INVESTIGATION OF HYPOXIA AND HYPERGLYCAEMIA MEDIATED DNA DAMAGE IN HUMAN ENDOTHELIAL CELLS

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Ph.D. 2008

Investigation of hypoxia and hyperglycaemia mediated DNA damage in human endothelial cells

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A thesis submitted in partial fulfilment of the requirements of
The Robert Gordon University
for the degree of
Doctor of Philosophy

The Robert Gordon University
Aberdeen

March 2008

DECLARATION

This thesis has been composed by myself and has not been submitted in any previous application for a higher degree. The work of which it is a record has been done by myself. All verbatim extracts have been distinguished by quotation marks and the sources of information specifically acknowledged.

Anita E. Weidmann

To my parents

Contents

Figures

Tables

(i) Acknowledgements

I wish to express my sincere thanks to the following people for their help, guidance and encouragement during this project:

Dr. Rachel Knott, Director of Studies, School of Pharmacy, for her advice, guidance and support throughout the project and the preparation of this thesis.

Dr. Stuart Cruickshank, School of Pharmacy, for his advice and guidance on all mitochondria related issues as well as the preparation of this thesis.

Dr. Derek Stewart, School of Pharmacy and *Prof. Klaus Wahle*, School of Life Science, for the continued encouragement and advice.

Dorothy Moir, Margaret Brown and Vivienne Hamilton, School of Pharmacy, for their technical assistance.

Raymond Reid, School of Pharmacy, for help with the CZE analysis.

Dr. S. MacManus for his help with the chemical structures and reactions surrounding taxifolin.

Dr. Marie Goua, School of Life Sciences, for her help with Western blots and the occasional very useful bit of advice/ information.

Prof. Terry Healy, Head of School of Pharmacy, for the funding of this project.

Prof. Cherry Wainwright, School of Pharmacy, for the corrections and useful feedback of my interim reports.

Dr. Julie Young, for being the best friend a girl can ask for and for keeping me sane.

(ii) Abstract

Chronic complications of diabetes mellitus are a pathological consequence of unregulated levels of blood glucose leading to disorders of the vascular system. When investigating the vascular complications the changes in the endothelium are of particular significance as they are central to vascular function. The endothelium actively regulates vascular function by regulating permeability, vascular tone and inflammatory responses. Certain endothelium-derived molecules, such as nitric oxide, act as vasodilators as well as exhibiting anti-platelet, anti-proliferative, permeability decreasing and anti-inflammatory properties (Kawashima *et al.* 2004). Changes to the molecular processes or injury to the endothelium lead to endothelial dysfunction and the initiation and progression of certain diseases such as the development of atherosclerosis as well as micro and macro-angiopathy (De Caterina *et al.* 2000). In the present study, we examine the effect of glucose and oxygen on endothelial DNA damage derived within 24h of incubation and the cytoprotective effect of the flavonoid antioxidant, silymarin, and two of its constituents, taxifolin and silibinin.

Results show a significant increase in endothelial DNA damage in response to glucose and hypoxia which appears to be additive. These findings are supported by a significant rise in mitochondrial ractive oxygen species (ROS) production within 6h of exposure, while mitochondrial number, morphology and HUVEC size stay the same. A change in superoxide radical production in hypoxic conditions alone emphasises the important contribution of hypoxia in the pathogenesis of endothelial damage. Further investigation reveals a role for HIF-1α in the development of endothelial DNA damage which is hypoxia-dependent. Attempts to counteract the observed cytotoxicity to endothelial cells using the flavonoid antioxidant, silymarin, revealed that silymarin is a potent cytoprotective agent for hypoxia-induced DNA damage. This appears to be due to its strong mitochondrial radical scavenging activity. Further analysis into the mechanism of silymarin reveals that the impurity, taxifolin,

possesses more potent cytoprotective activity than the formerly known active component silibinin.

Taken together, the results strongly emphasise the important contribution of hypoxia in the early development of endothelial changes, which have the potential to accumulate to vascular complications in diabetes since its cytotoxic effect appears to be additive to that of glucose. Flavonoid antioxidants, such as silymarin and taxifolin, show promising potential to prevent or slow the progression of these changes.

(iii) Abbreviations

AGE	advanced glycation end products
AHEAD	Action for Health in Diabetes
ala	α-lipoic acid
ANG-II	angiotensin–II
ANOVA	analysis of variance
ARI	aldose reductase inhibitor
AS	antisense
ATM	ataxia telangiectasia, mutated
ATP	adenosine triphosphate
ATR	ATM and Rad3-related
AUC	area under the curve
BER	base excision repair
β_2Mg	β_2-Microglobulin
CAT	catalase
Ca^{2+}	calcium
CHAOS	Cambridge Heart Antioxidant Study
cICAM-1	intercellular adhesion molecule 1
COX-2	cyclooxygenase - 2
CREB	cAMP response element-binding protein
Cu/Zn SOD	copper/zinc-superoxide dismutase
CV	cardiovascular
cVCAM-1	vascular cell adhesion molecule 1
DAG	diacylglycerol
DCCT	Diabetes Control and Complications Trial
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide

DNA	deoxyribonucleic acid
DPP	Diabetes Prevention Program
DSB	double-strand break repair
DSHP	di-sodium hydrogen phosphate
ECACC	European Collection of Cell Culture
EDIC	Epidemiology of Diabetes Intervention and Complications Study
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
ETC	electron transport chain
Fe^{2+}	iron
FCCP	carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone
FCS	foetal calf serum
GC-MS	gas chromatograhy/mass spectrometry
GF/SF	glucose free/serum free
GLUT	facilitative glucose transporters
GMEM	Glasgow Minimum Essential Medium
GSHPx	glutathione peroxidase
GTP	Guanosine-5'-triphosphate
GTPase	family of hydrolase enzymes that can bind and hydrolyze GTP
H_2O_2	hydrogen peroxide
HBSS	Hanks Balanced Salt Solution
HGTD-P	HIF-1α dependent pro-apoptotic molecule
HIF-1	hypoxia-inducible factor 1
HIF-1α	hypoxia-inducible factor-1 alpha
HIV	human immunodeficiency virus

HOPE	Heart Outcomes Prevention Evaluation study
HPLC	high pressure liquid chromatography
HRE	hypoxic response element
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule
IDF	The International Diabetes Federation
IFN-γ	Interferon-γ
IGF-2	insulin-like growth factor -2
IL-2	Interleukin-2
iNOS	inducible nitric oxide synthase
K_m	Michelis Menten constant
KCN	potassium cyanide
LDL	low density lipid
LMP	low melting point agarose
Mg^{2+}	magnesium
MAPK	mitogen activated protein kinase
MCF-7	human breast carcinoma cells
MI	myocardial infarction
MMR	DNA mismatch repair
Mn-SOD	manganese-superoxide dismutase
mPTP	mitochondrial permeability transition pore
mtDNA	mitochondrial DNA
mtNOS	mitochondrial nitric oxide synthase
N_2	nitrogen gas
Na_2SO_3	sodium sulfite anhydrous
NADPH	nicotinamide-adenine dinucleotide phosphate
NADH	nicotinamide-adenine dinucleotide

NADP+	the oxidized form of NADPH
NCV	nerve conduction velocity
NF-κB	nuclear factor – kappaB
NHS	National Health Service
NO	nitric oxide
$O_2^{\cdot-}$	superoxide radical
$OHOO^-$	peroxynitrite species
PARP	Poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDK1	pyruvate dehydrogenase kinase 1
PKC	protein Kinase C
PGC-1alpha	PPAR gamma coactivator 1-alpha
PHD	prolyl hydroxylases
PIs	phopshatidylinositides
PLC	phospholipase C
PLD	phospholipase D
PPAR	peroxisome proliferators-activated receptor
RAGE	receptor AGE binds to
RNA	ribonucleic acid
ROS	reactive oxygen species
SB	silibinin
SCGE	single cell gel electrophoresis
SDev	standard deviations
SDH	sorbitol dehydrogenase
SDS	sodium dodecyl sulphate

SEM	standard error of the mean
SM	silymarin
SOD	superoxide dismutase
TBS	tris buffered saline
TCA	tricarboxylicacid cycle
TNFα	tumour necrosis factor - alpha
TORC	transducer of regulated CREB activity
TX	taxifolin
UCP-1	uncoupling protein-1
UKPDS	UK Prospective Diabetes Study
VEGF	vascular endothelial growth factor
vHL	von Hippel-Lindau protein
VSMC	vascular smooth muscle
VEGF	vascular endothelial growth factor
vWF	von Willebrand Factor
WHO	World Health Organisation
2DG	2-deoxy-D-glucose
3OM	3-ortho-methyl glucose
2-DG-6P	2-d-glucose-6-phosphate
$\Delta\psi_m$	mitochondrial membrane potential
8-oHdG	8-hydroxy-2'-deoxyguanosine
8-oxo-dGuo	2'-deoxy-7,8-dihydro-8-oxoguanosine

CHAPTER ONE: Introduction

1.1. Diabetes Mellitus

Diabetes mellitus is a chronic metabolic disorder which is characterised by high blood glucose levels (hyperglycaemia) and is the fifth leading cause of death in most developed countries (International Diabetes Federation; IDF, 2006). Type 1 diabetes results from cellular-mediated autoimmune destruction of pancreatic islet beta-cells causing the loss of insulin production and Type 2 diabetes is characterized by insulin resistance and relative insulin deficiency (IDF, 2006). Currently 171 million people worldwide have been diagnosed with diabetes but many more are believed to remain undiagnosed. These figures published by the World Health Organisation (WHO) have risen from only 30 million in the year 1995 and are thought likely to more than double to an estimated of 366 million in the subsequent 25 years. There are strong signs for diabetes mellitus to become a global pandemic, accounting for approximately 9% of the global death toll (Wild *et al.* 2004).

In the United Kingdom, 1.7 million people are currently suffering from diabetes mellitus (World Health Organisation 2004), accounting for approximately 5% of total NHS expenditure. In December 2001, the Department of Health published the National Service Framework for Diabetes which sets the national standards for diabetes treatment and care (Department of Health, 2007). In addition to highlighting important building blocks of diabetes care, one of the main aims of this document is to identify diabetes as early as possible and thus prevent complications to improve the patient's quality of life (NHS Scotland, 2002). In October 2002, the Department of Health published a review of the current and future research on diabetes which identifies research questions still to be addressed. Emphasis is given to the understanding of molecular events involved in the pathogenesis of diabetes and diabetic complications (Department of Health, 2002).

1.2. Vascular complications of diabetes

Chronic complications of diabetes mellitus are a pathological consequence of unregulated levels of blood glucose leading to disorders of the vascular system. Not only are these complications a much greater burden on both diabetic patients and overall medical costs than diabetes itself (McMillan *et al.* 1997), they are also the major cause of morbidity and mortality in the diabetic population.

During the 1990s, two landmark trials, the Diabetes Control and Complications Trial (DCCT) (The Diabetes Control and Complications Trial Research Group, 1993) and the UK Prospective Diabetes Study (UKPDS); (UK Prospective Diabetes Study Group, 1998) investigated the effect of blood glucose control on the development of diabetic vascular complications. While the DCCT monitored the disease progression of 1,441 type 1 diabetes patients over the course of 10 years in the United States and Canada, the UKPDS study tracked the development of type 2 diabetes in 5,102 volunteers across England, Wales, Northern Ireland and Scotland. Both studies published evidence that intensive blood glucose control considerably slows the onset and progression of diabetic complications. Not only did they record a 44% reduction in the incidence of stroke (UKPDS), a 76% reduction in the development of diabetic eye disease and up to 60% reduction in kidney disease (DCCT), they also found a 32% reduction in the incidence of overal diabetes-related deaths (UKPDS). Although these findings still form the basis of modern diabetes treatment, to date the processes which led to the development of these debilitating vascular complications are not yet fully understood.

In general, diabetic vascular complications are classified into macro- and microvascular complications.

1.2.1. Macrovascular complications

Macrovascular complications are characterised by the formation of atherosclerotic plaques in arteries of diabetic patients. This is seen as the most serious complication of diabetes mellitus due to its high mortality rate (McMillan *et al.* 1997, Kammersgaard *et al.* 2006). Thus, cardiovascular (CV) disease is the most common cause of death for individuals with type 2 diabetes, accounting for approximately 80% of diabetes-related deaths (Williams *et al.* 1999). It is also responsible for an increase in mortality of patients with type 1 diabetes (Department of Health, 2002).

Hyperglycaemia is a major risk factor for the development of CV disease (Kannel *et al.* 1979) with other factors such as smoking, hypercholesterolaemia, duration of diabetes and age adding to the risk (Gray *et al.* 1997). Atherosclerosis can lead to myocardial infarction (MI), stroke, hypertension and leg/artery disease, requiring lower limb amputation in many cases. The development of atherosclerotic lesions is strongly associated with age while the duration of diabetes is of little significance (McMillan *et al.* 1997). Pathological changes in diabetes leading to atherosclerosis include the inactivation of nitric oxide (NO) (an endogenous vasodilator) (Giugliano *et al.* 1996), arterial wall thickening (Mohamed *et al.* 1999), plasma lipid disturbances, oxidation of low density lipid (LDL) cholesterol (Guillausseau *et al.* 1994, Gray *et al.* 1997) raised fibrinogen levels (McMillan *et al.* 1997, Gries *et al.* 1995) and haemodynamic changes (Gray *et al.*1997). There is also evidence to show an association between inflammation and its associated inflammatory markers and the pathogenesis of atherosclerosis (Gries *et al.* 1995).

1.2.2. Microvascular complications

Microvascular complications primarily affect the eyes, kidneys and the nervous system leading to highly disabling conditions such as blindness, renal failure and neuropathy.

1.2.2.1. Diabetic Retinopathy

Diabetic retinopathy is the leading cause of blindness in the Western world and requires continuous screening of diabetic patients (Cooper *et al.* 1997). Early abnormalities of the retinal vasculature include an increase in permeability, a thickening of the basement membrane, a reduction in blood flow and the loss of retinal pericytes (Mandarino *et al.* 1992). Basement membrane thickening and leucocyte activation lead to a reduction of perfusion through the vasculature, resulting in areas of hypoxia. As part of the body's repair mechanism, it is thought that new vessel growth attempts to revascularise the hypoxic areas. If these attempts are aborted because of an imbalance between pro- and anti-angiogenic factors, microaneurysms may result (Grant *et al.* 2004). Depending on the severity of the retinopathic changes microaneurysms (abortive neovascularization), cotton-wool spots (retinal infarcts) or severe haemorrhage, leading to retinal detachment and complete loss of vision, can be seen. Changes in the basement membrane structure and increased vascular permeability lead to cellular swelling and the formation of macular oedema (either focal or diffuse). These may lead to cell death and irreversible visual impairment (Forrester *et al.* 1997).

1.2.2.2. Diabetic Neuropathy

Diabetic neuropathy can affect many different neuropathic areas. The severity is related to the duration of diabetes and the degree of blood glucose control. Diabetic neuropathy is classified into acute mononeuropathy (reversible, affecting single nerves), diffuse, symmetrical neuropathy (progressive and irreversible), and pressure palsies (Ward *et al.* 1997). As with all diabetic vascular complications, a multitude of factors contribute to the pathogenesis of neuropathy. These include axonal loss, demyelination and regeneration, a reduction in nerve conduction velocity, basement membrane thickening, haematological changes, and decreased oxygen tension (Tesfaye *et al.* 2002), frequently leading to the development of severe ulceration and, in some cases, limb amputations (Vinik *et al.* 2006).

1.2.2.3. Diabetic Nephropathy

Diabetic nephropathy is a result of glomerular enlargement resulting in an increase in kidney volume and progressive loss of function. Diabetic nephropathy is the single most common cause of end-stage renal failure in the UK affecting 25% of all diabetic patients (Department of Health, 2004). Again, there is substantial evidence to suggest that the risk of developing nephropathy is related to the extent and duration of hyperglycaemia. Other causative factors include advanced glycated end products (AGE), impairing protein structures through the formation of protein cross links (Lehamn *et al.* 2001). Direct alteration of cell and cell component growth, activation of protein kinase C (PKC) enhancing the vascular permeability, haemodynamic disturbances and the activation of the "polyol pathway" (Trevisan *et al.* 1997); (Section 1.6.1).

1.3. Endothelial cell function and glucose uptake

When investigating vascular complications, changes in the endothelium are of particular importance as they present the first point of contact with the elevated concentrations of blood glucose. The response of the endothelium to adverse conditions is therefore important in understanding subsequent damage. Endothelial cells line the lumen of all vessels and act as a physical barrier between the blood and the tissue. Glucose is transported across the endothelial plasma membrane via membrane associated carrier proteins, also known as facilitative glucose transporters (GLUTs); (Bell *et al.* 1990). Once inside the cell, glucose is metabolised by glycolysis which drives oxidative phosphorylation in the mitochondria and subsequent generation of ATP (Fig.1.1). Very often, glucose analogues such as 2-deoxy-D-glucose (2DG) and 3-ortho-methyl (3OM) are used to determine the relative importance of D-glucose transport into the cell and its further metabolism by glycolysis and oxidative phosphorylation (Wood *et al.* 2007; Litherland *et al.* 2007; Cavallo-Perin *et al.*1985). 2-deoxy-D-glucose (2DG) has the 2-hydroxyl group replaced by hydrogen. Once inside the

cell the molecule is phosphorylated by hexokinases giving rise to 2-D-glucose-6-phosphate (2-DG-6P) during the first step of the glycolysis reaction. Because of its structure the phosphorylated molecule (2-DG-6P) cannot be metabolized further or diffuse outside the cell leading to the inhibition of glycolysis and the accumulation of the metabolised by-product (2-DG-6P) inside the cell. In contrast, 3-ortho-methyl glucose (3OM) is not metabolised at all and simply enters and leaves the cell. To date a family of thirteen related facilitative glucose transporters (GLUTs) has been identified all of which have a distinct tissue distribution and biochemical properties (Barnes *et al.* 1997); (Table 1.1).

GLUT-1 was the first glucose transporter to be isolated and is widely distributed throughout the body, with the highest levels found in erythrocytes and endothelial cells (Pardridge *et al.* 1990). Although GLUT-2 is found in pancreatic β-cells, where it acts as a glucose sensor (Chen *et al.* 1990), GLUT-4 is responsible for insulin-regulated glucose metabolism and is expressed in insulin-sensitive tissues (Olson *et al.* 1996). GLUT-4 is stored in specialized vesicles which, in the absence of insulin, move along a microtubule network below the plasma membrane. Once insulin binds to its receptor on the cell surface exterior, these vesicles are halted and fuse with the plasma membrane, where they release GLUT-4 which facilitates glucose transport into the cell (Lizunov *et al.* 2005).

Glucose transport into the cell is dependent on the Michelis Menten Constant (K_m) of the glucose tansporter, which inversely reflects the tissue affinity for glucose (Kahn *et al.* 1992). A low K_m (e.g. GLUT-1 ~ 2mmol/l) will lead to saturation of glucose transport at physiological levels, whilst a high K_m (e.g. GLUT-2 ~ 66mmol/l) will prevent saturation and ensure that glucose flux is directly proportional to the plasma glucose concentration. Therefore, the K_m of the glucose transporter is the rate-limiting step for glucose uptake into tissues (Resh *et al.* 1983; Olson *et al.* 1996) and is likely to be associated with the

accumulation of glucose in these tissues including the endothelium, and therefore the development of complications (Hirsch *et al.* 1999).

Glucose transporter isoform	Tissue distribution
GLUT-1	erythrocytes, endothelial cells, blood brain barrier, blood retina-barrier
GLUT-2	islet beta cells, hepatocytes, intestine
GLUT-3	neurons, placenta, heart
GLUT-4	adipose tissue, skeletal muscle, cardiac muscle
Class II – fructose transporter	GLUT-5, 7, 9 & 11
Class III	GLUT – 6,8,10, 12 and the H+/myoinositol transporter

Table 1.1: Facilitative glucose transporters and their characteristic tissue distribution.

Apart from glucose uptake, the above mentioned barrier function of endothelial cells is crucial in maintaining cellular homeostasis, and the provision of a balance between opposing physiological and molecular effects (Calles-Escandon *et al.* 2001). These include essential control of vascular tone through nitric oxide (NO)-mediated vasodilation and angiotensin–II (ANG-II) mediated vasoconstriction, maintenance of blood flow through activation of the proteolytic enzyme thrombin and the natural substrate fibrinogen, differentiation of VSMC through production of PGF and ANG-II and the regulation of inflammatory processes (Calles-Escandon *et al.* 2001, Crane *et al.* 2005; Shaw *et al.* 2005).

Endothelial dysfunction in type 1 diabetes is usually characterised by an increase in plasminogen inhibitors leading to an increase in blood clotting, inflammatory markers such as endothelial intercellular adhesion molecule 1 (cICAM-1) and vascular cell adhesion

molecule 1 (cVCAM-1), protein kinase C (PKC), fibronectin and von Willebrand Factor (vWF), a marker for endothelial dysfunction (Calles-Escandon *et al.* 2001), as well as a reduction in NO and NO-mediated vasodilation (Rodrígues-Nañas *et al.* 2003). Vascular changes and their detection seem more complex for type 2 diabetes as markers for endothelial dysfunction are usually present long before any damage occurs (Evans *et al.* 2002).

1.4. Cellular responses to hyperglycaemia

Since diabetes is characterized by abnormally high levels of circulating blood glucose, it follows that increased amounts of glucose enters the cell which results in more substrate being available for glycolysis and subsequent oxidative phosphorylation by the mitochondria (Fig. 1.1).

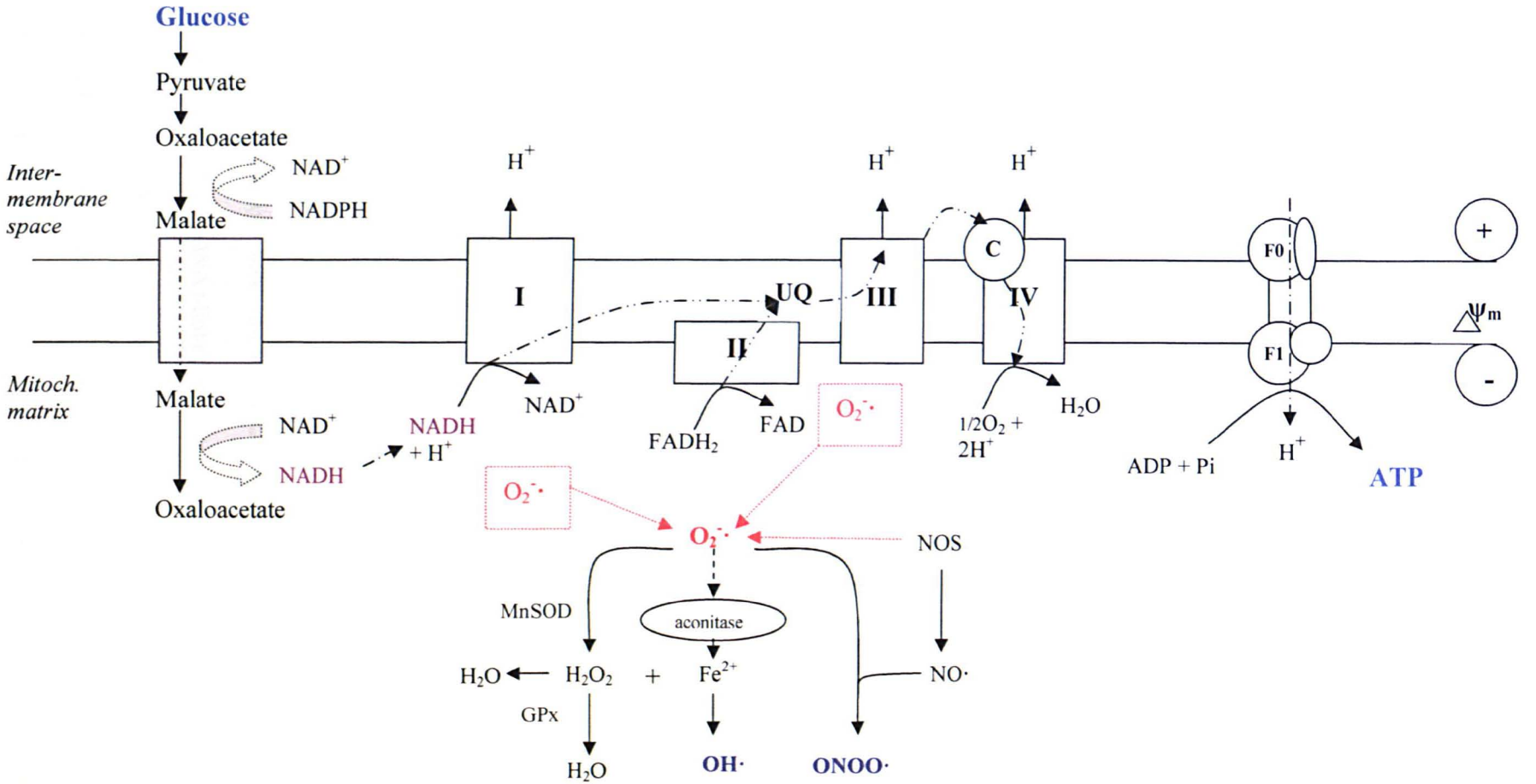


Fig. 1.1: Schematic diagram showing basic mitochondrial function and mitochondrial ROS generation. Electrons are removed from electron donors such as NADH and $FADH_2$ and passed down the mitochondrial electron transport chain (ETC) via a series of redox reactions. The energy generated is used to pump protons from the mitochondrial matrix into the inter membrane space, generating an electrochemical potential referred to as the mitochondrial membrane potential ($\Delta\Psi m$). This gradient drives ATP synthesis via the re-uptake of protons into the mitochondrial matrix.

1.4.1. Mitochondrial morphology and function

Mitochondria are remnants of a bacterial endosymbiont which are thought to have resided in a host cell over 1,500 million years ago (Margulis *et al.* 1986). They play essential and diverse roles in the metabolism and physiology of eukaryotic cells and provide energy through oxidative phosphorylation. Mitochondria are sub-cellular compartments for numerous essential metabolic reactions such as glycolysis, ROS detoxification, fatty acid transport and oxygenation and play central roles in apoptosis and aging (Scheffler *et al.* 2001).

The cellular function of mitochondria is reflected in their structure, which varies according to cell type, cell cycle stage and intracellular metabolic state (Westermann *et al.* 2002). The establishment and maintenance of an intracellular mitochondrial continuum by continuous membrane fusion and fission events is thought to be important for the distribution of energy in the cell (Westermann *et al.* 2002). Fusion of mitochondria serves to mix and unify mitochondrial compartments (Chan *et al.* 2006) important for the inheritance and maintenance of the mitochondrial genome encoding several polypeptides required for respiratory function and multiple cell signalling cascades. Proteins such as GTPases, kinases and phosphatases are involved in bi-directional communication between the mitochondrial reticulum and the rest of the cell, linking mitochondrial function and dynamics to the regulation of metabolism, cell-cycle control, development, antiviral responses and cell death (Neuspiel *et al.* 2005, McBride *et al.* 2006, Wang *et al.* 2006).

It has been proposed that mitochondrial fusion counteracts the manifestation of mitochondrial DNA (mtDNA)-linked diseases (Nakada *et al.* 2001) and cellular ageing (Chan *et al.* 2006). Mitochondrial DNA is highly susceptible to ROS-mediated damage, as it is subjected to a much higher level of free radicals compared to nuclear DNA (Kang *et al.* 2005). Multiple mitochondrial DNA deletions can be caused by an increased in ROS

(Wallace *et al.* 2002) and are thought to be associated with genetic disorders (Maassen *et al.* 2004). Research aimed at elucidating the role of mitochondria in cell death has become one of the fastest growing disciplines in biomedicine (Kim *et al.* 2007).

1.4.2. Mitochondrial regulation in hyperglycaemia and hypoxia

Calcium (Ca^{2+}) homeostasis is vital to the function and survival of cells and plays an essential role in mitochondrial regulation (Kannan *et al.* 2000). It helps to regulate the tricarboxylic acid cycle involved in fuel molecule catabolism and ATP production (Duchen *et al.* 2004) and mitochondrial membrane potential ($\Delta\psi_m$); (Richter e*t al.* 1998). There is a close relationship between Ca^{2+} concentration and cell death, with the disruption of Ca^{2+} homeostasis being responsible for most endothelial dysfunction associated with oxidative stress (Dreher *et al.* 1995).

Under normal physiological conditions, the $\Delta\psi_m$ drives Ca^{2+} accumulation in the mitochondria through its passive diffusion through the ruthenium red-sensitive uniporter (Jornot *et al.* 1999). A sharp increase in Ca^{2+}, oxidants and inorganic phosphate levels occurs as a result of hyperglycaemia and an increase in oxidative stress stimulates Ca^{2+} release (Richter *et al.* 1998) via the mitochondrial membrane permeability transition pore (mPTP) (Duchen *et al.* 2004). This results in a loss of $\Delta\psi_m$, leakage of the mitochondrial membrane, inhibition of ATP synthesis, mitochondrial damage and cell death (Fig.1.1).

Under hypoxic conditions, there is a reduction in mitochondrial respiration due to the lack of available molecular oxygen. This results in a reduction in cellular Ca^{2+} cycling (Gnaiger *et al.* 2000). In effect, a much slower rise in intracellular Ca^{2+}, a reduction in mitochondrial ROS production and subsequent closing of the mPTP, resulting in a reduced rate of mitochondrial depolarisation. One paper even suggests a functional switch of mitochondrial NO synthase (mtNOS) in endothelial cells from a NO generating enzyme under normoxic

conditions to a superoxide radical ($O_2^{\cdot-}$) generating enzyme under hypoxic conditions (Du *et al.* 1999). Since the intracellular Ca^{2+} concentration is known to regulate eNOS activity (Dröge *et al.* 2002) it is important to also consider the role of NO in the responses to hyperglycaemia-induced mitochondrial dysfunction.

It has been shown on many occasions that NO can combine with $O_2^{\cdot-}$ to form highly reactive peroxynitrite ($ONOO^-$); (Green *et al.* 2004) (Fig. 1.1). The formation of peroxynitrite is thought to be a critical step in the apoptotic process in endothelial cells in response to hyperglycaemia (Du *et al.* 1999). Some of the cytotoxic and pro-apoptotic downstream effects of peroxynitrite include: mitochondrial membrane depolarisation, activation of caspases 9 and 8, inactivation of ATP synthase, aconitase and creatine kinase (Waldorf *et al.* 2004), lipid peroxidation, oxidation of protein sulphydryls, nitration of tyrosines and DNA damage (Du *et al.* 2003). Therefore, peroxynitrite is a critical effector of apoptosis. Once formed, peroxynitrite inhibits NADH: ubiquinone reductase activity (complex I) and the ATP synthetase (complex V); (Waldorf *et al.* 2004). This will not only alter the permeability transition of the mitochondrial membrane but also the redox balance of the cell.

Despite contradictory reports about the oxidant properties of NO (Liu *et al.* 2001, Matsunaga *et al.* 2004, Brown *et al.* 2007), more recent studies have suggested that NO modulates the expression of the peroxisome proliferators-activated receptor (PPAR) gamma coactivator 1-alpha (PGC-1alpha), thereby regulating the mitochondrial ROS detoxification system (Valle *et al.* 2005, Borinquel *et al.* 2006). Interestingly, Wu *et al.* (2006) has identified the transducer of regulated CREB (cAMP response element-binding protein) binding protein (TORC) 1, as an upstream regulator of PGC-1 alpha, the master regulator of the ROS detoxification system (Wu *et al.* 2006). TORCs have been shown to be essential upstream regulators of CREB activity in beta cells (Conkright *et al.* 2003) and are thought of as promising new therapeutic targets for the treatment of diabetes mellitus (Patil *et al.* 2005).

