodes frequently reached the level of 2 mmol/L, although the lowest value obtained was 1.1 mmol/L. Hyperglycaemia was more frequent in patients with type 1 diabetes, although the values improved as pregnancy progressed. This resulted in a narrower mean range of glucose levels towards the end of pregnancy compared to the beginning. Using the continuous glucose monitoring system (CGMS), high variability in glucose levels have been previously demonstrated in pregnant women with type 1 diabetes [Kerssen et al., 2004].

3.5.3 Glucose levels in type 2 diabetes complicating pregnancy

Patients with type 2 diabetes had lower levels of hyperglycaemia compared to type 1 diabetes, resulting in a narrower range of glucose levels throughout pregnancy. In contrast to type 1 patients, no significant improvement in hyperglycaemia occurred as pregnancy progressed. Hypoglycaemic levels of 2 mmol/L were also seen in patients with type 2 diabetes.

3.5.4 Glucose levels in gestational diabetes

Of the 51 patients with gestational diabetes, 30 patients were treated with insulin and 21 patients by dietary measures alone. All newly diagnosed GDM patients were on diet control initially and started on insulin if subsequent blood glucose levels remained high. This explains the small but significant lowering of hyperglycaemia in the last readings compared to the first in women treated with insulin. Insulin requirement has been associated with the time of diagnosis of GDM; women diagnosed earlier being more likely to require insulin than those diagnosed later on in their pregnancy [Svare et al., 2001].

3.5.5 Adverse outcomes

Adverse outcome was defined as stillbirth or admission in the special care baby unit (SCBU) for greater than 24 hours. Reasons for admission included neonatal hypoglycaemia, jaundice, surfactant-deficient lung disease, low Apgar score and congenital defects.
3.5.5.1 Type 1 and 2 diabetes

27% of patients with type 1 diabetes had an adverse outcome compared to 31% of patients with type 2 diabetes. Although glucose levels improved throughout pregnancy in women with type 1 diabetes, adverse pregnancy outcomes were still high in this group. In women with type 1 diabetes, high levels of HbA1C early in pregnancy have been associated with a raised fetal malformation rate in numerous studies [Hanson et al., 1990; Nielsen et al., 1997; Temple et al., 2002; Ylinen et al., 1984].

In addition to elevated glucose levels in women with type 1 diabetes, this group also had the greatest degree of fluctuations between high and low blood glucose levels. However, it is unknown if these fluctuations contribute to the complications of diabetes in pregnancy. When women with normal and adverse outcome were compared, the glucose and HbA1C levels demonstrated a significant drop from week 10 to week 34 in both groups. However, the relatively small number of patients studied prevented statistical or epidemiological conclusions from being reached.

Adverse outcomes in pregnancy have been previously compared between type 1 and 2 diabetes. A large study examining 389 type 1 and 146 type 2 diabetes patients demonstrated a higher rate of serious adverse pregnancy outcome in type 2 compared to type 1 diabetes [Roland et al., 2005]. However, a larger study with 2359 patients has recently reported similar prevalence of congenital anomalies and perinatal mortality in patients with type 1 and 2 diabetes [Macintosh et al., 2006]. Comparing outcome measures between studies is often challenging due to differences in the level of care (both pre-conception and ante-natal), ethnicity and the socio-economic background of patients. The Diabetes Control and Complications Trial has reported that in pregnant women with type 1 diabetes, intensive control of glucose levels was associated with lowering of the congenital malformation rate to normal levels [DCCT Group, 1996]. As a corollary to this, daily self-monitoring of glucose levels in patients with type 1 diabetes has been associated with a reduction in adverse outcomes [Jensen et al., 2004].
3.5.5.2 Gestational diabetes

In this study, patients with GDM had the lowest rate of adverse pregnancy outcome (7.8%). Additionally, these women had more stable glucose levels than women with type 1 diabetes. Unlike type 1 and 2 diabetes, considerable debate exists regarding whether better control of blood glucose improves pregnancy outcome in gestational diabetes and Impaired Glucose Tolerance (IGT). It has been demonstrated that dietary advice, blood glucose monitoring, and insulin therapy (as required) in women with gestational diabetes reduced the rate of serious perinatal morbidity, but increased the rate of admission to the special care baby unit [Crowther et al., 2005]. An important limitation of this study by Crowther et al. was that no assessment was made of the actual glucose levels attained by GDM patients in the treated group. In contrast to type 1 diabetes [Jensen et al., 2004], self-monitoring of blood glucose has not been associated with improved outcome in gestational diabetes [Homko et al., 2002]. A study has shown no adverse outcome for impaired glucose tolerance (defined as a fasting plasma glucose between 6.1 and 7 mmol/L), but increased macrosomia in GDM [Nordin et al., 2006]. However, a larger study in China has found an association between IGT and high fetal birth weight [Yang et al., 2002]. It is hoped that the ongoing HAPO study will clarify the association between hyperglycaemia in GDM and adverse pregnancy outcome [HAPO Group, 2002].

3.5.6 HbA1C and glucose levels

In type 1 diabetes, HbA1C levels improved as pregnancy progressed; reflecting a similar trend in blood glucose levels. As described previously, raised HbA1C values in early pregnancy may play an important role in the pathogenesis of complications in pregnancy. HbA1C has been used as the surrogate marker for assessing blood glucose levels in the vast majority of epidemiological studies in diabetes. An important reason for its predominance in the literature is its reproducibility and ease of analysis compared to collating individual blood glucose levels. This also enables cross-comparisons between studies. However, its importance in the field of diabetes in pregnancy is less certain. In the current study, a significant drop was noticed between 10 and 34 weeks in type 1 patients with both a normal and adverse outcome in pregnancy.
In contrast to type 1 diabetes, no improvement in HbA$_{1C}$ levels was noted in women with type 2 and gestational diabetes on performing matched comparisons during pregnancy. A lack of improvement was also seen in the maximum blood glucose levels of these two groups.

3.5.7 Limitations of study

The relatively small number of patients examined (compared with population studies) prevents associations from being drawn between the parameters measured and pregnancy outcome. Furthermore, maximum and minimum glucose levels were assessed, which do not represent typical blood glucose levels in these patients. However, the main aim of this study was to identify a clinically relevant range of glucose levels to guide subsequent analysis of vascular function in pregnancy. The majority of studies have used HbA$_{1C}$ as a surrogate marker for actual blood glucose levels, necessitating the present investigation of glucose levels in a representative study population. Larger epidemiological studies measuring actual glucose levels (as opposed to HbA$_{1C}$) are required to examine a possible link between diurnal variations in blood glucose levels and pregnancy outcome.

Although this study has identified a clinically relevant range of glucose levels in women with diabetes in pregnancy, the duration of these aberrant glucose levels could not be assessed due to limitations of the SMBG method. The recently developed continuous glucose monitoring system (CGMS) has distinct advantages over SMBG in measuring the time span of these abnormal glucose levels. Using CGMS, Buhling et al. demonstrated the mean duration of hyperglycaemia (± standard deviation) above 8.9 mmol/L/24 h to be 9.3 ± 25 minutes in healthy non-pregnant women, 7.5 ± 14 minutes in healthy pregnant women and 14 ± 21 minutes in GDM [Buhling et al., 2004].

3.5.8 SUMMARY

Diabetes in pregnancy, despite being treated at a tertiary centre, was associated with aberrant blood glucose levels, which were more marked in type 1 diabetes. A high rate of adverse pregnancy outcome was noticed in patients with type 1 diabetes, who
also had the greatest fluctuation in glucose levels. The lower quartile of minimum glucose levels was 3 mmol/L, whereas the upper quartile of the maximum glucose levels was 11 mmol/L. These findings suggest that clinically-relevant glucose concentrations range from 2 to 12 mmol/L glucose in diabetes complicating pregnancy. Identifying this range provided a basis for all subsequent experiments investigating the effect of glucose levels on endothelial function in pregnancy.
CHAPTER 4

The effect of glucose on endothelial function in healthy non-pregnant and pregnant women
4.1 Introduction

The previous chapter demonstrated fluctuating levels of both hypoglycaemia and hyperglycaemia in women with diabetes in pregnancy. As discussed in Section 1.9.7, studies investigating hyperglycaemia have yielded conflicting results, which may be attributable to differences in the degree and duration of hyperglycaemia, agonists used, techniques employed and vascular beds studied. Therefore, the ideal method to investigate the effects of glucose on endothelial function would be to study the effect of a fixed number of glucose levels using a single technique on a variety of vascular beds. This would eliminate some ambiguity in this area of research by allowing comparison of effects between vascular beds at particular levels of glucose.

Clinically relevant glucose levels in women with diabetes in pregnancy have been identified in the preceding section. In healthy pregnant women, levels have been demonstrated to vary approximately between 3 to 6.8 mmol/L glucose (see Section 1.8). Using the new technique of continuous glucose monitoring system (CGMS), Buhling et al. recently illustrated that the prevalence of hyperglycaemia in healthy pregnant women was higher than previously reported, with levels greater than 8.9 mmol/L seen for short periods of 7.5 ± 14 minutes (mean ± standard deviation, over 24 hours) [Buhling et al., 2004]. In non-pregnant healthy individuals, similar levels of hyperglycaemia lasted for 9.3 ± 25 minutes. These novel findings signify that marked fluctuations in blood glucose level are seen even in normal pregnancy.

Myometrial arteries of the uterus play a vital role in facilitating blood flow to the growing fetus. However, the effects of the aforementioned glucose levels on these vessels are unknown in both normal and diabetic women. Furthermore, it is unclear whether the state of pregnancy or the agonist used for vasoconstriction influences the response to altered glucose levels. It is therefore necessary to characterise
vascular responses in myometrial arteries from both healthy pregnant and non-pregnant women prior to examining these effects in women with diabetes in pregnancy.

In this study, the effects of four clinically relevant glucose concentrations (2, 5, 8 and 12 mmol/L) were studied for a single duration of time (30 minutes), using wire myography with two vasoconstrictors (60 mmol/L potassium and U46619). Constriction and endothelium-dependent relaxation were evaluated in myometrial arteries isolated from both healthy pregnant and non-pregnant women.

4.2 Aims

1. To examine the effect of a 30 minute exposure to 2, 5, 8 and 12 mmol/L glucose on maximal constriction in myometrial arteries.

2. To examine the effect of a 30 minute exposure to 2, 5, 8 and 12 mmol/L glucose on endothelium-dependent relaxation in myometrial arteries.

3. To study whether any effect is agonist-dependent by using two different vasoconstrictors:
   A) 60 mmol/L KCl
   B) U46619

4. To study whether any effect is pregnancy-related by performing these studies in both non-pregnant and pregnant women.

5. To investigate the effects of a prolonged exposure of 2, 5, 8 and 12 mmol/L glucose lasting 2 hours on constriction and relaxation.

4.3 Materials and methods

These have been discussed in Chapter 2. The experimental protocols used were Protocol 1 and 2 (see Section 2.12.1 and 2.12.2). Arteries with maximum
constriction less than 1 mN/mm were excluded from analysis due to poor constriction.

**4.4 Results**

**4.4.1 Clinical Characteristics**

A summary of the clinical characteristics of healthy pregnant and non-pregnant women are given in Table 4.1. Subjects were divided into normal non-pregnant (N=15) and normal pregnant (N=30) groups. The normal pregnant group was further subdivided into the KCl group (N=16) and the U46619 group (N=14) depending on the vasoconstrictor used during experimentation. Patients were allocated these groups randomly. The BMI was based on the weight at the initial ante-natal clinic booking. Women in the non-pregnant group were significantly older and had a higher BMI than those in both pregnant KCl and U46619 groups (one-way ANOVA: Bonferroni post test p < 0.001). However, pregnant women in the KCl and U46619 groups had similar ages and BMI (one-way ANOVA: Bonferroni post test p > 0.05). The BMI of the non-pregnant women was in the obese range (> 30 kg/m²) and significantly higher than that of the pregnant women (one-way ANOVA: Bonferroni post test p < 0.05). The BMI of the healthy pregnant women was in the overweight range (25-30 kg/m²). Both gestational age at delivery and birthweight of the offspring were similar in the two groups of pregnant women (unpaired t-test, p > 0.05). In all groups, the blood pressure was in the normal range.
PARAMETERS | NORMAL NON-PREGNANT WOMEN (N = 15) | NORMAL PREGNANT WOMEN: KCL GROUP (N = 16) | NORMAL PREGNANT WOMEN: U46619 GROUP (N = 14)
---|---|---
Age (years) | 43.0 ± 2.4 | 32.7 ± 1.6 | 30.6 ± 1.1
BMI (kg/m²) | 34.4 ± 3.4 | 26.6 ± 1.0 | 26.6 ± 1.3
Max. Systolic BP (mm Hg) | 120 ± 9 | 122 ± 2 | 120 ± 2
Max. Diastolic BP (mm Hg) | 77 ± 5 | 73 ± 2 | 70 ± 2
Gravidity | 2 ± 0.4 | 2.3 ± 0.3 | 2.2 ± 0.2
Parity | 1.7 ± 0.4 | 0.9 ± 0.2 | 0.9 ± 0.2
Gestational Age at Delivery (weeks) | - | 38.8 ± 0.2 | 39.0 ± 0.3
Birth Weight (g) | - | 3663 ± 130 | 3546 ± 144
Individualised Birth Ratio (centile) | - | 66 ± 9 | 52 ± 8

Table 4.1: Clinical characteristics of healthy individuals
All data expressed as mean ± SEM. The individual birthweight ratio is a centile which is dependent on maternal ethnicity, height, weight, parity and gestation at delivery, in addition to fetal birthweight and sex [Sanderson et al., 1994].

4.4.2 Arterial Diameters
The mean diameter of arteries from non-pregnant women had a diameter (mean ± SEM) of 426 ± 23 μm in the KCl group (N=9, 32 arteries) and 392 ± 22 μm in the U46619 group (N=6, 22 arteries). No significant difference was noted in the mean diameters of these two groups (Fig. 4.1; unpaired t-test, mean difference = 34.09 ± 34.01, 95% confidence interval = -34.22 to 102.4; p > 0.05). Arteries from healthy pregnant women in the KCl group (N= 16, 50 arteries) had a significantly larger diameter than that in the U46619 group (N=14, 50 arteries) (377 ± 15 μm vs. 335 ± 13 μm) [Fig. 4.2; unpaired t-test, mean difference = 42.72 ± 20.06, 95% confidence interval = 2.854 to 82.59, p < 0.05].
Fig. 4.1: Arterial diameters (non-pregnant group)
Myometrial arteries isolated from healthy non-pregnant women in two vasoconstrictor groups: 60 mmol/L KCl (circles; N=9, artery segments=32) and U46619 (triangles; N=6, artery segments =22). Horizontal bars represent mean values. *Unpaired t-test: mean difference = 34.09 ± 34.01, 95% confidence interval = -34.22 to 102.4

Fig. 4.2: Arterial diameters (normal pregnant group)
Myometrial arteries isolated from pregnant women in two vasoconstrictor groups: 60 mmol/L KCl (N=16, artery segments = 50) and U46619 (N=14, artery segments = 50). Horizontal bars represent mean values. *Unpaired t-test: p < 0.05
4.4.3 Constriction: effect of glucose
(non-pregnant group - KCl)
Myometrial arteries from healthy non-pregnant women were constricted with 60 mmol/L potassium. No significant difference in active effective pressure was seen at 2, 5, 8 or 12 mmol/L glucose (Fig. 4.3, paired t-test: p > 0.05).

4.4.4 Endothelium-dependent relaxation: effect of glucose
(non-pregnant group - KCl)
No significant difference was noted in endothelium dependent relaxation of myometrial arteries of non-pregnant women after incubation at 2, 5, 8 and 12 mmol/L glucose for 30 minutes (Fig. 4.4, repeated measures ANOVA: p > 0.05).

4.4.5 Constriction: effect of glucose
(non-pregnant group - U46619)
Myometrial arteries from healthy non-pregnant women were constricted with $10^{-6}$ M U46619. No significant difference in active effective pressure was seen at 2, 5, 8 or 12 mmol/L glucose (Fig. 4.5, paired t-test: p > 0.05).