1.4.3. Oxidative stress, DNA damage and repair

Oxidative stress was first recognised by Conger and Fairchild in 1952 (Conger *et al.* 1952) and is now thought to be an integral component of the development of vascular complications in diabetes mellitus (McMillan *et al.* 1997). There is evidence to suggest that an increase in plasma free-radical concentration impairs insulin action, which in turn contributes to the development of hyperglycaemia and further production of free radicals (Ceriello *et al.* 2000). This observation of a continued cycle of metabolic stress and free-radical production leads to the hypothesis that oxidative stress represents a pathway linking hyperglycaemia to reduced insulin action (Baynes *et al.* 1991, Giugliano *et al.* 1996, Ceriello *et al.* 2000). Further, increased production of reactive oxygen species in response to hyperglycaemia is closely linked to an increase in oxidative DNA damage (Dandona *et al.* 1996). Oxygen and ROS have been shown to induce many different types of DNA damage, including single- and double-strand DNA breaks, base and sugar modifications and DNA protein cross links. If left un-repaired these mutations may have disastrous consequences for the organism (Tsuzuki *et al.* 2007).

DNA repair is a hierarchical process which is initiated by signalling proteins. Two enzymes governing serine-threonine kinases which regulate the response to DNA damage, thereby acting as cell cycle checkpoints (Rehman *et al.* 1999), are ataxia telangiectasia (ATM), mutated ATM and Rad3-related (ATR). These protein kinases respond to distinct types of DNA damage such as single or double strand breaks (Walworth *et al.* 2000). To date, the highly selective processes by which cells detect DNA damage, signal its presence and recruit the correct cellular mechanisms to effect its repair are not fully understood (Jackson *et al.* 2001). What is known is that once expressed these signalling proteins activate a number of DNA repair enzymes, such as DNA polymerase enzymes which catalyze the polymerization of deoxyribonucleotides alongside a DNA strand through the addition and subsequent elongation of free nucleotides to the 3' end of the newly forming strand. DNA polymerases

continuously scan the DNA for damage and on detection will initiate an appropriate repair pathway. Single strand breaks are mainly repaired via base excision repair (BER), in which the altered base is removed by the enzyme DNA glycosylase and the resulting sugar phosphate excised (Walworth *et al.* 2000). Double strand breaks are potentially more dangerous lesions as they leave no template for repair and if left unattended may lead to the breakdown of chromosomes. Double strand repair takes place mainly during non homologous end-joining (also known as DNA mismatch repair; MMR) and homologous end-joining (double-strand break repair; DSB (Walworth *et al.* 2000). During non-homologous end-joining, the broken ends of the double helix structure are juxtaposed and rejoined by DNA ligation, thus allowing for the complete DNA sequence to be restored. In order to facilitate DNA repair, cellular progression through the cell cycle is delayed until DNA repair is complete to avoid cell death or the formation of cellular mutations during replication (Barzilai *et al.* 2004; Alberts *et al.* 2001).

To prevent attacks from ROS and other free radicals, living cells employ a number of defences including low molecular weight compounds and antioxidants such as vitamin C and E which scavenge free radicals. More complex systems such as the enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase have also evolved to limit the damage induced by ROS. The ROS that causes oxidative damage can be divided into two categories: free radicals ($^{\cdot}OH$, NO & $^{\cdot}O_2^-$) and non-radical ROS (H_2O_2); (Shackelford *et al.* 2000).

Free radicals induce DNA lesions by attacking at the 8'position of guanine since it is the most oxidisable base (Rehman *et al.* 1999). The hydroxyl radical first reacts with guanine to form a C8-OH adduct followed by the loss of an electron and proton to generate 8-oxo-d-guanine (8-oHdG) (Slupphaug *et al.* 2003). Unless repaired prior to DNA replication, 8-oHdG residues can result in GC to TA transversion, which may ultimately lead to mutagenesis (Griffiths *et al.* 2002). The detection of 8-oHdG has been shown to be a suitable

biomarker for the detection of DNA damage in response to oxidative stress (Peoples *et al.* 2005) and is being used for the detection of oxidative DNA damage in diabetic patients (Leinonen *et al.* 1997, Park *et al.* 2001) using chromatographic methods such as HPLC and GC-MS. However, 8-oHdG is an unstable molecule which is further hydrolyzed to 8-oHG resulting in the analysis being marred by the risk of auto-oxidation during sample preparation and manual induction of false positive background readings (Gedik *et al.* 2002). Although suggested improvements in the method aim at eliminating the oxidation of guanine during handling, the measurement of the more stable hydrolyzed form 8-oHG has been proposed (Gedik *et al.* 2002).

Since the first results directly associating an increase in ROS with an increase in DNA damage in type 1 and type 2 diabetic patients (Dandona *et al.* 1996, Hinokio *et al.* 1999), many more studies have looked at the evidence for ROS-related DNA damage in diabetes and its complications (Donnini *et al.* 1996, Hannon *et al.* 1998). Most, if not all, of this research has been carried out in diabetic individuals with long-standing diabetes (10 years or more), some of whom already have established signs of vascular damage. This implies the need for caution when interpreting these results, as oxidative damage (Lorenzi *et al.* 1987, Méplan *et al.* 2000) is also increased with age and concurrent disease (De Bont *et al.* 2004, Lieber *et al.* 2004). However, the amount of evidence showing an increase in oxidative DNA damage in diabetic patients is overwhelming and has been accepted among the scientific community.

1.5. Cellular responses to hypoxia

The ability to sense and respond to changes in O_2 concentration is a fundamental property of all cells. Tissue oxygenation is strictly regulated to ensure the availability of substrate for oxidative phosphorylation through the mitochondrial electron transport chain while

minimizing the production of ROS which can damage cellular DNA, lipids and proteins (Lahiri *et al.* 2006). In 1993, Wang *et al.* published details of the isolation of the transcription factor HIF-1 (hypoxia-inducible factor 1) which binds to the hypoxic response element (HRE) at the 5' UTR of hypoxia regulated genes, thereby facilitating the adaptation and survival of cells in response to changes in oxygen tension (Goldberg 1988, Semenza *et al.* 2000 and Coulet *et al.* 2003).

HIF-1 is a basic helix-loop-helix heterodimer which consists of two subunits HIF-1α and HIF-1β (Wang *et al.* 1995). Both of these subunits contain the basic-helix-loop-helix domain which is required for dimerization and DNA binding. Under normoxic conditions the HIF-1β subunit is constitutively expressed while the HIF-1α subunit is subjected to proteasomal degradation. It is this HIF-1α subunit which is tightly regulated by the cellular oxygen concentration and presents the most crucial step in the activation of HIF-1 (Wang *et al.* 1995). It is only when both subunits are activated that HIF-1 translocates into the nucleus and leads to the increased transcription of genes such as glucose transporters, glycolytic enzymes, VEGF, erythropoietin, inducible nitric oxide synthase (iNOS) and insulin-like growth factor -2 (IGF-2) (Lando *et al.* 2000 and Semenza, 2000). It is now thought that HIF-1 controls several hundred hypoxia responsive genes in the human genome (~5%) (Manalo *et al.* 2005).

Remarkable progress has been made in understanding HIF-1 signalling and cellular oxygen sensing. In the late 1990s, it was discovered that HIF-1α degradation is controlled by binding of the von Hippel-Lindau protein (vHL) which is the recognition component of the E3 ubiquitin-protein ligase which targets HIF-1α proteasomal degradation (Huang *et al.* 1998, Kallio *et al.* 1999 and Maxwell *et al.* 1999). vHL binding is dependent on the hydroxylation of HIF-1α proline residues (402 & 564) for which prolyl hydroxylases (PHD) are responsible (Jaakkola *et al.* 2001, Ivan *et al.* 2002 and Fandrey *et al.* 2006). This

provides a mechanism through which changes in oxygen concentration can be directly translated into changes in gene expression. However, many other factors have been shown to play a role in oxygen sensing and HIF-1 activation, such as asparaginyl hydroxylase (Lando *et al.* 2002a), cytokine activation, growth factors and environmental stimuli (Ke *et al.* 2006 and Lee *et al.* 2000) via the activation of the MAPK or PI3K signalling pathway (Li *et al.* 2004).

1.5.1. HIF-1α and the mitochondria

Despite being a key promoter for the adaptive responses to cell survival in hypoxia, HIF-1α paradoxically also mediates hypoxic cell death and apoptosis. Several pro-apoptotic genes induced by HIF-1α have been reported (Bruick *et al.* 2000, Chen *et al.* 2003, Kim *et al.* 2004 Harada *et al.* 2006). In 2004, Lee *et al.* showed that the HGTD-P gene (HIF-1α dependent pro-apoptotic molecule), formerly known as a novel gene expressed in human dendritic cells, acts as a novel HIF-1α responsive pro-apoptotic molecule. It activates mitochondrial apoptotic cascades through the induction of the mPTP, thus transmitting apoptotic signals which are sensed by HIF-1α to the mPTP (Lee *et al.* 2004).

It has been proposed that HIF-1 activity requires a functional mitochondrial electron transport chain (Guzy *et al.* 2005). Mitochondrial generation of superoxide radicals (at complex I and III of the ETC) and the subsequent formation of hydrogen peroxide are required for the induction of HIF-1 activity and the transcription of its downstream targets in hypoxic cells (Chandel *et al.* 1998). Furthermore, the mitochondrial antioxidant, Mn-SOD, has been shown to be responsible for the conversion of superoxide radicals into hydrogen peroxide (Wang *et al.* 2005). The induction of HIF-1α and subsequent VEGF expression is suppressed in human breast carcinoma MCF-7 cells. This would indicate that mitochondrial overproduction of ROS, with a subsequent rise in Mn-SOD activity is detrimental rather than beneficial for the induction of the cellular HIF-1 response to hypoxia.

These findings are in agreement with the second hypothesis established by Ehleben *et al.* (1998) who suggested that an NAD(P)H oxidase of unknown origin converts O_2 into superoxide radicals and onwards into hydrogen peroxide via the enzyme SOD. A reduction in O_2 concentration would lead to a reduction in ROS and subsequently reduce the cellular signalling pathways which lead to the activation of HIF-1. In accordance with this hypothesis, Vaux *et al.* (2001) published evidence for the HIF-1 activation in hypoxia even in the absence of a functional mitochondrial respiratory chain as well as showing a reduction in cellular ROS production in response to hypoxia.

1.5.2. Tissue hypoxia and disease progression

Due to the realisation that hypoxia has a strong impact on cell biology and signalling processes, there has been an enormous interest in the biology of HIF-1 and its role in human disease (Semenza *et al.* 2000). Tissue hypoxia plays a crucial role in cardiovascular disease, tumour growth and diabetic retinopathy due to hypoxia-mediated remodelling of the vasculature (Ikeda *et al.* 2005). Angiogenesis is one of the most important adaptive responses to hypoxia as it attempts to restore oxygen transport to ischemic tissues by increasing vessel growth. HIF-1-mediated activation of VEGF promotes new vessel growth in diabetic retinopathy in an attempt to circumvent the hypoxic area and restore blood flow (Ferrara *et al.* 2003). Vascular remodelling can, however, also contribute to disease progression, such as conferring resistance to chemotherapy and radiotherapy through the remodelling of the tumour vasculature, by a direct change in the cellular phenotype (Kobayashi *et al.* 2006), and abortive vessel growth during the proliferative stage of diabetic retinopathy leading to hemorrhage, retinal detachment and loss of vision (Cukiernik *et al.* 2004). New vessel growth has been associated with a reduction in the luminal diameter of the pulmonary artery, resulting in an increased resistance to blood flow and progressive heart failure (Semenza *et al.* 2001). In addition, it has been linked to cerebral ischemia (Marti *et*

al. 2000) and pregnancy disorders, such as pre-eclampsia and intra-uterine growth retardation (Rajakumar *et al.* 2004 and Malamitsi-Puchner *et al.* 2005).

1.6. Intracellular pathways involved in cellular response to hyperglycaemia and hypoxia

1.6.1. The polyol pathway

The polyol pathway converts glucose into sorbitol and onwards into fructose (Fig.1.2). Under normal physiological conditions, this pathway only accounts for a small percentage of the total blood glucose metabolism since the conversion of glucose into glucose-6-phosphate by hexokinase (glycolysis) is the favoured mechanism for the normal metabolism of glucose. As the blood glucose levels rise, hexokinase becomes saturated and a two to four fold increased flux through the polyol pathway can be observed (Hotta *et al.* 1995). The selectivity of this mechanism is due to the high K_m of aldose reductase, the rate limiting enzyme.

During the polyol pathway, glucose is converted into sorbitol by the enzyme aldose reductase (Fig.1.2). This reaction utilises nicotinamide-adenosine dinucleotide phosphate (NADPH) as a hydrogen donor, converting NADPH into nicotinamide adenine dinucleotide phosphate, oxidized form ($NADP^+$). Sorbitol is then converted into fructose by the enzyme, sorbitol dehydrogenase (SDH). In contrast to the first reaction, nicotinamide-adenosine nucleotide (NAD+) is favoured as a hydrogen acceptor, converting NAD+ into NADH (King *et al.* 1996). Sorbitol is thought to accumulate in the tissue and thereby contribute to the pathogenesis of chronic diabetic vascular complications (Hotta *et al.* 1995). However, despite the investigations into the direct consequence of the accumulation of sorbitol and fructose, it appears that chronic vascular damage is the result of many factors which

contribute to a change in cellular redox state rather than a direct toxicity effect (Gries *et al.* 1995).

A direct consequence of the altered redox state within the cell is a depression in reduced glutathione levels and the resulting loss of the intrinsic protection against oxidative stress (Williams *et al.* 1999), as well as the reduction in myoinositol, leading to a reduced Na+/K+ - ATPase activity (Gries *et al.* 1995).

1.6.2. Advanced Glycation End Products (AGE)

As seen with the polyol pathway, glucose can lead to macromolecular changes. Advanced glycation end products (AGE) are produced by glucose-mediated changes to macromolecules. It is believed that the modification of tissue proteins by reducing sugars contributes to the pathological changes which lead to diabetic vascular complications (Baynes *et al.* 1999).

In the very early stages of the disease and in the presence of high glucose concentrations, glucose attaches rapidly to the amino groups of proteins forming Schiff bases (Maillard reaction) (Yim *et al.* 1995). These rearrange quickly to form more irreversible Amadori products and early glycation products. The last step in this protein glycation cascade is the formation of stable and irreversible advanced glycation end-products from early glycation products (Giardino *et al.* 1997, Mohamed *et al.* 1999). Since AGE products are irreversible, they will accumulate within the tissues or vessel walls over time and contribute to the chronic pathological changes in the vasculature. The more advanced the protein rearrangement, the more permanent these changes become. In addition, these early glycation products can generate free radicals due to their ability to undergo auto-oxidation (Ahmad *et al.* 1998, Ahned *et al.* 2005). This will contribute to protein cross-linking and various other forms of cellular damage in diabetes (Goldin *et al.* 2006).

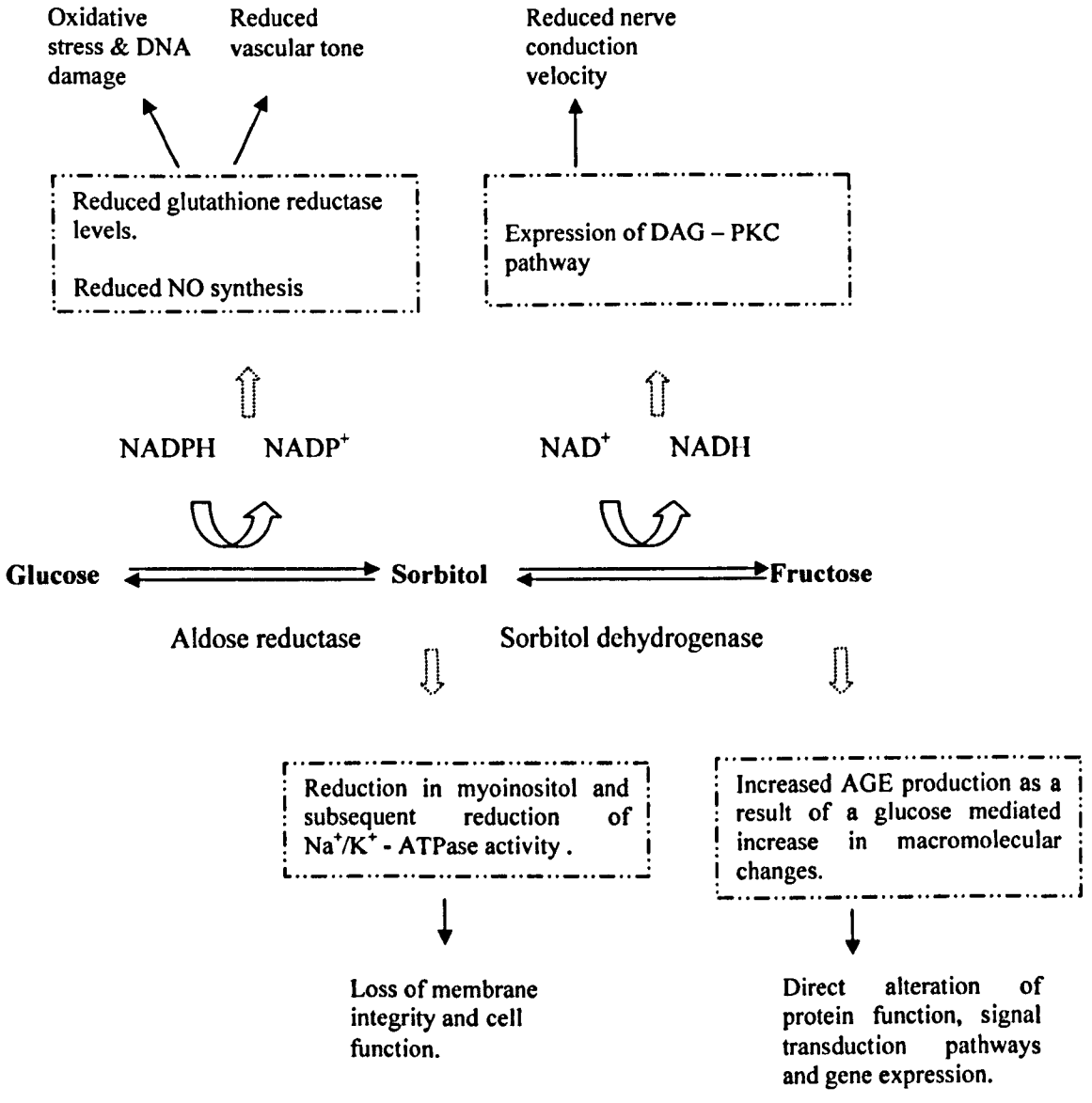


Fig.1.2: Illustration showing the dependence of the biochemical pathways on changes of the cellular redox sate in response to hyperglycaemia.

AGE can directly alter protein function and associated signal transduction and gene expression (King *et al.* 1996). Three mechanisms of AGE play an important part in the generation of tissue damage, all of which may lead to a change in vessel structure, reduction in their elasticity, abolishion of vessel tone and increased fluid filtration across the vessel walls (King *et al.* 1996). Consequences include the impaired regulation of blood flow (Rösen *et al.* 2001) and a contribution to the development of atherosclerosis (King *et al.* 1996) through the highly thrombogenic and pro-coagulant state of the vessel wall (Rösen *et al.* 2001), all of which are characteristic clinical signs within the population of diabetic subjects.

Associated with the above effects is the concept of AGE-induced acute oxidative stress (Baynes *et al.* 1991, King *et al.* 1996, Mohamed *et al.* 1999, Rösen *et al.* 2001). Binding of AGE to its receptor (RAGE) and the subsequent mediation of signal transduction leads to the generation of ROS (King *et al.* 1996, Mohamed *et al.* 1999) and hence depletion of the antioxidant defence mechanisms. Increased oxidative stress levels result in the activation of the free-radical sensitive transcription factor, NF-κB. NF-κB regulates the expression of many genes associated with haemodynamic control and cellular microcirculation of tissues (Mohamed *et al.* 1999, Rösen *et al.* 2001). Therefore the activation of NF-κB is critical in the pathogenesis and progression of diabetes and atherosclerosis (Mohamed *et al.* 1999).

1.6.3. PKC pathway

One of the most extensively researched pathways activated by high circulating blood glucose levels is the expression of protein kinase C (PKC). Its activation by elevated glucose concentration has to date been confirmed in nearly all tissues (King *et al.* 1996, Ways *et al.* 2001), but the extent of its involvement in the pathogenesis of vascular complications remains unclear (Mohamed *et al.* 1999). PKC belongs to the group of serine/threonine kinases. The entire PKC family is involved in a large number of cellular processes, ranging from host defence mechanisms to the development of neural function required for long-term

memory (Gomperts *et al.* 2003). During normal cellular processes, PKC catalyses the phosphate group transfer from adenosine triphosphate (ATP) to multiple cellular proteins required for normal cellular function (Rösen *et al.* 2001). PKC isoforms can be classified into three subfamilies, namely conventional (or classical), novel and atypical based on their second messenger requirements. Conventional PKCs (isoforms α, β_1, β_2, γ) are diacylglycerol (DAG) and Ca^{2+} sensitive, whereas novel PKCs (isoforms δ, ε, η, θ, μ) are DAG-sensitive but Ca^{2+} insensitive (Koya *et al.* 1998). DAG is synthesised in response to either the hydrolysis of phopshatidylinositides (PIs) or from the metabolism of phosphatidylcholine (PC) by phospholipase C (PLC) or D (PLD) (Koya *et al.* 1998). In response to hyperglycaemia there is an increased flux through the glycolytic pathway resulting in an increased production of DAG and a subsequent change in the NADH/NAD^+ ratio. Atypical PKCs (isoforms ξ, ι, λ) require neither Ca^{2+} nor DAG for activation (Koya *et al.* 1998).

Since high levels of PKC, mainly the β- and δ-isoforms, are present in most diabetic tissues, a number of pathogenic mechanisms have been proposed by which PKC contributes to the development of vascular changes (Meier *et al.* 2000). These include:

- impaired vascular reactivity through the inhibition of NO mediated vasodilation (Ramzy *et al.* 2006)
- reduced cellular Na^+/K^+ - ATPase activity leading to a disruption of cellular integrity and nerve conduction velocity (NCV)
- changes in vascular permeability (PKCα) (Hempel *et al.* 1997)
- basement membrane thickening and a change in vascular blood flow

Evidence of these changes can be found in all major tissues contributing to atherosclerosis (Yuan *et al.* 2000), retinopathy and neuropathy (Eichberg *et al.* 2002) as well as providing a link to many other pathogenic mechanisms associated with the vascular complications of

diabetes. It has been suggested that the activation of the DAG-PKC pathway is a common downstream pathway through which high circulating blood glucose levels exert their pathogenic effect (Aleksandrovski *et al.* 1998, Koya *et al.* 1998, Opara *et al.* 2002).

1.6.4. p42/44 MAPK and PI3K/akt pathways

P42/44 MAPK belongs to a family of highly conserved serine/threonine protein kinases which are involved in a number of cellular regulatory events such as differentiation, cell survival and migration (Cobb *et al.* 1999). The p42/44 MAP kinase pathway is a protein kinase cascade which links growth and differentiation signals with a resulting effect on cell proliferation (Ho *et al.* 2000). In addition, p42/44 phosphorylation and activation during reperfusion has been shown to be beneficial in limiting reperfusion-induced injury (Brar *et al.* 2000). It is though that ischaemic preconditioning, the most powerful protection against infarction, activates PI3Kinase (PI3K)/AKT and P42/44MAPK (Wynne *et al.* 2005).

PI3K and Akt are downstream effectors of insulin signaling (Galetic *et al.* 1999) and are important in the regulation of angiogenesis (Jiang *et al.* 2000) proliferation (Varma *et al.* 2005), microvascular permeability (Lal *et al.* 2001) and survival (Franke *et al.* 1997) of endothelial cells. High glucose is thought to induce HUVEC apoptosis via PI3K/Akt signaling as well as causing the generation of ROS via a PI3K/Akt dependent pathway (Sheu *et al.* 2005) while hypoxic induction of PI3K/Akt plays an important role in tumour angiogenesis through the induction of vascular endothelial growth factor (Mitzukami *et al* . 2006; Kobayashi *et al.* 2006).

1.7. Therapeutic targets for vascular complications in diabetes mellitus

As the treatment options for diabetic vascular complications are limited, the development of novel therapeutic agents and approaches is an important focus of diabetes research. One of

the first targets identified as having great potential for the treatment of diabetic microvascular complications was the polyol pathway and its rate-limiting enzyme, aldose reductase (Fig. 1.2). Aldose reductase inhibitors were first developed in 1973 (Dvornik *et al.* 1973) and together with flavonoid antioxidants, which have also been proposed to have aldose reductase inhibiting potential (Varma *et al.* 1977), formed the first generation of promising therapeutic agents for the treatment and prevention of vascular complications of diabetes which are still being investigated to date. However, due to our continuously evolving appreciation of the biochemical mechanisms involved, focus has since shifted to other promising agents such as PKCβ inhibitors (Kunisaki *et al.* 1994) and most recently poly(ADP-ribose) polymerase (PARP) inhibitors (Soriano *et al.* 2001).

1.7.1. Aldose reductase inhibitors

Since the first isolation of rat lens aldose reductase (Hayman *et al.* 1965) aldose reductase and its inhibitors have been extensively researched as potential therapeutic agents for the treatment and/or prevention of diabetic vascular complications. Despite showing promising results in animal and in vitro studies, aldose reductase inhibitors have failed to show any outstanding benefit in the multitude of clinical trials that have been conducted with the exception of some beneficial effects on neuropathy.

The greatest benefits to humans were seen in the prevention of a reduction in nerve conduction velocity in diabetic neuropathy (Goto *et al.* 1990). This has led to the licensing of certain aldose reductase inhibitors (tolrestat, epalrestat) in some European countries (Hotta *et al.* 1995) for the prevention of diabetic neuropathy. In addition aldose reductase inhibitors (ARI) investigated by the Sorbinil Retinopathy Trial Research Group (Sorbinil Retinopathy Trial Research Group (1990)) have shown encouraging results in the prevention of diabetic retinopathy (Zenon *et al.* 1990).

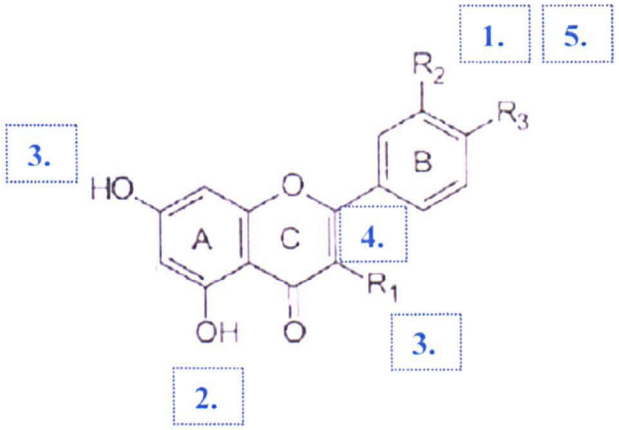
Consequences of inhibiting the rate-limiting enzyme of the polyol pathway can easily be extrapolated from our insight into the outcomes of polyol pathway expression. Research carried out in this area (Costantino *et al.* 1999) has shown that aldose reductase inhibitors improve nerve conduction velocity and endoneurial blood flow, improve endothelial NO-mediated vascular relaxation, improve nerve and vascular smooth muscle cell Na^+/K^+ - ATPase activity, lead to an incremental increase in the oxygen supply of peripheral nerves and reduce the incidence of capillary micro-aneurysms in the retina.

The limited data obtained from clinical trials suggests that the clinical role of the aldose reductase inhibitors is confined to a slowing of the pathogenic progression rather than the reversal of vascular damage (Costantino *et al.* 1999). This suggestion is further confirmed by studies which have shown that early administration of ARIs to galactose-fed rats can slow cataract formation. This suggests that further investigation of the beneficial effects of ARIs should focus on early intervention and prevention of vascular dysfunction (Kador *et al.* 1985) rather than the treatment of a manifested vascular complications.

1.7.1.1. Structural requirements of flavonoid aldose reductase inhibitors

Aldose reductase inhibitors can broadly be categorized into four structural groups: carboxylic acids, flavonoids, cyclic imides (mostly hydantoins) and "other compounds" (Sato *et al.* 1990). As none of the flavonoid aldose reductase inhibiting compounds have made it to the clinical trial stage, more potent and less toxic aldose reductase inhibitors need to be identified. Special attention is paid to the NADPH binding conformation and the identification of the structural pharmacophoric and stereometric properties.

In 2002 Matsuda *et al.* identified essential structural requirements of potent flavonoid ARI. These include:



1.	7 – hydroxyl and/or catechol moiety at the B-ring (3',4'-dihydroxyl moiety) which exhibits strong activity
2.	5 – hydroxyl moiety - has no effect on activity
3.	3 – hydroxyl and 7-O-glucosyl moieties-reduce activity
4.	2-3 double bond enhances activity
5.	catechol moiety at the B ring exhibits stronger activity than a pyrogallol moiety (3',4',' – trihydroxyl moiety)

Fig. 1.3: Core structure of a flavonoid comound and identified essential structural requirements for a potent flavonoid ARI

The more water-soluble flavonoid antioxidant compounds contain the 4-oxo-4H-chromen ring system which is essential for improved ARI activity. These compounds interact with the enzyme at a site which is different from that of the substrate. Conformational change of the enzyme is achieved by nucleophilic attack at the carbonyl group of the inhibitor (Kador *et al.* 1983). It is because of these effects that many antioxidants have been investigated for their therapeutic potential in the treatment of the vascular complications of diabetes.

Overall, the data obtained from studies with aldose reductase inhibitors do not live up to their promise as potent effective treatments for all vascular complications in diabetes. Research into these novel therapeutic agents is still ongoing (Hotta *et al.* 2001, Bril *et al.* 2006), although attention has now shifted to other more promising therapeutic targets such as PKCβ and PARP inhibitors.

1.7.2. PKCβ inhibitors

Recent developments focus on the usefulness of specific PKC-isoform inhibitors as novel therapeutic agents in the treatment and prevention of diabetic vascular complications (Cooper *et al.* 1997, Ways *et al.* 2001, Bloomgarden *et al.* 2002), and although they seem to show some improvement, one main hurdle is to establish their long-term consequences, since PKC is involved in a multitude of normal cell processes.

In 1996 the PKCβ inhibitor LY333531 was one of the first to be synthesised and shown to be a competitive reversible inhibitor of PKCβ_I and β_{II} (Ishii *et al.* 1996). Since then, it has been extensively tested against the hypothesis that PKCβ-induced ROS underlies the vascular dysfunction in diabetes (Zhou *et al.* 2006). LY333531 has since been shown to ameliorate diabetes-induced retinal hemodynamic abnormalities (Aiello *et al.* 2006), and calcium homeostasis of diabetic sensory neurons (Tahara *et al.* 2006) as well as being beneficial in preventing diabetes associated CV complications (Boyle *et al.* 2005; Zhou *et al.* 2006) in rats. LY333531, also known as Ruboxistaurin, is quickly emerging as a potential novel therapeutic agent for the treatment of diabetic microvascular diseases (Joy *et al.* 2005, Avignon *et al.* 2006) such as peripheral neuropathy (Casellini *et al.* 2007).

1.7.3. Antioxidants

As mentioned above, antioxidants have been investigated as potential therapeutic agents for diabetes since Varma *et al.* (1977) hypothesised a potential role for them as aldose reductase

inhibitors. Antioxidants are free-radical scavengers which prevent or delay the oxidation of substrates. Two types of endogenous antioxidants exist, antioxidant enzymes and small antioxidant molecules. In addition, antioxidants are ingested in a healthy, balanced diet and in some cases vitamin supplementation.

To date, a large number of studies have given conclusive evidence that free radical-generated cellular damage plays an important role in the pathogenesis of many chronic diseases (Hannon *et al.* 1998). As such, three of the four largest and most important long-term clinical trials carried out in diabetes have had nutritional intervention as a major element in their investigation. These include the Epidemiology of Diabetes Intervention and Complications Study (EDIC) (a follow-on study of the DCCT (NIDDK National Diabetes Information Clearinghouse), the Diabetes Prevention Program (DPP) (World Health Organisation 1990) and the Look AHEAD (Action for Health in Diabetes 2002) study.

As explained earlier, ROS play an important role in the pathogenesis of diabetic vascular damage (Section 1.4). It has therefore been hypothesised that the use of antioxidants as therapeutic agents could be beneficial in the treatment and prevention of diabetic vascular complications and other chronic diseases. Particular attention has been paid to antioxidants such as Vitamin E and C in the prevention of cardiovascular disease. Results from large-scale clinical trials are controversial depending on the study size, risk groups investigated (primary or secondary prevention), race, and dose of Vitamin E administered. The most interesting evidence was produced by the CHAOS (Cambridge Heart Antioxidant Study) study in 1996 (Stephens *et al.* 1996) which looked at the effect of Vitamin E on the secondary prevention and improvement in mortality in CV disease patients. Results revealed a 60% reduction in non-fatal myocardial infarctions. A second landmark trial, the Heart Outcomes Prevention Evaluation (HOPE) study (Kleinert *et al.* 1999), in contrast, found that long-term vitamin E supplementation does not prevent major cardiovascular events in

patients with vascular disease or diabetes mellitus, and may even increase the risk for heart failure (Lonn *et al.* 2005). This highlights the potential importance for the use of antioxidants in diabetes and the prevention of CV complications.

Alpha lipoic acid (another powerful anti-oxidant) has been shown to be particularly suited for the prevention and/or treatment of diabetic complications that arise from the overproduction of ROS (Packer *et al.* 2001). A randomised controlled trial investigating the usefulness of alpha lipoic acid, selenium and D-alpha-tocopherol in 80 randomly assigned diabetic patients confirmed that oxidative stress played a role in promorting the development of long-term diabetic complications (Kähler *et al.* 1993). Its combination with an aldose reductase inhibitor (ARI) has been suggested to be a suitable future treatment strategy (Cameron *et al.* 1999).

Although many different types of antioxidants exist, flavonoid antioxidants have largely been the focus of antioxidant research and will now be reviewed in more detail.

1.8. Flavonoids

Flavonoids are a group of polyphenolic antioxidant compounds, of which 3000 varieties are known to date. They can be found in a variety of plants, fruits and a number of beverages such as teas, wine and beer (Gökmen, 2002; Miller, 2002).

The core structure of all flavonoids includes two benzene rings on either side of a 3-carbon ring (Fig. 1.3). Depending on the further chemical structure, flavonoids are divided into five sub-groups: anthocyanidins, flavones/flavonoles, flavanones, catechins/leucoanthocyanidins and proanthocyanidins (Gökmen, 2002). These structural differences are responsible for the multitude of therapeutic activities that flavonoids have been shown to possess.

The therapeutic properties of flavonoids have been explored for centuries with the health benefit of teas and plant extracts well known to ancient Chinese medicine. Scientific interest in flavonoids has led to many studies being carried out since the 1940s. These studies have shown that flavonoids have antibacterial, anti-inflammatory (Nair *et al.* 2002), antiallergic, antiviral, anti-thrombic and vasodilatory activity (Narayana *et al.* 2001, Kris-Etherton *et al.* 2002). Most of these effects can be attributed to their potent antioxidant and radical scavenging capacity, as oxidative stress is an underlying factor in many of these diseases (Jovanovic *et al.* 2000, Cotelle *et al.* 2001). Flavonoids also inhibit a number of enzymes including aldose reductase, xanthine oxidase, phosphodiesterase, Ca^{2+} ATPase, lipo-oxygenase and cyclooxygenase and regulate certain hormones (Wang *et al.* 2000), making them applicable to a wide range of therapeutic approaches. In principle, the potent anti-oxidant activity of flavonoids is thought to be attributable to two main mechanisms of action: a radical scavenging activity which occurs through the donation of hydrogen atoms from free hydroxyls found on the flavonoid nucleus, and a metal chelating activity which is mediated through the phenolic OH groups (Sugihara *et al.* 1999). Research into the structure-activity relationship of flavonoids has identified three basic criteria which predict the potency of the flavonoids' radical scavenging ability. These include (i) the ortho-dihydroxyl structure in the B-ring, (ii) the 2,3-double bond allowing electron delocalization from the B-ring, and (iii) 3- and 5-OH with 4-oxo function in the A- and C-rings. In general, research suggests that flavonoids act as antioxidants in biological systems through their radical scavenging ability. However, in the presence of certain metal ions, flavonoids will form complexes which will dictate their effect as either anti- or pro-oxidants (Sugihara *et al.* 1999, Areias *et al.* 2001).

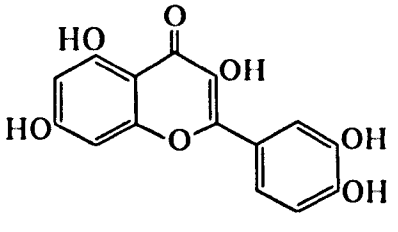
A Finnish prospective study which looked at the relationship between average dietary flavonoid intake and the development of chronic diseases in 10,054 men and women between 1966 and 1972 found an inverse relationship between flavonoid intake and the development of disease (ischaemic heart disease, cerebrovascular disease, lung & prostate

cancer, type 2 diabetes and asthma), with most of the beneficial effects attributed to quercetin (Knet *et al.* 2002). This inverse relationship between average dietary flavonoid intake and cardiovascular risk has also been summarised in a review by Cotelle in 2001.

Despite the multitude of scientific data, there is controversy over the supplementation of our diet with flavonoid extracts. As there is no evidence to suggest that a lack in polyphenols causes serious harm (unlike some vitamin deficiencies), the consensus seems to be that dietary intake of flavonoids through fruit and vegetables is more advisable than essential supplementation (Duthie *et al.* 2003).

1.9. Quercetin and the French Paradox

Quercetin is one of the most extensively studied flavonoids and is deemed to be one of the most potent antioxidants among the flavonoid group (Fig.1.4) (Formica *et al.* 1995). Large amounts of quercetin can be found in apples (Boyer *et al.* 2004), onions, kale, french beans, broccoli, lettuce and tomatoes as well as red wine, grape, lemon and tomato juice. Because of its wide distribution, quercetin constitutes an unavoidable component of our diet which is positive given the extensive health benefits attributable to flavonoids.



Quercetin [$C_{15}H_{10}O_7$]

Fig. 1.4: Illustration showing the chemical structure of the flavonoid quercetin

(Adapted from: Dictionary of Natural Products (DNP) on CD-Rom, Version 9:2. Copyright (c) 1982-2001 Chapman & Hall/CRC.)

Quercetin is said to be of particular benefit to the cardiovascular system (Boyer *et al.* 2004) as well as having a positive effect on inflammatory and vascular diseases, atherosclerotic plaque formation and viral and carcinogenic activity (Formica *et al.* 1995). However, other reports question the ability of quercetin to reverse diabetic oxidative stress overall (Sanders *et al.* 2001).

Despite the convincing results which confirm the health benefits of dietary flavonoids, something referred to as the "French paradox" (Criqui *et al.* 1994) questions our understanding of their mechanism of action. The mediterranean diet is saturated with flavonoids and other antioxidants and vitamins through the high dietary intake of fish, vegetables, fruit and red wine. This can be correlated to the low incidence in cardiovascular disease, cancer, diabetes and obesity in these countries. However studies show that flavonoids (especially quercetin) are susceptible to microbiological degradation in the lower bowel (Renauld *et al.* 1992) and poor absorption with only 0.3%-0.5% of quercetin being absorbed from the gut (Formica *et al.* 1995). Thus, it remains unclear as to how the health benefits can be achieved with the relatively low concentration of active compound reaching the affected area.

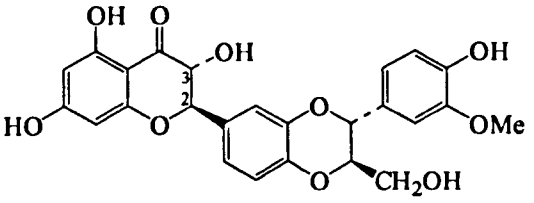
1.10. Silymarin

Silymarin or *Silibum marianum* is a biennial herb commonly known as milk thistle. The Greeks and Romans used the extracts and derivatives of the milk thistle in the 1st century, and evidence for this is found in the writings of many famous physicians of the past such as Dioscorides, Pliny the Elder, Hieronymus Bock, Jacobus, Theodorus and Culpepper

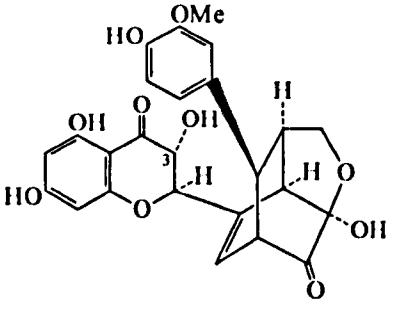
(Pepping *et al.* 1999). Historically, it is used for conditions of the liver, spleen and gallbladder.

Silymarin became available for clinical use in Europe and America in 1969. Until then, silymarin was thought to be a pure compound. Detailed analysis revealed that silymarin is made up of a number of different flavonoid isomers: silibinin, isosilibinin, silidianin and silichristin (Frömming *et al.* 1999) (Fig. 1.5). Of these, silibinin is thought to be responsible for the majority of therapeutic effects seen with silymarin (Zhao *et al.* 1999). Further HPLC analysis revealed that silymarin also contained taxifolin (Fig.1.6), another flavonoid compound structurally closely related to quercetin (Fig.1.4), as well as linoleic and oleic acids (approx. 20% of dry weight) (Carrier *et al.* 2004). The seeds of the milk thistle are said to contain a number of other flavonolignans such as dehydrosilybin, desoxysilcristin, desoxysilydyanin, silandrin, silybinome, silyhermin and neosilyhermin. Nothing is known about their therapeutic effectiveness.

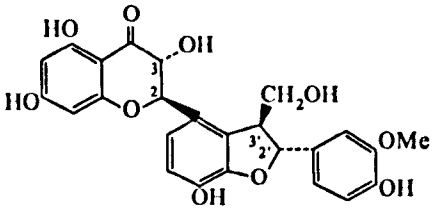
A recent analysis of the active components of silymarin suggests that silymarin contains impurities which have a more potent antioxidant capacity than any of the flavonoid isomers themselves, indicating that the higher the purity of the milk thistle extract the lower its antioxidant capacity (Kvasnička *et al.* 2003).



silymarin/silibinin [$C_{25}H_{22}O_{10}$]



silydianin [$C_{25}H_{24}O_{10}$]



silychristine [$C_{25}H_{22}O_{10}$]

Fig.1.5: Illustration showing the different flavonoid structures found in the silymarin compound (Adapted from: Dictionary of Natural Products (DNP) on CD-Rom, Version 9:2. Copyright (c) 1982-2001 Chapman & Hall/CRC).

1.10.1. Therapeutic properties of silymarin

Silymarin is mainly used as a hepatoprotective drug. It is marketed as Legalon70® by the German company Madaus (Köln) and is licensed for the treatment of toxic liver damage and the supplementary treatment of chronic inflammatory liver disease and liver cirrhosis.

Despite the large amounts of convincing research investigating the usefulness of milk thistle in liver disease, its mechanism of action is still poorly understood. To date it is thought that silymarin exerts its hepatoprotective effect through four main mechanisms (Fraschini *et al.* 2002), namely as a potent antioxidant radical scavenger, a cell membrane stabiliser and permeability regulator, a promoter of ribosomal RNA synthesis and an inhibitor of the transformation of stela hepatocytes into myofibroblasts.

Since silymarin is a flavonoid antioxidant, its theoretical benefits for a multitude of chronic diseases is evident. Despite research focusing on silymarin in the treatment of liver disease, other useful properties have been discovered over the years and research attention has widened to include other diseases such as cancer, inflammatory diseases and diabetes.

The use of silymarin in cancer treatment and prevention is a field of growing interest. Particular attention has been paid to its ability to protect against skin cancer development through the reduced phosphorylation of the Shc gene (Ahmad *et al.* 1998) as well as the inhibition of MAPK/ERK1&2 pathway (Zi *et al.* 1999) and subsequent cell cycle arrest (G2-M1 phase). Silymarin has also been shown to inhibit VEGF expression in cancerous HUVEC cells (Jiang *et al.* 2000) and has a potential anti-angiogenic effect in colon cancer (Yang *et al.* 2003).

Since inflammation is a contributory factor in many disease pathologies, research has also investigated the potential anti-inflammatory properties of silymarin (De La Puerta *et al.*

1996). Evidence has been published showing silymarin is able to suppress the action of NF-κB (Saliou *et al.* 1998, Saliou *et al.* 1999), inhibit the action of TNFα-induced adhesion molecules, VCAM-1, ICAM-1, E-selectin and the TNFα induced binding of NF-κB/Rel (Kang *et al.* 2003). It has also been shown to prevent LDL oxidation and expression of iNOS (Kang *et al.* 2003) making it a potential therapeutic agent for diseases such as arthritis (Gupta *et al.* 2000) asthma (Breschi *et al.* 2002) and cardiovascular diseases. This is supported by additional findings showing a reduction in plasma cholesterol levels (Škottová *et al.* 1998, Škottová *et al.* 1999, Kang *et al.* 2003), platelet aggregation in rat blood (Yao-Cheng *et al.* 1991) and cytoprotective and cell regenerating effects (Valenzuela *et al.* 1994). Since diabetes is a major risk factor for CV disease silymarin might be a suitable agent to slow the onset and progression of diabetes associated macrovascular complications (Psotova *et al.* 2002).

1.10.2. Use of silymarin in diabetes

The usefulness of silymarin in diabetes prevention and treatment was first suggested in the early 1990s (Zhang *et al.*1993). Since then only a few research reports have taken a closer look at the issues surrounding diabetes mellitus. In 1998 the first ever study was published which specifically looked at the treatment of alloxan-induced diabetic rats with silymarin (Soto *et al.* 1998). The study showed that silymarin prevents a rise in plasma glucose concentration and lipid-peroxidation as well as showing an increase in pancreatic and plasma glutathione levels. Following on from this study the same research group published further evidence in 2003, suggesting that silymarin restores the levels of antioxidant enzymes SOD, glutathione peroxidase (GSHPx) and catalase (CAT) in the pancreas of alloxan-induced diabetic rats. In addition, they observed a recovery of normal blood glucose levels. Their explanation is based on previous work carried out by Sonnenbichler *et al.* (1976), who reported that transcription of RNA is accelerated in the liver of rats and mice in the presence of silymarin. This leads to an increase in the rate of protein synthesis. The prevention of

pancreatic β-cell destruction through the maintenance or increase of glutathione levels is thought to be responsible for the restoration of the blood sugar levels observed (Shafer *et al.* 2001).

Another study investigating the long-term (23 months) effects of silymarin administration in cirrhotic diabetes patients found a significant decrease in fasting and mean daily blood glucose levels as well as a significant reduction in the mean exogenous insulin requirements (Velussi *et al.* 1997). These results were confirmed by a subsequent study looking at the compound silybin-beta-cyclodextrin in patients with type two diabetes mellitus and concurrent chirrosis (Lirussi *et al.* 2002).

Despite the limited amount of research which has gone into silymarin, both the *in vitro* and *in vivo* results seem to be promising. From research reports carried out on other tissues, certain mechanisms have been identified which strengthen the argument for research into the usefulness of silymarin in diabetes. Silymarin has, for example, been shown to be a potent radical scavenger, preventing radical-mediated DNA damage (Anderson *et al.* 1994, Yu *et al.* 1997, Kang *et al.* 2003) and a depression of antioxidant enzymes (Soto *et al.* 2003). It has also been shown to prevent lipid peroxidation (Soto *et al.* 2003, Kang *et al.* 2003) and to protect cellular glutathione levels (Shafer *et al.* 2001, Soto *et al.* 2003). Other effects such as the inhibition of VEGF (Jiang *et al.* 2000), NF-κB (Saliou *et al.* 1998), and the production of iNOS (Kang *et al.* 2003) could also have implications for the development of diabetic vascular disease. Despite intense research, however, the exact mechanism(s) of action of silymarin remain uncertain and the need for further research into the usefulness of silymarin and its flavonoid isomers such as silibinin and taxifolin in the prevention and treatment of diabetes mellitus and its complications remains. The main question raised is whether silymarin acts by improving the cellular environment due to its antioxidant capacity or if it

acts through a different mechanism which is independent of its antioxidant activity all together.

1.10.3. Silymarin as a possible aldose reductase inhibitor

One proposed mechanism of action for silymarin suggests that silibinin, the most therapeutically active component of silymarin is an aldose reductase inhibitor based on findings in 14 type 1 diabetic patients (Zhang *et al.* 1993). Hyperglycaemia is known to result in sorbitol production. The inhibition of aldose reductase as the rate-limiting enzyme of this pathway has a number of positive effects with respect to alteration of NADP/NAD ratio (King *et al.* 1996) (Section 1.6.1) which is thought to be associated with the manifestation of cellular dysfunction. The ARIs will theoretically restore the redox state, leading to the restoration of protein tyrosine phosphatase activity (Evans *et al.* 2002), normalisation of glutathione levels and improved membrane integrity as a result of improved myoinositol mediated Na+/K+ ATPase activity. Other knock-on effects would include the prevention of PKC and AGE activation as well as MAPK-mediated phosphorylation of transcription factors and the subsequent alteration of gene expression patterns which contribute to changes in DNA damage and the subsequent development of vascular complications (Tomlinson *et al.* 1999).

Silymarin has also been shown to depress glutathione levels (GSH) in the liver (Soto *et al.* 2003) and inhibit iNOS production in HUVECs (Kang *et al.* 2003). Furthermore, it has been shown to improve nerve conduction velocity in diabetic neuropathy (Zhang *et al.* 1993), act as a membrane stabiliser (Fraschini *et al.* 2002) and prevent ROS-induced DNA damage (Yu *et al.* 1997). Although all of these effects were observed in a multitude of different cell types using many different methods, they seem to tentatively support the argument for the action of silymarin as an aldose reductase inhibitor, bearing in mind that silymarin also acts through other mechanisms to reduce glutathione.

1.11. Silibinin

Silibinin (3,5,7-trihydroxy-2-[3-(4-hydroxy-3-methoxyphenil)-2-hydroxymethyl-1,4-benzodioxan-6-il]-chronan-4-one) is the main polyphenolic flavonoid isomer of silymarin and is mainly thought to be responsible for the antioxidant properties of the silymarin compound (Valenzuela *et al.* 1994). Some of the identified actions of silibinin include, an anti-inflammatory action by inhibiting intrahepatic NF-κB and subsequent activation of TNF, IFN-γ, IL-2 and iNOS (Dehmlow *et al.* 1996, Schümann *et al.* 2003), iron chelation useful in the treatment of chronic iron overload such as Cooley's anaemia (Borsari *et al.* 2001), a potent antioxidant action which shows protection against oxidative injury and an increase in protein synthesis while improving lipid peroxidation and reducing triglyceride levels (Lirussi *et al.* 2002).

Studies which looked at the benefits of silibinin in diabetes revealed that it improved nerve conduction velocity in type 2 diabetic patients (Zhang *et al.* 1993) as well as preventing the onset of diabetic neuropathy by inhibiting excessive mono-ADP-ribosylation in rat cervical ganglia (Gorio *et al.* 1996). A more recent study looking at the effect of silibinin on red blood cell sorbitol levels in diabetic patients suggests that silibinin might be a powerful aldose reductase inhibitor and therefore valuable in the prophylaxis and treatment of diabetic complications (Zhang *et al.* 2002).

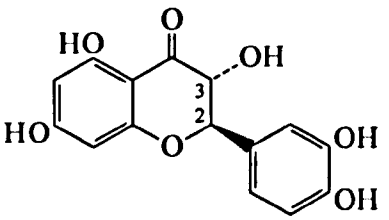
Studies looking at the effect of silibinin on blood glucose levels reported no beneficial effect. However, using the new oral formulation silybin-beta-cyclodextrin a reduction in blood glucose levels in 42 type 2 diabetic patients (with associated chronic liver disease) was observed after six months of oral administration (Lirussi *et al.* 2002). This is thought to be due to a reduction in lipid peroxidation and improved insulin activity as a result of the improved bioavailability of silibinin (SALAM Research, 2005). Silibinin is a water-soluble flavonoid which is a proven liver protectant (Dehmlow *et al.* 1996). Similar to other

flavonoids it is not well-absorbed and has been converted into a more lipid-compatible molecular complex. As such it is able to transition from a hydrophilic environment into the lipid-friendly environment of the outer cell membrane, and from there into the cell, finally reaching the blood (Kidd *et al.* 2005). Silibinin-beta-cyclodextrin is an oligosaccharide widely used in drug delivery formulation. It alters the release pattern, solubility and stability characteristics of the drug (Timmy *et al.* 2002).

More recently, silibinin has been investigated as a therapeutic agent for diseases other than hepatic disorders the main focus being on oncological and chemotherapeutic applications, such as prostate (Singh *et al.* 2006), skin (Gu *et al.* 2006) and bladder cancer (Tyagi *et al.* 2006). Numerous new effects of silibinin have been discovered, such as the modulation of drug transport via P-glycoproteins (Patel *et al.* 2004), its activity on oestrogen receptors (Crocenzi *et al.* 2001) and its effect of NF-kB and caspase regulated endothelial cell apoptosis (Yoo *et al.* 2004), where it is thought to affect the mitochondrial regulated apoptosis cascade (Mohan *et al.* 2004).

1.12. Taxifolin

Taxifolin is a polyphenolic flavanone compound with a catechol ring similar to quercetin. It is referred to as dihydroquercetin as it is the hydrophilic non-planar equivalent to quercetin (planar, hydrophobic) (Fig. 1.4; Fig. 1.6).



(2R,3R)-form

Taxifolin (dihydroquercetin) [$C_{15}H_{12}O_7$]

Fig. 1.6: Structure of taxifolin, a flavonoid compound found in silymarin and structurally closely related to quercetin. Taxifolin is also known as dihydroquercetin.
(Adapted from: Dictionary of Natural Products (DNP) on CD-Rom, Version 9:2. Copyright (c) 1982-2001 Chapman & Hall/CRC).

Taxifolin can commonly be found in citrus fruits, such as oranges and grapefruit and, like all flavonoids, it has been found to possess anti- and pro-oxidant effects. Research suggests that it is a weaker antioxidant than quercetin which is in accordance with the structural prediction criteria for flavonoid radical scavenging ability mentioned in Section 1.8 above. In addition, taxifolin binds more strongly to Fe^{2+} than quercetin (Areias *et al.* 2001), which ultimately determines its pro-oxidant activity.

Taxifolin was found to have a similar anti-oxidant activity profile to alpha-tocopherol (Tesekin *et al.* 1996), inhibiting superoxide anion production, protecting mitochondria from peroxyl radical damage and inhibiting the activation of NADPH-dependent cytochrome P_{450} reductase induced by microsomal lipid peroxidation (Haraguchi *et al.* 1996). Recently, taxifolin has been investigated by the food industry for its usefulness as a prophylactic measure against oxidative stress. It is thought to be valuable since it lacks adverse effects such as embryotoxicity, tetratogenicity, allergenicity and mutability (Tiukavkina *et al.* 1997). However, with respect to its potential pro-oxidant activity, the use of taxifolin as a dietary supplement needs to be dealt with cautiously.

1.12.1. Therapeutic properties of taxifolin

Only a limited amount of research has investigated the properties and mechanisms of taxifolin. Some studies have shown its therapeutic potential and the first suggestion for a possible signalling mechanism has been made. In 1996, an Italian research team found a cytoprotective effect for both quercetin and dihydroquercetin which could protect neurovascular structures in the skin from oxidative stress. This effect was enhanced when quercetin was paired with ascorbic acid. Taxifolin remained unaffected by the presence of ascorbic acid (Skaper *et al.* 1996). A few years later, a Korean research team also managed to show a neuroprotective effect of quercetin and taxifolin against oxidative injuries in cortical cells induced by hydrogen peroxide and xanthine / xanthine oxidase (Dok-Go *et al.* 2003).

The most interesting research, however, was carried out at the University of Berkeley in 2002 (Bito *et al.* 2002). The team investigated the effect of taxifolin on the expression of the intercellular adhesion molecule, ICAM-1, which is known to be important in mediation of inflammatory reaction. ICAM-1 is a fundamental component of all immune-related processes. It is associated with receptors of the integrin family mediating cell-cell interactions and allowing signal transduction. The paper presents evidence that taxifolin significantly inhibits tyrosine phosphorylation of JAK1 (a member of the Janus family of tyrosine kinases which is an integral component of the IFNγ signalling cascade), thereby inhibiting the INFγ-induced expression of ICAM-1. This suggests that the JAK-STAT pathway may be a potential molecular site of action for taxifolin (Bito *et al.* 2002). This is in contrast to quercetin, which inhibits ICAM-1 expression in endothelial cells by inhibiting the JNK pathway (Kobuchi *et al.* 1999). Due to the differences in molecular pathways and antioxidant capacity of the two flavonoids, it is thought that the effect observed in taxifolin is independent of its antioxidant capacity. As inflammatory changes are an underlying cause in many chronic diseases, there might be a place for the therapeutic benefits of taxifolin in their

treatment. These results raise the critical question of how much, if any, of the beneficial effect seen with silymarin in the treatment of acute and chronic liver diseases is attributable to taxifolin.

Taking current literature into consideration, there appears to be a need to further our understanding of the molecular contributions which lead to the development of endothelial changes early in the development of diabetes-related vascular complications. Since oxidative stress appears to play a crucial role in this pathology, it is important to further understand how oxidative stress contributes to endothelial changes which will inevitably provide pointers to potential new drug targets such as silymarin which can be explored in this context.

1.13. AIM AND OBJECTIVES OF STUDY

This study aims to determine the effect of hyperglycaemia and hypoxia on DNA damage in a human endothelial cell line and to determine if silymarin is able to ameliorate any observed changes.

Objectives

1. Determine the effect of hypoxia and/ or glucose on the level of DNA damage in an endothelial cell line.
2. Correlate findings with changes in cellular mitochondrial activity and the production of reactive oxygen species in response to changing glucose and oxygen concentrations.
3. Determine the role of antioxidants on the cellular parameters indicated above.

CHAPTER TWO: Materials and Methods

2.1. Materials

All chemicals were obtained from Sigma-Aldrich (Poole Dorset, UK) unless otherwise stated.

2.2. Methods

2.2.1. Human umbilical vein endothelial cell (HUVEC)

A transformed HUVEC line was purchased from the European Collection of Cell Culture (ECACC) and stored in liquid nitrogen. An immortalised cell line differs from a primary cell line, as it has acquired the ability to proliferate indefinitely (Chaudry *et al.* 2004) and serves as a model systems for studies of endothelial function, in response to changes in glucose and oxygen levels (Zanetti *et al.* 2001; Fang *et al.* 2005). HUVEC are isolated from normal human umbilical vein, cryopreserved at the end of primary culture and propagated. Cells were used from passage $\geq$ 24 onwards for the duration of the study and showed a consitent response to conditions. HUVEC are responsive to cytokine stimulation in the expression of cell adhesion molecules. These cell systems are commonly used for physiological and pharmacological investigations, such as macromolecule transport, blood coagulation, and fibrinolysis (Furie *et al.* 1988, Wojta *et al.* 1993). The cells are positive for factor VIII-related antigen expression and DiI-Ac-LDL uptake. Each batch is tested for HIV, Hepatitis B and Hepatitis C as well as mycoplasma, bacteria, yeast, fungi (Personal communication, ECACC Technical services).

2.2.2. HUVEC storage and resucitation

The cells were resuscitated by incubating the vial in a 37°C water bath under gentle agitation for 3 - 4min until completely thawed. The resulting cell suspension (1ml) was pipetted into a 25ml Sterilin (Fisher, Loughborough) and 4ml of standard cell culture media containing glucose-free Glasgow Minimum Essential Medium x 1 (GMEM), 10% (v/v) foetal calf

serum (FCS), L-glutamine (200mM), penicillin (10 000 U/ml) and streptomycin (10mg/ml) (all supplies obtained from Gibco, Paisley); (this is subsequently referred to as standard medium). All media are warmed to 37°C before use. The resulting 5ml cell suspension was centrifuged at 4000*g* for 10min (room temperature) to pellet the cells. The supernatant was discarded and the cell pellet re-suspended in 1ml GMEM cell culture media (5mM glucose, 10% (v/v) FCS) before seeding an appropriate volume of the cell suspension in 25cm^2, 75cm^2 flasks, 6 well or 96 well plates as required (Corning, Lutherworth).

2.2.3. Counting of HUVECs

The HUVEC pellet was re-suspended in 1ml of appropriate cell culture media (GMEM). A 10µl aliquot was removed and added to an Eppendorf tube containing 20µl cell culture media and 30µl of 0.4% (v/v) Trypan blue (GIBCO, Paisley). Trypan blue will stain dead or dying cells dark blue as their cell membrane is damaged and more permeable to the staining solution. The tip of the pipette containing the cell suspension was directed between the glass plate and the cover slip of a Neubauer Improved Haemocytometer and the cell suspension taken up by capillary action before obtaining a cell count within the defined areas. From the cell count the appropriate seeding density for the experimental setup was determined. The following seeding densities were used: $0.1x10^6$/ml for 96 well plates; $0.05x10^6$/ml for 2-well chamber slides; $0.1x10^4$ cells/ml for 16 well chamber slides and $2x10^4$ cells/gel for the comet assay.

2.2.4. Propagation of HUVECs

Flasks were placed in the incubator and maintained at 37°C in normoxia (5%CO_2/ 20%O_2/ 75% air, subsequently referred to as normoxia) until confluent. Further passage of the cells occurred at approximately 80% confluency. The cell culture media was discarded and cells washed three times with 5ml HBSS without Ca^{2+} and Mg^{2+} (Gibco, Paisley). Three ml trypsin 0.25% (v/v) was added to the flask (25cm^2 flasks) and replaced in the incubator for

10min. HUVECs were lifted by gentle shaking and the suspension decanted into a 25ml sterile container. Two ml of 10% (v/v) FCS was added to the cell suspension (2% (v/v)) to neutralise the action of trypsin before centrifugation at 4000g for 10min (room temperature). The supernatant was poured off and the cell pellet re-suspended and seeded into the cell culture flask as described above.