4.4.6 Endothelium-dependent relaxation: effect of glucose
(non-pregnant group – U46619)
Exposure to glucose levels of 2, 8 and 12 mmol/L for 30 minutes had no effect on endothelium-dependent relaxation when U46619 was used to constrict the arteries (Fig. 4.6, repeated measures ANOVA: p > 0.05).
Fig. 4.3A-D: Constriction: effect of glucose (non-pregnant group – KCl)

The effect of changes in glucose concentration from 5 mmol/L glucose on active effective pressure in myometrial arteries from healthy non-pregnant women (N=9). Vessels constricted with 60 mmol/L KCl. Active effective pressure assessed at:

Fig. 4.3A: 5 and 2 mmol/L glucose (n=7 arteries)
Fig. 4.3B: 5 mmol/L glucose (n=9 arteries)
Fig. 4.3C: 5 and 8 mmol/L glucose (n=8 arteries)
Fig. 4.3D: 5 and 12 mmol/L glucose (n=8 arteries)

*Paired t-test: p > 0.05*
Fig. 4.4A-D: Relaxation: effect of glucose (non-pregnant group – KCl)

Endothelium-dependent relaxation at 5 mmol/L glucose and after 30 minute incubation at 2, 5, 8, and 12 mmol/L glucose in myometrial arteries from healthy non-pregnant women (N=9). Vessels constricted with 60 mmol/L potassium and relaxed with $10^{-10}$ to $10^{-6}$ M bradykinin. Relaxation assessed at:
Fig. 4.4A: 5 (circles) and 2 (triangles) mmol/L glucose (n=7 arteries)
Fig. 4.4B: 5 mmol/L glucose (circles) (n=9 arteries)
Fig. 4.4C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=8 arteries)
Fig. 4.4D: 5 (circles) and 12 (squares) mmol/L glucose (n=8 arteries)

Repeated measures ANOVA: $p > 0.05$
Fig. 4.5A-D: Constriction: effect of glucose (non-pregnant group – U46619)

The effect of changes in glucose concentration from 5 mmol/L glucose on active effective pressure in myometrial arteries from healthy non-pregnant women (N=6). Vessels constricted with $10^{-6}$ M U46619. Active effective pressure assessed at:

- Fig. 4.5A: 5 and 2 mmol/L glucose (n=6 arteries)
- Fig. 4.5B: 5 mmol/L glucose (n=6 arteries)
- Fig. 4.5C: 5 and 8 mmol/L glucose (n=5 arteries)
- Fig. 4.5D: 5 and 12 mmol/L glucose (n=6 arteries)

*Paired t-test: $p > 0.05$*
Fig. 4.6A-D: Relaxation: effect of glucose (non-pregnant group – U46619)

Endothelium-dependent relaxation at 5 mmol/L glucose and after 30 minute incubation at 2, 5, 8, and 12 mmol/L glucose in myometrial arteries from healthy non-pregnant women (N=6). Vessels constricted with $10^{-6}$ M U46619 and relaxed with $10^{-10}$ to $10^{-6}$ M bradykinin. Relaxation assessed at:

Fig. 4.6A: 5 (circles) and 2 (triangles) mmol/L glucose (n=6 arteries)
Fig. 4.6B: 5 mmol/L glucose (circles) (n=5 arteries)
Fig. 4.6C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=5 arteries)
Fig. 4.6D: 5 (circles) and 12 (squares) mmol/L glucose (n=6 arteries)

Repeated measures ANOVA: $p > 0.05$
4.4.7 Constriction: effect of glucose (pregnant group - KCl)
Myometrial arteries from healthy pregnant women were constricted using 60 mmol/L KCl. The active effective pressure was calculated at the peak of constriction. No significant difference in active effective pressure was seen at 2, 5, 8 or 12 mmol/L glucose (Fig. 4.7, paired t-test: p > 0.05).

4.4.8 KCl - Endothelium-dependent relaxation: effect of glucose (pregnant group - KCl)
Exposure to glucose levels of 2, 8 and 12 mmol/L for 30 minutes had no effect on endothelium-dependent relaxation when 60 mmol/L KCl was used to constrict the arteries (Fig. 4.8, repeated measures ANOVA: p > 0.05).
Fig. 4.7A-D: Constriction: effect of glucose (pregnant group - KCl)
The effect of changes in glucose concentration from 5 mmol/L glucose on active effective pressure in myometrial arteries from healthy pregnant women (N=16). Vessels constricted with 60 mmol/L KCl. Active effective pressure assessed at:
Fig. 4.7A: 5 and 2 mmol/L glucose (n=13 artery segments)
Fig. 4.7B: 5 mmol/L glucose (n=14 artery segments)
Fig. 4.7C: 5 and 8 mmol/L glucose (n=12 artery segments)
Fig. 4.7D: 5 and 12 mmol/L glucose (n=10 artery segments)
Paired t-test: p > 0.05
**Fig. 4.8A-D:** Relaxation: effect of glucose (pregnant group - KCl)

Endothelium-dependent relaxation at 5 mmol/L glucose and after 30 minute incubation at 2, 5, 8, and 12 mmol/L glucose in myometrial arteries from healthy pregnant women (N=16). Vessels constricted with 60 mmol/L potassium and relaxed with $10^{-10}$ to $10^{-6}$ M bradykinin. Relaxation assessed at:

Fig. 4.8A: 5 (circles) and 2 (triangles) mmol/L glucose (n=11 arteries)
Fig. 4.8B: 5 mmol/L glucose (circles)(n=15 arteries)
Fig. 4.8C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=12 arteries)
Fig. 4.8D: 5 (circles) and 12 (squares) mmol/L glucose (n=11 arteries)

Repeated measures ANOVA: $p > 0.05$
4.4.9 Constriction: effect of glucose (pregnant group – U46619)

Myometrial arteries from healthy pregnant women were constricted with $10^{-6}$ M U46619. Similar to the results obtained using 60 mmol/L potassium, no significant difference in active effective pressure was seen at 2, 5, 8 or 12 mmol/L glucose (Fig. 4.9, paired t-test: $p > 0.05$).

4.4.10 Endothelium-dependent relaxation: effect of glucose (pregnant group – U46619)

In the control arteries at 5 mmol/L glucose, an enhancement of endothelium-dependent relaxation was observed with time (Fig. 4.10B, repeated measures ANOVA: $p < 0.05$). Bonferroni post-hoc testing revealed significant differences in relaxation at $10^{-8}$ M ($p < 0.01$) and $10^{-7}$ M ($p < 0.05$) bradykinin. Similarly, significant enhancement of relaxation was also present after incubation at 8 and 12 mmol/L glucose (Fig. 4.10C and 4.10D, repeated measures ANOVA: $p < 0.05$). Bonferroni post-hoc testing did not reveal a significant difference at any specific concentration of bradykinin ($p > 0.05$). However after exposure to 2 mmol/L glucose for 30 minutes, this enhancement was absent, with no difference in relaxation (Fig 4.10A, repeated measures ANOVA: $p > 0.05$). This implies an impairment of endothelium-dependent relaxation at 2 mmol/L. This was more clearly illustrated on unpaired comparisons at different glucose levels. In Fig. 4.11, similar endothelium-dependent relaxation was present in arteries incubated at 5 mmol/L glucose (two-way ANOVA, $p > 0.05$). However, when these arteries were incubated at 2, 5, 8 and 12 mmol/L glucose for 30 minutes, impaired relaxation was noted at 2 mmol/L glucose compared with control at 5 mmol/L glucose (Fig. 4.12; two-way ANOVA, $p < 0.05$). Bonferroni post-hoc testing did not reveal a significant difference at any specific concentration of bradykinin (at $10^{-6}$ M bradykinin, mean difference = 1.28, 95% confidence interval = -41.98 to 16.72).
Fig. 4.9A-D: Constriction: effect of glucose (pregnant group – U46619)

The effect of changes in glucose concentration from 5 mmol/L glucose on active effective pressure in myometrial arteries from healthy pregnant women (N=14). Vessels constricted with 10^{-6} M U46619. Active effective pressure assessed at:

Fig. 4.9A: 5 and 2 mmol/L glucose (n=13 arteries)
Fig. 4.9B: 5 mmol/L glucose (n=14 arteries)
Fig. 4.9C: 5 and 8 mmol/L glucose (n=7 arteries)
Fig. 4.9D: 5 and 12 mmol/L glucose (n=12 arteries)

*Paired t-test: p > 0.05*
Endothelium-dependent relaxation at 5 mmol/L glucose and after 30 minute incubation at 2, 5, 8, and 12 mmol/L glucose in myometrial arteries from healthy pregnant women (N=14). Vessels constricted with $10^{-6}$ M U46619 and relaxed with $10^{-10}$ to $10^{-6}$ M bradykinin. Relaxation assessed at:

Fig. 4.10A: 5 (circles) and 2 (triangles) mmol/L glucose (n=10 arteries)
Fig. 4.10B: 5 mmol/L glucose (circles) (n=13 arteries)
Fig. 4.10C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=5 arteries)
Fig. 4.10D: 5 (circles) and 12 (squares) mmol/L glucose (n=11 arteries)

Repeated measures ANOVA: $p < 0.05$ in 5 (control), 8 and 12 mmol/L glucose (time effect)

Repeated measures ANOVA: $p > 0.05$ at 2 mmol/L glucose
Fig. 4.11: Relaxation at 5 mmol/L glucose (pregnant group – U46619)

Endothelium-dependent relaxation in arteries all initially at 5 mmol/L glucose prior to changing glucose levels

Two-way ANOVA: p > 0.05
4.12 Relaxation at 2, 5 and 12 mmol/L Glucose

**Fig. 4.12** Relaxation at 2, 5 and 12 mmol/L glucose (pregnant group – U46619)

Endothelium-dependent relaxation after change of glucose level to 2, 5 and 12 mmol/L glucose. Impaired relaxation at 2 mmol/L compared to control at 5 mmol/L glucose. Relaxation at 8 mmol/L glucose overlapped that at 5 and 12 mmol glucose and was omitted for clarity.

*Two-way ANOVA: p < 0.05*
4.4.11: Comparison of endothelium-dependent relaxation in non-pregnant and pregnant groups

The effect of pregnancy on myometrial artery function was assessed by comparing endothelium-dependent relaxation in non-pregnant and pregnant groups at 5 mmol/L glucose (Fig. 4.13 A, B, C). No significant difference was noted in the endothelium-dependent relaxation between the two groups, irrespective of whether 60 mmol/L KCl (Fig. 4.13A) or U46619 (Fig. 4.13B) was used as a vasoconstrictor (two-way ANOVA: p > 0.05). Furthermore, analysis of maximum relaxation at the highest concentration of bradykinin (10^-6 M, Fig. 4.13C) revealed no significant difference in relaxation between any of the groups (one-way ANOVA: p > 0.05).
Fig. 4.13A-C: Relaxation: non-pregnant and pregnant groups

Comparison of endothelium-dependent relaxation in myometrial arteries from healthy non-pregnant and pregnant women at 5 mmol.L glucose

Fig. 4.13A after constriction with KCl
Fig. 4.13B after constriction with U46619

*Two-way ANOVA, p > 0.05*

Fig. 4.13C Maximum relaxation at highest concentration of bradykinin (10⁻⁶ M)

*One-way ANOVA: p > 0.05*
4.4.12 Effect of prolonged exposure (2 hours) of 2, 5, 8 and 12 mmol/L glucose

To examine whether more prolonged exposures to altered glucose levels affected vascular function, Protocols 1 and 2 were used (see Section 2.12) with the only difference being that the vessels were exposed to 2, 5, 8 and 12 mmol/L glucose for 2 hours instead of 30 minutes, with periodic washes as before. The majority of myometrial arteries from both healthy pregnant and non-pregnant women demonstrated the following aberrant vascular responses after 2 hours:

a) marked reduction in maximum constriction (Fig. 4.13)

b) transient constriction with spontaneous relaxation

Atypical responses were obtained in both control arteries as well as arteries incubated at different glucose levels. Spontaneous relaxation without addition of a vasodilator prevented the assessment of endothelium-dependent relaxation. However, arteries with responses similar to that seen after 30 minutes incubation were also seen, as well as arteries with varying degrees of the above anomalous responses.
Fig. 4.14: Two hour incubation: aberrant responses

Actual raw data trace illustrating the effect of two hour incubation on vascular function. The four concentration-response curves are marked by arrows (A-D). After two hour incubation at 8 mmol/L glucose, arteries demonstrated markedly diminished constriction and endothelium-dependent relaxation. This was illustrated by the small amplitude of curves C and D compared to the initial two curves A and B. Similar results were obtained in the control vessel.
4.5 Discussion

4.5.1 Summary of results

The results are summarised in Table 4.2

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>Effect of glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy non-pregnant</td>
<td>KCl - Constriction</td>
<td>No effect</td>
</tr>
<tr>
<td>(N=15 women, n=54 arteries)</td>
<td>KCl - Relaxation</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>U46619 - Constriction</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>U46619 - Relaxation</td>
<td>No effect</td>
</tr>
<tr>
<td>Healthy pregnant</td>
<td>KCl - Constriction</td>
<td>No effect</td>
</tr>
<tr>
<td>(N=30 women, n=100 arteries)</td>
<td>KCl - Relaxation</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>U46619 - Constriction</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>U46619 - Relaxation</td>
<td>Impaired relaxation at 2 mmol/L glucose</td>
</tr>
</tbody>
</table>

Table 4.2: Summary of results

4.5.2 Low glucose concentration and impaired relaxation

Exposure to 2 mmol/L glucose for 30 minutes resulted in impaired endothelium-dependent relaxation in myometrial arteries from healthy pregnant women when constricted with U46619 (Fig. 4.12), an effect not present when the vessels were constricted with 60 mmol/L KCl (Fig. 4.8). In contrast, constriction and relaxation in myometrial arteries from healthy non-pregnant women were unaltered at 2, 8 or 12 mmol/L of glucose, irrespective of the vasoconstrictor used.
This represents the first demonstration that hypoglycaemia inhibits endothelium-dependent relaxation in any vascular bed. Studies examining the effect of hypoglycaemia on vascular function are extremely limited. Swafford et al. studied rat middle cerebral artery and found that reducing the glucose concentration from 5.5 mmol/L to 1.0 or 0.5 mmol/L for 1.5 hours each had no significant effect on the diameter of the arteries [Swafford et al., 1998]. A study by Watanabe and colleagues postulated that hypoglycaemia stimulated prostacyclin production in human umbilical vein endothelial cells [Watanabe and Jaffe, 1995]. However, the incubation period of 24 hours and the cell culture model may not accurately reflect the in vivo setting.

The mechanisms responsible for impairment of relaxation with hypoglycaemia were not investigated in the present study. The diameter of arteries constricted with U46619 was marginally lower than those constricted with KCl (Fig. 4.2), although it is uncertain if this influenced the results.

**4.5.2.1 The effect of vasoconstrictors**

Impairment of endothelium-dependent relaxation was noted (at 2 mmol/L glucose) when the arteries were constricted with U46619, but not with potassium, implying that this effect may be agonist-specific. As discussed in Section 1.5.5, a compensatory mechanism of the endothelial vasodilators has been demonstrated to exist which can rectify the absence of one particular mediator. Endothelium-dependent relaxation in myometrial arteries of healthy pregnant women has been previously demonstrated to be preserved in the presence of 25 mmol/L potassium, an inhibitor of EDHF [Kenny et al., 2002]. The explanation given for this result was that NO-mediated relaxation was enhanced in such conditions, compensating for the absence of EDHF-mediated relaxation. Furthermore, concurrent blockade of NO and EDHF resulted in significantly impaired relaxation. These findings of Kenny et al. in the myometrial vascular bed may also explain the results of the present study. The preservation of endothelium-dependent relaxation noted here (when arteries were constricted with 60 mmol/L potassium) may be due to increased NO-mediated relaxation, which counterbalanced the lack of EDHF-mediated relaxation. This enhancement of NO was present only when EDHF was blocked; on using U46619,
the lack of EDHF blockade may have prevented an increase in NO-mediated relaxation. This may also explain why hypoglycaemia was not associated with impaired relaxation in arteries from non-pregnant women as the aforementioned compensatory mechanism has noted to be absent in this group [Kenny et al., 2002].

4.5.2.2 The effect of pregnancy
The impaired relaxation with hypoglycaemia was specific to pregnancy, as reducing glucose concentrations had no effect on arteries from non-pregnant women irrespective of the vasoconstrictor used (Fig. 4.10 and 4.12). This may represent a physiological adaptation in pregnancy to counter-act the deleterious effects of reduced blood glucose levels. During hypoglycaemia, shunting of blood into the splanchnic circulation to maintain blood flow to vital organs such as the liver has been previously reported [Braatvedt et al., 1991; Parker et al., 1999]. However, it is uncertain if the impaired endothelium-dependent relaxation noted when myometrial arteries were exposed to hypoglycaemia is an adaptive response to maintain visceral circulation.

4.5.3 Other findings

4.5.3.1 Constriction
Constriction was observed to be unaltered irrespective of the glucose level, the vasoconstrictor used or whether vessels were obtained from non-pregnant (Fig. 4.3 and 4.5) or pregnant women (Fig. 4.7 and 4.9). This signifies that constriction, an endothelium-independent vascular function, is not affected by acute hypo- and hyperglycaemia.

4.5.3.2 Effect of pregnancy on endothelium-dependent relaxation
Irrespective of whether 60 mmol/L KCl or U46619 was used as the vasoconstrictor, endothelium-dependent relaxation was similar in both pregnant and non-pregnant groups. Although relaxation was greater with U46619 at $10^{-8}$ and $10^{-7}$ M bradykinin, the maximum relaxation at $10^{-6}$ M was similar in all groups. These findings corroborate those by Kenny et al., who found similar relaxation in myometrial arteries of pregnant and non-pregnant women [Kenny et al., 2002]. The
physiological changes in vessel function during pregnancy have been discussed in Section 1.3.2. It has been previously demonstrated that endothelium-dependent relaxation is enhanced in normal pregnancies [Faber-Swensson et al., 2004; Saarelainen et al., 2006]. However, these studies were performed using brachial artery flow-mediated dilation: a limitation of this technique is that it assesses function in larger conduit arteries (Section 1.5.6.2). Therefore, these results may not be applicable to the resistance arteries in the uterine vascular bed. The present study demonstrated no difference in maximal endothelium-dependent relaxation of myometrial arteries from non-pregnant and pregnant women. This finding emphasizes the importance of studying relevant vascular beds directly, as vascular function can vary in different tissue regions.

4.5.3.3 Effect of prolonged incubation

Incubation of arteries for two hours resulted in aberrant vascular responses which were not reproducible (Fig. 4.13). The defects in constriction and relaxation signified a loss of viability of these arteries when mounted for a prolonged time in a wire myograph. Dissected arteries stored at 4° C prior to mounting on a wire myograph have been demonstrated to be viable [Ang et al., 2002]. However, viability appears to be impaired after wire insertion and hours of experimentation at 37° C. The prolonged stretching of the vessel by the wires that occurs even in the resting state during experimentation may have resulted in damage to the arterial wall, leading to the abnormal responses. Viability after 2-hour incubation was considerably less with mice uterine arteries, probably because these vessels had a much thinner wall compared to human myometrial arteries. Although function was preserved in approximately 50% of the myometrial arteries examined, the high failure rate prevented four different glucose levels from being assessed, thereby limiting the value of the study. Therefore the incubation period in subsequent experiments was limited to 30 minutes, as no loss of viability was observed for this duration of time.
4.5.3.4 High glucose concentrations

This study has also demonstrated that exposure to physiologically relevant high glucose concentrations for a short period of time had no effect on endothelium-dependent relaxation in healthy individuals (Fig. 4.4, 4.6, 4.8 and 4.10). This was seen irrespective of the vasoconstrictor or whether arteries were from pregnant or non-pregnant women.

4.5.4 Explanations for the lack of effect of high glucose levels

4.5.4.1 Inadequate duration of high glucose levels

Most studies have studied the effect of glucose levels for longer durations lasting up to 24 hours [Houben et al., 1996]. However, as discussed in Section 1.9.4, such a long duration of hyperglycaemia is not usually seen clinically, limiting the physiological relevance of such studies. In healthy pregnant and non-pregnant women, the mean duration of hyperglycaemia (± standard deviation) greater than 8.9 mmol/l over 24 hours has been reported to be 7.5 ± 14 minutes and 9.3 ± 25 minutes respectively [Buhling et al., 2004]. Due to this observation and also due to methodological considerations, the time duration chosen for this study has been limited to 30 minutes. However, this may have been an insufficient time for the deleterious effects of a high glucose concentration to be manifested, as studies demonstrating impaired relaxation with hyperglycaemia have used longer durations of up to 6 hours (see Table 1.3).

4.5.4.2 Inadequate degree of high glucose levels

As discussed previously, most studies investigating the vascular effects of hyperglycaemia have examined high levels such as 44 mmol/L [Cipolla et al., 1997]. However, such high glycaemic levels are rarely seen clinically. In healthy women, the blood glucose levels have been reported to range between 3 to 6.8 mmol/L (see Section 1.8.). Even in diabetic women, the mean level of hyperglycaemia does not usually reach levels as high as 44 mmol/L; in the previous chapter the highest reading was 28 mmol/L glucose. In another study, the mean peak glucose level (± standard deviation) in women with type 1 diabetes in
pregnancy was reported to be 10.1 ± 3.2 mmol/L [Ben-Haroush et al., 2004]. The aim of this study was to examine the effects of glucose at physiologically relevant levels and extremely high concentrations of glucose were therefore not investigated. However, it is possible that higher levels of hyperglycaemia may result in altered endothelial function.

4.5.4.3 Systemic Factors

It is not yet clear if hyperglycaemia per se causes vascular dysfunction or whether it causes dysfunction by triggering associated systemic factors. These factors could include: neural mechanisms, hyperinsulinemia and lipid alterations, as discussed in Chapter 1. In this study the in vitro method of wire myography was used, thereby eliminating the effect of such systemic factors on vascular function. Systemic neurohumoral factors such as circulating hormones and vascular innervation may be important in mediating vascular responses in the myometrial vascular bed. In humans, the smaller branches of uterine arteries have been observed to be densely innervated, emphasizing the importance of neural regulation in uterine blood flow [Akerlund, 1994]. Shear stress from blood flow, an important trigger for NO-mediated relaxation in vivo, is not reproduced in wire myography. Arteries may exhibit different characteristics in vivo from those demonstrated here, due to the various biological interactions influencing vascular function. An advantage of using in vivo methods such as flow-mediated dilation is that such interactions are taken into account when assessing vessel responses. However, a limitation of such studies is that a precise level of hyperglycaemia is often difficult to maintain and characterise. For example, studies by Kawano et al. and Title et al. used glucose tolerance tests to induce hyperglycaemia [Kawano et al., 1999; Title et al., 2000]. However, this technique is associated with variable levels of hyperglycaemia over different periods of time. An important advantage of wire myography is that it allows four precise levels of glucose to be studied simultaneously for a specific time period in the same patient.
4.5.4.4 Endothelial mediators of vasodilation

Previous studies have used 25 mmol/L potassium to block EDHF-mediated relaxation [Kenny et al., 2002]. Although the rationale for using 60 mmol/L potassium in the present study was to constrict arteries by depolarization, a corollary effect of employing this agonist was the inhibition of EDHF-mediated relaxation. In such arteries, the endothelium-dependent relaxation observed is therefore restricted to the actions of NO and prostacyclin. Nevertheless, maximal relaxation in the absence of EDHF was similar to that in the presence of EDHF, when U46619 was used for constriction (Fig. 4.13C). Hyperglycaemia has been associated with reduced bioavailability of NO and impaired endothelium-dependent relaxation (Section 1.6.3). Therefore, vascular beds where NO contributes significantly to relaxation may have a greater degree of impairment. The effect of hyperglycaemia on prostacyclin and EDHF-mediated relaxation is yet to be clearly outlined. Although some of the earlier studies attributed the impaired endothelium-dependent relaxation with hyperglycaemia to reduced NO, the EDHF component was often not adequately blocked [Williams et al., 1996]. There may also be a compensatory mechanism occurring between the various pathways, as discussed previously, making assessment of individual mediators of relaxation difficult. This mechanism may circumvent the effect of hyperglycaemia on one particular pathway. The proportion of relaxation due to NO, EDHF and prostacyclin has been demonstrated to vary in different vascular beds (Section 1.6.4). In the myometrial vascular bed, endothelium-dependent relaxation has been mainly attributed to NO and EDHF [Kenny et al., 2002]. The present study has demonstrated that a high glucose concentration was not associated with alterations in maximum relaxation, either in the presence or absence of EDHF. However, it is possible that a compensatory enhancement of NO-mediated relaxation may occur on blocking EDHF.

4.5.4.5 Bradykinin

Although bradykinin is an effective endothelium-dependent vasodilator in vitro its physiological role in vivo is less well-characterised. In the physiological setting, bradykinin is inactivated by a host of peptidases, of which angiotensinogen converting enzyme is particularly important. Bradykinin has been demonstrated to have little effect on the pulmonary vascular bed as a result of this [Bonner et al.,
1990]. Inactivation of bradykinin also occurs within the arterial wall [Skidgel, 1992], which may limit its effectiveness in certain vascular beds. In the myometrial arterial bed, however, various studies have demonstrated reproducible efficacy in using bradykinin as an endothelium-dependent relaxing agent [Kenny et al., 2002; Myers et al., 2006]. A study in diabetic rats has reported that bradykinin can reduce oxidative stress in hyperglycaemic conditions [Mikrut et al., 2001]. Therefore, although bradykinin produces reproducible relaxation in myometrial arteries, it is uncertain if aberrant glucose levels can affect its potency to mediate endothelium-dependent relaxation.

4.5.4.6 Branch-order

Vascular function, whether constriction or relaxation, is largely determined by the calibre of the artery. As previously discussed (Section 1.5.2.3), the relative contribution to relaxation from NO, EDHF or prostacyclin varies depending on vessel diameter. Shimokawa et al. has demonstrated in the rat that expression of eNOS was the highest in the aorta and the lowest in the distal mesenteric arteries [Shimokawa et al., 1996]. Similarly, in human arteries the contribution of EDHF has been shown to increase as the vessel diameter decreased [Urakami-Harasawa et al., 1997]. Therefore, vascular function of any artery needs to be considered in the context of its location in the vascular tree. Most of the studies demonstrating impaired endothelium-dependent relaxation with hyperglycaemia have been performed on large vessels such as brachial arteries (Table 1.3). Studies investigating hyperglycaemia in the smaller resistance arteries are rare. As the role of the endothelium-dependent mediators NO and EDHF have been demonstrated to vary depending on vessel size, it may not be possible to generalise the results obtained in large-calibre arteries to resistance vessels. Therefore the effect of hyperglycaemia at this branch-order of the arterial tree is uncertain.

4.5.4.7 Age

In Section 1.5.4.1, evidence for the deterioration of endothelial function with age has been discussed. One limitation of this study is that the subjects in the pregnant group and in the non-pregnant group were not age matched, as hysterectomies were
normally performed only in women who had completed their families. The mean (± SEM) age of women in the pregnant and non-pregnant group was 31.5 (± 0.9) years and 43 (± 2.4) years respectively. The age of individuals in studies that have demonstrated impaired relaxation with hyperglycaemia vary from 25 years [Title et al., 2000] to 62 years [Kawano et al., 1999]. The effect of hypo- and hyperglycaemia on endothelial function at different age groups is yet to be elucidated, as no known study has investigated this clinically important subject. Endothelial function has been reported to deteriorate with age [Gerhard et al., 1996]. It is therefore possible that the effects of hyperglycaemia on endothelial function become more pronounced with age. The lack of effect of high glucose concentrations on endothelial function may be due to the relatively young age of the patients in the present study.

4.5.4.8 Nature of vascular bed
As described in Section 1.6.4, aberrant glucose levels do not have the same effect in all vascular beds. It is also uncertain whether the onset of endothelial dysfunction occurs concurrently in all vascular systems of the body. It is possible to speculate from the conflicting results of studies investigating hyperglycaemia (Section 1.9.7) that the effect of hyperglycaemia may vary depending on the nature of the vascular bed and its metabolic requirements. The majority of studies have examined upper-limb circulation, an arterial bed with vastly different vessel characteristics and metabolic demands than the uterine circulation in pregnancy. This highlights the importance of studying hyperglycaemia in the myometrial arteries directly, instead of extrapolating findings obtained in other vascular beds in pregnancy.

4.5.4.9 Gender
As noted in Section 1.5.2.1, gender plays an important role in NO-mediated relaxation, which has been related to circulating estradiol [Hayashi et al., 1992]. In female mice, EDHF was demonstrated to be more important than NO and PGI2 in mediating relaxation, whereas the converse was true in males [Scotland et al., 2005]. Studies investigating hyperglycaemia and vascular function have had a mixed study population, which may obscure gender-specific differences in
endothelial function. As the current study is limited to females, it restricts comparisons between studies. Hyperglycaemia may be affected by the type of endothelial mediator pre-dominant in the gender examined.

4.5.5 SUMMARY

Incubation of myometrial arteries from healthy pregnant women at 2 mmol/L glucose for 30 minutes was associated with impaired endothelium-dependent relaxation when constricted with U46619. However, this effect was absent on constricting arteries using 60 mmol/L potassium, signifying an agonist-specific effect. Constriction of myometrial arteries was not affected by changes in glucose level in either the pregnant or non-pregnant group. Furthermore, exposure to 2, 5, 8 and 12 mmol/L for 30 minutes had no effect on endothelium-dependent relaxation in arteries from healthy non-pregnant women. These results suggest that acute exposure to low glucose levels can impair endothelial function in healthy pregnant women, an effect which is specific to both the agonist used and the state of pregnancy. Characterisation of the effects of glucose levels on endothelial function in normal pregnancy provides a basis for examining these effects in diabetes complicating pregnancy, which is dealt with in the next chapter.
CHAPTER 5

The effect of glucose on endothelial function in diabetes complicating pregnancy
THE EFFECT OF GLUCOSE ON ENDOTHELIAL FUNCTION IN DIABETES COMPLICATING PREGNANCY

5.1 Introduction:

The previous chapter demonstrated that reduced glucose levels can cause endothelial dysfunction in myometrial arteries of healthy pregnant women. In diabetes, endothelial dysfunction has been demonstrated in various vascular beds such as the coronary [Nitenberg et al., 1993] and renal circulation [Shestakova et al., 2005]. However, no known study has examined the myometrial arterial bed in pregnancies complicated by diabetes. Studies in diabetes have often used the upper-limb circulation as a marker for endothelial dysfunction. However, as discussed in Section 1.6.4, the nature of the vascular bed plays a pivotal role in determining endothelial responses. The preceding chapter demonstrated unique endothelial responses in myometrial arteries distinct from those seen in other vascular beds. For example, maximal vasodilation in these arteries was not found to be enhanced in pregnancy, in contrast with reports in brachial arteries [Faber-Swensson et al., 2004]. Furthermore, as the time of onset of endothelial dysfunction in diabetes and its rate of progression in various vascular beds are unknown, it is possible that normal endothelial function at one site may not be representative of vascular responses at other sites. For example, the endothelial responses in mesenteric and femoral arteries of diabetic rats were markedly different from that seen in the carotid artery of the same animals [Shi et al., 2006]. Because of the heterogeneity of responses, studying the relevant vascular bed directly is crucial in assessing vascular function in diabetes.

The complications of diabetes in pregnancy are multi-factorial in nature. However, as diabetes is a vascular disease, endothelial dysfunction in the arteries that regulate blood flow to the growing fetus may be an aetiological factor in these complications. Myometrial resistance arteries are pivotal in this regard as they determine blood flow to the placenta, and unlike placental arteries have been affected by maternal diabetes and aberrant glucose levels for the entire duration of the disease.
For this study, patients with type 1, type 2 and gestational diabetes were recruited using the criteria discussed in Section 2.2.2. Similar experimental protocols were followed as in the previous chapter, to facilitate comparison of vascular function between healthy and diabetic pregnant women.

The findings in Chapter 3 suggest that glucose levels between 2 to 12 mmol/L glucose represent clinically relevant values for women undergoing treatment for diabetes in pregnancy, although poorly controlled diabetes is associated with much higher levels of hyperglycaemia. The duration of different glucose levels has been limited to 30 minutes to assess whether acute changes in glucose concentration influence vessel function. This time interval has been demonstrated to reflect a clinically relevant duration of hyperglycaemia in pregnancies complicated by diabetes [Buhling et al., 2004].

### 5.2 Aims

1. To examine the effect of exposure to 30 minutes of 2, 5, 8 and 12 mmol/L glucose on maximal constriction (as measured by active effective pressure) in myometrial arteries from women with diabetes complicating pregnancy.

2. To examine the effect of exposure to 30 minutes of 2, 5, 8 and 12 mmol/L glucose on endothelium-dependent relaxation in these myometrial arteries.

6. To study whether effects are agonist-dependent by using two different vasoconstrictors:
   
   A) 60 mmol/L KCl
   
   B) U46619

3. To compare constriction and endothelium dependent relaxation of myometrial arteries between healthy pregnant women and women with gestational diabetes
5.3 Materials and methods

These have been discussed in Chapter 2. The experimental protocols used were Protocol 1 and 2 (see Section 2.12.1 and 2.12.2). Data obtained from healthy pregnant women described in the preceding chapter were used for comparison with diabetic patients.

5.4 Results

5.4.1 Clinical characteristics - gestational diabetes

Details of patients with gestational diabetes recruited in this study are given in Table 5.1. To enable correlation of clinical characteristics with vascular function, GDM patients have been sub-divided into KCl and U46619 groups; depending on the vasoconstrictor used. Overlap of these groups was present, as arteries from the same patient were used in both groups in five cases. Patients in both groups were obese and also had high birth weights. These patients had near-normal HbA$_{1C}$ levels (normal reference range < 6.5 %). Patients in the two groups had no significant difference in their age, BMI, birth weight of babies or HbA$_{1C}$ (unpaired t-test, p > 0.05).
<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>GDM KCl GROUP (N = 14)</th>
<th>GDM U46619 GROUP (N = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>33.6 ± 1.2</td>
<td>34.1 ± 1.7</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>35.0 ± 2.3</td>
<td>34.5 ± 2.5</td>
</tr>
<tr>
<td>Max. Systolic BP (mm Hg)</td>
<td>134 ± 5</td>
<td>133 ± 6</td>
</tr>
<tr>
<td>Max. Diastolic BP (mm Hg)</td>
<td>85 ± 4</td>
<td>83 ± 4</td>
</tr>
<tr>
<td>Gravidity</td>
<td>3 ± 0.3</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>Parity</td>
<td>1.9 ± 0.3</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Antenatal Blood Glucose (low reading, mmol/L)</td>
<td>4.1 ± 0.2</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>Antenatal Blood Glucose (high reading, mmol/L)</td>
<td>9.7 ± 0.7</td>
<td>11.4 ± 1.2</td>
</tr>
<tr>
<td>Mean Antenatal HbA_1C (%)</td>
<td>6.4 ± 0.2</td>
<td>6.9 ± 0.3</td>
</tr>
<tr>
<td>Mother’s blood glucose at delivery (mmol/L)</td>
<td>4.9 ± 0.4</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>Baby’s blood glucose at delivery (mmol/L)</td>
<td>2.2 ± 0.2</td>
<td>2.0 ± 0.05</td>
</tr>
<tr>
<td>Gestational Age at Delivery (weeks)</td>
<td>38.5 ± 0.2</td>
<td>38.8 ± 0.2</td>
</tr>
<tr>
<td>Birth Weight (gm)</td>
<td>4010 ± 143</td>
<td>4171 ± 257</td>
</tr>
<tr>
<td>Individualised Birth Ratio (centile)</td>
<td>81 ± 7</td>
<td>79 ± 10</td>
</tr>
</tbody>
</table>

**Table 5.1: Clinical characteristics – gestational diabetes**

Data shown as mean ± SEM. Normal reference range for HbA_1C = < 6.5% . The individual birthweight ratio is a centile which is dependent on maternal ethnicity, height, weight, parity and gestation at delivery, in addition to fetal birthweight and sex.
5.4.2 Diameter of arteries- gestational diabetes

The mean diameter (± SEM) of arteries from women with gestational diabetes was 398 (± 18) µm in the KCl group and 347 (± 21) µm in the U46619 group (Fig. 5.1A). There was no significant difference in diameter between the two groups (unpaired t-test: p > 0.05). To assess whether this result was due to multiple observations in arteries obtained from the same patient, the mean diameter of all arteries obtained from a single patient was also plotted and compared (Fig. 5.1B). Here also, no significant difference was seen between the KCl (N=14) and U46619 (N=8) groups (unpaired t-test: p > 0.05).