Where further treatment of the cells was required, this was carried out at approximately 70% confluence. The cell culture media was discarded and the cells washed three times using 5ml of HBSS (GIBCO, Paisley) before the addition of 5ml of standard glucose free/ serum free (GF/SF) medium. Flasks were returned to the incubator and maintained at 37°C under normoxic atmosphere (5%CO_2/ 20%O_2/ 75% air) overnight. The following morning, the media was taken off and cells washed 3 x with HBSS before the addition of standard glucose-free GMEM cell culture media, supplemented with either 5mM or 20mM filter/sterilised D-glucose. Where appropriate, the cells were supplemented with silymarin 50μM, silibinin 25μM, taxifolin 600μM, α-lipoic acid 100μM, and $PKC_{\beta II}$/EGFR Inhibitor (4,5-bis(4-Fluoroanilino)-phthalimide) 2μM (Calbiochem, Nottingham) and 2-deoxy-d-glucose (5mM/ 20mM), 3-ortho-metyhl-glucose (5mM/ 20mM), PD98059 (2'-Amino-3'-methoxyflavone, MEK inhibitor) 1μM respectively (Calbiochem, Nottingham). Where appropriate, dimethylsulfoxide was used as the solvent at a final concentration of ≤ 0.7% (v/v). Cell culture flasks were then placed in the incubator and maintained at 37°C in normoxia (5%CO_2/ 20%O_2/ 75% air) or hypoxia (5%CO_2/ 5%O_2/ 90% air subsequently referred to as hypoxia) for time periods specified in the relevant texts.

2.2.5. Cell proliferation assay

Cell proliferation was determined using the AlamarBlue™ assay as described by Fields *et al.* (Fields *et al.* 1993). The assay is based on the chemical reduction of the colorimetric growth indicator, AlamarBlue™ (Serotec, Oxford) during cellular metabolism. The extent of the dye

reduction is directly proportional to the cell proliferation rate and allows monitoring of cells over extended periods of time. Cells were seeded into a 96 well plate at a density of 0.1×10^6/ml and made up to a total volume of 200µl/per well with the appropriate cell culture media alongside the relevant media controls (Fig.2.1). Cells were incubated in standard medium in normoxia overnight to allow attachment of the cells before the addition of 20µl of AlamarBlue™ to each well. A baseline absorbance reading (t = 0h) was taken using a FL600 Microplate Fluorescence Reader (Biotek, Warminster, UK) set at a wavelength of 570nm. The plate was then returned to the incubator and analysed for up to 48h. Any alteration to the culture conditions is specified in the relevant texts.

	GMEM control	GMEM control	5mM Glucose	5mM Glucose	20mM Glucose	20mM Glucose	5mM SM	20mM SM			
	GMEM control	GMEM control	5mM Glucose	5mM Glucose	20mM Glucose	20mM Glucose	5mM SM	20mM SM			
	GMEM control	GMEM control	5mM Glucose	5mM Glucose	20mM Glucose	20mM Glucose	5mM SM	20mM SM			
	GMEM control	GMEM control	5mM Glucose	5mM Glucose	20mM Glucose	20mM Glucose	5mM SM	20mM SM			
	GMEM control	GMEM control	5mM Glucose	5mM Glucose	20mM Glucose	20mM Glucose	5mM SM	20mM SM			
	GMEM control	GMEM control	5mM Glucose	5mM Glucose	20mM Glucose	20mM Glucose	5mM SM	20mM SM			

Fig.2.1: Example of a 96 well plate layout used in an AlamarBlue™ assay. A parallel plate was used to incubate in hypoxia (5%CO_2/ 5%O_2/ 90% air).

2.2.6. Statistical analysis

All experiments were carried out in triplicate (to assess and control for intra-assay variability) and on three separate occasions (unless otherwise stated) to assess variability amongst prearations, resulting in an n-value of 3. Non-parametric analysis throughout is used

to reflect the limitations in sample size as it makes no assumptions about the sample distribution. Unlike their parametric equivalents non-prametric tests do not compare the means of the sample groups but tests equality of population medians among groups. For multiple samples groups a Kruskal-Wallis analysis of ranks is used, which is an extension of the Mann-Whitney U test used when two samples are compared.

For some data sets which show considerable variance in the control group the data was normalised to 100% (control) to reflect any consistent changes in the mean of the test group compared to control. As the process of normalisation ranks the data, the control group has zero variance making the use of a parametric test a more appropriate method of analysis as the mean of the test group is compared to 100% (Mean – 100%/ SEM = t(df n-1). A t-test was carried out for all data of this nature and where multiple sample means were compared a One way analysis of variance (ANOVA) was carried out with a post-hoc Tukey's test.

The level of significance was expressed as *$p<0.05$, **$p<0.01$, p***<0.005. No significant difference was assigned to samples where $p>0.05$.

2.3. Resolution of DNA using agarose gel electrophoresis

DNA was extracted using a commercially available column-based DNA extraction kit (GenElute™ Mammalian Genomic DNA Purification Miniprep Kit G1N-70). Sample DNA concentration was determined using spectrophotometric analysis measuring the absorbance at 260nm based on the assumption that a DNA concentration of 1mg/ml gives an absorption of 20. The purity of the DNA was determined by calculation of the ratio at 260/280nm and was routinely found to be between approximately 1.8 and 2.1.

Agarose gel electrophoresis was established in 1960s (Rapp *et al.* 1968). It is based on the separation of DNA fragments in a porous gel that acts like a filter when exposed to an electric current. Linear DNA gives a characteristic DNA banding pattern from which the fragmentation extent can be identified and the size of each individual fragment can be calculated based on the principle that there is an inverse relationship between the distance moved and the size of the DNA fragment in [log bp].

To each standardised DNA sample (50μg), 10μl of standard loading dye (15% Ficoll® 400, 0.03% bromophenol blue and xylene cyanolFF, 0.4% orange G, 10mM Tris-HCl (pH7.5) and 50mM EDTA) (New England BioLabs, Ipswich, UK) was added and mixed thoroughly. Each sample was loaded into the wells of a 1.2% (w/v) agarose gel (Biological grade) (Loughborough, UK) in 1 x TBE buffer (45mM Tris Base, 44mM Boric acid, 1.3mM EDTA (Na salt)). The gel contained 50μg/ml ethidium bromide to visualise DNA fragments after electrophoresis. Electrophoresis was carried out at 100V for 1h and DNA fragmentation analysed using a UVItec Transilluminator with camera support (Cambridge, UK). DNA fragments were sized by comparison with simultaneous resolution of DNA fragments of known size (Promega, Southampton) (Table 2.1).

This long-established method has, with time, evolved into a more sophisticated method, namely the single cell gel electrophoresis (SCGE) or comet assay (Singh *et al.* 1988). This method allows the investigation of DNA fragmentation within individual cells embedded on agarose covered microscope slides. It is widely used and can be applied to any cell population, provided they have been isolated from tissue without degradation (Lovell *et al.* 1999).

DNA markers 50-1000bp		**DNA markers 125 – 2313bp**	
Fragment size	Number of base pairs (bp)	Fragment size	Number of base pairs (bp)
1	1000	7	23,130
2	750	8	9,416
3	500	9	6,557
4	300	10	4,361
5	150	11	2,357
6	50	12	2,057
		13	564
		14	125

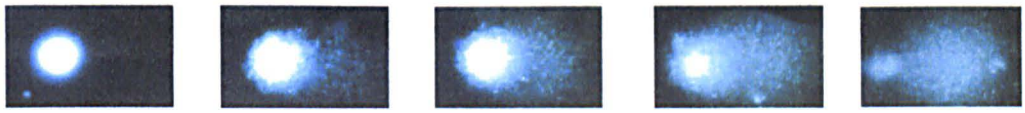
Table 2.1: DNA markers used for the determination of DNA fragments if size unknown.

2.4. Comet Assay

The single cell electrophoresis assay is a sensitive method for detecting DNA strand breaks and was first developed by Ostling and Johanson in 1984. As the original protocol only allowed the detection of double stranded DNA breaks, Singh *et al.* modified the protocol four years later to include alkaline conditions (pH > 13) (Singh *et al.* 1988). The comet assay enables the supercoiled DNA to relax and unwind, allowing the investigation of DNA fragmentation within individual cells through electrophoresis. The DNA of each cell migrates through the gel of an agarose covered microscope slide giving a characteristic pattern that correlates with the degree of fragmentation.

HUVECs were cultured as described above (Section 2.2). After 1, 6 and 24h incubation the cells were trypsinised as previously described. Cells were lifted by adding 1ml of standard medium, which was gently pipetted to avoid exogenous cell damage.

A 1ml cell suspension containing $1x10^5$ cells/ml was transferred to an Eppendorf tube and placed on ice prior to centrifugation at 200*g* at 4°C for 10min. The supernatant was poured off and the cell pellet re-suspended in ice-cold PBS. Cells were washed a further two times. A positive DNA damage control sample was prepared using hydrogen peroxide (100μM H_2O_2 in phosphate buffered saline; PBS) for 5min. HUVECs ($2x10^4$ cells/gel) were suspended in 1% (w/v) low melting point agarose (LMP); (Invitrogen, Paisley) in dH_2O (37°C) and pipetted onto pre-coated (1% (w/v) agarose in dH_2O) microscope slides (Fisher, Loughburough). Each gel was covered with a cover slip (18x18mm, Fisher-Scientific, Loughburough). The agarose gels were allowed to set in the fridge for 5min, the cover slips were removed and the slides immersed in lysis solution (2.5M NaCl, O.1M EDTA, 10mM Tris, 10mM NaOH pH10, 1% (v/v) Triton X-100) at 4°C for at least 1hr. Cells were washed with dH_2O after lysis to prevent NaCl residues affecting the electrophoresis conditions. Slides were then placed in a 210mm wide electrophoresis tank (Thistle Scientific, Glasgow) containing cold (4°C) electrophoresis buffer (0.3M NaOH, 1mM EDTA) for 40min. Electrophoresis was carried out at 21V for 30min at 4°C. Slides were washed in a neutralising solution (0.4M Tris, conc. HCl to pH 7.5) (Fisher-Scientific, Loughburough) for 5 min (repeated three times) before staining with 20μl of 1μg/ml DAPI (4'6-diamidine-2-phenylindol dihydrochloride) immediately before scoring (22 x 22mm cover slip) (Fisher-Scientific, Loughburough). Fluorescent nucleoids were scored visually using a Leica DML fluorescent microscope. Prior to quantitation the identity of the slides were coded by an independent individual and only revealed after the scoring had been completed. One hundred comets from each gel (selected at random) were assigned a number between 0 (undamaged) and 4 (maximal damage) (Fig. 2.2). The total score for 100 comets could therefore range from 0 (all undamaged) to 400 (all maximally damaged) and is expressed as arbitrary units (au).



0 1 2 3 4

Fig. 2.2: Illustration of different comet types and visual scoring allocation (Collins *et al.* 2004).

2.4.1. Statistical analysis

Each experiment was performed in triplicate and repeated on three separate occasions. The test distribution was assumed not to be normal to account for the limitations in sample size. Statistical significance was determined using a Kruskal-Wallis test or Mann-Whitney U test against the test hypothesis (H_0) that there are no differences between the means of the samples. Where $p < 0.05$ the H_0 must be rejected and the H_A (there is a difference between the means of the samples) accepted. Where data was normalised (100%) a Student's t-test (Mean – 100%/ SEM) and/or ANOVA (with Tukey's post-hoc test) was carried out. The level of significance is expressed as $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$. No significant difference was assigned to samples where $p > 0.05$.

2.5. Mitochondrial staining

2.5.1. MitoTracker Green FM and RedoxSensor Red CC-1 fluorescent staining of HUVECs

Fluorescent dyes are widely used to monitor changes in mitochondrial function. MitoTrackerGreen FM (5μM in DMSO) (Molecular Probes, Paisley, UK) is a mitochondrial specific green fluorescent dye which allows the localisation of mitochondria within cells. RedoxSensor Red CC-1 (1μM in DMSO) (Molecular Probes, Paisley, UK) is a redox sensitive red fluorescent dye which allows the determination of the redox state of the cell. Any red fluorescent dye present in the mitochondria indicates the proliferative state of the

cell, while accumulation of the dye outside the mitochondria indicates lysosomal staining of quiescent cells.

HUVECs were cultured on round plastic cover slips (40 x 40mm supplied by Intracel, Royston) using the method described in Section 2.2. After 24h incubation with the standard cell culture medium MitoTracker Green FM and RedoxSensor Red CC-1 fluorescent dye(s) were added to the medium and the cover slips returned to the incubator for 10min. The media was then taken off and the cover slips washed three times with PBS to remove any excess dye before fitting the cover slips into a flow through chamber (Intracel, Royston) which allows the continuous perfusion of HUVECs with the appropriate cell culture medium. The medium was perfused at 0.1ml/min (37°C) while fluorescent dye(s) distribution was determined using a Leica DML fluorescent microscope and Leica QWin imaging software. MitoTracker Green FM was imaged using an excitation and emission wavelength of 490/515nm while RedoxSensor Red CC-1 utilised an excitation /emission wavelength of 546/590nm filter. A 400x magnification was used throughout.

For cells grown and analysed in hypoxia, the standard cell culture medium substituted with 5mM or 20mM filter sterilised D-glucose used with the continuous perfusion system was bubbled with Nitrogen gas (N_2) and its pO_2 concentration monitored using a pO_2 meter (Strathkelvin Instruments, Model 782, Glasgow). When the pO_2 concentration had dropped to 5%, 1mM sodium sulphite anhydrous (Na_2SO_3) was added to induce chemical hypoxia and maintain the pO_2 concentration of 5% throughout the experiment. The pH of the culture media was checked using a pH meter (Mettlet Toledo, Leicter) to ensure that neither the N_2 nor the addition of Na_2SO_3 contributed to a pH change which could influence the results. The pH of the cell culture media was recorded to be 7.5 before and after the addition of Na_2SO_3. Na_2SO_3 has been shown to induce $O_2^{\cdot}$ in human neutrophils within 5min of exposure while affecting gene expression without altering any biological processes after long periods of

substitution (Labbé *et al.* 1998). Cells were monitored for any morphological changes for the duration of the short-term exposure.

2.5.2. Quantitation and Statistical analysis of fluorescence intensity

Image analysis software (ImageJ freeware) was used to capture and analyse images obtained using MitoTracker Green FM (5μM in DMSO) and RedoxSensor Red CC-1 (1μM in DMSO) fluorescent staining (using Leica QWin software). The background of all pictures was subtracted before measuring cell size using the "freehand selection" tool to identify the circumference of each individual cell. The output detailed the circumference of the cell expressed as number of pixels and the total fluorescence intensity of MitoTracker Green FM expressed as mean grey level intensity. The fluorescence intensity of RedoxSensor Red CC-1 in the same area was measured. Mean cell size values (pixel) were calculated and any values above and below two standard deviations (SDev) identified were excluded. In order to standardise fluorescence intensity (mean grey level per unit area) for both MitoTracker Green FM and RedoxSensor Red CC-1 fluorescence, values were adjusted for cell size before calculating the mean and standard error of the mean (SEM) for all samples.

All experiments were carried out in triplicate with a total of 50 cells per sample. Analysis was carried out by converting the mean grey level intensity (and pixel) into percentage values to illustrate the relative percentage change between samples (inter-sample variation) compared to 5mM glucose normoxia (100%). The test distribution was assumed not to be normal to account for the limitations in sample size. Statistical significance was determined using a Kruskal-Wallis test or Mann-Whitney U test against the test hypothesis (H_0) that there are no differences between the means of the samples. Were $p< 0.05$ the H_0 must be rejected and the H_A (there is a difference between the means of the samples) accepted. Where data was normalised (100%) a Student's t-test (Mean – 100%/ SEM) and/or ANOVA with Tukey's post-hoc test was carried out. The level of significance is expressed as *p<

0.05, **p< 0.01 or ***p< 0.001. No significant difference was assigned to samples where p > 0.05.

2.5.3. Co-localisation of fluorescent staining

Images showing the distribution of MitoTracker Green FM (5μM in DMSO) and RedoxSensor Red CC-1 (1μM in DMSO) fluorescent dye(s) in identical cells (obtained using Leica QWin software) were superimposed using ImageJ freeware. Co-localised staining was evident as yellow staining and occurs in proliferating cells while accumulation of RedoxSensor Red CC-1 stain outside the mitochondria accumulates in lysosomes in quiescent cells. To confirm the distribution of both dyes the images were converted to Stacks (ImageJ freeware) and identical cross-sections analysed after subtraction of the background. Pixel values obtained for each cross section were normalised to 100 pixels which allows both sets of values to be superimposed and allows the identification of any shift in RedoxSensor Red CC-1 staining from mitochondrial to lysosomal.

2.5.4. Mitochondrial measurement

Images of HUVECs loaded with MitoTracker Green FM were coded by an independent individual and selected at random. A standardised section (6mm length) obtained from a 400x magnified calibration bar was selected from each picture (at random) and individual mitochondria measured using the "freehand line selection" tool in ImageJ freeware. The output obtained was given as "length - no of pixel". A total of six cells per sample (selected at random) were analysed. The test distribution was assumed not to be normal to account for the limitations in sample size. Statistical significance was determined using a Kruskal-Wallis test or Mann-Whitney U test. Where data was normalised (100%) a Student's t-test (Mean – 100%/ SEM) and/or ANOVA with Tukey's post-hoc test was carried out. The level of

significance is expressed as *p< 0.05, **p< 0.01 or ***p< 0.001. No significant difference was assigned to samples where p > 0.05.

2.6. Superoxide radical activity measurement

2.6.1. HUVEC preparation for use with SOD Assay Kit -WST

Cells ($9x10^5$) were grown and treated as described in Section 2.2. After 24h incubation with standard medium cells were trypsinised (Section 2.2.4), harvested and centrifuged at 2000*g* for 10min at 4°C. The supernatant was discarded and the cells washed four times by adding 1ml PBS and centrifuging at 1000*g* for 10min at 4°C. After removal of the PBS HUVECs were broken up using the freeze-thaw method (-20°C for 20min, 37°C for 10min, repeated x 2). One ml of PBS was added to the cell pellet and cells centrifuged at 10 000*g* for 15min at 4°C. The supernatant was carefully taken off and 10µl of a 1mM potassium cyanide (KCN) in dH_2O solution (Fisher-Scientific, Loughburough) was added to block the cytoplasmic fraction of Cu/Zn SOD. Twenty µl of the resultant supernatant was removed and added to a 96 well plate.

A commercially available superoxide radical activity testing kit (SOD Assay Kit -WST, NBS Biological, Huntington) was used to measure superoxide radical formation. The kit is based on the reduction of water-soluble tetrazolium salts (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) by superoxide radicals resulting in a colorimetric reaction. The amount of coloured product formed is proportional to the amount of superoxide radical present (Ukeda *et al.* 2005). All solutions where made up according to kit instructions. A 20µl volume of the sample solutions and all control solutions were added to the 96 well plate in triplicate. This was followed by 200µl of WST working solution (NBS Biological, Huntington) and 20µl of enzyme solution (0.1M xanthine oxidase) and incubated

for 20min and kept at 37°C in either normoxia or hypoxia, as previously described. The level of activity was determined by measurement of absorbance at 450nm using a FL600 Microplate reader (Biotek, Warminster, UK).

2.6.2. Statistical analysis

The mean absorbance values (n = 3) were converted into a percentage value to illustrate the relative change between samples compared to total superoxide radical formation in 5mM glucose normoxia (100%) samples. The normalised data was analysed using either a Student's t-test (Mean – 100%/ SEM) and/or ANOVA with Tukey's post-hoc test was carried out. The level of significance is expressed as *$p< 0.05$, **$p< 0.01$ or ***$p< 0.001$. No significant difference was assigned to samples where $p > 0.05$.

2.7. Hypoxia inducible factor-1α (HIF-1α) antisense

In order to determine the role played by HIF-1α in the induction of DNA damage, a HIF-1α antisense oligonucleotide probe was used. HUVECs were seeded into a 96 well plate at three different concentrations ($0.1x10^5$, $0.5x10^4$, $0.1x10^4$ cells/ml) and the plate analysed at intervals of 24h, 48h and 72h in order to determine the optimal seeding density of HUVECs onto 16 well chamber slides and this was used in all subsequent antisense experiments. Absorbance values were obtained for three independent samples each with seven replicates.

HUVECs were prepared as previously outlined and seeded into a 16 well chamber slide using standard media. A fluorescently labelled control oligonucleotide (2μM) (Biognostik®, Göttingen) was added to the cells for periods of 1, 2, 4, 8, 24 and 48 hour intervals. After the incubation was complete, the media was removed and the slides were fixed using 4% paraformaldehyde $(CH_2O)_n$ /GMEM for 5min, before dehydrating slides using a graded

series of ethanol (C_2H_6O) (70% - 100%) (Fisher-Scientific, Loughborough). The uptake time of the oligonucleotide was identified by detecting the intensity of the fluorescent FITC (450/515nm; excitation/emission) signal using a Leica DML fluorescent microscope. The efficacy of HIF-1α antisense to reduce protein levels in the HUVECs was determined by immunocytochemical detection of HIF-1α protein following the addition of HIF-1α antisense (2μM); (Biognostik®, Göttingen) for 2, 8 and 24hours.

2.7.1. Immunocytochemistry

Following incubation of HUVECs with HIF-1α antisense (2μg) for 24h, cells were fixed in 4% paraformaldehyde for 5 minutes and frozen at -20°C until required for subsequent immunocytochemical detection of HIF-1α protein. Prior to use, the slides were removed from the freezer and allowed to thaw at room temperature for 60 minutes. This was followed by a further 5 minute incubation in Tris buffered saline pH 7.6 (TBS) (0.05M tris/HCl, 0.15M NaCl Buffer, to 1L in dH_2O). This latter process was repeated twice. A 1:200 dilution of primary antibody (HIF-1α); (Affinity Bioreagents, Cambridge, UK) in TBS was added to each cell chamber section and left at 4°C overnight. The following morning, the excess liquid was removed and the slides washed in TBS for a further 3 x 5 min at room temperature. A 1:300 dilution of secondary antibody (rabbit α-mouse biotinylated) (ABR, Cambridge, UK) was prepared in TBS and added to respective cell samples. Cells were again washed in TBS for 10min and this was repeated twice. The StreptABComplex (DAKO Ltd, Cambridgeshire, UK) was prepared as recommended by the manufacturer, added to the samples and incubated for 30min. The sections were washed in TBS for 3 x 10min followed by the addition of 50μl of substrate (20mM naphtol AS-MX disodium salt, 18mM Fast red, 14mM levamisol) prepared in veronal acetate buffer (48mM sodium acetate dehydrate, 8mM Sodium diethyl barbiturate, 0.1M HCL to pH 9.2) for 1 to 3min. Slides were washed in water to remove any non-specific staining. Slides were again washed in dH_2O, allowed to air dry and mounted in

aqueous permanent mounting medium (DAKO Ltd, Cambridgeshire). Analysis of the staining intensity and distribution was carried out using a Leica DML light microscope.

2.8. Reverse Transcriptase - Polymerase chain reaction (RT-PCR)

HUVECs were grown and treated as described in section 2.2. RNA was isolated using Qiashredders and the Qiagen RNeasy kit (Qiagen, Crawford, UK) from the cells that had previously been incubated under standard conditions and from cells incubated in the presence of HIF1α antisense, as previously described. After 24h incubation, cell culture flasks were placed on ice and washed three times using sterile PBS before the addition of 600μl of RLT lysis buffer (Qiagen, Crawford, UK) containing 10μl mercaptoethanol (0.1μM)/1ml RLT buffer. The resulting cell lysate was collected using a cell scraper and any clumps dispersed by gentle pipetting. All solutions used in the isolation of RNA were treated with diethylpyrocarbonate (DEPC) 0.1% prior to use to remove any endogenous RNases.

A fixed volume of 450μl of the collected cell lysate was added to each Qiashredder column (Qiagen, Crawford) and centrifuged for 2min at 8000*g* to homogenise the lysate. This was followed by the addition of 70% ethanol (Fisher-Scientific, Loughborough) to the homogenised lysate before transferring 600μl of the homogenate to each RNeasy mini column. A further 600μl of RW1 buffer was added to the column and centrifuged for 15sec at 8000*g* before transferring the column into a new collection tube. Cells were washed by adding 500μl of RPE buffer followed by centrifugation at 8000*g* for 15sec. After discarding the flow through, a further 500μl of RPE buffer was added to the column and centrifuged at 8000*g* for 2min to dry the RNeasy membrane. Again the column was placed into a new collection tube and centrifuged at maximum speed for a further 1min. Finally 30μl of RNase-free water was directly pipetted onto the RNeasy membrane and the column centrifuged for

1minute at 8000*g*. The purity of the sample was checked by running a 10µl sample of RNA through a 1.2% (w/v) agarose electrophoresis gel. In all samples, 18S and 28S ribosomal RNA (rRNA) bands were evident, with no evidence of RNA degradation.

2.8.1. RNA Quantitation

RNA sample concentration was determined spectophotometrically, assuming that one optical density unit is equivalent to 40µg/ml RNA. The purity of the RNA was determined by measuring the absorbance at 280nm and determining the 260nm/ 280nm ratio. This was routinely found to be within the range 1.9 - 2.1. The RNA was further concentrated by the addition of 3 times the volume of ice cold ethanol (100%) and sodium acetate at 52mM (pH 5.2) followed by incubation at -20°C for at least 2h. This was followed by centrifugation at 8000g for 5min before washing the pellet twice using 70% (v/v) DEPC treated ethanol and centrifuged at 8000g. The pellet was allowed to air dry before adding 20µl of RNAse free water and the RNA stored at -20°C prior to use.

2.8.2. Preparation and amplification of cDNA

To each sample tube (thin walled PCR tube) containing 5µg of re-precipitated RNA 1mg/ml of oligo – deoxythymidine (13mer) (Sigma Genosys, Poole) was added and incubated at 70°C for 10min. The PCR tubes were placed on ice and all sample droplets around the edge of the tube were collected using a pulse-spin. To each tube 4µl of reaction buffer, 25mM of nucleotide mix (Roche, Burges Hill, UK), 0.1M DTT and 100units of reverse transcriptase (Qiagen, Crawley) were added and mixed gently. The PCR reaction mixes were incubated at room temperature for 10min before placing the PCR tubes in the Thermocycler and incubating them at 42°C for 50min followed by 95°C for 5min when the reaction was completed. To each tube 80µl of DEPC treated water was added and samples were stored at -20°C prior to use.

Primer sequences specific for the amplification of HIF-1α were determined using the database facilities of the Human Genome Mapping Project and the specificity was determined by BLAST search analysis. To 5μl of purified cDNA, 5μl (10mM concentration) of HIF-1α primer (Sigma Genosys, Poole) or β_2-Microglobulin primer (Sigma Genosys, Poole) (Table 2.2) were added. Samples were placed in the Thermocycler (1cycle, 94.5°C, 2min; 33cycles, 55°C, 1min; 1cycle, 72°C, 2min). The samples were stored at -20°C prior to resolution using agarose gel electrophoresis if not used immediately.

Primer	Sequence	Product size (bp)
HIF-1α	5' TGCTTCACTCATCCCATTCA 3' 3' TTTTGCTCTTTGTGGTTGGA 5'	385
β2-Microglobulin	5' CCTTGAGGCTATCCAGCGTACTCC 3' 5' CCATGATGCTGCTTACATGTCTC 3'	321

Table 2.2: DNA sequences of oligonucleotide PCR primers.

A 1.2% (w/v) ultrapure agarose (Biological Grade Agarose) gel in 1 x TBE buffer was prepared. Ethidium bromide (50μg) was added to the resulting solution to visualise DNA fragments in the gel. Loading dye (2.5μl) and dH_2O (5μl) was added to each 5μl sample of amplified cDNA and loaded into the wells. DNA fragments of known size (Promega, Southampton, UK) were added as reference markers which act as controls allowing the size determination of amplified DNA fragments (Table 2.1). Electrophoresis was carried out at 100V for 1hr. Following amplification the size of the resulting fragments was determined by comparison with the known standards. The specificity of the amplification was subsequently confirmed by sequencing (Aberdeen University Sequencing Unit) and shown to be > 98% homologous with predicted sequence (Wang *et al.* 1995).

2.9. Capillary Zone Electrophoresis

2.9.1. Apparatus

Capillary electropherograms were obtained using a Hewlett-Packard 3D capillary electrophoresis system (Waldborn, Germany). Electrophoresis and sweeping experiments were performed in fused silica capillaries 50μm x 65cm obtained from Composite Metal Services (Ilkeley, UK). The detection wavelength was set at 200 and 280nm.

2.9.2. Sample preparation

Stock solutions of 400mM sodium dodecyl sulphate (SDS) ((BDH, Poole) and 250mM di-sodium hydrogen phosphate (DSHP), pH 2; (Fisher Scientific, Loughborough) were made up in water and diluted as required for the preparation of running buffer. The running buffer was prepared by diluting 2.0 ml of SDS and 2ml of DSHP with 2.5ml of HPLC grade methanol (0.1M) (Fisher Scientific, Loughborough) and making up to a final volume of 10ml. Stock solutions of silymarin, taxifolin and silibinin (1M) were made up in HPLC grade methanol and further diluted in 50mM DSHP to give secondary stock solutions of 1:25 silymarin, 1:25 silibinin and 1:625 taxifolin.

2.9.3. Capillary electrophoresis procedure

Prior to each analysis, the capillary was flushed with 0.1M sodium hydroxide for 2min followed by the running buffer (80mM SDS; 50mM DSHP; 25% Methanol) for 3min. The sample was then injected for 300 seconds using pressure (50mbar) and a voltage of 30kV was applied until all peaks were separated (30min). Calculation of individual components could then be determined using the area under the curve (AUC).

CHAPTER THREE: Effect of glucose concentration and oxygen tension on DNA integrity in human umbilical vein endothelial cells

3.1. Hypothesis

Changes in glucose concentration and oxygen tension have an effect on the DNA integrity of human umbilical vein endothelial cells.

3.2. Introduction

Oxidative stress plays a significant role in the development of diabetic complications with ROS-related DNA damage levels elevated in both type 1 and 2 diabetic patients (Dandona *et al.* 1999). It has been shown that high glucose (20mM) prolongs cell cycle traversal in cultured human macrovascular endothelial cells (Lorenzi *et al.* 1987), which is important when investigating the contribution of high blood glucose levels in the development of vascular complications. Changes in cellular oxygen tension also have to be taken into account. Aerobic life is dependent on molecular oxygen for ATP generation and cell viability (Michiels *et al.* 2002), but the range at which optimal oxygen tension is achieved is very narrow. An increase in oxygen tension leads to a simultaneous increase in ROS and increased DNA damage, while a reduction in cellular oxygen tension impairs energy availability, thereby impacting on cell viability (Michiels *et al.* 2002). Until recently, both events (hyperglycaemia and hypoxia) have been investigated as separate mechanisms in the pathogenesis of diabetes mellitus.

If we are to fully understand the processes which lead to the development of these detrimental vascular changes, we need to take both factors into consideration. This notion supported by two recent publications confirms that both hypoxia and hyperglycaemia are two separate events which could have a potentially additive effect on the molecular changes in microvascular endothelial cells (Catrina *et al.* 2004, Nyengaard *et al.* 2004).

Thus, the effect hypoxia and hyperglycaemia on cell proliferation and endothelial DNA damage over a 24h period were examined.

3.3. Materials and Methods

The effect of glucose concentration and oxygen tension on cell growth was determined using the Alamar blue proliferation assay (Fields *et al.* 1993) (Section 2.2.5). Any effect of these conditions on DNA integrity was determined using agarose gel electrophoresis (Section 2.3) and the comet assay (Section 2.4). HUVECs challenged with equimolar concentrations of glucose and mannitol (latter used to control for changes in osmolarity) for 24h were cultured as described in Section 2.2.4 and their DNA extracted as described in section 2.3. A further challenge of HUVECs with 1mM H_2O_2 for 24h was included as positive control. This high concentration of H_2O_2 was used to induce maximal DNA damage (Astley *et al.* 1999; Lockett *et al.* 2006). Comet scores are expressed as arbitary units (au) unless otherwise stated.