5.4.3 Constriction: effect of glucose (GDM - KCl)

Myometrial arteries from women with gestational diabetes were constricted using 60 mmol/L KCl, initially at 5 mmol/L glucose. The active effective pressure was calculated at the peak of constriction. After 30 minutes incubation in 2, 5, 8 or 12 mmol/L glucose, constriction was calculated again (Fig. 5.2). No significant difference in active effective pressure was present at any of the glucose levels examined (paired t-test: p > 0.05 in all comparisons; at 12 mmol/L glucose: mean difference = 0.65, 95% confidence interval = -0.42 to 1.72).
Fig. 5.1A & B: Arterial diameters - GDM

Fig 5.1A: Myometrial arteries from women with gestational diabetes divided into two groups based on the vasoconstrictor used: KCl (N=14, 51 arteries) and U46619 (N=8, 24 arteries). Horizontal bars represent mean values.

Fig 5.1B: Mean of the diameter of all arteries obtained from individual patients

*Unpaired t-test: p > 0.05*
Fig. 5.2A-D: Constriction: effect of glucose (GDM- KCl)

The effect of changes in glucose concentration on active effective pressure in myometrial arteries from women with Gestational Diabetes (N=14). Vessels were constricted with 60 mmol/L KCl. Active effective pressure assessed at:

Fig. 5.2A: 5 (circles) and 2 (triangles) mmol/L glucose (n=14 arteries)

Fig. 5.2B: 5 (circles) mmol/L glucose (n=13 arteries)

Fig. 5.2C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=11 arteries)

Fig. 5.2D: 5 (circles) and 12 (squares) mmol/L glucose (n=13 arteries)

*Paired t-test: p > 0.05*
5.4.4 Endothelium-dependent relaxation: effect of glucose (GDM - KCl)

Myometrial arteries were constricted using 60 mmol/L KCl and endothelium-dependent relaxation of arteries at 5 mmol/L was compared to that after 30 minutes of incubation at 2, 5, 8 and 12 mmol/L glucose (Fig. 5.3). Acute changes in glucose concentration had no significant effect on endothelium-dependent relaxation in arteries from patients with GDM at any glucose level studied (repeated measures ANOVA: p > 0.05).

5.4.5 Constriction: effect of glucose (GDM - U46619)

When myometrial arteries from women with gestational diabetes were constricted with U46619, no significant difference in active effective pressure was present at any of the glucose levels examined (Fig. 5.4, paired t-test: p > 0.05).

5.4.6 Endothelium-dependent relaxation: effect of glucose (GDM - U46619)

Acute changes in glucose concentration had no significant effect on endothelium-dependent relaxation when myometrial arteries from patients with GDM were constricted with U46619 (Fig. 5.5, repeated measures ANOVA: p > 0.05).
**Fig. 5.3 A-D: Relaxation: effect of glucose (GDM-KCl)**

Endothelium-dependent relaxation in myometrial arteries from women with gestational diabetes (N=14). Arteries were constricted with 60 mmol/L KCl. Relaxation assessed at:

Fig. 5.3A: 5 (circles) and 2 (triangles) mmol/L glucose (n=14 arteries)
Fig. 5.3B: 5 (circles) mmol/L glucose (n=13 arteries)
Fig. 5.3C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=11 arteries)
Fig. 5.3D: 5 (circles) and 12 (squares) mmol/L glucose (n=13 arteries)

Repeated measures ANOVA: $p > 0.05$
The effect of changes in glucose concentration on active effective pressure in myometrial arteries from women with gestational diabetes (N=8). Vessels were constricted with $10^{-6}$ M U46619. Active effective pressure assessed at:

**Fig. 5.4A:** 5 (circles) and 2 (triangles) mmol/L glucose (n=5 arteries)
**Fig. 5.4B:** 5 (circles) mmol/L glucose (n=8 arteries)
**Fig. 5.4C:** 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=5 arteries)
**Fig. 5.4D:** 5 (circles) and 12 (squares) mmol/L glucose (n=6 arteries)

*Paired t-test: $p > 0.05$*
Fig. 5.5 (A-D): Relaxation: effect of glucose (GDM – U46619)
Endothelium-dependent relaxation in myometrial arteries from women with gestational diabetes (N=8). Arteries were constricted with 10^{-6} M U46619. Relaxation assessed at:
Fig. 5.5A: 5 (circles) and 2 (triangles) mmol/L glucose (n=5 arteries)
Fig. 5.5B: 5 (circles) mmol/L glucose (n=8 arteries)
Fig. 5.5C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=4 arteries)
Fig. 5.5D: 5 (circles) and 12 (squares) mmol/L glucose (n=5 arteries)
Repeated measures ANOVA, p > 0.05
5.4.7 GDM: Effect of vasoconstrictor on endothelium-dependent relaxation

Endothelium-dependent relaxation was compared in myometrial arteries from women with GDM constricted with KCl and U46619 at 5 mmol/L glucose (Fig. 5.6). Relaxation with KCl was significantly impaired compared to that with U46619 (two-way ANOVA: p < 0.001). Bonferroni post-hoc test revealed significant differences at $10^{-8}$, $10^{-7}$ and $10^{-6}$ M bradykinin (p < 0.001).

5.6 GDM: Effect of vasoconstrictor

![Graph showing the effect of vasoconstrictor on endothelium-dependent relaxation](image)

**Fig. 5.6:** Endothelium-dependent relaxation in GDM: effect of vasoconstrictor

Endothelium-dependent relaxation in myometrial arteries from women with gestational diabetes after constriction with 60 mmol/L potassium and $10^{-6}$ M U46619. Significantly impaired relaxation when KCl used

*Two-way ANOVA, p < 0.001*

*Bonferroni post-hoc test: p < 0.001 at $10^{-8}$, $10^{-7}$ and $10^{-6}$ M bradykinin*
5.4.8 Constriction: effect of glucose
(Type 1 and 2 DM: KCl)

As suitable numbers of pregnant women with type 1 and 2 diabetes (N= 4 and 3 respectively) could not be obtained, only a limited study of vascular function was possible in these two groups. Changes in glucose levels had no significant effect on maximal constriction (paired t-test: p > 0.05), when myometrial arteries from women with type 1 and 2 diabetes were constricted with 60 mmol/L KCl (Fig. 5.7 and 5.8).

5.4.9 Endothelium-dependent relaxation: effect of glucose
(Type 1 and 2 DM: KCl)

No significant difference in endothelium-dependent relaxation was noted in either type 1 or 2 diabetes groups on altering glucose levels to 2, 5, 8 or 12 mmol/L (Fig. 5.9; repeated measures ANOVA: p > 0.05).
Fig. 5.7 & 5.8: Constriction: effect of glucose (type 1 and 2 diabetes – KCl)

The effect of changes in glucose concentration from 5 mmol/L glucose on active effective pressure in myometrial arteries from pregnant women with type 1 diabetes (N=4, Fig 5.6) and type 2 diabetes (N=3, Fig 5.7). Vessels constricted with 60 mmol/L KCl, and those achieving constriction < 1 mN/mm excluded

Fig. 5.6: Type 1 diabetes Active Effective Pressure at 2, 5, 8 and 12 mmol/L glucose
Fig. 5.7: Type 2 diabetes Active Effective Pressure at 2, 5, 8 and 12 mmol/L glucose

Paired t-test: $p > 0.05$
Endothelium-dependent relaxation in myometrial arteries from women with type 1 diabetes (N=4). Arteries were constricted with 60 mmol/L KCl. Relaxation assessed at:

- Fig. 5.9A: 5 (circles) and 2 (triangles) mmol/L glucose (n=4 arteries)
- Fig. 5.9B: 5 (circles) mmol/L glucose (n=4 arteries)
- Fig. 5.9C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=3 arteries)
- Fig. 5.9D: 5 (circles) and 12 (squares) mmol/L glucose (n=4 arteries)

Repeated measures ANOVA: $p > 0.05$
5.10A  5 vs 2 mmol/L Glucose

5.10B  Control: 5 mmol/L Glucose

5.10C  5 vs 8 mmol/L Glucose

5.10D  5 vs 12 mmol/L Glucose

**Fig. 5.10(A-D):** Relaxation: effect of glucose levels (Type 2 diabetes – KCl)

Endothelium-dependent relaxation in myometrial arteries from women with type 2 diabetes. Arteries were constricted with 60 mmol/L KCl. Relaxation assessed at:

Fig. 5.10A: 5 (circles) and 2 (triangles) mmol/L glucose (n=3 arteries)

Fig. 5.10B: 5 (circles) mmol/L glucose (n=3 arteries)

Fig. 5.10C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=2 arteries)

Fig. 5.10D: 5 (circles) and 12 (squares) mmol/L glucose (n=3 arteries)

*Repeated measures ANOVA: p > 0.05*
5.4.10 Comparison between GDM and healthy pregnant women: Constriction

The maximum constriction of myometrial arteries, expressed as active effective pressure, was compared between healthy pregnant women and patients with GDM at 5 mmol/L glucose (Fig. 5.11 and 5.12).

5.4.10.1 Using KCl

No significant difference was noted in the active effective pressure between the two groups (Fig. 5.11; unpaired t-test: p > 0.05).

5.4.10.2 Using U46619

The active effective pressure was significantly higher in the GDM group compared to healthy pregnant women (Fig. 5.12; unpaired t-test: p < 0.05).

5.4.11 Comparison between GDM and healthy pregnant women: Endothelium-dependent relaxation

5.4.11.1 Using KCl

Myometrial arteries from healthy pregnant individuals and women with GDM were constricted with 60 mmol/L KCl and endothelium-dependent relaxation compared at 5 mmol/L glucose. Relaxation in arteries from women with GDM were significantly impaired compared to normal (Fig. 5.13, two-way ANOVA: p < 0.001). Bonferroni post-hoc tests showed significantly reduced relaxation (p < 0.001, mean difference = 11.74, 95% confidence intervals = 5.86 to 17.61) for the last concentration of bradykinin \(10^{-6} \text{ M}\). There was no significant difference in bradykinin sensitivity between the two groups as \(EC_{50}\) values were similar (normal = \(1.18 \times 10^{-7} \text{ M}\) and GDM = \(1.45 \times 10^{-7} \text{ M}\)).

5.4.11.2 Using U46619

Myometrial arteries from healthy pregnant women and women with GDM were constricted with \(10^{-6} \text{ M}\) U46619 and endothelium-dependent relaxation was compared (Fig. 5.14). No significant difference was seen between the two groups (Two-way ANOVA, p > 0.05)
5.11  **KCl Constriction: Normal vs GDM**

![KCl Constriction: Normal vs GDM](image)

**Fig 5.11:** KCl Constriction: Normal vs GDM

Arterial constriction using KCl in normal pregnant (N=16, n=49 arteries) and GDM (N=14, n=51 arteries) groups. Horizontal red bars represent mean values. *Unpaired t-test, p > 0.05*

5.12  **U46619 Constriction: Normal vs GDM**

![U46619 Constriction: Normal vs GDM](image)

**Fig 5.12:** U46619 Constriction: Normal vs GDM

Arterial constriction using 10⁻⁶ M U46619 in normal pregnant (N=14, n=46 arteries) and GDM (N=8, n=24 arteries) groups. Horizontal red bars represent mean values. *Unpaired t-test, p < 0.05*
**Fig. 5.13**: Endothelium-dependent relaxation – Normal vs GDM: KCl

Comparison of endothelium-dependent relaxation in myometrial arteries from normal pregnant women (N=16) and women with gestational diabetes (N=14). Arteries constricted with 60 mmol/L KCl at 5 mmol/L glucose. Relaxation significantly impaired in GDM.

*Two-way ANOVA, p < 0.001*

*Bonferroni post-hoc test: 10^{-6} M bradykinin  * p < 0.001*
Fig 5.14: Endothelium-dependent relaxation - GDM and normal: U46619
Comparison of endothelium-dependent relaxation in myometrial arteries from normal pregnant women and women with gestational diabetes. Arteries constricted with $10^{-6}$ M U46619 at 5 mmol/L glucose. No significant difference was seen in relaxation.

*Two-way ANOVA, $p > 0.05$*
5.5 Discussion

5.5.1 Summary of results

The findings of this section have been summarised in Table 5.2 (GDM, type 1 and 2 diabetes) and Table 5.3 (comparison between normal and GDM).

### Table 5.2: Summary of results - effect of change in glucose levels

<table>
<thead>
<tr>
<th>Diabetes Group</th>
<th>Effect of change in glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gestational Diabetes</strong></td>
<td></td>
</tr>
<tr>
<td>(N=17 women, n=75 arteries)</td>
<td>No effect</td>
</tr>
<tr>
<td>Constriction: KCl &amp; U46619</td>
<td></td>
</tr>
<tr>
<td>Endothelium-dependent relaxation</td>
<td></td>
</tr>
<tr>
<td><strong>Type 1 and 2 Diabetes</strong></td>
<td></td>
</tr>
<tr>
<td>(N=7 women, n=21 arteries)</td>
<td>No effect</td>
</tr>
<tr>
<td>Constriction: KCl</td>
<td></td>
</tr>
<tr>
<td>Endothelium-dependent relaxation</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5.3: Summary of results – comparison between normal and GDM

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vasoconstrictor</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constriction</td>
<td>KCl</td>
<td>No difference</td>
</tr>
<tr>
<td></td>
<td>U46619</td>
<td>Enhanced constriction GDM</td>
</tr>
<tr>
<td>Endothelium-dependent relaxation</td>
<td>KCl</td>
<td>Impaired relaxation GDM</td>
</tr>
<tr>
<td></td>
<td>U46619</td>
<td>No difference</td>
</tr>
</tbody>
</table>
5.5.2 Endothelial dysfunction in GDM

The main finding of this section was that endothelium-dependent relaxation was significantly impaired in myometrial arteries of women with gestational diabetes compared to normal (Fig. 5.12). This aberrant function was present despite these patients having a near-normal HbA1C, a marker for diabetes control. Endothelial dysfunction has been previously demonstrated in the subcutaneous arteries of women with GDM [Knock et al., 1997]. However the present study is the first demonstration of abnormal vascular function in the myometrial vascular bed, which is more relevant to foetal outcome.

The impairment in relaxation was evident only when the arteries were constricted with 60 mmol/L KCl, and was absent when U46619 was used. This signifies that these effects are specific to the agonist used. As demonstrated in the previous chapter, myometrial arteries of healthy pregnant women also demonstrated agonist-specific effects (see Section 4.4.10).

Hypoglycaemia was not associated with impaired endothelium-dependent relaxation in GDM, a result at variance with that demonstrated in healthy pregnant women. However, similar results were obtained in myometrial arteries from non-pregnant women. This suggests that the pregnancy-associated alterations to the effects of glucose in myometrial arteries may be inhibited in gestational diabetes.

5.5.2.1 Explanations for impaired relaxation

A) EDHF

An absence of EDHF-mediated relaxation may be responsible for the impaired vasodilation with 60 mmol/L KCl, as it has been demonstrated that high concentrations of potassium inhibit the EDHF response [Chen and Suzuki, 1989]. When vessels were constricted with U46619 the presence of EDHF-mediated relaxation may have normalised endothelium-dependent relaxation. Diabetes has been associated with impairment in both NO [Bolego et al., 2006] and EDHF mediated relaxation [Matsumoto et al., 2006]. However, as the diameter of arteries becomes smaller, EDHF plays a more important role in mediating endothelium-dependent relaxation than NO [Shimokawa et al., 1996]. Thus, at the resistance
artery level, EDHF may play a greater role in endothelium-dependent relaxation than nitric oxide. Furthermore, in the rat model of type 1 diabetes, EDHF has been shown to compensate for reduced bio-availability of NO [Shi et al., 2006]. Kenny et al. have demonstrated the importance of both NO and EDHF in the myometrial arterial bed in healthy pregnant women [Kenny et al., 2002], although their relative contribution in diabetes is uncertain. The present study suggests that EDHF may play an important role in endothelium-dependent relaxation in the myometrial vascular bed in women with gestational diabetes.

B) Obesity
Patients in the gestational diabetes group were obese, with a higher BMI than the normal pregnant group. The impaired endothelium-dependent relaxation noted may be due to the association between obesity and endothelial dysfunction (see Section 1.6.5.2). However, reports conflict on this association and it is not clear whether obesity in GDM independently affects endothelial function. A reduction of visceral fat in non-diabetic obese women has been associated with an improvement in endothelial function [Park and Shim, 2005]. In contrast to this finding, two studies examining endothelial function in obese women with previous GDM have not found obesity to be associated with altered endothelial function. In the first study, equal impairment in brachial artery flow-mediated dilation was observed in obese and non-obese women with previous GDM [Anastasiou et al., 1998]. Another study demonstrated that moderate weight loss and therapeutic lowering of LDL cholesterol was associated with improvement of endothelial function in obese women with previous GDM; whereas a similar improvement was not seen with weight loss alone [Bergholm et al., 2003]. This suggests that LDL levels, rather than obesity per se may have a stronger influence on endothelial function. It is therefore uncertain whether the maternal obesity noted here is independently associated with the endothelial dysfunction demonstrated in GDM patients. However, as obesity is frequently associated with gestational diabetes, this distinction may not be clinically relevant.
5.5.2.2 Implications of findings

Endothelial dysfunction is associated with functional impairment of target organs in diabetes such as the heart [Esper et al., 2006] and kidney [Shestakova et al., 2005]. In humans, endothelial dysfunction in type 2 diabetes has been related to altered hemodynamics in the renal circulation, with aberrant capillary blood flow [Futrakul et al., 2006]. However, it is unknown whether the endothelial dysfunction demonstrated in the present study is associated with aberrant uterine blood supply during pregnancy in GDM. As discussed in Section 1.4.2, even minimal impairment of arterial diameter can be associated with a marked reduction of blood flow, as flow is proportional to the fourth power of radius. Reduced blood flow may also be a factor in the pathogenesis of other complications of diabetes in pregnancy such as infertility. A study has demonstrated that an absence of sub-endometrial blood flow results in a poor success rate during in-vitro fertilization [Zaidi et al., 1995].

Endothelial dysfunction in diabetic rats has been positively correlated with the duration of diabetes [Pieper, 1999]. However, a study has demonstrated that the degree of diabetes control may be a more important factor influencing endothelial dysfunction (as estimated by the concentration of asymmetric dimethylarginine) than the duration of diabetes [Xiong et al., 2005]. Gestational diabetes has a relatively shorter duration than type 1 diabetes, and in the patients studied the mean glycated haemoglobin was close to normal levels. Nevertheless, the presence of endothelial dysfunction in this group indicates that factors other than diabetes duration and glycaemic control are important. One such factor is insulin resistance, which has been noted even in lean women with gestational diabetes [Kautzky-Willer et al., 1997]. The link between insulin resistance and endothelial dysfunction may involve NO, as endothelial nitric oxide synthesis has been positively correlated with insulin sensitivity [Petrie et al., 1996]. Insulin resistance has also been demonstrated to be an important factor in the endothelial dysfunction of type 2 diabetes [Pistrosch et al., 2004].

GDM is associated with a higher rate of pre-eclampsia [Nordin et al., 2006]. The aberrant endothelial function reported in pre-eclampsia [Savvidou et al., 2003] may form a link between these two conditions. Increased insulin resistance has also been
noted in pre-eclampsia [Berkowitz, 1998], further strengthening the association with gestational diabetes.

5.5.2.3 Limitations of study

Compared to studies utilising techniques such as plethysmography, this study was limited by a relatively small number of patients, despite recruitment from three large hospitals (see Section 2.14.1). However, the number of patients studied exceeds that of other reports investigating vascular function in diabetic patients [Ang et al., 2002; Smits et al., 1993]. As noted in Chapter 3, only a small proportion (11.7% in the cohort studied) of women with gestational diabetes underwent elective Caesarean section, which restricted tissue availability. Patients with potential confounders such as hypertension and medication, pre-term deliveries and emergency Caesarean sections were excluded, further reducing patient numbers. This resulted in an inability to group these patients based on factors such as ethnicity, BMI and mode of treatment (insulin or diet control). Endothelium-independent relaxation was not systematically investigated in this study. However, previous studies have demonstrated no association between hyperglycaemia and endothelium-independent relaxation [Reed et al., 2004; Williams et al., 1998]. As cholesterol levels were not routinely checked in all patients, it was uncertain if hypercholesterolaemia was present, which may affect endothelial function (see Section 1.5.4.4). However, hypercholesterolaemia was absent in patients whose cholesterol level had been measured.

5.5.3 Patient characteristics

In this study, despite demonstrating impaired endothelial function in myometrial arteries, the mean birth weight of babies in the GDM group was higher than normal. One of the earliest explanations of macrosomia in diabetes is the Pedersen hypothesis, which states that maternal hyperglycaemia stimulates the fetal pancreas to secrete excess insulin, resulting in increased adiposity and macrosomia [Pedersen, 1954]. However, in the subsequent decades since this was first postulated, a more complex picture for the aetiology of large babies in gestational diabetes has emerged. In both diabetic and non-diabetic pregnant women, maternal obesity is associated with fetal size [Ehrenberg et al., 2004; Mathew et al., 2005; Yogev et al., 2005]. A study has demonstrated that macrosomia was not related to gestational age,
ethnic group or insulin treatment, but was associated with high fasting plasma glucose levels, non-pregnant weight and weight gain during pregnancy [Hardy, 1999]. These findings have been corroborated by other studies [Bo et al., 2003; Yang et al., 2004]. Factors such as maternal weight may therefore be of more importance in the pathogenesis of macrosomia than uterine blood flow. Furthermore, it has been shown that decreased uterine blood flow in the diabetic pregnant rat does not modify the augmented glucose transfer to the fetus [Palacin et al., 1985]. It is therefore possible that impaired endothelial function in myometrial arteries does not influence the aetiology of macrosomia, which is multi-factorial and may be attributable to the high BMI of the patients with GDM in this study.

5.5.4 The effect of glucose levels on vascular function

5.5.4.1 Constriction

Changing glucose levels from 5 mmol/L to 2, 5, 8 and 12 mmol/L had no significant effect on maximum constriction (Fig. 5.2 A-D). Identical results were obtained in both healthy non-pregnant and pregnant women, signifying that myometrial artery constriction (an endothelium-independent function) was not influenced by the glucose levels studied.

No significant difference was noticed in maximum arterial constriction between arteries from healthy individuals or those with GDM when constricted with KCl (Fig. 5.10). This implies that myometrial arteries from this group of diabetic patients had similar smooth muscle function as those from healthy pregnant women. This is an important observation, as impairment in endothelium-dependent relaxation can theoretically be attributed to aberrant smooth muscle function. Constriction was significantly enhanced in the GDM group compared to normal when U46619 was used (Fig. 5.11), although a smaller number of GDM patients was studied (N=8).

5.5.4.2 Endothelium-dependent relaxation

Irrespective of the vasoconstrictor used, exposure to glucose levels of 2, 5, 8 or 12 mmol/L had no effect on endothelium-dependent relaxation in myometrial arteries.
from women with gestational diabetes (Fig. 5.3 and 5.5). This result is at variance from that seen in healthy pregnant women, where levels of 2 mmol/L glucose were associated with impaired endothelium-dependent relaxation when U46619 was used (Fig. 4.8). It was postulated that blocking EDHF may have enhanced NO-mediated relaxation in healthy pregnant women. However, this mechanism may be absent in women with gestational diabetes. This suggests that arteries from women with GDM respond differently to stimuli such as hypoglycaemia. However, the relatively small number of patients in the GDM-U46619 group prevents firm conclusions from being drawn.

Levels of 12 mmol/L glucose had no effect on endothelium-dependent relaxation in both the normal pregnant group and the GDM group. In the preceding chapter, possible explanations for the lack of effect of high glucose concentrations in the normal pregnant group had been discussed (Section 4.5.4). Most of these arguments may also be relevant in explaining the lack of effect of high glucose concentrations in the GDM group. Vascular NO has been demonstrated to be reduced after exposure to 16.6 mmol/L glucose for 1 hour in rat vessels [Lash et al., 1999]. The level and duration of high glucose concentrations studied may be insufficient to affect NO bio-availability. Alternatively, it is possible that impairment in NO may be compensated for by other mediators such as EDHF. Such a compensatory mechanism has been demonstrated to exist in myometrial arteries from normal women [Kenny et al., 2002]. Little is known about the association between hyperglycaemia and mediators such as EDHF and PGI₂, as existing research has mainly focused on NO. Increased production of PGI₂ has been reported in the early stages of diabetes in mice injected with streptozotocin [Shen et al., 2003]. The association between aberrant glucose levels and the endothelial mediators has been explored in the following chapter.

5.5.5 Type 1 diabetes and type 2 diabetes

The insufficient numbers of type 1 and 2 diabetes patients recruited (reasons for which have been described in Section 2.14.1) prevent conclusions from being made regarding the effect of glucose on endothelial function in these two types of diabetes. However, data were consistent with a lack of effect of glucose levels on
either constriction or endothelium-dependent relaxation was seen, similar to the results obtained in the GDM group.

5.5.6 SUMMARY

When constricted with 60 mmol/L potassium, myometrial arteries from women with gestational diabetes demonstrated impaired endothelium-dependent relaxation compared to those obtained from normal women. This effect was absent when U46619 was used as an agonist. Exposure to 2, 5, 8 and 12 mmol/L glucose for 30 minutes had no effect on either arterial constriction or endothelium-dependent relaxation in GDM irrespective of the vasoconstrictor used. Limited availability of tissue from healthy women and patients with diabetes prevented investigation of the mechanisms of endothelial function. This limitation was overcome and the mechanisms examined by utilising an animal model of normal pregnancy and diabetes in pregnancy, which is discussed in the next chapter.
CHAPTER 6

The effect of glucose on endothelial function in non-pregnant and pregnant mice
THE EFFECT OF GLUCOSE ON ENDOTHELIAL FUNCTION IN NON-PREGNANT AND PREGNANT MICE

6.1 Introduction

The preceding two chapters have illustrated that human research is often limited by tissue availability, a restriction which can curtail the scope of a study. Researchers have overcome this obstacle by utilising animal models to understand both physiological and pathological processes. As discussed in Chapter 1, investigations in animal models of normal pregnancy have resulted in a greater understanding of the normal physiological changes occurring in pregnancy. Furthermore, animal models of diabetes have facilitated investigations of the aetiology of diabetes and its complications, including those occurring in pregnancy.

Animal studies have usually been conducted independent of human studies: this often makes extrapolation of findings to humans difficult. Few studies have concurrently examined the effects of an identical stimulus in both human and animal physiology. This limits cross-comparison between species, hindering attempts at understanding physiological differences which may be of importance in animal models.

For the above reasons, a study of the effect of glucose levels on endothelial function in animal models would provide valuable insight into whether inter-species variations of glucose effects exist; as well as allowing investigations which could not be performed in humans due to limited tissue availability. Little is known about uterine vascular function in pregnant diabetic mice, as studies have concentrated on other vascular beds such as mesenteric, carotid and the aorta. Constriction and relaxation in the main uterine artery of normal pregnant and non-pregnant mice were evaluated using similar methods as described in Chapter 2. This helped to minimise methodological differences between human and animal studies.

Due to the limited number of diabetic patients in this study, the creation of an animal model of diabetes was undertaken, as described in Section 2.14. However,
diabetes could be induced only in a minority of mice, whereas most were found to be resistant to the diabetogenic actions of streptozotocin. Baseline experiments using previously described protocols were carried out in the pregnant mice not responding to streptozotocin (streptozotocin-controls), to act as a control group for future studies in diabetic pregnant mice.

6.2 Aims

1. Examine the effect of 2, 5, 8 and 12 mmol/L glucose for 30 minutes duration, on constriction and endothelium-dependent relaxation in uterine arteries from non-pregnant, pregnant and streptozotocin-control mice
2. Examine the effect of pregnancy on constriction and relaxation by comparing results from non-pregnant and pregnant mice
3. Examine the effect of hyper-osmolarity on endothelium-dependent relaxation
4. Examine the role of the mediators of endothelium-dependent relaxation in the uterine artery of normal pregnant mice
5. Examine whether aberrant glucose concentrations altered the contribution of these mediators.

6.3 Materials and methods

These were previously described in Chapter 2. The experimental protocols used were Protocol 1, 2, 3 and 4 (see Section 2.12). Uterine arteries were constricted with phenylephrine and relaxed with acetylcholine. Arteries with maximum constriction less than 0.2 mN/mm were excluded from analysis.
6.4 Results

6.4.1 Arterial diameters

The mean diameter of uterine arteries from pregnant mice (250 ± 4 μm; N=18, 62 arteries) was significantly greater than that from non-pregnant mice (184 ± 3 μm; N=15, 44 arteries) [Fig. 6.1, unpaired t-test: p < 0.0001].

Fig. 6.1: Arterial diameters – normal non-pregnant and pregnant mice
Diameter of uterine arteries from normal non-pregnant and pregnant mice. Horizontal red bars represent mean values. Mean diameter significantly greater in pregnant mice.
Unpaired t-test: p < 0.0001
6.4.2 Constriction: normal non-pregnant mice

Exposure of uterine arteries from normal non-pregnant mice to 2, 5, 8 and 12 mmol/L glucose for 30 minutes had no effect on arterial constriction (Fig. 6.2(A-D); paired t-test: p > 0.05 in all comparisons; at 12 mmol/L glucose: mean difference = -0.95, 95% confidence intervals = -1.97 to 0.07).

6.4.3 Endothelium-dependent relaxation: normal non-pregnant mice

Endothelium-dependent relaxation in uterine arteries of normal non-pregnant mice was not altered by exposure to 2, 5, 8 or 12 mmol/L glucose for 30 minutes (Fig. 6.3(A-D); repeated measures ANOVA: p > 0.05). The similarity in relaxation was also illustrated in Fig. 6.4 where the relaxation at all four glucose levels are shown.
Fig. 6.2A-D: Constriction: effect of glucose (normal non-pregnant mice)
Constriction at 5 mmol/L glucose and after 30 minute incubation at 2, 5, 8, and 12 mmol/L glucose in uterine arteries from normal non-pregnant mice (N = 15). Vessels constricted with 10^{-5} M phenylephrine. Constriction assessed at:
Fig. 6.2A: 5 (circles) and 2 (triangles) mmol/L glucose (n=11 arteries)
Fig. 6.2B: 5 mmol/L glucose (circles) (n=14 arteries)
Fig. 6.2C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=6 arteries)
Fig. 6.2D: 5 (circles) and 12 (squares) mmol/L glucose (n=10 arteries)
No significant difference in constriction noted.
*Paired t-test: p > 0.05*
Fig. 6.3A-D: Relaxation: effect of glucose (normal non-pregnant mice)
Endothelium-dependent relaxation at 5 mmol/L glucose and after 30 minute incubation at 2, 5, 8, and 12 mmol/L glucose in uterine arteries from normal non-pregnant mice (N = 15). Vessels constricted with 10⁻⁵ M phenylephrine. Relaxation assessed at:
Fig. 6.3A: 5 (circles) and 2 (triangles) mmol/L glucose (n=11 arteries)
Fig. 6.3B: 5 mmol/L glucose (circles) (n=14 arteries)
Fig. 6.3C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=6 arteries)
Fig. 6.3D: 5 (circles) and 12 (squares) mmol/L glucose (n=10 arteries)
No significant difference in relaxation noted.
Repeated measures ANOVA, p > 0.05
**6.4 Relaxation: Non-Pregnant Mice**

![Graph showing relaxation effect of glucose levels in non-pregnant mice](image)

**Fig. 6.4: Relaxation: effect of glucose (normal non-pregnant mice)**

Unpaired analysis of data in Fig 6.3. Endothelium-dependent relaxation at 2 (blue), 5 (red), 8 (green), and 12 (orange) mmol/L glucose in uterine arteries from normal non-pregnant mice (N = 15). Vessels constricted with $10^{-5}$ M phenylephrine.

No difference in relaxation noted.