Each experiment was performed in triplicate with three internal replicas per sample. The test distribution was assumed not to be normal to account for the limitations in sample size. Statistical significance was determined using a Kruskal-Wallis test or Mann-Whitney U test against the test hypothesis (H_0) that there are no differences between the means of the samples. Were $p< 0.05$ the H_0 must be rejected and the H_A (there is a difference between the means of the samples) accepted. Where data was normalised (100%) a Student's t-test was used (Mean – 100%/ SEM). The level of significance is expressed as $*p< 0.05$, $**p< 0.01$ or $***p< 0.001$. No significant difference was assigned to samples where $p > 0.05$.

3.4. Results

3.4.1. Effect of glucose concentration and oxygen tension of HUVEC proliferation

The effect of glucose concentration and oxygen tension on HUVEC proliferation was determined over a period of 24h. The rate of cell growth was significantly increased in the

presence of high glucose concentration (20mM; 0.30 ± 0.006) when compared to growth in 5mM (0.20 ± 0.007) ($p=0.004$) and further increased with a reduction in oxygen tension (20mM H) (0.40 ± 0.007) ($p=0.003$) (Fig.3.1) when compared to respective glucose controls.

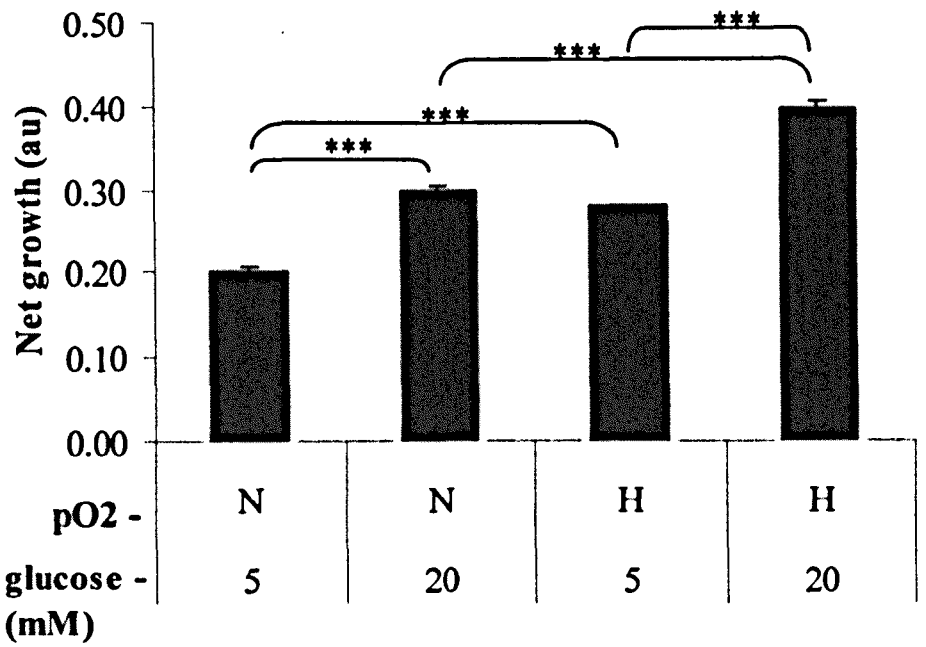


Fig 3.1: Effect of 5mM & 20mM glucose, normoxia and hypoxia on cell proliferation of human umbilical vein endothelial cells as determined by Alamar blue assay after 24h. Significance of conditions on cell growth was determined using a Kruskal-Wallis test and level of significance at $p < 0.001$ is denoted ***.

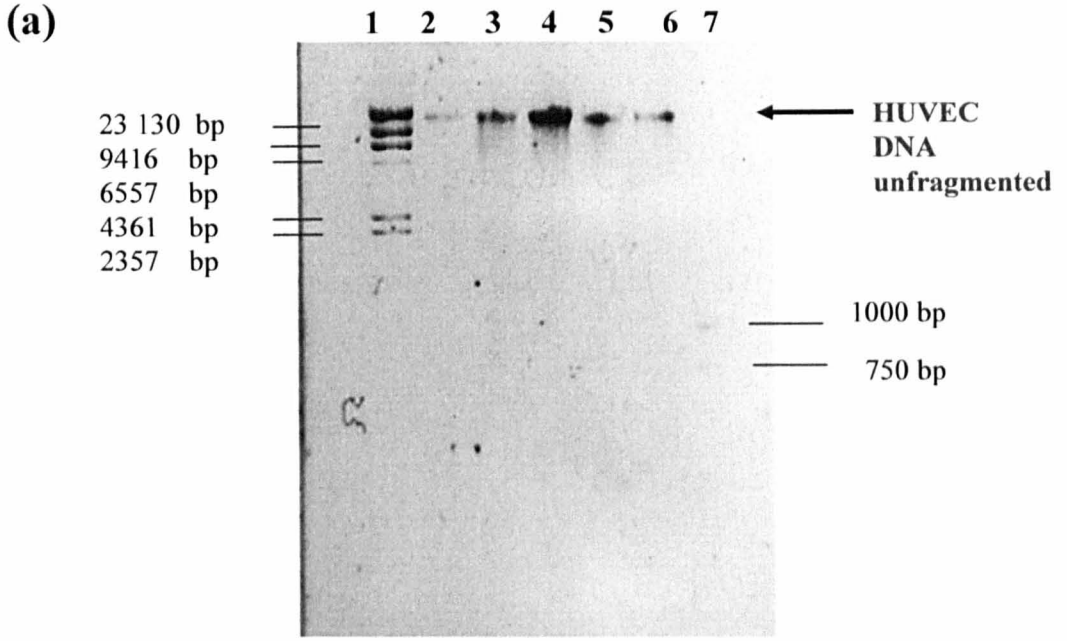
3.4.2. Effect of glucose concentration and oxygen tension on DNA integrity

DNA was extracted and purified from HUVECs and 50μg resolved by agarose gel electrophoresis. From visual inspection, there was no evidence of DNA degradation in normoxia (Fig.3.2a), while DNA damage was evident in HUVECs cultured in 20mM glucose in hypoxia for 24h as shown by intense fluorescence in regions of gel corresponding to < 500bp (Fig.3.2b). Samples incubated with equimolar concentrations of mannitol show no comparable DNA damage.

The comet assay was used to examine in more detail the effect of glucose concentration and oxygen tension on DNA fragmentation. Blinded analysis showed that HUVECs exposed to 1mM H_2O_2 presented with a high score in the comet assay (343 ± 9) which were significantly higher than the control (37 ± 9) ($p < 0.001$) (Fig.3.3b). Fig.3.3a illustrates comets representative of this score. H_2O_2 treatment was used as a positive control and incorporated in every subsequent experiment. The positive control routinely showed high levels of DNA damage ($p > 0.05$). The data presented is representative of three separate experiments with difference between experimental means of 5.4 (au).

DNA damage that may be attributed to the processing of the cells was determined using DNA purified from cells prior to incubation and is subsequently referred to as t = 0h. There was no significant increase in DNA fragmentation between cells at t = 0h and cells incubated in 5mM glucose normoxia for 24h. Hypoxic conditions increased DNA damage levels significantly when compared both to t = 0h (45 ± 3.6) ($p = 0.003$) and normoxia (70 ± 5) ($p=0.004$) at an equivalent time period (Fig.3.4). DNA damage values stayed below a total score of 100 (au) indicating minimal overall DNA damage. Inter-sample variation was small (t = 0h: 60±2.9; t=24h N: 59±3.7; t=24h H: 84± 5.4).

Fig. 3.2: Agarose gel showing DNA isolated from HUVECs exposed to a range of glucose concentrations and oxygen tension. The DNA was visualised using UV light. From left to right **(a)** Normoxia (5%CO_2/ 20%O_2/ 90% air): 1. DNA marker 125-2313bp, 2. t = 0h, 3. 5mM glucose, 4. 20mM glucose, 5. 5mM mannitol, 6. 20mM mannitol, 7. DNA marker 50-1000bp. **(b)** Hypoxia (5%CO_2/ 5%O_2/ 90% air): 1. DNA marker one 125-2313bp, 2. t = 0h, 3. 20mM mannitol, 4. 5mM mannitol, 5. 20mM glucose, 6. 5mM glucose, 7. DNA marker two 50-1000.


(a)
1 2 3 4 5 6 7
23 130 bp
9416 bp
6557 bp
4361 bp
2357 bp
HUVEC
DNA
unfragmented
1000 bp
750 bp

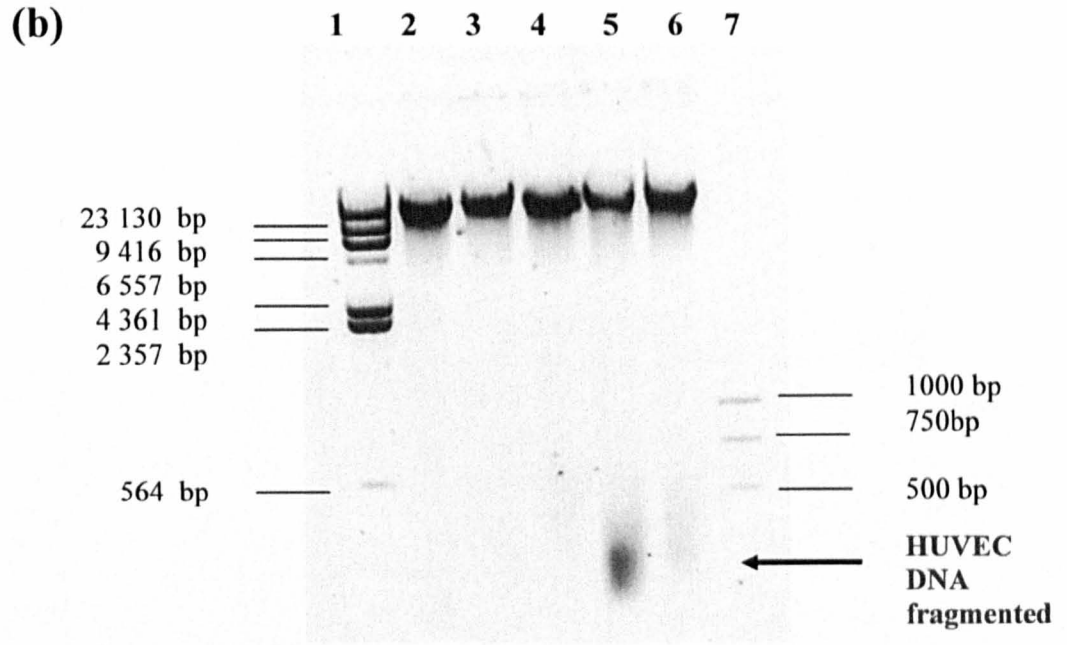
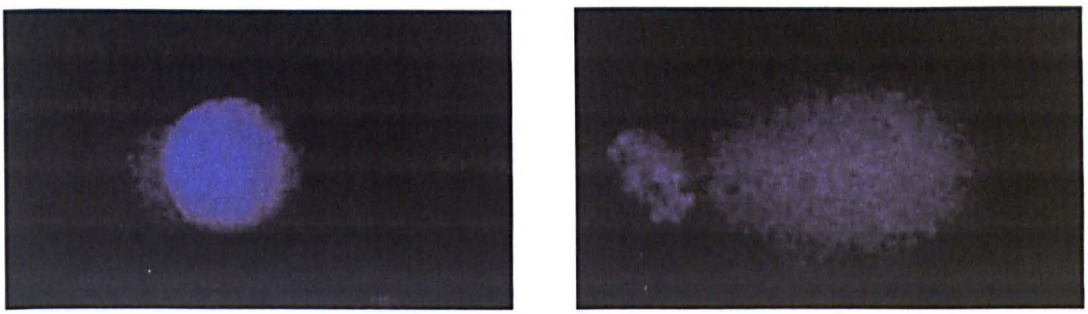
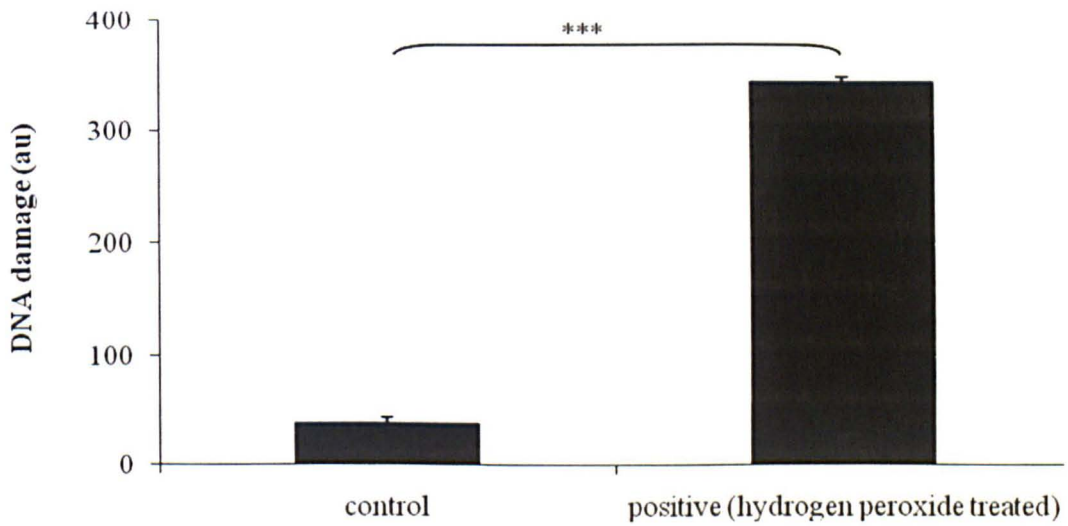

(b)
1 2 3 4 5 6 7
23 130 bp
9 416 bp
6 557 bp
4 361 bp
2 357 bp
564 bp
1000 bp
750bp
500 bp
HUVEC
DNA
fragmented

Fig 3.3: Effect of H_2O_2 (1mM) on HUVEC DNA damage using the comet assay. **(a)** Illustration of representative comets for minimal and near to maximal DNA damage as seen with the positive control using the comet assay. **(b)** quantitative analysis of the DNA damage induced by H_2O_2 cells. The results are expressed as mean ± SEM from three separate experiments (n = 3) (Mann-Whitney U test: ***$p < 0.001$).

(a)



(b)


400
300
200
100
0
DNA damage (au)
***
control
positive (hydrogen peroxide treated)

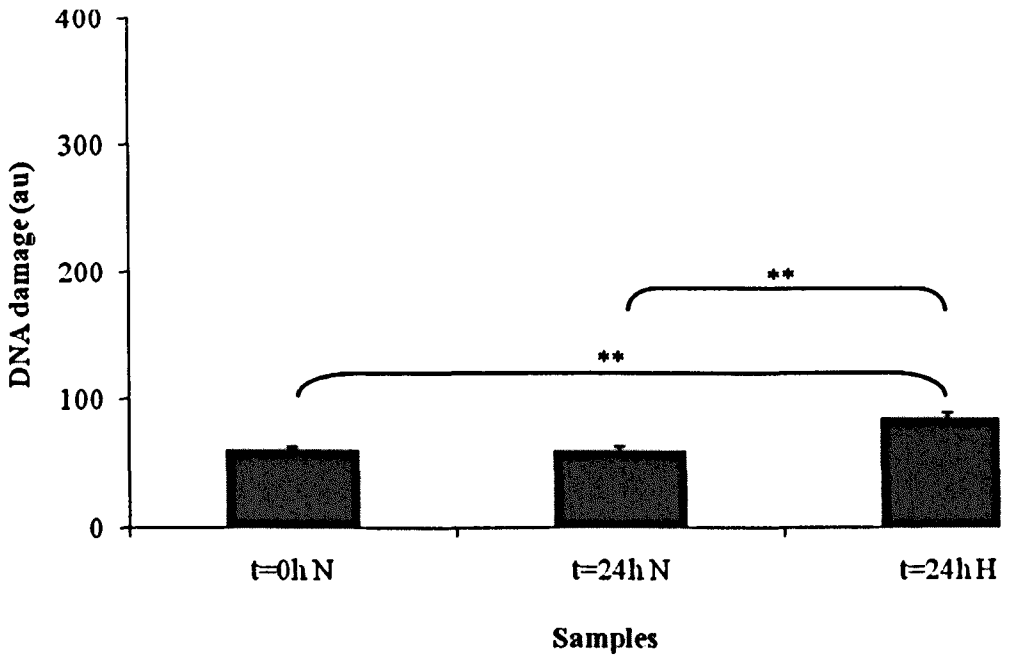


Fig. 3.4: DNA damage in HUVECs for 24h. Cells were cultured in 5mM glucose normoxia or hypoxia for 24h and analysed using the comet assay. The results are expressed as mean ± SEM from three separate experiments (n = 3) (Kruskall-Wallis test: $^{**}p < 0.01$).

A change in DNA integrity was determined in response to high glucose and low oxygen culture conditions for 24h. High glucose concentration (20mM) significantly increased DNA damage in HUVECs (119±7) when compared to 5mM glucose in normoxia (70±5) ($p<0.001$) (Fig.3.5). Furthermore, cells incubated in high glucose conditions (20mM) in hypoxia showed a further significant increase in DNA damage when compared to normoxia (237±15) ($p < 0.001$). The greatest increase in DNA damage can be seen in cells incubated in both high glucose (20mM) and low oxygen tension suggesting an additive effect of DNA damage by these conditions (237±15) ($p < 0.001$).

To identify the effect of time on the observed DNA damage DNA integrity was analysed after 24h & 48h in cells incubated in hyperglycaemic conditions (20mM). There was a significant increase in DNA damage both in cells incubated in normoxic (166±2) as well as hypoxic (200±1) conditions after 48h when compared to 24h ($p < 0.001$) (Fig.3.6). Further, the effect of re-oxygenation on cells exposed to hypoxic conditions for 24h was determined by incubating the cells in normoxic conditions for a further 24h (199±9) ($p < 0.001$). The DNA damage observed was not significantly different from the level of DNA damage found in HUVECs exposed to hypoxic conditions for a total of 48 h.

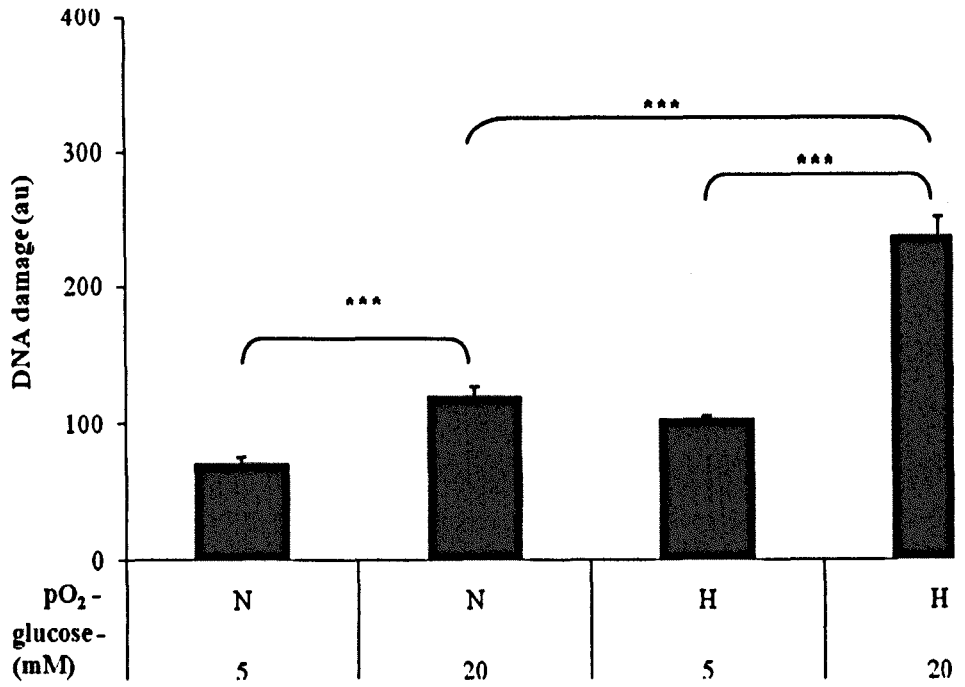


Fig. 3.5: DNA damage in HUVECs due to glucose concentration (5mM & 20mM) and/ or oxygen tension (N = normoxia and H = hypoxia) as determined by the comet assay. The results are expressed as mean ± SEM from three separate experiments (n = 3) (Kruskal-Wallis test: ***$p < 0.001$).

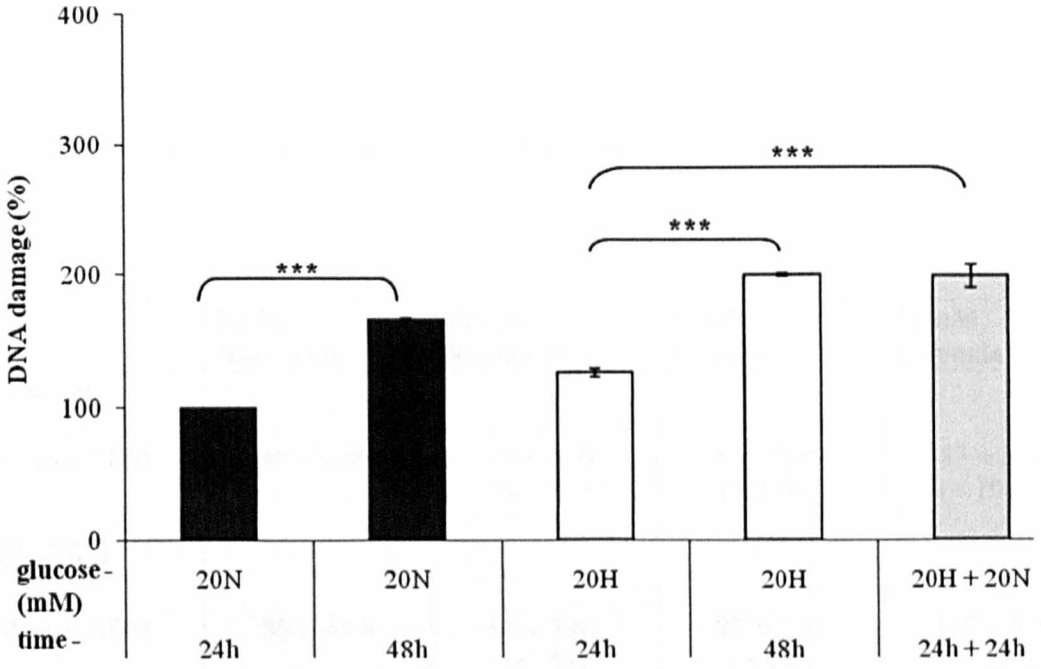


Fig. 3.6: Effect of incubation time and re-oxygenation (N = normoxia and H = hypoxia) on HUVEC DNA damage. Cells were stepped down overnight in glucose free/serum free media prior to incubation and DNA damage is determined by the comet assay. The change in DNA damage is expressed relative to control (20N = 100%). The results are expressed as mean ± SEM from three separate experiments (n = 3) (Student's t-test: ***$p<0.001$).

To determine the significance and the specificity of glucose mediated DNA damage, HUVECs were treated with equimolar concentrations of mannitol (5mM & 20mM) and compared to glucose treated HUVECs (Table 3.1). HUVECs treated with 20mM glucose showed a significant increase in (%) DNA damage when compared to 5mM glucose (Normoxia: $p < 0.01$; Hypoxia: $p < 0.001$) confirming earlier observations (Fig. 3.5). The DNA damage score seen in HUVECs induced with equimolar concentrations of mannitol is a lot higher throughout (88±4.98) when compared to samples treated with glucose (40±0.99). When adjusted for baseline (5N = 100%) however, there is no significant change between mannitol treated samples (Kruskal-Wallis test: $p > 0.05$).

	5mM Normoxia	**20mM Normoxia**	**5mM Hypoxia**	**20mM Hypoxia**
Glucose				
Mean ± SEM	40 ± 0.99	53 ± 4.38 (+ 33%)	55 ± 2.89 (+ 37%)	83 ± 5.03 (+ 108%)
Mannitol				
Mean ± SEM	88 ± 4.98	94 ± 3.67 (+ 7%)	87 ± 5.63 (+ 9%)	107 ± 5.48 (+ 22%)
Negative control	39 ± 3	**Positive control**	379 ± 1	

Table 3.1: DNA damage induced by equal concentrations of mannitol & glucose (5mM & 20mM) on HUVECs, as determined by the comet assay. The relative percentage difference is compared to each 5mM glucose or 5mM mannitol sample incubated in normoxia (100%). The results are expressed as mean ± SEM from three separate experiments (n = 3).

3.5. Discussion

The analysis of DNA integrity and proliferation of HUVECs in response to glucose concentration and oxygen tension in an established cell culture model has yielded significant findings. Results demonstrated that both high glucose and low oxygen significantly increases HUVEC proliferation. Accelerated endothelial cell proliferation in response to hypoxia is well established in the literature since it is a characteristic feature of tumour cell growth. Increasing tumour mass results in progressively more nutrient deprived and hypoxic areas (Scheurer *et al.* 2004). In order to improve oxygen tension a number of adaptive responses are triggered including the activation and translocation of the transcription factor hypoxia inducible factor (HIF-1). HIF-1 increases vascular endothelial growth factor (VEGF) transcription, which results in new vessel growth and accelerated cell proliferation (Zgouras *et al.* 2003).

Accelerated proliferation rates in response to 20mM glucose, however, is not consistent with other observations (Curcio *et al.* 1992; Varma *et al.* 2005). Most studies report a reduction in primary HUVEC proliferation rate over time when treated with glucose (Varma *et al.* 2005). This is believed to be due to the pro-apoptotic effects of glucose resulting from oxidative stress-regulated signalling pathways (Buttke *et al.* 1994; Vaux *et al.* 1996). Involvement of ROS in the induction of apoptosis by high glucose has been demonstrated in cultured human endothelial cells (Du *et al.* 1999). More recently, it was shown that high glucose concentration, induces NF-kappaB-related up-regulation of COX-2 via PI3K/Akt signalling, which in turn triggers caspase-3 activity that facilitates HUVEC apoptosis and reduced cell proliferation (Sheu *et al.* 2005). Most studies however, use primary isolates of HUVECs or excessive concentrations of glucose (up to 40mM) (Varma *et al.* 2005) it is therefore difficult to establish a direct comparison to our results.

The combination of high glucose and low oxygen concentration induced the most significant increase in HUVEC proliferation rate. The effect of simultaneous exposure of hyperglycaemia and hypoxia on HIF-1α protein stability in primary dermal fibroblasts and endothelial cells has been reported (Catrina *et al.* 2004). They suggest that hyperglycaemia abolishes the hypoxia afforded protection of the HIF-1α subunit against proteasomal degradation. This may effect a loss of HIF-1α, and subsequent HIF1 activity leading to a reduction in cell proliferation rate due to the loss in VEGF expression. However, this is not supported by the current data. Furthermore, since cellular proliferation rates seem to be closely linked to the production of ROS and apoptosis, the determination of cellular DNA damage and repair has also been of great interest (Chai *et al.* 2000). Several studies looking at DNA damage in diabetes mellitus have reported an increase in DNA damage in both type 1 and type 2 diabetes patients (Dandona *et al.* 1996).

In our agarose gel electrophoresis results the effect of high glucose (20mM) or low oxygen tension in HUVECs for 24h failed to show any DNA fragmentation (Fig. 3.2 and Fig. 3.5), while the combined effects of high glucose concentration and low oxygen tension showed significant evidence of DNA degradation (Fig. 3.2 and Fig. 3.5). Since agarose gel electrophoresis requires the cumulative extract of cultured cellular DNA there are many possible explanations for the lack of fragmentation observed with glucose alone. Given the very short time frame in which the HUVECs were exposed to the detrimental conditions it is most likely that the level of DNA damage inflicted is too small to be measurable by agarose gel electrophoresis. A more sensitive method of analysis such as the single cell gel electrophoresis assay is required to determine the extent of DNA damage inflicted.

An increase in HUVEC DNA damage using the comet asay has previously been reported after five days exposure to 45mM glucose supplemented culture medium (Shimoi *et al.*

2001) but no-one has reported damage in the combined conditions of hypoxia and hyperglycaemia over a 24hour period.

Although hyperglycaemia and hypoxia are known to cause cell damage (Shimoi *et al.* 2001), little has been reported about the combined and acute effect of both these conditions on endothelial cells. One of the earliest studies carried out in HUVECs reported that hyperglycaemia induced apoptosis after 72h chronic exposure to 30mM glucose (Baumgartner-Prazer *et al.* 1995). This was supported by further work which reported a rapid rise in ROS after only 2-6h exposure of HUVECs to 30mM glucose concentrations (Du *et al.* 1999). Furthermore, hypoxia induced a significant increase in DNA damage which appears to be independent to that of glucose. Although the effect of hypoxia and its highly developed adaptive responses in endothelial cells has been well documented for CV disease (Giordano *et al.* 2005; Verrier *et al.* 1996) and cancer (Fuks *et al.* 2005), the consequences of hypoxia on the development of diabetic vascular complications has been less well investigated.

One possible indication of the mechanisms involved lies in the observation that the DNA damaging effects of glucose and hypoxia seem to be additive (Fig. 3.5). It is documented that glucose induces a state of "pseudohypoxia" due to its effect on NO and the free cytosolic NADH/NAD+ ratio (Williamson *et al.* 1993). The known additive effects of high glucose and low oxygen concentrations can be rationalised as cytosolic NADH levels are increased by both hypoxia and hyperglycaemia-mediated pathways via different yet additive mechanisms (Nyengaard *et al.* 2004). Since the net energy charge of the cytosol seems to be an indicator of a cells commitment for further proliferation, the observed increase in cell proliferation rate seem to be in line with this hypothesis (Breiter-Hahn *et al.* 1998).

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To add an even more interesting dimension to these results, Fig. 3.6 shows that the DNA damage inflicted within the first 24h is not reversed by subjecting cells to an equal time

period of re-oxygenation. There appears to be a steady increase in DNA damage over time an observation which is of immense importance with respect to the cumulative damage inflicted on endothelial cells in hyperglycaemic or/and hypoxic conditions.

3.6. Limitations of the study

As mentioned before comparison of the current data to most published research in the area is limited by the fact that this study uses an established HUVEC line. The use of a cell line is a well established model system for preliminary studies of endothelial function in response to changes in glucose and oxygen levels (Zanetti *et al.* 2001; Nachman et al. 2004; Fang *et al.* 2005) often providing important information to justify the progression of the research to a primary cell model or an *in vivo* setting. A cell line can be distinguished from primary cells in that they possess the ability to proliferate indefinitely. This allows their use to much higher passage numbers but is arguably limited by the use of cultured clones. The use of endothelial cells in culture, whether primary or a cell line, are not likely to be entirely reflective of the processes which occur in the tissues as they lack the interaction with other tissue factors, a limitation avoided by the use of an *in vivo* model (Bricknell, 2004). However, the data provides important preliminary information on which to base further studies to elucidate the processes involved.

A further limitation is the experimental definition of hypoxia and normoxia when using vein endothelial cells. Normal oxygen pressure in human umbilical vein blood is 3.2% to 5.1% O_2 (ie, pO_2=22 to 35 mm Hg) (Casanello *et al.* 2005), therefore the use of 5%O_2 in hypoxia and 20% in normoxia is not entirely reflective of the physiological conditions but it does provide a stable system for the investigation of the effect of reduced oxygen availability and facilitates the investigation of the mechanisms underlying this phenomenon. Stable oxygen levels below 5% are notoriously difficult to achieve with the equipment used and although true hypoxia is not achieved the use of 5%O_2 is widely established in the literature (Namiki

et al. 1995; Buras *et al.* 2000) and it is the response to the relative reduction of oxygen tension that is of interest in this study.