*Repeated measures ANOVA: $p > 0.05$*
6.4.4 Constriction: normal pregnant mice

In contrast to findings in non-pregnant mice, a significant increase in constriction was seen in arteries from normal pregnant mice when exposed for 30 minutes to 2, 8 or 12 mmol/L glucose for 30 minutes (Fig. 6.5(A-D); paired t-test: \( p < 0.05 \)). This effect was not seen in the control vessel at 5 mmol/L glucose (Fig. 6.5B; paired t-test: \( p > 0.05 \)). Mean values (± SEM) of active effective pressure were:

- 5 and 2 mmol/L glucose = 16.3 (± 1.2) and 17.3 (± 1.3) kPa
- 5 and 5 mmol/L glucose = 17.5 (± 1.0) and 18.1 (± 1.1) kPa
- 5 and 8 mmol/L glucose = 15.5 (± 0.9) and 17.0 (± 1.15) kPa
- 5 and 12 mmol/L glucose = 15.0 (± 0.7) and 15.8 (± 0.8) kPa

6.4.5 Endothelium-dependent relaxation: normal pregnant mice

Endothelium-dependent relaxation demonstrated impairment with time at 5 mmol/L glucose (control vessel), 2 and 8 mmol/L glucose (Fig. 6.6(A-C); repeated measures ANOVA: \( p < 0.05 \)). However, at 12 mmol/L glucose, no significant impairment with time was seen (Fig. 6.6D; repeated measures ANOVA: \( p > 0.05 \)), signifying an enhancement of endothelium-dependent relaxation at this glucose level. This effect was more clearly demonstrated by plotting endothelium-dependent relaxation at the four different glucose concentrations together (Fig. 6.7). In contrast to findings in non-pregnant mice, relaxation was significantly enhanced at 12 mmol/L glucose compared to 5 mmol/L (two-way ANOVA, \( p < 0.001 \)). Bonferroni post-hoc testing revealed a significant difference at 10⁻⁷ M acetylcholine (\( p < 0.01 \), mean difference = -18.22, 95% confidence intervals = -36.71 to 0.27).
Fig. 6.5A-D: Constriction: effect of glucose levels (normal pregnant mice)

Constriction at 5 mmol/L glucose and after 30 minute incubation at 2, 5, 8, and 12 mmol/L glucose in uterine arteries from normal pregnant mice (N = 18). Vessels constricted with 10^{-5} M phenylephrine. Constriction assessed at:

Fig. 6.5A: 5 (circles) and 2 (triangles) mmol/L glucose (n=16 arteries)

p < 0.05, paired t-test

Fig. 6.5B: 5 mmol/L glucose (circles) (n=17 arteries) p > 0.05, paired t-test

Fig. 6.5C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=11 arteries)

p < 0.05, paired t-test

Fig. 6.5D: 5 (circles) and 12 (squares) mmol/L glucose (n=18 arteries)

p < 0.05, paired t-test
Fig. 6.6A-D: Relaxation: effect of glucose levels (normal pregnant mice)
Endothelium-dependent relaxation at 5 mmol/L glucose and after 30 minute incubation at 2, 5, 8, and 12 mmol/L glucose in uterine arteries from normal pregnant mice (N = 18). Vessels constricted with $10^{-5}$ M phenylephrine. Relaxation assessed at:
Fig. 6.6A: 5 (circles) and 2 (triangles) mmol/L glucose (n=16 arteries)
Fig. 6.6B: 5 mmol/L glucose (circles) (n=17 arteries)
Fig. 6.6C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=11 arteries)
Fig. 6.6D: 5 (circles) and 12 (squares) mmol/L glucose (n=18 arteries)
Fig. 6.7: Relaxation: effect of glucose (normal pregnant mice)

Unpaired representation of data from Fig. 6.6. Endothelium-dependent relaxation at 2 (blue), 5 (red), 8 (green), and 12 (orange) mmol/L glucose in uterine arteries from normal pregnant mice (N = 18). Vessels constricted with $10^{-5}$ M phenylephrine. No significant difference was seen at 2 or 8 mmol/L glucose compared to control (two-way ANOVA: $p > 0.05$). Relaxation was significantly enhanced at 12 mmol/L glucose compared to control at 5 mmol/L glucose (two-way ANOVA: $p < 0.01$). Bonferroni post-hoc testing revealed significant difference at $10^{-7}$ M acetylcholine (* $p < 0.01$)
6.4.6 Effect of pregnancy on constriction and endothelium-dependent relaxation

Uterine arteries from pregnant mice demonstrated enhanced constriction (Fig. 6.8; unpaired t-test: $p < 0.05$) as well as enhanced endothelium-dependent relaxation (Fig. 6.9; two-way ANOVA: $p < 0.05$) compared to arteries from non-pregnant mice.

6.4.7 Effect of hyper-osmolarity - pregnant mice

No difference in endothelium-dependent relaxation was seen in a normal osmolar medium (at 5 mmol/L glucose) compared to a hyper-osmolar medium (with an additional 7 mmol/L mannitol) (Fig. 6.10; two-way ANOVA: $p > 0.05$).

**Fig. 6.8: Constriction: normal non-pregnant vs pregnant mice**

Comparison of constriction in uterine arteries at 5 mmol/L glucose in normal non-pregnant (triangles; N=15, n=41 arteries) and pregnant mice (inverted triangles; N=18, n=62 arteries) and Vessels were constricted with $10^{-5}$ M phenylephrine. Horizontal bars represent mean values. Constriction was significantly enhanced in arteries from pregnant mice

*Unpaired t-test: $p < 0.001$
**Relaxation: Non-pregnant vs Pregnant mice**

Comparison of endothelium-dependent relaxation in uterine arteries at 5 mmol/L glucose in normal non-pregnant (triangles; N=15, n=41 arteries) and pregnant mice (circles; N=18, n=62 arteries) and. Vessels were constricted with $10^{-5}$ M phenylephrine. Endothelium-dependent relaxation was significantly enhanced in arteries from pregnant mice.

*Two-way ANOVA: $p < 0.001$*

*Bonferroni post-hoc test: at $10^{-6}$ M acetylcholine  * $p < 0.001$*
**6.10 Effect of Hyper-osmolarity**

![Graph showing the effect of Acetylcholine M on % Maximum Constriction under different osmolar solutions.]

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**Fig. 6.10**: The effect of hyper-osmolarity:

Endothelium-dependent relaxation in uterine arteries from pregnant mice (N=3) compared in two different solutions

- Normal osmolar solution (= 5 mmol/L glucose PSS; solid circle)
- Hyper-osmolar mannitol solution (= 5 mmol/L glucose PSS + 7 mmol/L of Mannitol; open square).

No significant difference was noted in endothelium-dependent relaxation

*Two-way ANOVA: p < 0.05*.
6.4.8 Normal pregnant mice: effect of endothelial blockers at 5 and 12 mmol/L glucose

At both 5 mmol/L (Fig. 6.11) and 12 (Fig. 6.12) mmol/L glucose, indomethacin had no effect on endothelium-dependent relaxation compared to control (two-way ANOVA: p > 0.05). L-NNA was associated with a significant impairment of relaxation at both 5 and 12 mmol/L glucose (two-way ANOVA: p < 0.05). Impaired relaxation compared with control was also present when L-NNA and indomethacin were used in combination at 12 mmol/L glucose (two-way ANOVA: p < 0.05). Endothelium-dependent relaxation was predominantly due to a non-NO/non-prostanoid mechanism, most likely EDHF, although this was not specifically tested.

6.4.9 Normal pregnant mice: effect of glucose levels on endothelial blockers

Endothelium-dependent relaxation mediated by NO (as blocked by L-NNA, Fig. 6.13B) and prostanoids (as blocked by indomethacin, Fig. 6.13C) was similar at both 5 mmol/L and 12 mmol/L glucose (two-way ANOVA, p > 0.05). The non-NO/non-prostacyclin component of relaxation was also similar at both levels (Fig. 6.13D, two-way ANOVA: p > 0.05).
Fig. 6.11: The effect of blockers on relaxation – 5 mmol/L glucose
The effects of specific blockers to endothelium-dependent relaxation in uterine arteries of pregnant mice (N=13) were evaluated at 5 mmol/L glucose. Vessels constricted with $10^{-5}$ M phenylephrine.
Control (circles, n=8 arteries) L-NNA (inverted triangles, n=13 arteries) indomethacin (squares, n=9 arteries) L-NNA & indomethacin (triangles, n=12 arteries)
Control vs indomethacin: two-way ANOVA, $p > 0.05$
Control vs L-NNA + indomethacin two-way ANOVA, $p > 0.05$
Control vs L-NNA two-way ANOVA, $p < 0.05$
The effects of specific blockers to endothelium-dependent relaxation in uterine arteries of pregnant mice (N=15) were evaluated at 12 mmol/L glucose. Vessels constricted with $10^{-5}$ M phenylephrine.

Control (circles, n=11 arteries) L-NNA (inverted triangles, n=14 arteries) indomethacin (squares, 13 arteries) L-NNA & indomethacin (triangles, n=15 arteries)

Control and indomethacin: two-way ANOVA, $p > 0.05$

Control and L-NNA: two-way ANOVA, $p < 0.05$

Control and L-NNA + indomethacin: two-way ANOVA, $p < 0.05$

(Bonferroni post-hoc test= $10^{-6}$ M ACh, $p < 0.05$)
Fig. 6.13A-D: The effect of blockers at 5 and 12 mmol/L glucose
Comparison of endothelium-dependent relaxation at 5 and 12 mmol/L glucose after addition of blockers
Fig. 6.13A: Control at 5 (solid circles) and 12 (open circles) mmol/L glucose
Fig. 6.13B: L-NNA at 5 (solid inverted triangles) and 12 (open inverted triangles) mmol/L glucose
Fig. 6.13C: Indomethacin at 5 (solid squares) and 12 (open squares) mmol/L glucose
Fig. 6.13D: L-NNA + Indomethacin at 5 (solid triangles) and 12 (open triangles) mmol/L glucose
No significant difference in endothelium-dependent relaxation was seen at any glucose level (two-way ANOVA, p > 0.05)
6.4.10 Blood glucose in mice

Mean (± SD) blood glucose of mice before streptozotocin injection was 6.8 (± 0.5) mmol/L. Values obtained after injection of either streptozotocin or vehicle alone are illustrated in Fig. 6.14. Very few mice (approximately 17%) developed diabetes (two examples shown: D1-2), with mean (± SD) blood glucose levels of 16.2 (± 2.2) mmol/L glucose. Mice not responding to streptozotocin (STZ-control, S1-S8) had blood glucose levels of 7.4 (± 1.2) mmol/L, whereas mice injected with vehicle alone (vehicle-control, two examples shown: V1-2) had levels of 6.7 (± 0.9) mmol/L glucose. There was no significant difference in glucose levels between STZ-control and vehicle-control mice (one-way ANOVA, Bonferroni post test: p > 0.05). Diabetic mice had significantly higher blood glucose levels than both streptozotocin-controls and vehicle controls (one-way ANOVA, Bonferroni post test: p < 0.001). Due to the extremely poor success rate for diabetes induction and the resultant time constraints, only results from streptozotocin-controls (S1-8) have been demonstrated in subsequent experiments.
Fig. 6.14: Blood glucose: mice
Glucose levels measured in mice after injection of streptozotocin or vehicle:
S1 - 8 = Streptozotocin –controls: mice injected with streptozotocin, non-diabetic
D1-2 = Diabetic mice: mice injected with streptozotocin, diabetic
V1-2 = Vehicle-controls: mice injected with vehicle only

6.4.11 Pregnant streptozotocin-control mice

6.4.11.1 Constriction: effect of glucose levels (pregnant streptozotocin-control mice)

Maximum active effective pressure was compared before and after acute changes in glucose concentration in uterine arteries of pregnant mice (N=8) that had been injected with streptozotocin, but did not develop diabetes (Fig. 6.15). A significant increase of active effective pressure was noted in the control arteries (5 mmol/L glucose) as well as after incubation at 8 and 12 mmol/L glucose (paired t-test: p < 0.05). However, no significant difference was noted after incubation at 2 mmol/L
glucose (paired t-test: p > 0.05). Mean values (± SEM) of active effective pressure were:

5 and 2 mmol/L glucose = 14.6 (± 0.8) and 15.9 (± 0.6 kPa)
5 and 5 mmol/L glucose (control) = 18.2 (± 0.8) and 20.0 (± 0.6) kPa
5 and 8 mmol/L glucose = 18.8 (± 1.7) and 21.1 (± 1.8) kPa
5 and 12 mmol/L glucose = 19.0 (± 0.7) and 19.9 (± 0.8) kPa

6.4.11.2 Relaxation: effect of glucose levels (pregnant streptozotocin-control mice)

Endothelium-dependent relaxation in uterine arteries of pregnant non-diabetic mice treated with streptozotocin was not altered by exposure to 2, 5, 8 or 12 mmol/L glucose for 30 minutes (Fig. 6.16, repeated measures ANOVA, p > 0.05). The similarity in relaxation has also been demonstrated in Fig. 6.17 where relaxation at all four glucose levels have been shown.
Fig. 6.15A-D: Constriction: effect of glucose levels (pregnant STZ-controls)

Constriction at 5 mmol/L glucose and after 30 minute incubation at 2, 5, 8, and 12 mmol/L glucose in uterine arteries from pregnant mice not responsive to streptozotocin (N = 8). Vessels constricted with $10^{-5}$ M phenylephrine.

Constriction assessed at:

Fig. 6.15A: 5 (circles) and 2 (triangles) mmol/L glucose (n=8 arteries)

Fig. 6.15B: 5 mmol/L glucose (circles) (n=7 arteries) $p < 0.05$, paired t-test

Fig. 6.15C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=8 arteries)

Fig. 6.15D: 5 (circles) and 12 (squares) mmol/L glucose (n=8 arteries)

$p < 0.05$, paired t-test
**Fig. 6.16A-D: Relaxation: the effect of glucose (pregnant STZ-controls)**
Endothelium-dependent relaxation at 5 mmol/L glucose and after 30 minute incubation at 2, 5, 8, and 12 mmol/L glucose in uterine arteries from pregnant mice not responsive streptozotocin (N = 8). Vessels constricted with $10^{-5}$ M phenylephrine. Relaxation assessed at:

Fig. 6.16A: 5 (circles) and 2 (triangles) mmol/L glucose (n=8 arteries)
Fig. 6.16B: 5 mmol/L glucose (circles) (n=7 arteries)
Fig. 6.16C: 5 (circles) and 8 (inverted triangles) mmol/L glucose (n=8 arteries)
Fig. 6.16D: 5 (circles) and 12 (squares) mmol/L glucose (n=8 arteries)

*Repeated measures ANOVA: $p > 0.05$*
6.17 Relaxation: Pregnant STZ-Controls

![Graph showing relaxation effect of glucose levels](image)

**Fig. 6.17: Relaxation: effect of glucose levels (pregnant STZ-controls)**

Unpaired representation of data from Fig. 6.14. Endothelium-dependent relaxation at 2 (blue), 5 (red), 8 (green), and 12 (orange) mmol/L glucose in uterine arteries from pregnant STZ non-responding mice (N=8). Vessels constricted with 10^{-5} M phenylephrine.

*Two-way ANOVA: p > 0.05*
6.5 Discussion

6.5.1 Summary of findings

The findings in this section have been summarised in Table 6.1 below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>Effect of glucose levels</th>
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<tr>
<td>arteries)</td>
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<td></td>
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<tr>
<td></td>
<td>Endothelium-dependent relaxation</td>
<td>No effect</td>
</tr>
<tr>
<td>Normal pregnant mice (N=18 mice, n=62</td>
<td>Constriction</td>
<td>Enhanced at 2, 8 and 12 mmol/L glucose</td>
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<tr>
<td>arteries)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Endothelium-dependent relaxation</td>
<td>Enhanced at 12 mmol/L glucose</td>
</tr>
<tr>
<td>STZ- control pregnant mice (N=8 mice, n=31</td>
<td>Constriction</td>
<td>Attenuated at 2 mmol/L glucose</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endothelium-dependent relaxation</td>
<td>No effect</td>
</tr>
</tbody>
</table>

Table 6.1: Summary of findings

6.5.2 Enhanced vasodilation at 12 mmol/L glucose

The main finding of this study was that 12 mmol/L glucose lasting 30 minutes was associated with enhanced endothelium-dependent relaxation in the uterine artery of normal pregnant mice. This effect was specific to pregnancy, as no such change was noticed in arteries from non-pregnant mice. It was also independent of hyper-osmolarity, as a hyper-osmolar mannitol solution had no effect on endothelium-dependent relaxation in pregnant mice.
Studies reporting endothelium-dependent vasodilation with hyperglycaemia have been previously discussed (Table 1.4). These studies varied in the precise glucose levels examined; therefore the threshold above which raised glucose concentrations start affecting vessel function is unknown. In the uterine arteries of pregnant mice, vasodilation was noted at 12 mmol/L glucose but not at 8 mmol/L. This narrows the range at which the vasoactive effects of glucose become evident in this vascular bed. Exposure to 12 mmol/L glucose for 30 minutes represents one of the lowest and shortest degrees of hyperglycaemia for which vascular effects have been demonstrated. Interestingly, studies demonstrating vasodilation with hyperglycaemia (Table 1.4) generally had a duration of hyperglycaemia of an hour or less [Hoffman et al., 1999; van Veen et al., 1999], whereas those demonstrating impaired relaxation (Table 1.3) had a duration of 1-6 hours [Affonso Fde et al., 2003; Kawano et al., 1999; Williams et al., 1998]. Therefore, it may be possible that hyperglycaemia has a biphasic effect on vascular function: enhancing vasodilation during short-term exposures, but causing impairment during longer exposures. The vasodilation observed in the uterine artery at high glucose concentrations may enhance blood flow to the offspring in the post-prandial period. The longer exposures to hyperglycaemia may uncouple the endothelial Nitric Oxide Synthase (eNOS) enzyme; resulting in the generation of free radicals and diminished NO availability [Santilli et al., 2004]. NO has been demonstrated to be reduced after exposure to 16.6 mmol/L glucose for 1 hour in rat spinotrapezius arterioles [Lash et al., 1999].

A limitation of the present study is that the effect of a more prolonged exposure to high glucose concentrations was not examined. This was due to the methodological limitations of using wire myography: after a 2-hour exposure to high glucose concentrations, ex-vivo uterine arteries were found to have diminished viability with erratic responses. Similar aberrant responses were seen in human myometrial arteries (see Section 4.4.12). Therefore it is uncertain whether the enhanced endothelium-dependent vasodilation continues to be present after a longer exposure to high glucose concentrations.
6.5.3 Glucose requirements in different vascular beds

In normal individuals, hyperglycæmia has been associated with both vasodilation [Hoffman et al., 1999; van Veen et al., 1999] and conversely, the impairment of endothelium-dependent vasodilation [Title et al., 2000; Williams et al., 1998]. In animals, Cipolla et al. has shown that hyperglycaemia (44 mmol/L for 1-2 hours) induces endothelium-dependent vasodilation in cerebral arteries of rats [Cipolla et al., 1997]. As glucose is a crucial substrate for many cellular processes, hyperglycaemia may have different effects at different vascular beds depending on the metabolic requirements of that tissue region. Therefore, studies in physiologically relevant vascular beds may be of more value in understanding the pathophysiology of hyperglycaemia. As the glucose requirements are vastly different in the cerebral or uterine circulation compared to forearm musculature or skin, these different effects may be an adaptive response to limited substrate availability. The vasodilation observed in the uterine artery at high glucose concentrations may therefore represent a vascular adaptation to promote blood flow and the delivery of nutrients to the offspring in the post-prandial period.

6.5.4 Hyperglycaemia and hyper-osmolarity

It has been previously reported that hyper-osmolarity is associated with vasodilation, mediated via the endothelium [Masset et al., 2000; Sasaki et al., 1986]. Vasodilation seen in this study is an effect specific for high glucose concentrations, as incubation in an equi-osmolar mannitol solution did not alter endothelium-dependent relaxation. Furthermore, at 12 mmol/L glucose, vasodilation was seen only in arteries from pregnant mice and not in those from non-pregnant mice. The change in osmolarity induced by increasing glucose levels from 5 to 12 mmol/L is minimal: studies have shown the effect of hyper-osmolarity to become evident only at much higher levels, such as 36 mmol/L glucose [Wang et al., 1998]. Other studies have also demonstrated that alterations in vascular function with hyperglycaemia are not due to hyper-osmolarity [Affonso Fde et al., 2003; Gomes et al., 2004; Sercombe et al., 2004].
6.5.5 Hyperglycaemia and systemic factors

Systemic factors have been implicated in the modulation of vascular function by hyperglycaemia. These include alterations in neural activity and increased insulin levels in the blood. Acute hyperglycaemia in normal individuals has been associated with increased sympathetic neural activation and vasodilation [Hoffman et al., 1999]. Hyperosmolarity may have been responsible for this vasodilation, as mannitol was found to produce effects similar to glucose. This is in contrast to the results demonstrated in this section, where mannitol had no vasoactive effect. Hyperinsulinemia has been associated with vasodilation involving NO release [Scherrer et al., 1994]. Renaudin et al. reported arteriolar vasodilation in a rat trapezius muscle preparation with hyperinsulinaemia alone, but vasoconstriction when hyperglycaemia and hyperinsulinaemia were present [Renaudin et al., 1998]. Systemic hyperinsulinaemia has been demonstrated to be more important than local hyperinsulinaemia in mediating vasodilation [Cardillo et al., 1998]. These studies suggest that the concurrent increase in insulin level with hyperglycaemia may play an important role in mediating vasodilation in vivo. However, the present study has demonstrated that high glucose concentrations can enhance vasodilation by a local action independent of systemic factors such as hyperosmolarity, hyperinsulinaemia and neural input.

6.5.6 Effect of pregnancy

Vasoconstriction, as measured by active effective pressure, was significantly higher in uterine arteries from pregnant mice compared to non-pregnant mice, signifying that the state of pregnancy is associated with an enhanced constrictor response to phenylephrine. Enhanced endothelium-dependent relaxation was also seen in uterine arteries from pregnant mice compared to those obtained from non-pregnant mice. Similar augmentation of vasodilation in pregnancy has been previously reported in human uterine arteries [Nelson et al., 1998] and the rat aorta [Honda et al., 1996]. As demonstrated in Chapter 4, no such enhanced vasodilation during pregnancy was present in human myometrial arteries, highlighting the differences in artery function at disparate vascular beds in pregnancy. These findings also raise the possibility of inter-species variation in the effect of pregnancy on vasculature.
6.5.7 Effect of glucose on constriction

Exposure to 30 minutes of 2, 8 and 12 mmol/L glucose was associated with an increase in constriction in uterine arteries of normal pregnant mice; an effect not present either in the control arteries at 5 mmol/L glucose or at any glucose level examined in normal non-pregnant mice. Although this enhanced constriction was statistically significant, the actual change in active effective pressure was small. The physiological relevance of this finding is therefore uncertain.

The above findings signify that unique alterations occur in mouse uterine arteries during pregnancy. Morphological changes include increased diameter whereas functional changes comprise of alterations in the physiological effects of glucose. Pregnancy was also associated with an increase in maximal constriction and endothelium-dependent relaxation of uterine arteries.

6.5.8 Endothelial mediators of vasodilation

This study demonstrated that in small-calibre arteries, endothelium-dependent relaxation is mainly mediated by a non-NO/non-prostacyclin mediator; most likely EDHF, although this was not specifically blocked. NO played a less important role in this vascular bed, whereas prostacyclin did not mediate endothelium-dependent relaxation in the uterine artery of pregnant mice. These findings corroborate other studies that have documented the dominance of EDHF-mediated relaxation in small arteries [Shimokawa et al., 1996; Urakami-Harasawa et al., 1997]. In contrast, NO has been associated with relaxation in larger arteries such as the aorta in rats [Vizioli et al., 2005]. Current research in the field of endothelium-dependent relaxation in hyperglycaemia is predominantly directed at the contribution of NO, with relatively few studies examining the role of EDHF. This is because hyperglycaemia is associated with increased free radical formation and diminished bio-availability of NO, which contributes to endothelial dysfunction in diabetes [Marfella et al., 2000; Monnier et al., 2006]. The present study has demonstrated no alteration in NO-mediated relaxation after exposure to mildly raised glucose concentrations for 30 minutes. As EDHF was not specifically blocked, the effects of glucose on this mediator are uncertain. Resistance arteries play an important role in modulating blood flow to target organs and EDHF has been demonstrated to be the predominant
mediator of endothelium-dependent relaxation in these vessels [Shimokawa et al., 1996]. Reduced EDHF has been recently associated with endothelial dysfunction in streptozotocin-induced diabetic mice [Matsumoto et al., 2006]. Hence characterization of EDHF by future studies may help to discern its contribution to endothelial function during hyperglycaemia.

As described in Section 1.6.4, there is considerable variation in the proportion of endothelium-dependent relaxation mediated by the three endothelial mediators depending on the vascular bed. When Crauwels et al. examined these mediators in C57BL/6 mice (also used in the present study), endothelium-dependent relaxation was demonstrated to be mediated almost exclusively by nitric oxide in the carotid artery [Crauwels et al., 2000]. In the femoral artery of these mice, relaxation was pre-dominantly mediated by NO with a smaller contribution from a non-prostanoid/non-NO factor. No prostanoid-mediated relaxation was seen in the carotid or femoral arteries. These results are at variance to those demonstrated in the present section, where NO had a relatively small role in mediating relaxation in the uterine arteries. This emphasizes the variation in activity of the endothelial mediators at different vascular beds.

A number of studies have unravelled a more complex inter-relationship between the endothelial vasorelaxants than originally envisaged. Nitric oxide and cyclooxygenase derivatives can substitute for each other in producing relaxation in human small omental arteries [Ohlmann et al., 1997]. Therefore, blocking one pathway may enhance the vasodilation mediated by another pathway. As discussed in Chapter 3, a similar mechanism has been observed in myometrial arteries of normal pregnant women [Kenny et al., 2002]. In mesenteric arteries of eNOS-knockout mice eNOS (-/-), endothelium-dependent relaxation was mediated by upregulation of EDHF in the absence of NO [Waldron et al., 1999]. Similar results have been reported in muscle arterioles of female eNOS-knockout mice [Huang et al., 2001]. In addition, the proportion of relaxation mediated by prostacyclin, EDHF and NO has been demonstrated to vary depending on gender; as discussed in Section 1.5.2.1. [Scotland et al., 2005]. Estimating the true role of a specific pathway in relaxation can therefore be challenging.
The enhanced vasodilation demonstrated in the present study could not be attributed to enhancement of a particular endothelium-dependent mediator. This may be because EDHF was not specifically blocked. One of the reasons for this was that previous studies had demonstrated an important role for NO in mediating aberrant responses to hyperglycaemia [Giugliano et al., 1997; Trachtman et al., 1997], making it the main area of interest in this study. Furthermore, the relative importance of NO, PGI2 and EDHF in this vascular bed was unknown, as this has not been previously reported in the literature. There were also technical limitations of the myograph apparatus: the addition of 25 mmol/L potassium to block EDHF would envisage a fifth artery studied in parallel, whereas a maximum of 4 arteries can be studied in the myograph used. This preliminary characterisation of vascular function in this vascular bed, has however pointed to the importance of examining EDHF in future studies. This can be done by utilising specific blockers such as a combination of apamin and charybdotoxin, which abolishes EDHF-mediated relaxation [Coleman et al., 2004].

6.5.9 Streptozotocin treated non-diabetic mice (STZ-controls)

As discussed in Section 2.14.7, the majority of mice injected with streptozotocin failed to develop diabetes. Failure to conceive was noted in diabetic mice, in addition to deterioration in health during pregnancy. Due to these factors, only data from vascular studies on streptozotocin treated non-diabetic mice has been presented.

Possible reasons for failure to induce diabetes include differences in the β-cell responsiveness to streptozotocin, as well as the age of mice. Streptozotocin has been demonstrated to induce more beta-cell destruction in young animals than in older mice [Riley et al., 1981]. The mice used in the present study were 8 weeks old at the time of injection of streptozotocin. In C57BL/6 mice, diabetes has been induced by streptozotocin from the age of 4 weeks [Shen et al., 2003] to 5 months [Fahim et al., 1999]. It is therefore uncertain how important age is in determining resistance to the diabetogenic actions of streptozotocin in C57BL/6 mice. Differences in β-cell responsiveness to the action of streptozotocin has also been demonstrated between different strains of mice [Abramovici and Agarwal, 1985; Hayashi et al., 2006].
At the control level of glucose (5 mmol/L), uterine arteries of STZ-control mice demonstrated a small, but significant increase in constriction during the second part of the experiment, implying a time effect. This was also seen at 8 and 12 mmol/L glucose, although the increase was not significant at 2 mmol/L glucose. However, these changes were minimal, and as noted in normal pregnant mice, may not be biologically significant.

In these mice, endothelium-dependent relaxation was not affected by changes in glucose levels, in contrast to the vasodilation noted in normal pregnant mice at 12 mmol/L glucose. The relatively small number of streptozotocin-control mice studied (N=8) compared to the normal pregnant mice (N=18) prevents a valid interpretation of these results. Furthermore, it is not known if streptozotocin alters vascular function independent of its diabetogenic properties, due to the marked lack of reports on vessel function in non-diabetic streptozotocin-control mice. These results may therefore be important in examining whether streptozotocin can influence vascular function independent of diabetes.

_Postscript:_ Work on the mice model of diabetes in pregnancy has been subsequently carried forward by the postgraduate student Joanna Stanley, with experiments being performed in vehicle-control, streptozotocin-control and diabetic mice.

**6.5.10 SUMMARY**

Pregnancy in normal mice is associated with changes in uterine vascular reactivity to glucose. Exposure to 12 mmol/L glucose for 30 minutes enhanced endothelium-dependent vasodilation. This effect was not seen in uterine arteries of non-pregnant mice. The endothelium-dependent relaxation was mainly due to a non-NO/non-prostanoid mediator and to a lesser degree NO, with no contribution from prostacyclin in this vascular bed. This indicates that short exposures to elevated glucose concentrations can influence vascular function of the uterine arteries during pregnancy. This effect is mediated locally and is independent of osmotic, hormonal or neural mechanisms. The development of the animal model of diabetes in pregnancy will facilitate future investigations into the effects of glucose on vascular function.
CHAPTER 7

DISCUSSION
7.1 Overview of study

The main purpose of this study was to examine if acute changes in glucose concentration affected endothelial function in normal pregnancies and pregnancies complicated by diabetes. A limitation of earlier studies was that theoretical rather than empirical concentrations had been used to examine the effects of hyperglycaemia. Furthermore, extremely few studies have examined the effect of hypoglycaemia on endothelial function. The first part of this study was to therefore identify clinically-relevant glucose concentrations during diabetes in pregnancy. Although the duration of these aberrant glucose concentrations was not examined, results from earlier studies of diabetes in pregnancy were used to select a clinically-relevant duration of exposure. Arteries of the uterine vascular bed were directly studied at these glucose levels, thereby overcoming limitations of previous studies which examined vascular function in large conduit arteries at extremely high glucose levels. By studying \textit{ex-vivo} arteries using wire myography, vessel function could be characterised in both human and animal vascular beds over a range of glucose concentrations. This enabled the comparison of effects between species at standardised experimental conditions.

This work represents the first study of the effects of glucose concentration on endothelial function in human myometrial arteries and mice uterine arteries. Altered vascular function was observed at both high and low glucose concentrations. At 2 mmol/L glucose, myometrial arteries from normal pregnant women exhibited impaired endothelium-dependent relaxation when constricted with U46619; an effect not seen in arteries from non-pregnant women. This represents the first report of a possible link between hypoglycaemia and impaired endothelium-dependent relaxation. At 12 mmol/L glucose, enhanced endothelium-dependent relaxation was noted in uterine arteries of pregnant, but not non-pregnant mice. These findings have demonstrated for the first time that short exposures of clinically-relevant glucose levels in pregnant humans and mice can influence endothelial function, an effect not evident in the non-pregnant state. The novel results obtained suggest that
the state of pregnancy is associated with a unique modulation of endothelial responses to glucose in both animals and humans.

This study represents the first report on the role of NO, prostacyclin and EDHF in the uterine artery of pregnant mice. Endothelium-dependent relaxation in the uterine artery of pregnant mice was predominantly mediated by a non-nitric oxide/non-prostanoid mechanism, with a smaller contribution from nitric oxide, and no prostanoid-mediated relaxation. Another novel finding was that NO-mediated endothelium-dependent relaxation in mice uterine arteries was similar at 5 and 12 mmol/L glucose.

Myometrial arteries from women with diabetes in pregnancy have been examined for the first time in this study. Compared to healthy pregnant women, impaired endothelium-dependent relaxation was observed in these arteries from women with GDM. This effect was present when the arteries were constricted with 60 mmol/L potassium, but absent with U46619; indicating the important role of EDHF in normalising endothelial function in this vascular bed.

A unique feature of this study was that the glucose levels studied and their duration were chosen based on results of women with diabetes in pregnancy. Previous studies have examined the effects of hyperglycaemia on vascular function after prolonged and markedly high glucose concentrations. Although changes in function have been demonstrated by these studies, such conditions are rarely seen clinically. The aim of the present study was to therefore examine if more subtle changes in glucose concentrations altered endothelial function.

The findings of this study may be of clinical significance in the setting of diabetes in pregnancy. The endothelial dysfunction of myometrial arteries in GDM may influence blood supply to the fetus. Fluctuations in glucose level did not affect endothelial function in women with GDM, although the effect of these fluctuations in women with type 1 and 2 diabetes could not be examined fully due to a lack of patients. This study has also demonstrated that fluctuations in blood glucose levels as well as adverse outcomes were high in women with type 1 diabetes. These swings in glucose level have been demonstrated to exert a more specific triggering effect on
oxidative stress than chronic sustained hyperglycaemia [Monnier et al., 2006]. Further studies are required to investigate whether fluctuations between high and low glucose levels play an additional role in mediating the complications of diabetes in pregnancy.

**Figure 7.1:** Overview of study

- Blue: Previously established associations
- Green: Associations characterised further by this study
- Red: New associations demonstrated by this study
7.2 Key Findings

A summary of the key findings obtained from this project has been depicted in Figure 7.1. Novel associations demonstrated for the first time in this study include impaired endothelium-dependent relaxation at 2 mmol/L glucose and impaired endothelial function in myometrial arteries of women with GDM. Another new association was the enhancement of endothelium-dependent relaxation at 12 mmol/L glucose in uterine arteries of pregnant mice. In addition to these original findings, a number of existing associations have been characterised further by this study. These include the variation in endothelial function with the type of vascular bed, the role of prostacyclin, NO and EDHF in small arteries as well as the effect of pregnancy on endothelial function.

7.3 Hypothesis 1:

The effects of glucose concentrations on endothelial function vary in different vascular beds.

Previous studies examining the effects of glucose have differed in the methodology, species, concentration of glucose, duration of exposure and the vascular bed investigated. The large number of variables inherent in these studies has predictably led to heterogeneous results. This study has attempted to overcome some of these intrinsic ambiguities by utilising a single methodology, and by keeping the duration and concentration of glucose constant. By doing so, it has been demonstrated that exposure to the same glucose concentration can modulate endothelial function in disparate ways, depending on the vascular bed. The state of pregnancy and the species examined were found to be important in determining the effect of glucose on vascular function. This work therefore reveals a more complex relationship between glucose concentrations and endothelial function than previously envisaged. It also suggests that the effects of glucose should be considered in the context of the vascular bed studied, helping to explain some of the inconsistencies of past studies.
Regional variations in vascular responses to various stimuli have been reported in a number of studies. For example, both regional and species heterogeneity have been demonstrated in the vascular responses to the action of muscarinic agonists in the rat and frog [Leont'eva and Krivchenko, 2001]. The present study has demonstrated inter-species variation in the effects of 2 and 12 mmol/L glucose on endothelial function. These different responses may be due to the variations between species in counter-acting the effects of oxidative stress. Studies in C57BL/6 mice have suggested that there are differences in the response to oxidative stress, even between strains of mice [Agardh et al., 2000]. Differences in the balance between NO and superoxide generation have been demonstrated to be an important factor in the variation in vascular responses between C57BL/6 mice and other strains [Bendall et al., 2002]. Hyperglycaemia-related oxidative stress may induce endothelial dysfunction due to variations in anti-oxidant capabilities. Inter-species differences in endothelial mediators also exist, further influencing the interaction between glucose concentrations, oxidant stress and endothelial dysfunction. Additionally, it has been noted in humans that genetic variations between individuals can play a role in determining vascular reactivity [Henrion et al., 2002].