3.7. Conclusion

In conclusion, we can state that both glucose and oxygen exhibit distinct yet potentially additive effects on endothelial cells which results in an increase in cell proliferation, DNA damage a nd possibly DNA repair mechanisms over a 24hour period. The generation of oxidative stress in response to hyperglycaemia and the expression of potential hypoxia dependent mechanisms such as the expression of HIF-1 are now investigated to facilitate our understanding of these events.

CHAPTER FOUR: Role of mitochondrial ROS production in hypoxia- and hyperglycaemia-induced endothelial DNA damage

4.1. Hypothesis

Mitochondrial ROS production, play a role in hypoxia- and hyperglycaemia-induced endothelial DNA damage.

4.2. Introduction

A change in mitochondrial regulation, such as increased ROS production and mitochondrial DNA damage, has been shown to contribute to disease and ageing (Kang *et al.* 2005). Mitochondria are therefore fast emerging as a selective target for distinct therapeutic approaches (Armstrong *et al.* 2006). The first association of a defect in oxidative phosphorylation with diabetes was made as far back as 1959 by Bierman *et al.* Since then, a multitude of signalling pathways have been identified which all contribute to the excessive ROS production by the mitochondria during hyperglycaemia and more recently, hypoxia (Nyengaard *et al.* 2004). More recently, it has been suggested that mitochondrial superoxide production is the missing link between diabetic hyperglycaemia and the activation of a number of biochemical pathways known to be involved in the pathogenesis of diabetes such as PKC, AGE and the Polyol pathway (Nishikawa *et al.* 2003). Furthermore, damaged mtDNA amplifies oxidative stress by reducing the expression of certain proteins critical for electron transport and mitochondrial regulation. This initiates a cycle of ROS production and mitochondrial dysregulation which eventually triggers apoptosis and vascular damage (van Houten *et al.* 2006).

The events associated with the increased DNA damage seen in Chapter Three will be further elucidated. The effect that hypoxia and/or hyperglycaemia have on mitochondrial number, morphology as well as on ROS production in human umbilical vein endothelial cells is determined.

4.3. Materials and Methods

The effect of glucose concentration and oxygen tension on HUVEC size was determined using specialised imaging software (Section 2.5). The effects of increased glucose concentration and/or decreased oxygen tension on mitochondrial number and morphology were determined using MitoTracker Green FM fluorescent dye (Section 2.5) which was co-localised with the fluorescent dye RedoxSensor Red CC-1 to determine the cellular redox potential. The resulting images were analysed using ImageJ software. The cellular production of superoxide radical formation was also measured using a commercially available kit (Section 2.6). HUVECs challenged with glucose and oxygen for 24h were cultured as described in Section 2.2 and mitochondria were visualised as described in Section 2.5.1.

Each experiment was performed in triplicate and repeated on three separate occasions. The test distribution was assumed not to be normal to account for the limitations in sample size. Statistical significance was determined using a Kruskal-Wallis test or Mann-Whitney U test against the test hypothesis (H_0) that there are no differences between the means of the samples. Where $p < 0.05$ the H_0 must be rejected and the H_A (there is a difference between the means of the samples) accepted. Where data was normalised (100%) a Student's t-test (Mean – 100%/ SEM) and/or ANOVA (with Tukey's post-hoc test) was carried out. The level of significance is expressed as $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$. No significant difference was assigned to samples where $p > 0.05$.

4.4. Results

4.4.1. Co-localisation of ROS production with mitochondria

Co-localisation and cross-sectional distribution of both MitoTracker Green FM and RedoxSensor Red CC-1 was carried out to verify the specificity of the RedoxSensor Red CC-1 fluorescent stain for the mitochondria (Fig. 4.1). Co-localisation of both dyes occurs in proliferating cells while the accumulation of RedoxSensor Red CC-1 stain (a redox sensitive dye) outside the mitochondria accumulates in lysosomes, indicative of contact inhibited cells (Shukla *et al.* 2003).

4.4.2. Identification of ROS production from HUVEC mitochondria

To determine if the cellular ROS production is directly associated with the mitochondria, HUVECs were perfused with the protonophore carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) (1μM) (Fig. 4.2). The FCCP collapses the membrane potential (Aronis *et al.* 2002). This shows that the ability of the cell to take up the oxidised CC-1 product requires an intact membrane potential. Changes in the intensity of the RedoxSensor Red CC-1 fluorescence dye were visualised and measured using ImageJ freeware (Fig.4.3). Statistical analysis using a Mann-Whitney U test shows a significant reduction in fluorescence intensity in HUVECs perfused with FCCP (1.83±0.01) compared to untreated cells (8.74±0.12), ($p < 0.001$).

4.4.3. Changes in HUVEC size in response to glucose and oxygen

In order to investigate if glucose concentration and oxygen tension has an effect on cell morphology such as size and appearance, any potential changes in HUVEC size over time (6h & 24h) were determined using ImageJ freeware. This will help with the interpretation of any results (such as mitochondrial numbers and cellular ROS production) gathered in this context.

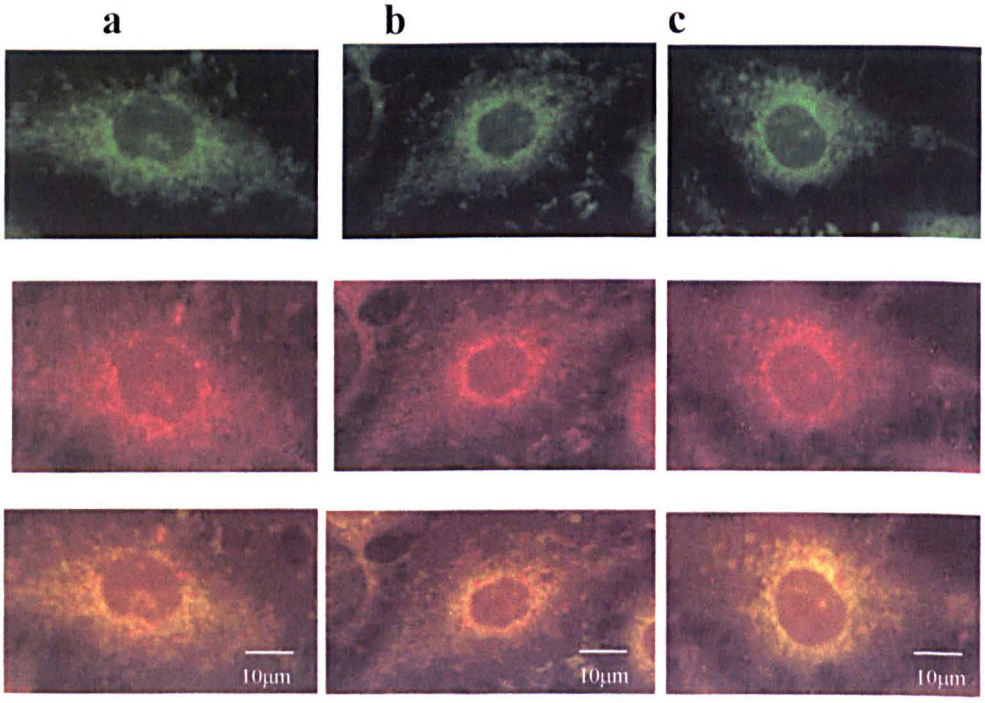
Changes in cell size were determined relative to control and expressed as a percentage of control (t = 0h) (Fig.4.4). Neither glucose (5mM & 20mM) nor oxygen concentration had any significant effect on HUVEC size in cells incubated for 6h (5N: 102±6; 20N: 108±1; 5H: 116±8; 20H: 110±7) and 24h (5N: 105±0; 20N: 108±4; 5H: 104±3; 20H: 111±5). These values were obtained using a Student's t-test in which changes in cell size were determined relative to control (t = 0h) at 6h and 24h. A calculation of the level of significance comparing samples with each other was carried out using an ANOVA plus Tukey's post-hoc test also failed to highlight any significant differences between samples ($p > 0.05$).

4.4.4. Changes in MitoTracker Green FM and RedoxSensor Red CC-1 fluorescence intensity in response to glucose and oxygen

Changes in both MitoTracker Green FM and RedoxSensor Red CC-1 fluorescence intensity were determined using ImageJ freeware. The percentage change in fluorescence intensity for both markers was determined relative to control (t = 0h 100%). Statistical analysis using a Student's t-test analysing the percentage changes in MitoTracker Green FM fluorescence intensity relative to control (t = 0h) showed neither glucose (5mM & 20mM) nor oxygen concentration (normoxia and hypoxia) to have a significant effect on MitoTracker Green FM fluorescence intensity after 6h (5N: 102±13; 20N: 122±37; 5H: 139±30; 20H: 108±12) or 24h (5N:114±33; 20N: 111±19; 5H: 81±16; 20H: 105±6) ($p > 0.05$) (Fig. 4.5). Additional analysis of the significance for each individual experiment was carried out using an ANOVA with a post-hoc Tukey's test to confirm that there was no significant change in MitoTracker Green FM fluorescence intensity at either time point ($p > 0.05$).

RedoxSensor Red CC-1 fluorescence intensity in HUVECs was analysed in an identical manner. There was a significant reduction in fluorescence intensity in HUVECs which had been incubated for 24h (5N: 133±56; 20N: 128±37; 5H: 141±21; 20H: 141±14) compared to

6h (5N: 337±26; 20N:427±46; 5H: 349±12; 20H: 450±63) ($p < 0.05$) in varying glucose (5mM & 20mM) and oxygen conditions (normoxia & hypoxia) when compared to control (Fig. 4.6). A Student's t-test, confirmed these findings ($p \leq 0.05$).


a
b
c
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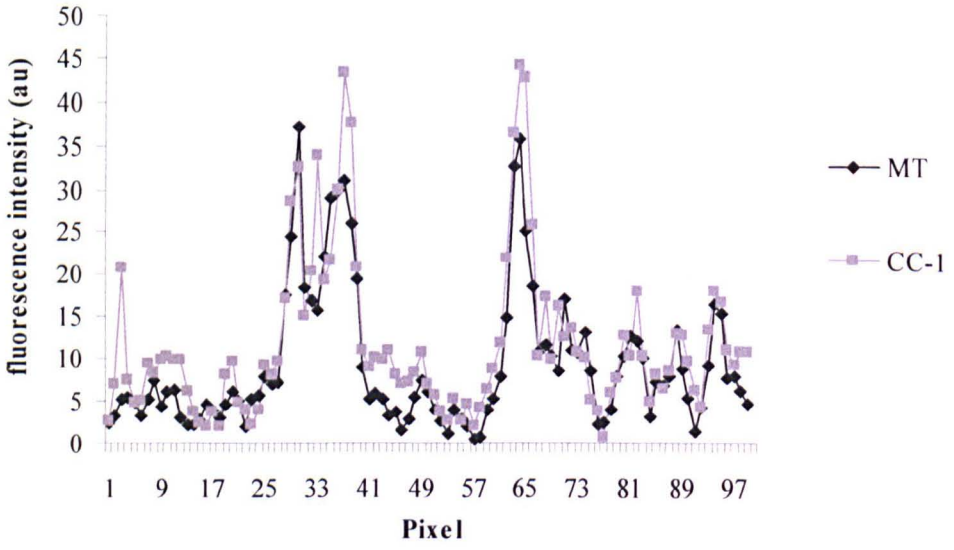
10μm

(a)


fluorescence intensity (au)
50
45
40
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30
25
20
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10
5
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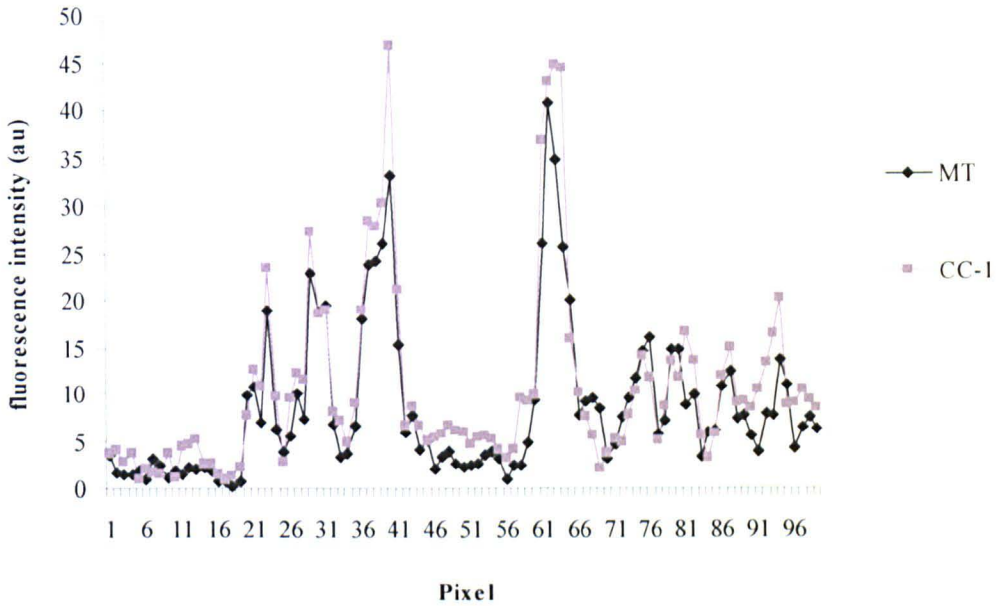
81
89
97
Pixel
MT
CC-1

(b)



(c)

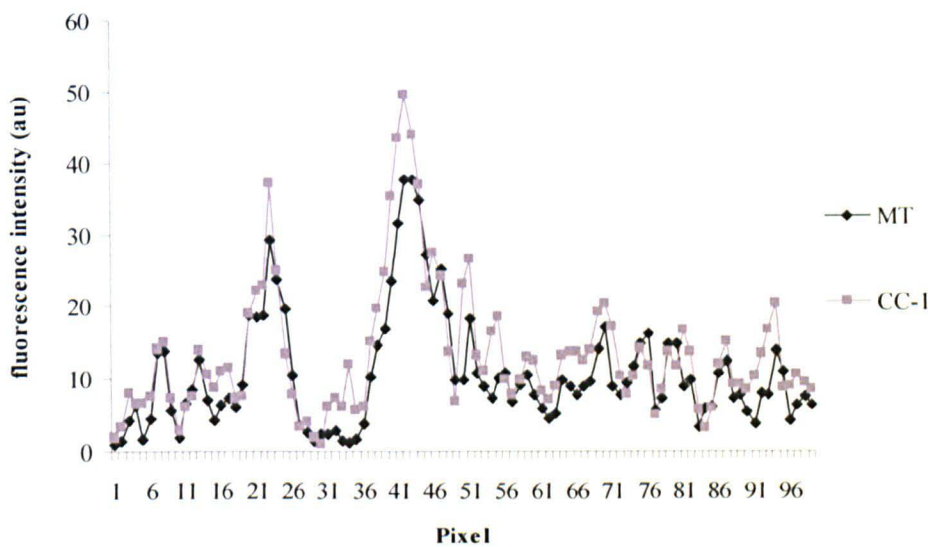
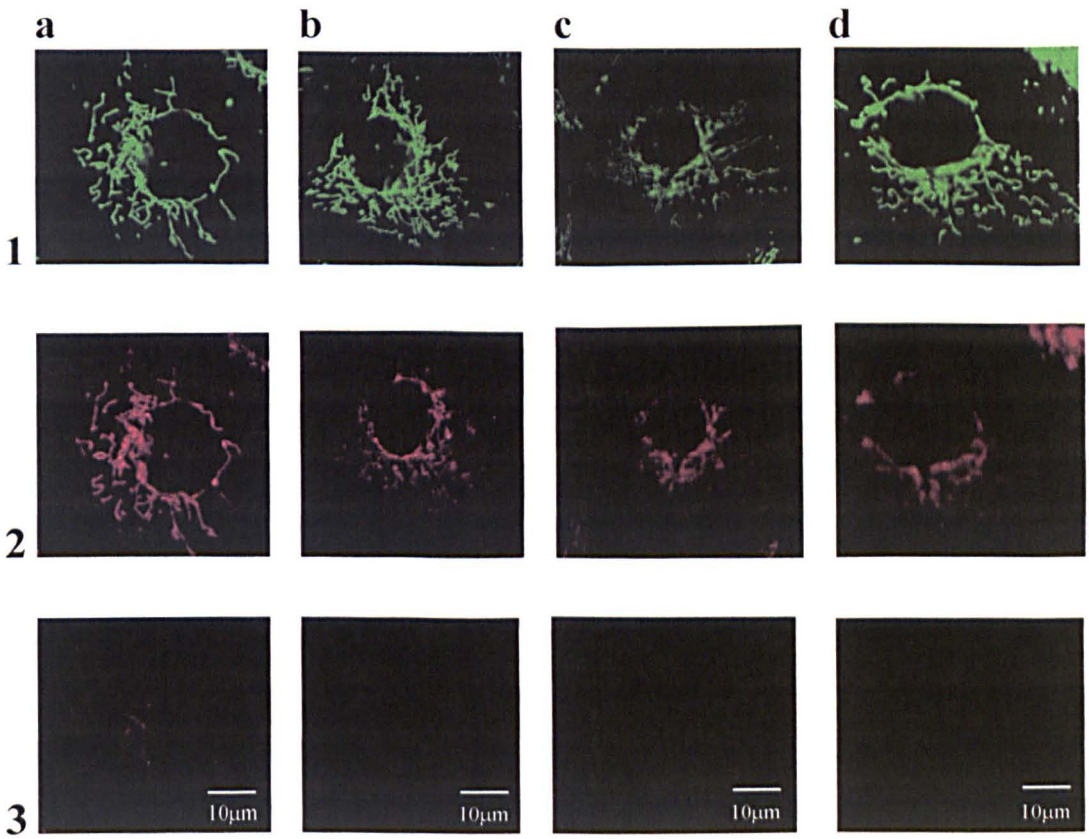


Fig. 4.1: Examples of co-localisation staining and cross-sectional distribution of both MitoTracker Green FM (5μM) stain and RedoxSensor Red CC-1 (1μM) stain in a human umbilical vein endothelial cell line (Magnification: 400x).

Fig. 4.2: Images showing the relationship between the ability of the mitochondria to take up oxidised CC-1 product and its membrane potential (400x Magnification). **1)** MitoTracker Green FM dye (5μM); **2)** RedoxSensor Red CC-1 (1μM); **3)** RedoxSensor Red CC-1 fluorescence after collapsing the membrane potential using carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (1μM). Samples analysed from left to right: **(a)** 5mM glucose Normoxia; **(b)** 20mM glucose Normoxia; **(c)** 5mM glucose Hypoxia; **(d)** 20mM glucose Hypoxia. The images are representative of samples from three different experiments (n = 3).


a
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1
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10μm
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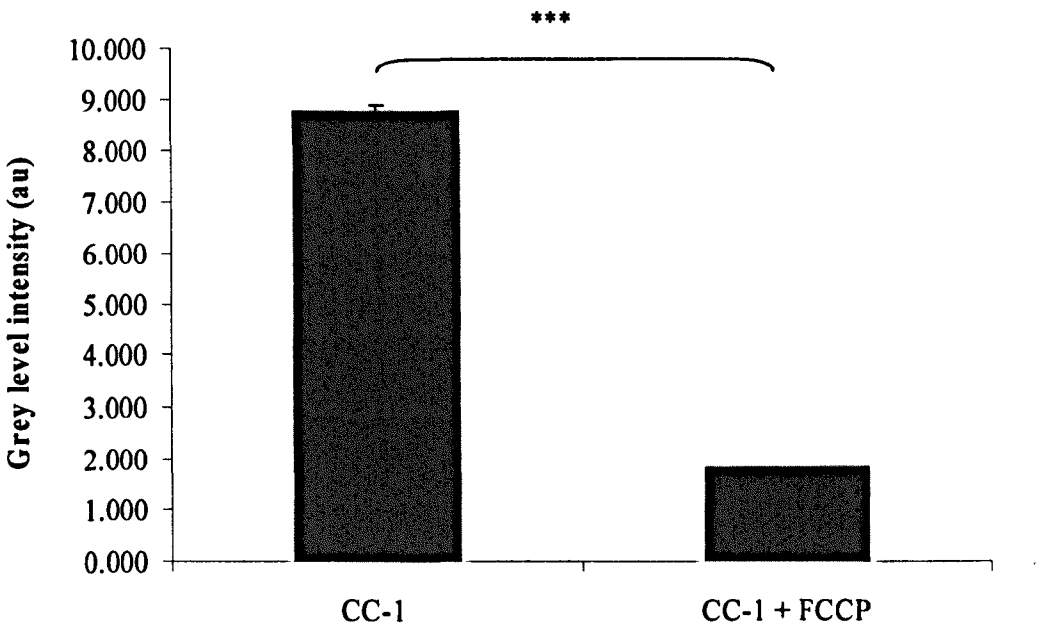


Fig. 4.3: Effect of the protonophore carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) (1μM) on RedoxSensor Red CC-1 (1μM) fluorescence intensity in HUVECs. The change in fluorescence intensity was analysed using a Leica DML fluorecent microscope and ImageJ freeware. The results are expressed as mean ± SEM from three separate experiments (n = 3 total of 150 HUVECs) (Mann-Whitney U test: ***$p < 0.001$).

Fig. 4.4: Determination of the effect of time, glucose (5mM & 20mM) and oxygen concentration (N = normoxia, H = hypoxia) on size of human umbilical vein endothelial cells. The change in cell size is expressed relative to control (t = 0h 100%). The graph is representative of the mean ± SEM of three separate experiments (n = 3) with 50 observations per sample (ANOVA plus post hoc Tukey's test: $p > 0.05$).

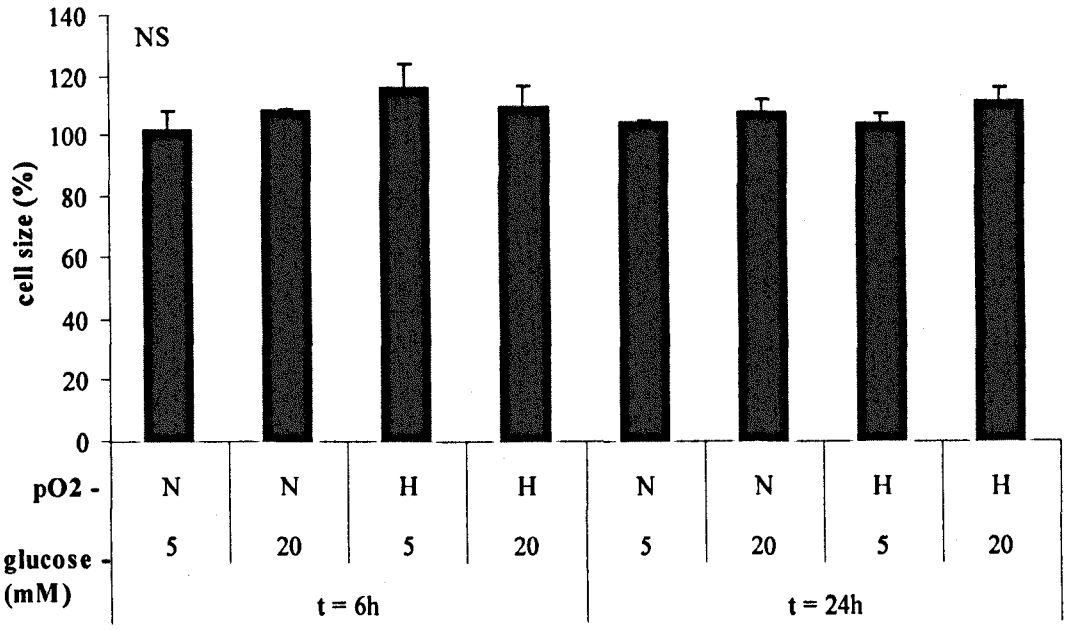

NS
140
120
100
80
60
40
20
0
cell size (%)
pO2 -
N
N
H
H
N
N
H
H
glucose -
(mM)
5
20
5
20
5
20
5
20
t = 6h
t = 24h

Fig. 4.5: Effect of time, glucose (5mM & 20mM) and oxygen concentration (N = normoxia and H = hypoxia) on MitoTracker Green FM fluorescence intensity in human umbilical vein endothelial cells. The change in fluorescence intensity is expressed relative to control (t = 0h 100%). The graph is representative of the mean ± SEM of three separate experiments (n = 3) with 50 observations per sample (ANOVA plus post hoc Tukey's test: $p > 0.05$).

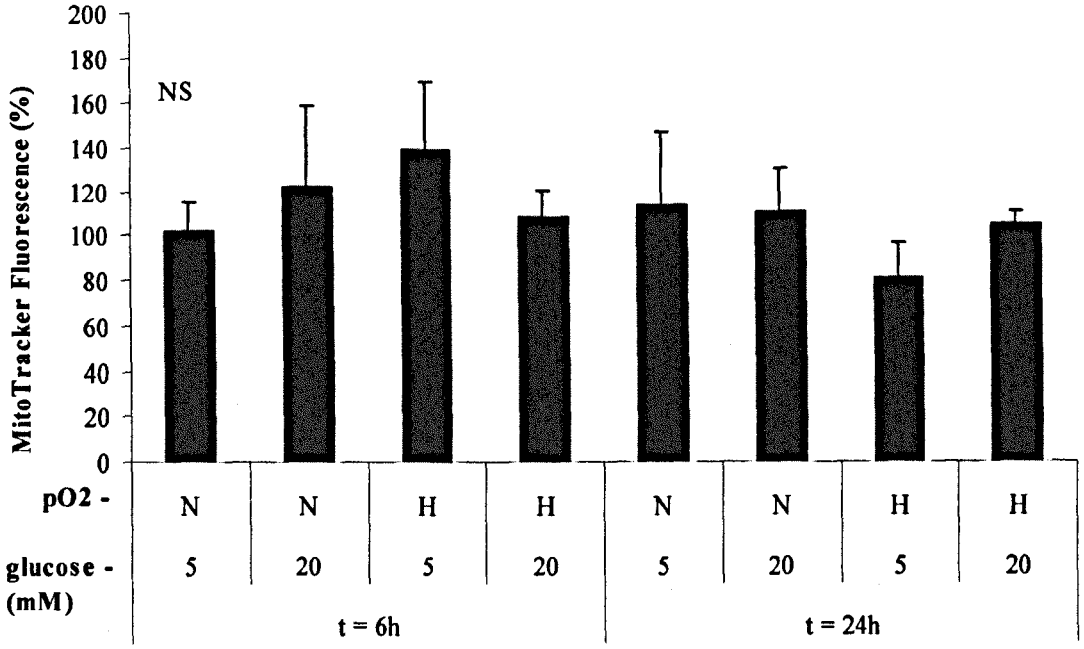
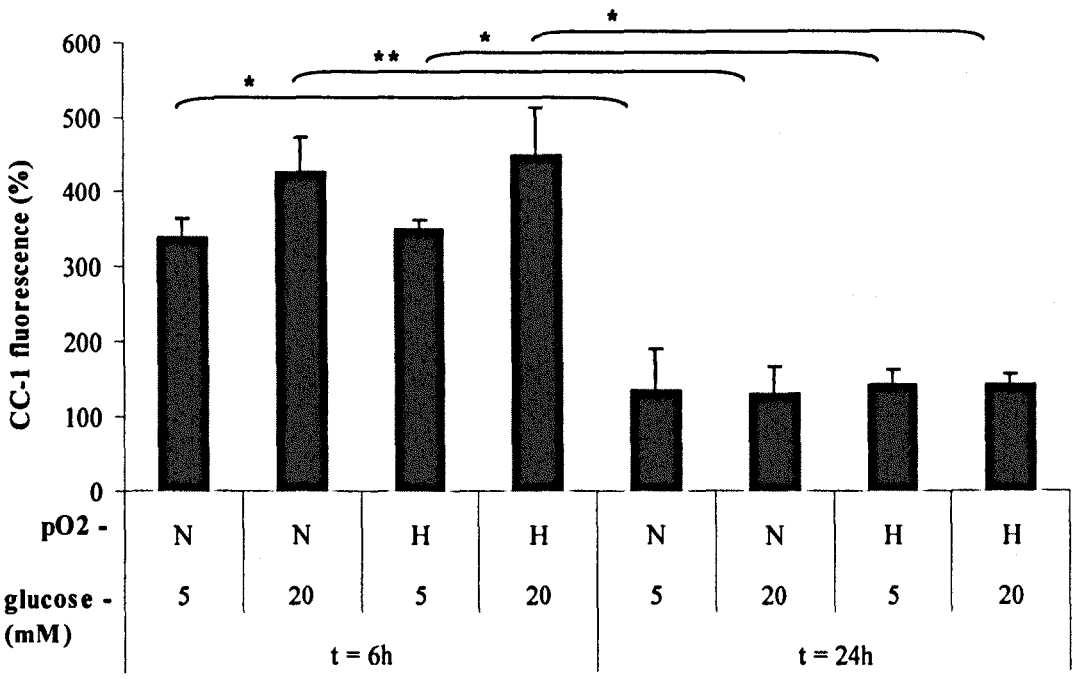

MitoTracker Fluorescence (%)
200
180
160
140
120
100
80
60
40
20
0
NS
pO2 -
N
N
H
H
N
N
H
H
glucose - (mM)
5
20
5
20
5
20
5
20
t = 6h
t = 24h

Fig. 4.6: Effect of time, glucose (5mM & 20mM) and oxygen concentration (N = normoxia and H = hypoxia) on RedoxSensor Red CC-1 fluorescence intensity in human umbilical vein endothelial cells. The change in fluorescence intensity is expressed relative to control (t = 0h 100%). The graph is representative of the mean ± SEM of three separate experiments (n = 3) with 50 observations per sample (ANOVA plus post hoc Tukey's test: *$p < 0.05$; **$p < 0.01$).


CC-1 fluorescence (%)
600
500
400
300
200
100
0
*
**
*
*
pO2 -
N
N
H
H
N
N
H
H
glucose - (mM)
5
20
5
20
5
20
5
20
t = 6h
t = 24h

4.4.5. Morphological changes of mitochondria in response to glucose and oxygen

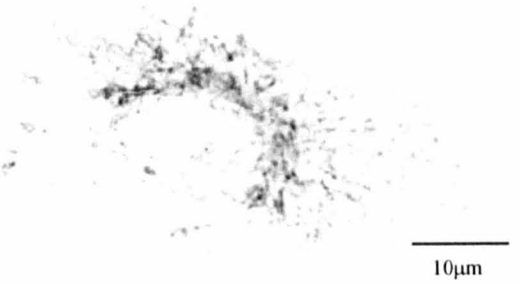
In a blinded analysis the effect of glucose (5mM & 20mM) and oxygen (normoxia and hypoxia) on mitochondrial morphology in HUVECs over time (6h & 24h) was assessed. Adaptive stress responses lead to changes in mitochondrial morphology (Fig. 4.7) and are indicative of cellular response mechanisms. A quantitative analysis of the mitochondrial length (Fig. 4.8) showed no significant changes in mitochondrial length between samples over time compared to control (6h – 5N:27±3; 20N: 37±5; 5H: 35±8; 20H: 52±8 & 24h – 5N: 35±5; 20N: 42±8; 5H: 36±10; 20H: 38±8) ($p > 0.05$). In addition no consistent changes in mitochondrial morphology with a change in culture conditions could be observed (Fig.4.7)

4.4.6. Superoxide radical production in response to glucose and oxygen

Using a commercially available kit, the total cellular superoxide radical production in response to 5mM and 20mM glucose as well as normoxic and hypoxic conditions was determined. The percentage proportion of superoxide radicals found in mitochondria and the cytoplasm was analysed. The results showed no significant change in total cellular superoxide radical production between samples (5N: 100±0; 20N: 100±2; 5H: 102±3; 20H: 103±2) ($p > 0.05$) (Fig.4.9a). Most of these superoxide radicals seem to be produced in the mitochondria with a significant increase in cells incubated in hypoxia (5mM glucose) compared to normoxia (5N: 66±3; 5H: 81±2) (Fig. 4.9b). This increase is reflected by a corresponding decrease seen with cytoplasmic radical formation in the same samples (5N: 34±3; 5H: 22± 1) ($p < 0.05$) (Fig. 4.9c).