The importance of glucose in metabolism is underscored by the fact that multiple homeostatic mechanisms are in place to ensure an adequate supply of glucose to different tissue regions depending on their metabolic activity. Glucose transport and its concentrations are closely regulated with varying degrees of complexity in all biological systems. This study has demonstrated differences in endothelial function to glucose in the pregnant and non-pregnant state within the same vascular bed in animals (uterine arteries) as well as humans (myometrial arteries). The heterogeneity in these responses may be related to the altered glucose demands and the vascular permutations occurring in pregnancy, which have been discussed in Section 1.3.2. Differences in vascular function in various vascular beds of the same animal have also been reported during pregnancy. The vascular sensitivity to U46619 was attenuated in small myometrial arteries of pregnant sheep compared with omental arteries, which may help preserve uterine blood flow [Anwar et al., 1999].
Arterial diameter may play an important role in determining regional responses to the effect of glucose concentrations. Endothelial mediators vary with the calibre of arteries; however, the effects of glucose on the action of these mediators (particularly EDHF and prostacyclin) are yet to be fully characterised. This study has highlighted the importance of EDHF at the resistance artery level, corroborating previous work [Shimokawa et al., 1996]. Furthermore, EDHF has been demonstrated to be the predominant endothelium-derived mediator in female mice, whereas NO and PGI₂ were more important in male mice [Scotland et al., 2005]. Variations in the potency of endothelial mediators at various vascular beds as well as differences in the response of these mediators to hypoglycaemia and hyperglycaemia may account for the results obtained.

7.4 Hypothesis 2:

Acute changes in glucose concentration from normal are associated with altered endothelial function

This study has demonstrated that short exposures to both high and low glucose levels can affect endothelial function in pregnancy. The changes in constriction and endothelium-dependent relaxation indicate that even subtle changes in glucose concentration may influence vascular function. An implication of this finding is that episodic rises in glucose levels (such as in the post-prandial state) may be associated with aberrant vascular function at the resistance artery level. Most of the earlier studies on the effects of glucose examined large conduit arteries, which differ from smaller arteries in the relative contribution of the individual endothelial mediators of relaxation. Results obtained in these studies cannot therefore be extrapolated to smaller arteries, where EDHF has a more prominent role than NO.

The mechanisms responsible for the altered endothelial function with glucose concentrations have been investigated in this study. No enhancement of endothelial mediators was found at 12 mmol/L glucose in mice uterine arteries, most likely due to a lack of specific EDHF blockade. Nevertheless, the findings provide a basis for
future studies investigating the mechanisms responsible for altered endothelial responses.

In contrast to previous studies, a high glucose concentration was not associated with impaired endothelium-dependent relaxation in any of the arterial beds studied. This suggests that longer exposures or higher glucose concentrations may be required for endothelial function to be disrupted by the deleterious effects of elevated glucose levels. This finding may be of consequence in determining the time course of hyperglycaemia-related changes to vascular function. Prolonged exposure to hyperglycaemia (lasting 1-6 hours) has been associated with impaired endothelium-dependent relaxation in previous studies (Table 1.3). This study therefore suggests that the effects of glucose should be considered in the context of the duration of exposure.

7.5 Hypothesis 3:

Diabetes in pregnancy is associated with endothelial dysfunction in myometrial arteries

When constricted with KCl, endothelium-dependent relaxation was impaired in myometrial arteries from women with GDM compared with normal. However, when U46619 was used as an agonist, endothelial function was preserved, indicating that EDHF (which was blocked with KCl) may play an important role in normalising endothelial function in diabetes. This study therefore brings in to focus the need for specifically analysing the role of EDHF and its relationship with changes in glucose concentrations.

Knock et al. demonstrated impaired relaxation to acetylcholine in subcutaneous arteries from women with gestational diabetes compared with normal pregnant women [Knock et al., 1997]. This study suggested that prostacyclin may be of more importance to relaxation than NO in subcutaneous arteries. However, prostanoids have been demonstrated to have little or no role in myometrial arteries of pregnant women, a vascular bed where endothelium-dependent relaxation is mediated by NO.
and EDHF [Ashworth et al., 1999; Kenny et al., 2002]. It is uncertain how diabetes in pregnancy influences the role of these mediators in myometrial arteries. This aspect could not be investigated further in the present study due to the limited availability of suitable patients.

In streptozotocin-induced diabetic C57BL/6 mice, relaxation mediated by EDHF has been reported to be impaired [Morikawa et al., 2005]. In rat mesenteric arteries, the impaired endothelium-dependent relaxation after exposure to 22.2 mmol/L glucose has been demonstrated to be due to impaired EDHF-mediated relaxation [Ozkan and Uma, 2005]. The importance of EDHF is also underscored by the demonstration that EDHF can substitute for NO in mediating relaxation in arteries of female eNOS-knockout mice [Huang et al., 2001]. Future research directed at the association between hyperglycaemia and EDHF may reveal important links between it and the pathophysiology of endothelial dysfunction in diabetes.

Women in the GDM group studied had relatively well-controlled diabetes, with near-normal mean HbA1C values. This may have influenced endothelial responses, as well-controlled diabetes has been associated with normal endothelial function in sub-cutaneous arteries of pregnant women with type 1 diabetes [Ang et al., 2002]. However, the fundamental differences between GDM and type 1 diabetes, as well as the difference in the vascular beds examined limit comparisons of endothelial function between these studies.

Obesity is associated with impaired endothelial function [Myers et al., 2006], and the women with GDM had a higher BMI than those in the normal group. However, this is unlikely to be the cause of impaired relaxation in myometrial arteries of women with GDM, as impairment was not present when U46619 was used as an agonist. Nevertheless, the elevated BMI of the GDM patients may explain the increased birth weight of babies in this group, as an association between the two has been previously demonstrated [Bo et al., 2003; Hardy, 1999].

Impaired endothelium-dependent relaxation has also been reported in the myometrial vascular bed in pre-eclampsia [Ashworth et al., 1997], a condition with a raised prevalence in gestational diabetes [Bryson et al., 2003]. This highlights the
importance of future investigations into endothelial dysfunction in GDM, which may lead to valuable insights into the pathophysiology of complications seen in this condition.

**7.6 Limitations of study**

This study was subject to certain limitations which need to be considered when interpreting the results. The methodological limitations of wire myography have been previously alluded to in Section 2.8.3. Studies performed in humans and mice differed in the vasoconstrictor and vasodilator used; the reasons for this having been discussed in Section 2.10.9. This may limit comparisons between species, although the protocols were identical in other respects. A time-related impairment of endothelium-dependent relaxation was observed in arteries from mice, but not from humans. However, the inclusion of a time control at 5 mmol/L glucose enabled accurate interpretation of the data despite this effect. Endothelium-independent relaxation was not systematically investigated in this study, thereby restricting the assessment of smooth muscle function. This was because the vast majority of previous reports have found no association between hyperglycaemia and endothelium-independent relaxation [Beckman et al., 2001; Reed et al., 2004; Williams et al., 1998]. The preservation of endothelium-independent relaxation has been demonstrated in disease states such as diabetes [Fukao et al., 1997; McNally et al., 1994]. An estimation of the integrity of smooth muscle function can be made from maximal constriction; which was found to be similar in myometrial arteries from both normal pregnant women and women with gestational diabetes.

In Chapter 1, the influence of factors such as age, BMI and ethnicity on endothelial function have been examined. The human subjects included in this study were not matched for these variables. This was because the evidence for an association between these factors and endothelial function is not conclusive. Acquiring myometrial arteries entails an invasive biopsy, which severely restricts the number of patients, even when recruiting simultaneously from three large hospitals. Furthermore, only a minority of pregnant women deliver by Caesarean section. Matching obese and non-obese patients with GDM is of questionable value, as
obesity and the subsequent insulin resistance are often necessary pre-requisites for the development of GDM. The limited availability of human biopsies was the main reason for attempting to create an animal model of diabetes.

When the endothelial mediators in uterine arteries of pregnant mice were examined, a specific blocker was not used for EDHF. Previous studies in this strain of mice had identified NO to be the most important mediator of relaxation in carotid and femoral arteries [Crauwels et al., 2000]. Furthermore, hyperglycaemia has been associated with reduced NO bioavailability [Giugliano et al., 1997; Trachtman et al., 1997], making this mediator the focus of interest in the present study. Although the results suggested that NO was unaffected by exposure to 12 mmol/L glucose, they also revealed the importance of EDHF in this vascular bed. This preliminary characterisation of the endothelial mediators in the uterine artery of mice may facilitate future vascular studies.

The effects of a more prolonged exposure to altered glucose concentrations (such as two hours) could not be fully investigated because of the loss of viability of arteries as described in Section 4.4.12. Techniques such as pressure myography may offer improved viability for prolonged durations of time, as vessels are not stretched as in wire myography. However in pressure myography, only a single vessel can be examined at a time; this limits its value in a study of this nature, where multiple glucose concentrations need to be investigated concurrently.

A successful model of diabetes in pregnancy was created by the author with the assistance of the University of Manchester staff. Although the initial batch of mice failed to develop diabetes, results from this group have been presented as streptozotocin-controls, to form a basis for further work. Diabetes was successfully induced in the second batch of mice, despite a high failure rate. Although results from diabetic mice could not be presented due to time constraints, it is hoped that the ongoing work on this model of diabetes in pregnancy will lead to further insights into this disorder.
7.7 Future avenues of research

The results obtained from this study have brought to light specific directions which future research may be able to take in ascertaining the effects of glucose on endothelial function in pregnancy and the influence of diabetes.

I. Determine the mechanisms responsible for impaired endothelium-dependent relaxation in gestational diabetes, and examine whether such changes occur in type 1 and type 2 diabetes in pregnancy.

Understanding the mechanisms responsible for endothelial dysfunction may help in developing novel therapies aimed at normalising vascular function in diabetes.

II. Investigate further the association between hypoglycaemia and endothelial dysfunction.

This may help determine whether hypoglycaemia plays a role in mediating vascular complications in diabetes.

III. Use the animal model of diabetes in pregnancy developed in this study to:

- examine the association of EDHF with hyperglycaemia and hypoglycaemia
  
  The response of EDHF to these stimuli may be more important than NO in mediating endothelial dysfunction in diabetes at the resistance artery level

- ascertain the link between endothelial dysfunction and fetal development.

  Animal models would enable characterisation of how the endothelial dysfunction demonstrated in diabetes can influence the fetus.
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don't know what it is


APPENDIX A: CONSENT FORM

Academic Unit of Obstetrics & Gynaecology and Reproductive Healthcare

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Maternal and Fetal Health Research Centre
Director: Professor Philip N Baker
Study Number: Telephone 0161 276 5460 Fax 0161 276 6134
Patient Identification Number for this trial

CONSENT FORM

(Pregnancy complicated by diabetes)

Drs Michael Taggart, Louise Kenay, Mike Maresk, Peter Selby, Professor Philip Baker

We would like to invite you to take part in a study of

Endothelium-dependent vascular behaviour in pregnancies complicated by diabetes

Please initial box

1. I confirm that I have read and understand the information sheet dated ....................
   (version ...........) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time,
   without giving any reason, without my medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked at by responsible
   individuals from the Maternal and Fetal Health Research Centre, or from regulatory
   authorities where it is relevant to my taking part in research. I give permission for
   these individuals to have access to my records.

4. I consent to a biopsy of my womb being taken at Caesarean section.

5. I agree to take part in the above study.

Name of Patient __________________________ Date __________________________ Signature __________________________

Name of Person taking consent (if different from researcher) __________________________ Date __________________________ Signature __________________________

Researcher __________________________ Date __________________________ Signature __________________________
APPENDIX B: PATIENT INFORMATION SHEET

Patient Information Sheet

(Pregnancy complicated by diabetes)

Drs Michael Taggart, Louise Kenny, Mike Maresh, Peter Selby, Professor Philip Baker
Tommy’s Campaign: Maternal and Fetal Health Research Centre, St. Mary’s Hospital
University of Manchester, Manchester M13 0JH
Tel: 0161 276 6461

We would like to invite you to take part in a study of:

Endothelium-dependent vascular behaviour in
pregnancies complicated by diabetes

Diabetes is a disease that affects pregnant women and can occasionally lead to serious problems for both the mothers and their unborn babies. We are studying very small blood vessels, including the blood vessels which supply the baby and placenta to investigate the possibility that abnormalities of these blood vessels are involved in these conditions. We are approaching you because your pregnancy has been complicated by Diabetes.

At the time of your Caearean Section we would like your permission to take a small 5mm x 5mm x 5mm biopsy (tissue sample) from your womb and a similar size from the underlying fat. The Obstetrician will take the biopsies after the delivery of your baby. It takes less than 5 minutes to remove the biopsies and the procedure will not affect healing or recovery after the operation.

We will use these samples in studies performed in our laboratories. Tissue samples will be destroyed once the study is complete. The study will be of no direct benefit to you. Our aim is to understand the blood vessel changes which occur in diabetes in pregnancy, in order to improve the way we care for pregnant women with diabetes.

Please feel free to discuss the study with the doctor/midwife explaining the project, and with your doctor, midwife family and friends. Should you prefer not to participate in the study, your normal care will not be affected in any way.
APPENDIX C: PRO FORMA FOR STUDY OF DIABETES IN PREGNANCY

Central Manchester and Manchester Children’s University Hospitals NHS Trust
Audit Title: Audit of the outcome of pregnancy with diabetes
Reg No: 624

Please keep all marks inside the boxes and complete the form in Black Ink, keeping free text to a minimum. Forms which do not comply with this cannot be scanned.

<table>
<thead>
<tr>
<th>Q1 Audit ID</th>
<th>Q2 Casenote number</th>
<th>Q3 Date Of Birth</th>
<th>Q4 Ethnicity</th>
<th>Q5 EDD</th>
<th>Q7 Height in cm</th>
<th>Q8 Weight in Kg</th>
<th>Q9 Parity</th>
<th>Q10 Type of diabetes</th>
<th>Q11 Pre-conception care</th>
<th>Q12 pre-conception therapy in pats with Type 2 diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Hypothyroid
Hyperthyroid
Thyroiditis
Addison’s disease
Hypertension- essential
Hypertension - gestational
Proteinuria
hyperlipidaemia

Q13 Management mode
Diet  Insulin

Q14 Insulin date

Q15 If insulin, from which week:

Q16 Insulin admin device
pen  syringe  infusion pump  Jet injector  Not applicable

Q17 Insulin type used
human  Analogue  Porcine  Beef  Not applicable

Q18 Insulin Regime
BD  basal  Basal bolus  Bolus  Pump infusion  not applicable

Q16. Parameters according to gestational age

<table>
<thead>
<tr>
<th>weeks</th>
<th>Precon</th>
<th>1st rec</th>
<th>10±1wk</th>
<th>20±1wk</th>
<th>34±1wk</th>
<th>Last rec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
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</table>

<table>
<thead>
<tr>
<th>Q19 Weight</th>
<th>HbA1C</th>
<th>min gluc</th>
<th>max gluc</th>
<th>Total insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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</table>

261
# APPENDIX C: PRO FORMA FOR STUDY OF DIABETES IN PREGNANCY

<table>
<thead>
<tr>
<th>Q20 Change in the form of insulin during the course of this pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Human</td>
</tr>
<tr>
<td>Analogue</td>
</tr>
<tr>
<td>Porcine</td>
</tr>
<tr>
<td>Beef</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Q21 Change insulin date 1</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Q22 Change insulin date 2</td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Hypopglycemic episodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q23 Minor</td>
</tr>
<tr>
<td>Q24 (1) trim 1</td>
</tr>
<tr>
<td>Q25 (1) trim 1</td>
</tr>
<tr>
<td>Q26 Major</td>
</tr>
<tr>
<td>Q24 (2) trim 2</td>
</tr>
<tr>
<td>Q25 (2) trim 2</td>
</tr>
<tr>
<td>Q24 (3) trim 3</td>
</tr>
<tr>
<td>Q25 (3) trim 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Q27 Precipitation of any diabetic complication</th>
</tr>
</thead>
<tbody>
<tr>
<td>D Nephropathy</td>
</tr>
<tr>
<td>D Retinopathy</td>
</tr>
<tr>
<td>D Neuropathy</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Q29 Other acute problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperemesis</td>
</tr>
<tr>
<td>DKA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Q30 Gestational age in weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperemesis Yes No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Q31 (1) Measurements for gestational weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age in weeks</td>
</tr>
<tr>
<td>Abdo &lt;10</td>
</tr>
<tr>
<td>Abdo 10-90</td>
</tr>
<tr>
<td>Abdo &gt;90</td>
</tr>
<tr>
<td>Fem &lt;10</td>
</tr>
<tr>
<td>Fem 10-90</td>
</tr>
<tr>
<td>Fem &gt;90</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Q32 Gestational age in weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q33 Date of Delivery/Termination</td>
</tr>
<tr>
<td>Q34 Method of delivery</td>
</tr>
<tr>
<td>Q35 Outcome</td>
</tr>
<tr>
<td>Q36 Gender</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Q37 Were there any congenital abnormalities noted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes No</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Q38 If yes, please detail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q39 If yes, reason</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Q40 date admitted to scbu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q41 Date of death</td>
</tr>
<tr>
<td>Q42 SCBU admission for &gt;24hrs</td>
</tr>
<tr>
<td>Q43 Discharge date</td>
</tr>
<tr>
<td>Q44 If yes, reason</td>
</tr>
<tr>
<td>Q45 Final comments - please keep brief</td>
</tr>
</tbody>
</table>

Q31 (2) Fetal anomaly

Q36 Birthweight

Q36 Gender: Male Female