Fig. 4.7: Illustration of the morphological changes seen during the mitochondrial fusion and fission process in HUVECs (400x magnification). **(1)** Mitochondria appear long and strand like **(2)** Fission i.e. break up and shortening of mitochondria is visible **(3)** Mitochondria appear punctate, fission is complete.

1.



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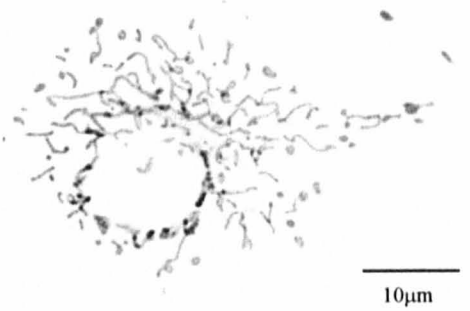


3.

Fig. 4.8: Quantitative blind analysis of mitochondrial length in response to changes in glucose (5mM & 20mM) and oxygen concentration (N = normoxia; H = hypoxia) in HUVECs. Twenty random mitochondria were measured per cell. The results are expressed as mean ± SEM from six separate cells (n = 6) with 10 observations per cell (Kruskal-Wallis test: $p > 0.05$).

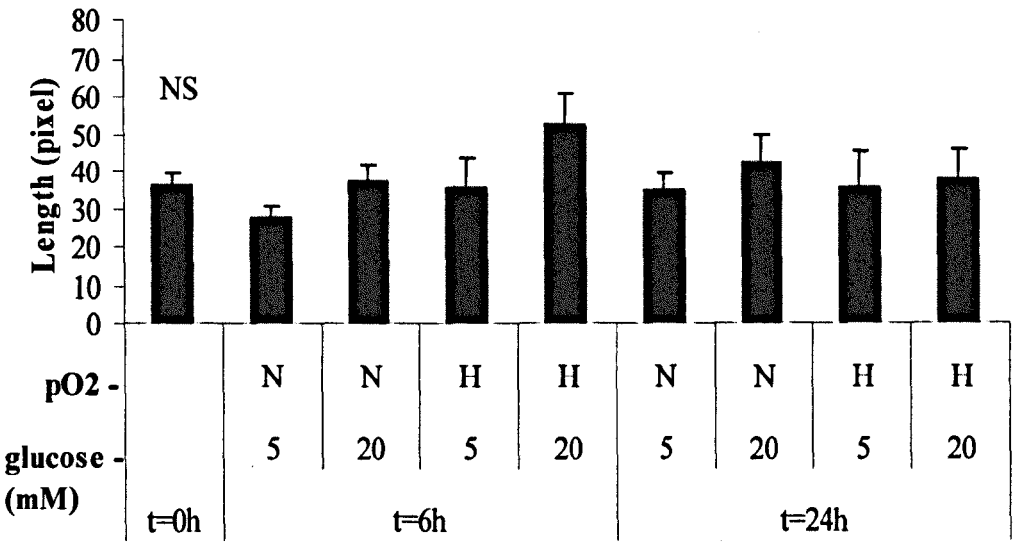
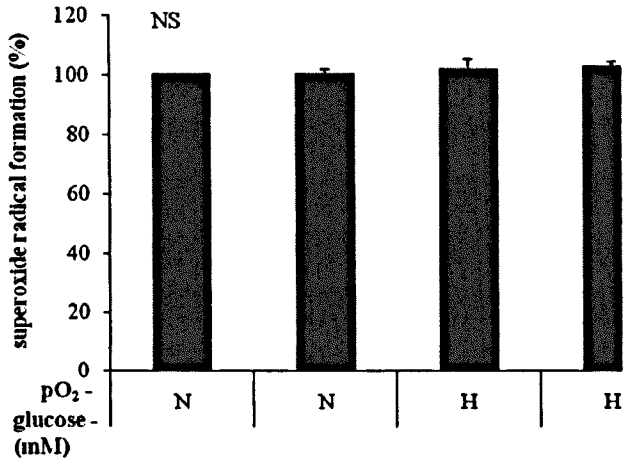
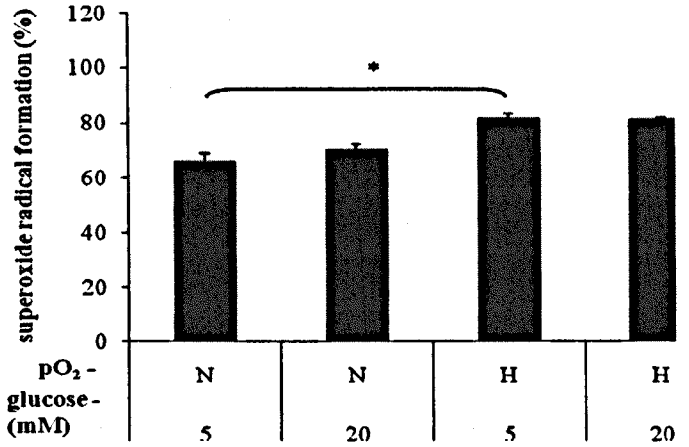

Length (pixel)
80
70
60
50
40
30
20
10
0
NS
pO2 -
glucose -
(mM)
N
H
5
20
t=0h
t=6h
t=24h

Fig. 4.9: Determination of changes in cellular superoxide radical formation in response to changes in glucose (5mM & 20mM) and oxygen concentration (N = normoxia; H = hypoxia) in HUVECs. The change in superoxide radical formation is compared relative to total cellular superoxide radical control (5N = 100%). **(a)** total superoxide radical formation in HUVECs **(b)** percentage proportion superoxide radical formation found in mitochondria **(c)** percentage proportion superoxide radical formation found in the cytoplasm. Samples were analysed using a SOD-WST kit. Each bar represents the mean ± SEM from three different experiments (n = 3) (ANOVA plus post hoc Tukey's test: * $p > 0.05$).

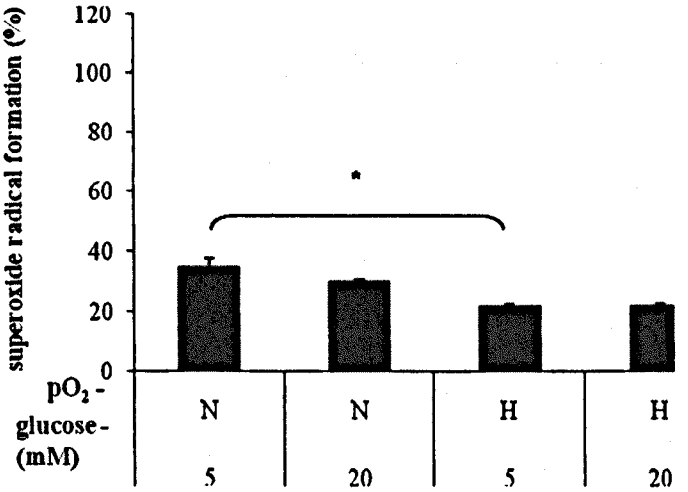
(a) total cellular



(b) mitochondrial



(c) cytoplasmic



4.5. Discussion

The focus of this chapter was to determine the effect that hypoxia and hyperglycaemia have on endothelial mitochondrial number, morphology and cellular ROS production to determine if this may offer an explanation for the effect of increased cell proliferation and increased DNA damage observed in the previous chapter. The type of quantitation carried out to determine the number of mitochondria and ROS production was highly dependent on a consistent cell size within the population studied. We demonstrated that, with different concentrations of glucose and oxygen, there was no significant difference in cell size ($p > 0.05$) between treatment groups after 24h exposure (Fig. 4.4). Changes in cell size are highly cell-type dependent, with some reports of changes in cell size, such as swelling (Schmiedl *et al.* 2001) and changes in cell structure (Mandal *et al.* 2006), in response to hyperglycaemia-induced oxidative stress. However, evidence corresponding to a time-frame of 24h is hard to find, as exposure times vary considerably. It would be unrealistic to expect cell size to stay constant, given the processes involved in oxidative stress such as apoptosis, but for the interpretation of our results it is important to know that cell size remains unaffected after only 24h incubation to allow for an accurate interpretation of the results which have been gathered from HUVECs treated in the various conditions.

Analysis of the mitochondrial content also showed no significant change between treatment groups after 6h and 24h (Fig. 4.5). However, the relatively large SEM values indicate a high degree of variability which is indicative of the heterogeneity of the mitochondrial population. Although mitochondria are known to form a reticulum, evidence exists that mitochondria within individual cells can also form morphologically heterogeneous and physically distinct entities (Collins *et al.* 2002). This allows the mitochondria to function independently with respect to their individual $\Delta\psi_m$, Ca^{2+} uptake and mPTP activation. It is therefore perceivable that the large variability in MitoTracker Green FM fluorescence is indicative of either **(a)** an increase in mitochondrial permeability, as a result of cell structure changes in response to

prolonged exposure to oxidative stress, **(b)** a possible increase in mitochondrial fragmentation or **(c)** a simple variability in dye uptake due to changes in $\Delta\psi_m$ (Keij *et al.* 2000, Buckman *et al.* 2001, Pendergrass *et al.* 2004).

It is known that mitochondrial morphology is regulated by the fusion and fission processes described earlier, which are strongly linked to oxidative stress. Therefore, changes in mitochondrial fragmentation in response to oxidative-stress-induced apoptosis (Frank *et al.* 2001) may lead to an increase in fluorescence intensity, depending on the extent of the fragmentation. Although this is pure speculation as our data cannot verify these assumptions we do see a modest reduction in MitoTracker Green FM fluorescence in hypoxia-treated samples but it does not reach statistical significance. Since hypoxia has been reported to increase mitochondrial length and reduce fission (Skulatchev *et al.* 2001) we would possibly expect to see a drop in fluorescence intensity. At this point, however, we cannot make any assumptions about changes in mitochondrial morphology and it is sufficient to bear in mind that total cellular MitoTracker Green FM fluorescence does not show any significant change after 24h exposure to hyperglycaemia and hypoxia (Fig. 4.5).

There is, however, a significant difference between mitochondrial ROS production at 6h compared to 24h ($p < 0.05$); (Fig. 4.6). The RedoxSensor Red CC-1 stain passively enters live cells where the non-fluorescent probe is either oxidised in the cytosol and accumulates in the mitochondria, or the probe is transported to the lysosomes where it is oxidised. The differential distribution of the oxidized product between mitochondria and lysosomes appears to depend on the redox potential of the cytosol (Chen *et al.* 2000) and on the proliferation rate, with predominantly mitochondrial staining in proliferating cells and lysosomal staining in contact-inhibited cells (Chen *et al.* 2000). The observed change in cellular redox potential in response to glucose and/or hypoxia is in agreement with the literature reports (Nakajima *et al.* 2006, Zhang *et al.* 2007). Co-localisation studies show that

most if not all of the oxidised product accumulates in the mitochondria (as seen in Fig. 4.1.). These findings are supported by Fig. 4.2 and Fig. 4.3, which demonstrate that the RedoxSensor Red CC-1 signal is significantly reduced when the mitochondrial membrane potential is artificially collapsed using FCCP (1μM) ($p < 0.001$). Artificially collapsing the membrane potential also identifies the close relationship between glucose- and/or hypoxia-generated oxidative stress and the mitochondrial membrane potential. Although it has been shown that mitochondrial ROS production is dependent on the $\Delta\psi_m$ with a high membrane potential seemingly favouring the production of ROS (Kadenbach *et al.* 2003, Zhang *et al.* 2007), no direct reports exist for this interdependence in response to hypoxia and/or hyperglycaemia.

Our findings confirm an early change in cellular redox potential in response to glucose and hypoxia within the first 6hours of exposure. Since we have shown that neither cell size nor mitochondrial numbers change under identical conditions, this rise cannot be explained by an increase in intensity-to-size ratio (i.e. smaller cells: increased fluorescence or more mitochondria: increased fluorescence) but could reflect either an impaired ability of the mitochondria to take up the oxidised CC-1 product or a decrease in the cellular redox potential.

The results from Chapter Three have demonstrated that HUVECs exhibit an increase in DNA damage after 24h incubation. Furthermore, the net increase in ROS seen here, together with the knowledge that overproduction triggers apoptosis almost as a fail-safe mechanism to prevent the cells from uncontrolled proliferation in the presence of oxidative stress (Kannan *et al.* 2000), may represent a distinct but related phenomena. It also needs to be considered that the significant increase in ROS production after 6h is a possible consequence of the pre-treatment culture conditions for HUVECs. HUVECs are incubated with glucose free/serum free media overnight in order to ensure all cells are in the same stage of the cell

cycle prior to treatment with different glucose and oxygen concentrations. The rise in ROS production could be a consequence of the HUVECs resuming "normal" cellular function. As there is no significant difference in ROS production between samples, cellular ROS production may not be the sole cause for the observed loss of DNA integrity with high glucose concentration and low oxygen tension.

It is, however, important to distinguish between cytoplasmic and intra-mitochondrial oxidative stress (Kristal *et al.* 1997). Mitochondrial DNA (mtDNA) is very susceptible to ROS-induced DNA damage, more so than nuclear DNA (nDNA). In 1974, Clayton *et al.* found that UV- induced pyrimidine damage was not repaired in mtDNA, a result interpreted as a complete absence of repair capacity in mitochondria. It has since been shown that mtDNA repair enzymes are encoded for in the nucleus and are transported into the mitochondria via the membrane potential (Bohr *et al.* 2002). Subsequently, any change in mitochondrial membrane potential will affect the transport efficiency and repair capacity of mtDNA repair enzymes.

An interesting study carried out as far back as 1997 reported that H_2O_2-induced mtDNA damage incurred after 15min was repaired efficiently within 1.5h after H_2O_2 withdrawal. However, extension of H_2O_2 treatment from 15 to 60min led to persistent mtDNA lesions refractory to repair up to 24h after treatment (Yakes *et al.* 1997). They observed that prolonged exposure to H_2O_2 damage correlated with a loss of mitochondrial function and eventually cell death. They hypothesised that a vicious cycle exists whereby oxidative mtDNA damage leads to faulty gene expression, deficiency of key electron transport enzymes, subsequent ROS production and ultimately cell death (van Houten *et al.* 2006). Therefore, it is fair to say that the redox status of the cell has a multilayered effect on cell survival and cell death.

As mentioned above mitochondrial fusion and fission are integral to the mitochondrial responses to oxidative stress (Section 4.2). A recent paper by Yu *et al.* (2006) observed that dynamic changes in mitochondrial morphology are associated with high glucose-induced overproduction of ROS. Mitochondria undergo rapid fragmentation with a concomitant increase in ROS formation after exposure to high glucose concentrations. They suggest that mitochondrial fragmentation mediated by the fission process is a necessary component for high glucose-induced respiration increase and ROS overproduction (Yu *et al.* 2006). Considering this hypothesis we analysed our samples for any evidence of increased fusion and fission processes. Figure 4.7 shows examples of cells exhibiting long and strand like mitochondria (a) and their increased level of fragmentation (b) until nearly all mitochondria are fragmented and fission is complete (c). However, although there is evidence of individual cells having undergone fission to a more or less advanced extent, the analysis of individual mitochondrial length did not result in any significant differences between samples and time points (Fig. 4.8). Since our results did not confirm the recent literature findings (Yu *et al.* 2006), we are unable to extrapolate whether a hyperglycaemia and/or hypoxia-induced rise in respiration with subsequent ROS overproduction is dependent on mitochondrial morphology.

One key aspect of cellular stress is the antioxidant defence enzymes, such as superoxide dismutase and glutathione peroxidase. They, alongside other antioxidants, ultimately determine the availability and increased production of ROS since they are very effective free-radical scavengers. In order to understand the effect that glucose and hypoxia have on their activity, mitochondrial superoxide radical formation was analysed in response to high glucose and low oxygen levels (Fig. 4.9).

Superoxide dismutase together with catalase is responsible for scavenging superoxide radicals ($O_2^{\cdot-}$) in the cytoplasm (Cu/Zn-SOD) and the mitochondria (Mn-SOD). The

overproduction of mitochondrial superoxide has been hypothesised to be the missing link between hyperglycaemia and the activation of the known biochemical pathways involved in the development of vascular complications (Nishikawa *et al.* 2000).

These data demonstrate that total cellular superoxide levels do not change in response to glucose and/or oxygen concentration alone. The highest level of $O_2^{\cdot-}$ radicals can be found in the mitochondria, where a significant increase in generation was observed in normoglycaemic samples exposed to hypoxia for 24h. The inverse is true for $O_2^{\cdot-}$ radical production in the cytoplasm (Cu/Zn-SOD). Although the high mitochondrial $O_2^{\cdot-}$ radical levels could be interpreted as confirmation that superoxide radicals are predominantely produced in the mitochondria, their levels do not seem to change in response to hyperglycaemia. On the contrary, mitochondrial $O_2^{\cdot-}$ levels are significantly increased in normoglycaemic cells when exposed to hypoxia for 24h, but remain unaffected in response to glucose. From these results, it appears unlikely that mitochondrial superoxide radical formation is the sole cause for oxidative-stress-mediated DNA damage in HUVECs, and other factors relating more specifically to hypoxia will have to be considered.

The study carried out by Brownlee *et al.* (2005) verified the hypothesis that hyperglycaemia induces superoxide radical formation through the mitochondrial electron-transport chain by over-expressing UCP-1 (collapsing the mitochondrial membrane potential) or Mn-SOD. The results indicated that hyperglycaemia does not increase ROS production when UCP-1 is over-expressed, verifying that ROS production is associated with the mitochondrial electron-transport chain. Secondly, over-expression of Mn-SOD led to a similar reduction in ROS production in response to hyperglycaemia indicating that the ROS produced are indeed superoxide radicals. In addition, they tested the effect of Mn-SOD and UCP-1 over-expression on the activation of all known biochemical mechanisms involved in causing

vascular complications (i.e. Polyol, AGE and PKC) and found that none of the pathways were expressed in response (Du *et al.* 2001, Brownlee *et al.* 2005).

4.6. Conclusion

This study has identified that mitochondria play an important role in the immediate responses to oxidative stress in endothelial cells. As the results do not support a correlation between hypoxia/hyperglycaemia and cellular ROS production on changes in DNA damage, the involvement of hypoxia in the endothelium mediated oxidative stress response will now be investigated. Thus, evidence for hypoxia-mediated survival signalling via the transcription factor HIF-1 will now be presented.

CHAPTER FIVE: Role of HIF-1α in hypoxia- and hyperglycaemia-induced DNA damage in HUVECs

5.1. Hypothesis

The transcription factor HIF-1α plays a role in hypoxia- and hyperglycaemia-induced endothelial DNA damage.

5.2. Introduction

HIF-1α mediated VEGF expression plays a crucial role in oxygen-dependent diabetic diseases, such as proliferative diabetic retinopathy (Arjamaa *et al.* 2006), diabetes-related cardiovascular disease (Marfella *et al.* 2002), neuropathy (Chavez *et al.* 2005) and diabetic wound healing (Ozawa *et al.* 2001). It has been proposed that HIF-1 activity requires a functional mitochondrial electron transport chain and that the generation of mitochondrial superoxide radicals (at complex I and III of the ETC) and subsequent formation of hydrogen peroxide are required for the induction of HIF-1 activity and the transcription of its downstream targets in hypoxic cells (Chandel *et al.* 1998).

Although HIF-1α expression is strongly hypoxia associated, evidence is accumulating which suggests that hyperglycaemia alone can impact on HIF-1α regulation (Catrina *et al.* 2004). It has been shown that high glucose concentration increases basal HIF-1α mRNA expression in streptozotocin diabetic rat heart (Marfella *et al.* 2002), as well as interfering with hypoxia induced proteasomal dependent stabilisation of HIF-1α and its transcriptional activation (Catrina *et al.* 2004). This implies that hyperglycaemia can directly regulate HIF-1α activity and stabilisation identifying a more complex regulatory mechanism for HIF-1α in diabetes highlighting a need for investigating the role of HIF-1α in the pathology of diabetes mellitus and its complications.

In this Chapter the contribution of HIF-1α to glucose and hypoxia mediated changes in endothelial cell DNA damage and mitochondrial ROS production was assessed to further elucidate the involvement of hypoxia in the endothelial mediated oxidative stress responses.

5.3. Materials and Methods

The optimisation of HUVEC seeding density used with 16 well chamber slides to ensure that the cells remain within the proliferation phase during the experiment was carried out in 96 well plates using the Alamar blue proliferation assay as described in Section 2.2.5 The uptake of FITC labelled control oligonucleotide using fluorescent imaging (Section 2.7) was also determined in order to optimise the time frame for incubation with the antisense. HIF-1α protein was detected using immunocytochemical analysis (Section 2.7.1) and HIF-1α mRNA was detected using the reverse transcriptase polymerase chain reaction (Section 2.8). The effect of glucose concentration and oxygen tension on DNA integrity, cell size, mitochondria number and cellular redox potential was determined as described in previous Chapters (Sections 2.4, 2.5).

HUVECs exposed to 5 or 20mM glucose and/or 5 or 20% oxygen for 24h were cultured as described in Section 2.2.

Each experiment was performed in triplicate with three internal replicas per sample. The test distribution was assumed not to be normal to account for the limitations in sample size. Statistical significance was determined using a Kruskal-Wallis test or Mann-Whitney U test against the test hypothesis (H_0) that there are no differences between the means of the samples. Were $p < 0.05$ the H_0 must be rejected and the H_A (there is a difference between the means of the samples) accepted. Where data was normalised (100%) a Student's t-test (Mean – 100%/ SEM) and/ or ANOVA plus Tukey's post hoc test was used. The level of

significance is expressed as *p< 0.05, **p< 0.01 or ***p< 0.001. No significant difference was assigned to samples where p > 0.05.

5.4. Results

5.4.1. Effect of HIF-1α antisense on the level of HIF-1α protein and mRNA

Optimal seeding density (Fig. 5.1) was determined for the immunocytochemical analysis of HIF-1α activity in HUVECs grown on 16 well chamber slides for 24h ± HIF-1α antisense oligonucleotide. An alamar blue assay was carried out to determine the optimal seeding density based on the proliferation rate profiles of three different HUVEC samples ($1x10^5$, $0.5x10^4$, $0.1x10^4$). As Fig.5.1 shows cells seeded at $1x10^5$ and $0.5x10^4$ reach a plateau in their proliferation phase between 24h & 48 h after which cell proliferation levels off while cells seeded at density of $0.1x10^4$ continue to grow throughout the 48 h period. Subsequent immunocytochemical detection of HIF-1α was carried out using a $0.1x10^4$ cells/well seeding density.

5.4.2. Uptake of control oligonucleotide

Optimal oligonucleotide incubation time was determined using a FITC labelled control oligonucleotide representative of the HIF-1α antisense oligonucleotide which will be used during the experiment. The fluorescently labelled oligonucleotide was maximally taken up into the cells within 6h and was still present after 24h (Fig. 5.2). All future results were conducted using a 24h incubation time.

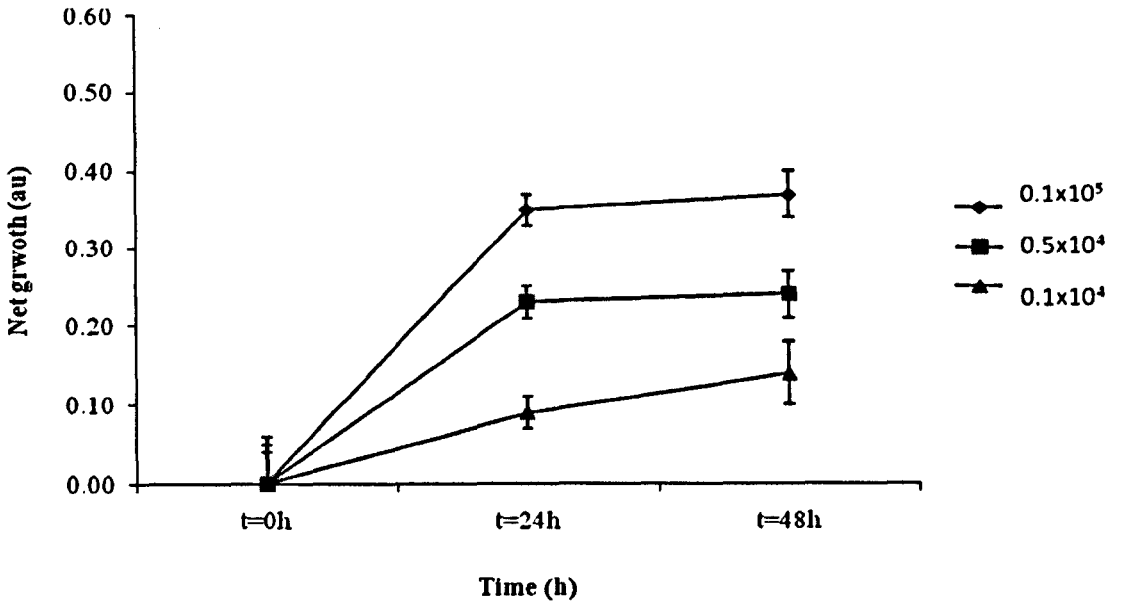
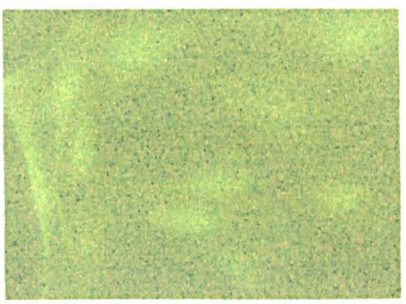


Fig. 5.1: Determination of the optimal seeding density of HUVECs to be used with 16well chamber slides. Cell proliferation of HUVECs seeded at three different concentrations ($0.1x10^5$, $0.5x10^4$, $0.1x10^4$) was determined using the Alamar blue assay. Each bar represents the mean ± SEM from six different experiments with six replicates per sample.

Fig. 5.2: Determination of the optimum incubation time for the cellular uptake of the FITC labelled control oligonucleotide (HIF-1α antisense control). **(a)** 0h, **(b)** 1h, **(c)** 6h, **(d)** 24h incubation. A 2μM concentration of FITC labelled control oligonucleotide was directly added to 16well chamber slides containing $0.1x10^4$ cells/well before analysis using a Leica DML Fluorescent microscope. The above images are representative of samples from six different batches of HUVECs (n = 6). Magnification: 200x

(a)



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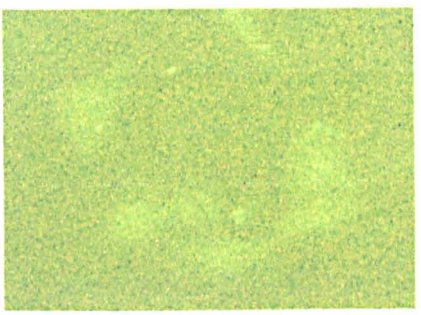

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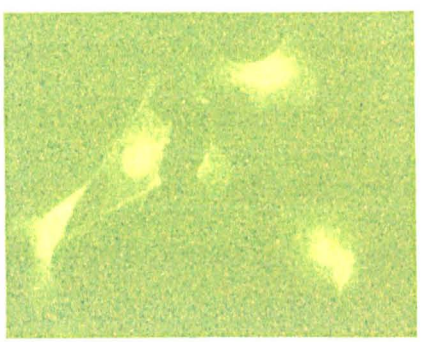
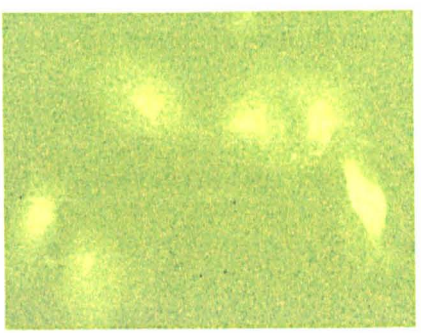


(d)



5.4.3. Effect of HIF-1α antisense on the level of HIF-1α protein and mRNA

Detection of the HIF-1α protein was carried out to confirm that the HIF-1α antisense is capable of blocking the expression of HIF-1α protein in HUVECs. As Fig. 5.3 illustrates, cells grown in hypoxic conditions for 24h without antisense show intense red staining corresponding to HIF-1α protein production in these cells (Fig. 5.3a). Fig. 5.3b shows the absence of staining in HUVECs incubated under identical conditions in the presence of HIF-1α antisense oligonucleotide. This confirms the ability of HIF-1α antisense oligonucleotide to block HIF-1α protein synthesis to the level of detection sensitivity of this assay after 24h, the chosen time point for all future experiments.

The level of HIF-1α mRNA was also determined using the reverse transcriptase polymerase chain reaction following cDNA preparation from RNA isolated from cells incubated in the presence of HIF-1α antisense. The integrity of the RNA was confirmed following electrophoretic separation and the amplification of β2 microglobulin from the cDNA (Fig.5.5a or b). Clear separation of both 18S and 28S ribosomal rRNA confirm the purity of the extracted RNA sample and confirm the suitability of this sample for use with PCR (Fig. 5.4). A HIF-1α PCR product was detected irrespective of the presence of HIF-1α antisense.

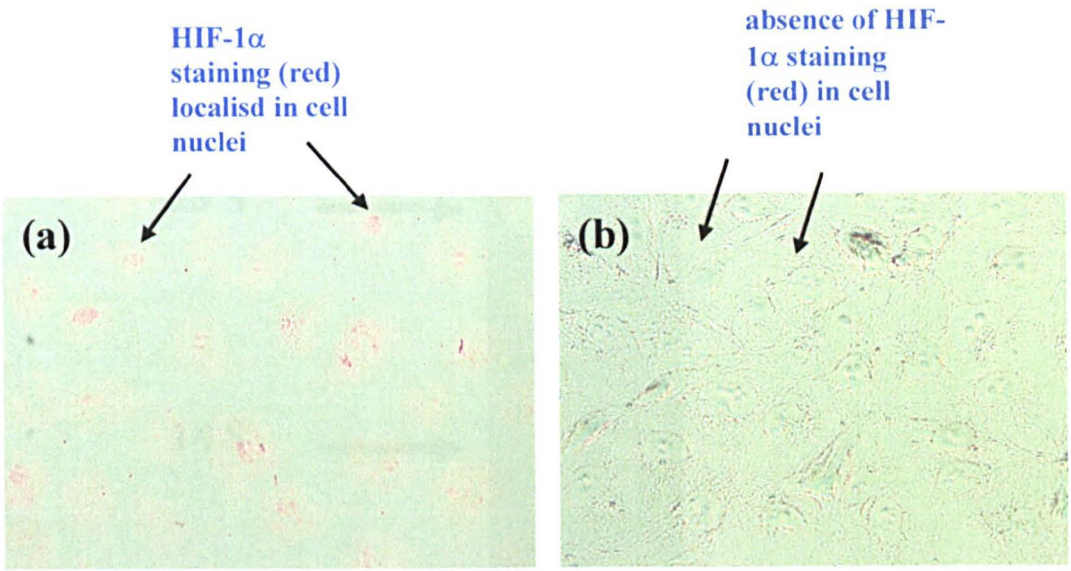


Fig. 5.3: Immunocytochemical detection of HIF-1α protein in human umbilical vein endothelial cells treated with and without HIF-1α antisense. **(a)** 20mM glucose hypoxia, no antisense (24h), **(b)** 20mM glucose hypoxia + HIF-1α antisense (24h), Magnification: 200x.

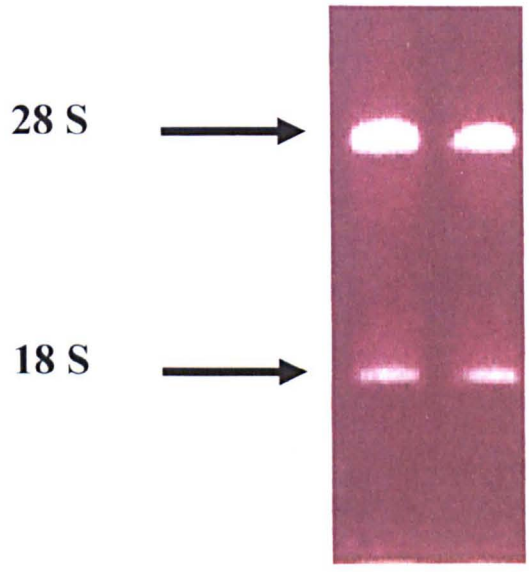


Fig. 5.4: Resolution of RNA isolated from HUVECs. Representative agarose gel (1.2% w/v) demonstrating separation of total RNA with no evidence of degradation of RNA sample. RNA was visualised by staining with ethidium bromide and viewed under ultraviolet light.

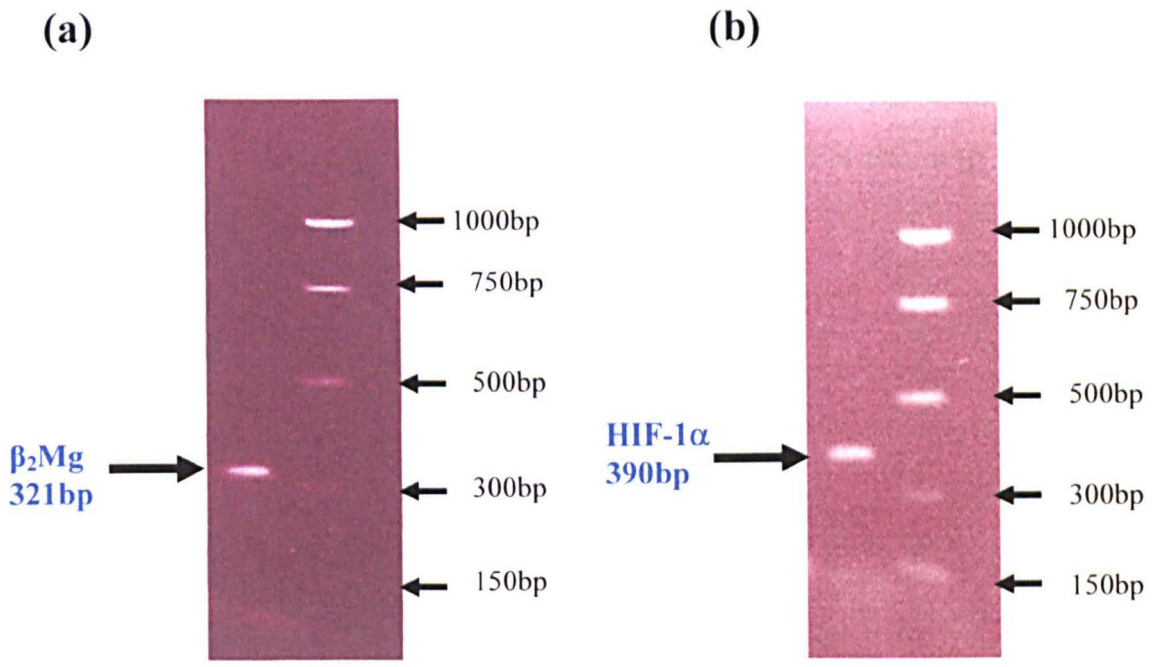


Fig. 5.5: Agarose gel (1.2% w/v) showing **(a)** β_2-Microglobulin (β_2Mg) and **(b)** HIF-1α PCR product compared to markers of known size. The products conformed to predicted size analysis of 321bp and 390bp for β_2Mg and HIF-1α respectively. The images are representative of three different experiments (n = 3).

5.4.4. Effect of HIF-1α antisense treatment on DNA damage

As reported in the previous chapters, DNA damage is increased in high glucose concentration and/or decreased oxygen concentration with the most significant increase in DNA damage observed in cells exposed to both conditions simultaneously ($p < 0.001$). These results are consistent with those obtained in Chapter Three (Fig. 3.5). In normoxic conditions, the addition of HIF-1α antisense to HUVECs had no effect on the level of DNA damage seen in response to high (20mM) glucose concentration ($p > 0.05$) (Fig. 5.6). However, HUVECs cultured in hypoxia in the prescence of HIF-1α antisense show significantly less DNA damage in response to both 5mM and 20mM glucose ($p < 0.001$).

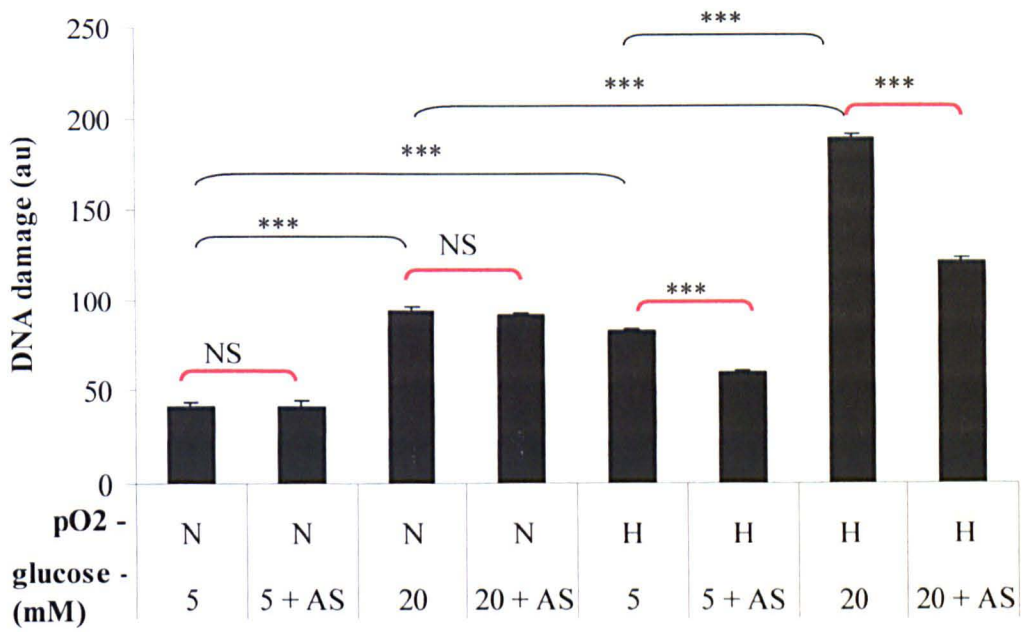


Fig. 5.6: Determination of the effect HIF-1α antisense (AS) (2μM) on glucose (5mM & 20mM) and oxygen (N = normoxia and H = hypoxia) induced HUVEC DNA damage. Cells were stepped down overnight in glucose free/serum free media and incubated for 24h prior to analysis with the comet assay. The results are expressed as mean of three experiments ± SEM (n = 3) (Kruskal-Wallis test: ***$p < 0.001$; NS: $p > 0.05$).

5.4.5. Effect of HIF-1α antisense on ROS production and sub-cellular location

The effect of glucose concentration and oxygen tension on HUVEC size over time was determined following HIF-1α antisense treatment of HUVECs using ImageJ freeware. The percentage change in cell size was determined relative to control (t = 0h 100%) (Fig.5.7) and was found to be non significant using a Student's t-test ($p > 0.05$). There also was no significant difference between sample groups ($p > 0.05$). Results show no change in HUVEC size in response to HIF-1α antisense treatment alone (6h – 5N: 99±1; 20N: 106±7; 5H: 104±4; 20H: 102±5; 24h – 5N: 98±3; 20N: 104±6; 5H: 102±5; 20H: 97±8) ($p > 0.05$) and when compared to untreated HUVECs (6h - 5N: 102±6; 20N: 108±1; 5H: 116±8; 20H: 110±7; 24h – 5N: 105±0; 20N: 108±4; 5H: 104±3; 20H: 111±1).

Changes in both MitoTracker Green FM and RedoxSensor Red CC-1 fluorescence intensity were determined using ImageJ freeware. The percentage change in fluorescence intensity for both markers was determined relative to control (t = 0h 100%) and analysed individually for all three experiments (Fig.5.8).

MitoTracker Green FM fluorescence was not affected by HIF-1α antisense treatment when compared to control (6h – 5N: 140±21; 20N: 94±19; 5H: 152±26; 20H: 113±17; 24h – 5N: 145±21; 20N: 130±13; 5H: 110±24; 20H: 112±59) (Fig.5.8) as determined by Student's t-test (6h & 24h $p > 0.05$). There was also no statistically significant difference between sample groups treated with HIF-1α antisense as analysed by ANOVA plus post-hoc Tukey's test.

RedoxSensor Red CC-1 fluorescence intensity in contrast does change significantly over time (Fig. 5.9). When compared to control there is a significant increase in RedoxSensor Red CC-1 fluorescence intensity in AS treated samples after 6h in normoxia (6h – 5N: 337±26; 5NAS: 499±77; 20N: 427±46; 20NAS: 517±62) ($p < 0.05$). The fluorescence intensity of

control samples was significantly reduced after 24h which is in agreement with earlier findings (Fig. 4.6) (5N: 133±56; 20N: 128±37; 5H:141±21; 20H:141±14). The addition of AS to normoxia samples after 24h leads to a significant reduction in CC-1 fluorescence intensity which contrasts the results obtained after 6h (5NAS: 75± 28; 20NAS: 69±8) ($p < 0.05$).

In hypoxia the addition of antisense significantly reduced CC-1 fluorescence intensity after 6h and 24h in 20mM glucose samples (6h – 20H: 450±63, 20HAS: 283±28; 24h – 20H: 141±21, 20HAS: 63±25) ($p < 0.05$). These results indicate that HIF-1α has a direct effect on ROS production in hypoxia which may or may not contribute to the DNA damage seen in these conditions. The mechanisms, through which this occurs is unknown but appears to be different from that in normoxic conditions.

Fig. 5.7: Determination of the effect HIF-1α antisense (AS) treatment (2μM) has on size of human umbilical vein endothelial cells compared to untreated (as shown previously in Fig. 4.3). Cells were stepped down overnight in glucose free/serum free media before incubation with glucose concentration of either 5mM or 20mM and oxygen concentrations (N = normoxia and H = hypoxia) for 24h. The change in cell size is expressed relative to control (t = 0h 100%). The above graph is representative of the mean ± SEM of three separate experiments (n = 3) with 50 observations (SEM values range from 1-8% within a group) per sample (Student's t-test and ANOVA plus post-hoc Tukey's test: $p > 0.05$).

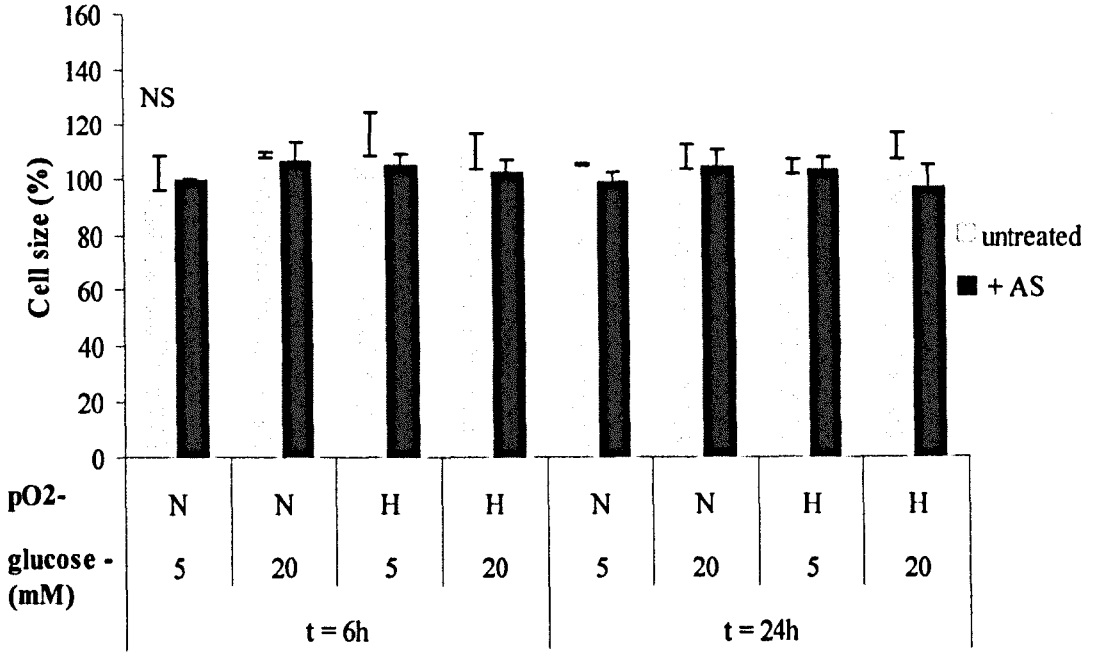

160
140
120
100
80
60
40
20
0
Cell size (%)
NS
untreated
+ AS
pO2-
N
N
H
H
N
N
H
H
glucose - (mM)
5
20
5
20
5
20
5
20
t = 6h
t = 24h

Fig. 5.8: Effect of HIF-1α antisense (AS) treatment (2μM) on MitoTracker Green FM fluorescence intensity in human umbilical vein endothelial cells compared to untreated (as shown previously in Fig. 4.4). Cells were stepped down overnight in glucose free/ serum free media before incubation in glucose concentrations of either 5mM or 20mM and oxygen concentrations (N = normoxia and H = hypoxia) for 24h. The change in fluorescence intensity is expressed relative to control (t = 0h 100%). The above graph is representative of the mean ± SEM of three separate experiments (n = 3) with 50 observations per sample (Student's t-test and ANOVA plus post-hoc Tukey's test: $p > 0.05$).

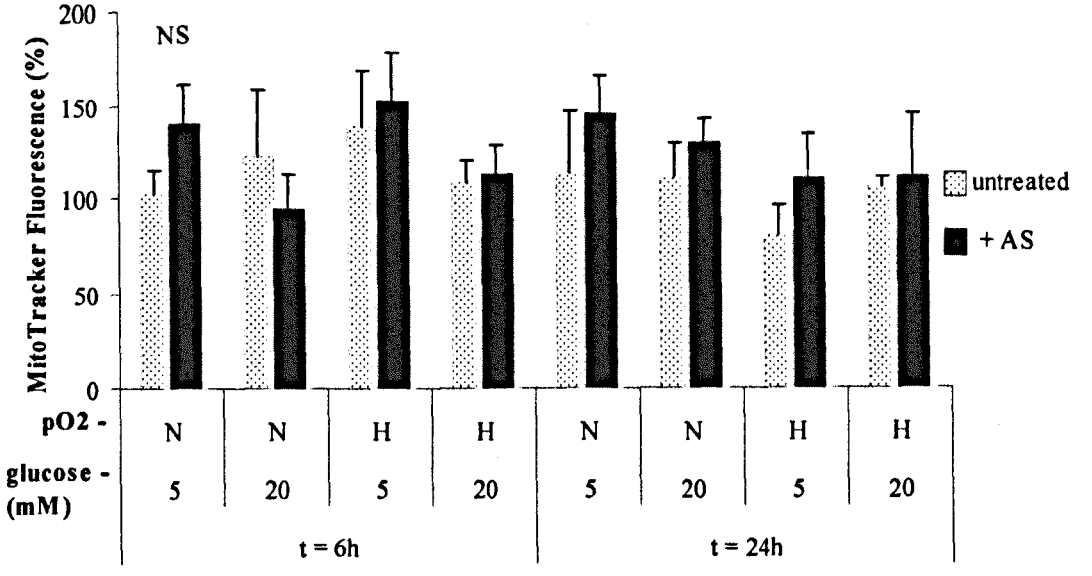
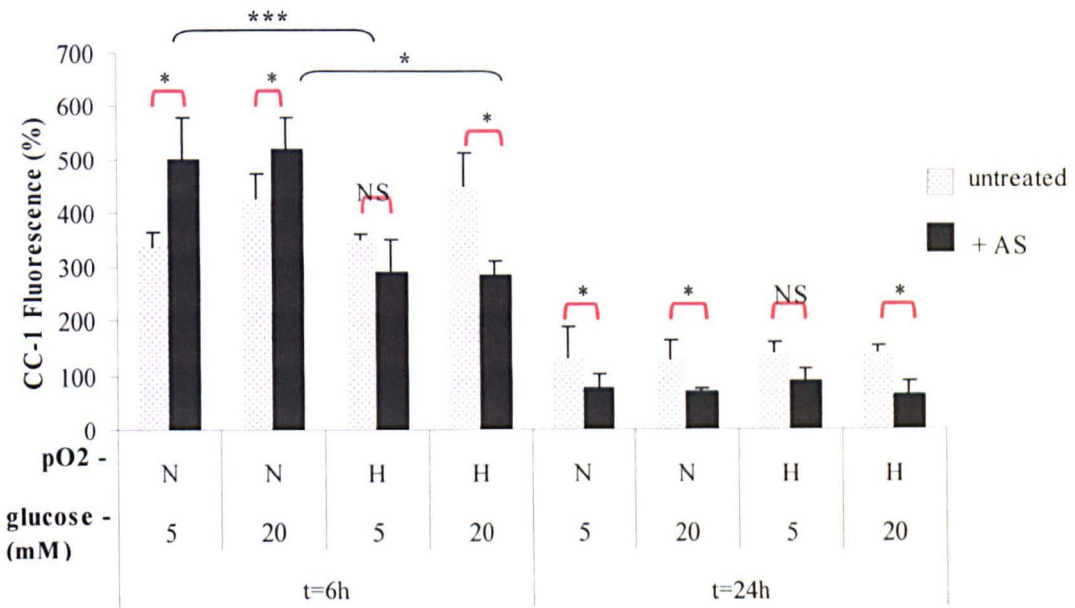

MitoTracker Fluorescence (%)
200
150
100
50
0
NS
pO2 -
glucose - (mM)
N
H
5
20
t = 6h
t = 24h
untreated
+ AS

Fig. 5.9: Effect of HIF-1α antisense (AS) treatment (2μM) on RedoxSensor Red CC-1 fluorescence intensity in human umbilical vein endothelial cells compared to untreated (as shown previously in Fig. 4.5). Cells were stepped down overnight in glucose free/ serum free media before incubation in glucose concentration of 5mM & 20mM and oxygen concentrations (N = normoxia and H = hypoxia) for 24h. The change in fluorescence intensity is expressed relative to control (t = 0h 100%). The above graph is representative of the mean ± SEM of three separate experiments (n = 3) with 50 observations per sample (Student's t-test and ANOVA plus post-hoc Tukey's test: *$p < 0.05$; **$p < 0.01$; ***$p<0.001$).


***
*
*
*
*
NS
*
*
*
NS
*
700
600
500
400
300
200
100
0
CC-1 Fluorescence (%)
untreated
+ AS
pO2 -
N
N
H
H
N
N
H
H
glucose - (mM)
5
20
5
20
5
20
5
20
t=6h
t=24h

5.5. Discussion

Although HIF-1α is recognized as a key mediator for a number of the cellular adaptive responses under hypoxic conditions (Section 5.2), little has been reported about its direct effect on endothelial DNA damage in response to hypoxia and/or hyperglycaemia. HIF-1α mediated adaptive responses appear to protect the cell in hypoxic conditions although its pro-apoptotic involvement has also recently been shown (Brunelle *et al.* 2002, Lee *et al.* 2004). HIF-1 has been shown to induce the expression of genes encoding glycolytic enzymes such as glucose transporter (GLUT-1) under hypoxic conditions (Semenza *et al.* 1994). It is suggested that increased glycolysis is necessary to produce energy when low oxygen will not support oxidative phosphorylation within the mitochondria. In contrast, Papandreou *et al.* (2006) found that, while HIF-1 stimulates enzymes required for glycolysis, it also actively represses mitochondrial function and oxygen consumption by inducing pyruvate dehydrogenase kinase 1 (PDK1). PDK1 phosphorylates and inhibits pyruvate dehydrogenase from using pyruvate to fuel the mitochondrial TCA cycle. This causes a drop in mitochondrial oxygen consumption and results in a relative increase in intracellular oxygen tension resulting in increased oxygen availability and decreased cell death. On the other hand HIF-1α has been shown to be pro-apoptotic with the activation of caspase-3, Apaf-1-mediated caspase 9 and the release of cytochrome c having been reported in several cell types under hypoxic conditions (Brunelle *et al.* 2002). It has even been demonstrated that HIF-1α is associated with the activation of the mitochondrial triggered apoptosis cascade (Weinmann *et al.* 2004).

Despite the intense research into the pro- & anti-apoptotic effects of HIF-1α, the literature does not indicate if HIF-1α is directly involved in acute oxidative stress induced endothelial DNA damage. Results obtained for HUVECs incubated in hypoxic and hyperglycaemic conditions for 24h and treated simultaneously ± HIF-1α antisense (Fig. 5.6) show significantly less DNA damage in cells incubated under hypoxic conditions, irrespective of

the glucose concentration ($p < 0.001$). HIF-1α antisense treatment has no effect on normoxia-induced DNA damage. These results suggest that HIF-1α contributes to acute hypoxia-induced endothelial DNA damage seen in Chapter Three (Fig. 3.5), although the action of HIF-1 does not appear to be cytotoxic as cell proliferation is not affected within this time frame (Fig. 3.1).

One likely site of this effect is the mitochondria. Since HIF-1α has been shown to influence mitochondrial function (Emerling *et al.* 2005), it is perceivable that a mechanism exists through which the activation of HIF-1α initially contributes to mitochondrial ROS mediated cell damage. A recent paper describes the requirement of mitochondrial ROS release (from complex III) into the cytoplasm for the hypoxic stabilisation of HIF-1α (Guzy *et al.* 2005) while a second paper describes how loss of mitochondrial function impairs cellular oxygen sensing and hypoxic HIF-α activation (Mansfield *et al.* 2005). Both papers seem to suggest that HIF-1α requires mitochondrial ROS production for its stabilisation and activitation. However, higher ROS levels also instigate more cell damage.

In order to verify if mitochondrial ROS production is linked to HIF-1α and the observed levels of DNA damage, mitochondrial ROS levels were determined in the presence of HIF-1α antisense (Fig. 5.9). There is an early change in cellular redox potential in response to glucose and or hypoxia measurable after 6h. This is in line with earlier findings of HUVECs treated without the addition of antisense (Fig. 4.6). In hypoxia, HUVECs treated with HIF-1α antisense for 6h show a significant reduction in RedoxSenor Red CC-1 flourescence intensity compared to identical untreated samples ($p < 0.05$). These observations suggests that hypoxia incurs a reduction in cellular redox potential in the absence of HIF-1α and a potential reduction in cellular ROS formation. Since blocking HIF-1α protein production has no effect on MitoTracker Green FM fluorescence intensity (Fig.5.8) or overall HUVEC size

(Fig.5.7), the reduction in cellular redox potential in hypoxia in the presence of HIF-1α antisense seems to support the suggestions that the activation of HIF-1α in hypoxia initially contributes to mitochondrial ROS production and possibly an associated increase in cell damage.

One possible mechanism through which the hypoxia stimulated release of ROS could contribute to the regulation of HIF-1α is through the accumulation of DAG. It has been reported that both hypoxia and high glucose concentration lead to DAG production (Rossi *et al.* 1991, Temes *et al.* 2004). This in turn leads to the accumulation of phosphatidic acid via the activation of diacylglycerol kinase. Phosphatidic acid in turn is thought to regulate HIF-1 expression (Temes *et al.* 2004, Aragones *et al.* 2001). Furthermore, DAG and phosphatidic acid also leads to the direct activation of PKC (Stasek *et al.* 1993). PKC induces cell damage by reducing the mitochondrial ATP production, leading to an overload of mitochondrial Ca^{2+} and loss of mitochondrial function (Costa *et al.* 2006). Although PKC has not been implicated in the hypoxia triggered accumulation of DAG and subsequent regulation of HIF-1 (Temes *et al.* 2004), loss of mitochondrial function could lead to the loss of HIF-1 function if the above hypothesis is correct.

Alternatively, the HIF-1α associated increase in endothelial DNA damage under hypoxic conditions (Fig. 5.6) could be an indication of a more direct involvement in the mediation of hypoxic cell death and apoptosis. Since Lee *et al.* (2004) were able to establish that the HGTD-P gene activates the mitochondrial apoptotic cascade through the induction of the mPTP a link has been established between HIF-1α and mitochodrial apoptosis signalling. Although our results clearly show the involvement of HIF-1α in mitochondrial redox changes (Fig. 5.9) (with the origin of the fluorescent RedoxSensor Red CC-1 marker confirmed as being mainly mitochondrial Fig. 4.2) we were also able to observe a proliferative increase under these same conditions (Fig. 3.1). As detailed in Chapter Three

however (Section 3.4.1), an increase in HUVEC proliferation under hypoxic conditions is associated with the increased transcription of VEGF, which in turn is mediated by HIF-1 (Zgouras *et al.* 2003).

In normoxia, a reduction in CC-1 fluorescence intensity can be observed in cells cultured in normoxic conditions for 24h when cultured in HIF-1α antisense compared to untreated (Fig. 5.9). This is in stark contrast to fluorescence levels seen at 6h where HIF-1α antisense seemingly induces mitochondrial ROS production significantly (Fig. 5.9). This suggests that HIF-1α plays a significant role in ROS production under normoxic conditions and although this could be considered to be in line with findings that the normoxic expression of HIF-1α is essential for macrophage function (Knowles *et al.* 2006) it also suggests that a reduction in hypoxia mediated DNA damage (Fig. 5.6) is unlikely to be due to a direct involvement of HIF-1α as the results are not consistent for both 5mM and 20mM glucose samples.

Clearly the reactions and interactions within the cell are complex. It is also likely that a plethora of other possible mechanisms activated during the oxidative stress response such as MAPK, PI3K, p42/44 and apoptosis will contribute to the observed effects of HIF-1α.

5.6. Conclusion

The present results have identified that that there seems to be a close relationship between HIF-1α activity, endothelial DNA damage and cellular ROS production in hypoxia. As ROS production is not significantly different in 5mM glucose samples in the presence of HIF-1α antisense it appears unlikely that the DNA protective effect of HIF-1α anisense in hypoxia is due to its direct effect on ROS production. It is more likely that HIF-1α is involved in

mitochondrial-mediated apoptosis signaling. More studies are required to corroborate this hypothesis.

CHAPTER SIX: Effect of glucose analogues and intracellular signalling inhibitors on endothelial DNA damage

6.1. Hypothesis

Endothelial DNA damage in response to hypoxia and hyperglycaemia is mediated through signalling pathways such as PKC and p42/44 activation or the metabolism of D-glucose.

6.2. Introduction

In order to elucidate the contributing mechanisms and signalling pathways involved in the development of hypoxia- and hyperglycaemia- induced loss of endothelial DNA integrity, DNA damage was assessed after the addition of glucose analogues (2-deoxy-D-glucose, 3-orthomethyl-glucose) and signalling inhibitors ($PKC_{\beta II}$/EGFR and PD98095 (p42/44 inhibitor)). 2-deoxy-D-glucose (2DG) and 3-ortho-methyl glucose (3OM) are structural derivatives of D-glucose. Once inside the cell, 2DG is phosphorylated by hexokinases giving rise to 2-D-glucose-6-phosphate (2-DG-6P) during the first step of the glycolysis reaction. Because of its structure, the phosphorylated molecule (2-DG-6P) cannot be metabolized further or diffuse outside the cell leading to the inhibition of glycolysis and the accumulation of the metabolised by-product (2-DG-6P) inside the cell. 3-ortho-methyl glucose (3OM) is not metabolised at all and simply enters and leaves the cell.

Common signalling pathways activated in hyperglycaemia- and hypoxia- mediated events include, amongst others, protein kinase C (PKC) and p42/44MAPK (Wynne *et al* 2005, Idris *et al.* 2006). Known inhibitors of these pathways include the highly selective PKC inhibitor LY333531 and the p42/44 inhibitor PD98095.

The assessment of the effect glucose analogues (2-deoxy-D-glucose, 3-orthomethyl-glucose) and the signalling inhibitors $PKC_{\beta II}$/EGFR and PD98095 (p42/44 inhibitor) have on endothelial DNA damage and cell proliferation after only 24h was carried out in order to elucidate the molecular processes which lead to the observed rise in DNA damage in response to high glucose concentration and low oxygen tension.

6.3. Materials and Methods

The effect of glucose concentration and oxygen tension on DNA integrity of HUVECs was determined using the comet assay (Section 2.4). This was carried out using equimolar concentrations of D-glucose and its analogues 2-deoxy-D-glucose and 3-ortho-methyl glucose as well as the signalling inhibitors $PKC_{\beta II}$/EGFR inhibitor (1μM) and PD98095 (2μM). HUVECs challenged for 24h were cultured as described in Section 2.2. and their DNA extracted as described in Section 2.3.

Statistical significance was determined using a Student's t-test (Mean – 100%/ SEM) and/ or ANOVA plus post hoc Tukey's test for normalised data. The level of significance expressed as *$p< 0.05$, **$p< 0.01$ or ***$p< 0.001$. A non significant difference (NS) was denoted for samples where $p > 0.05$.

6.4. Results

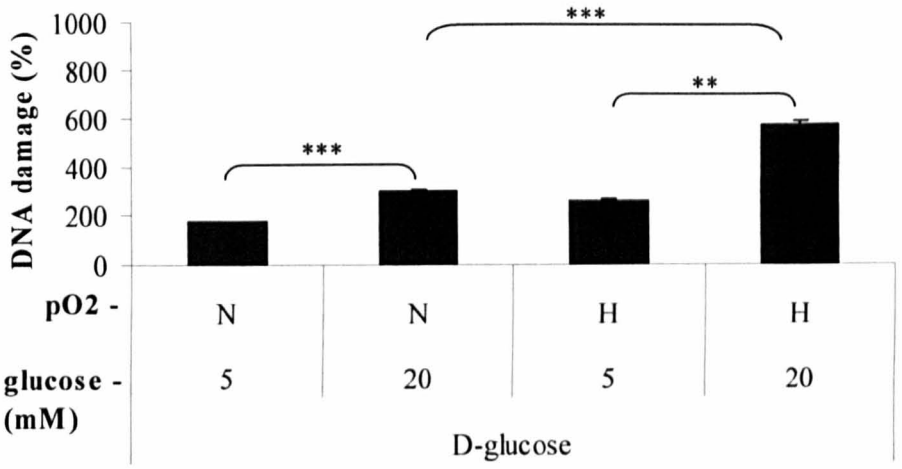
6.4.1. Effect of D-glucose, 3OM and 2-DG-6P on DNA integrity of HUVECs

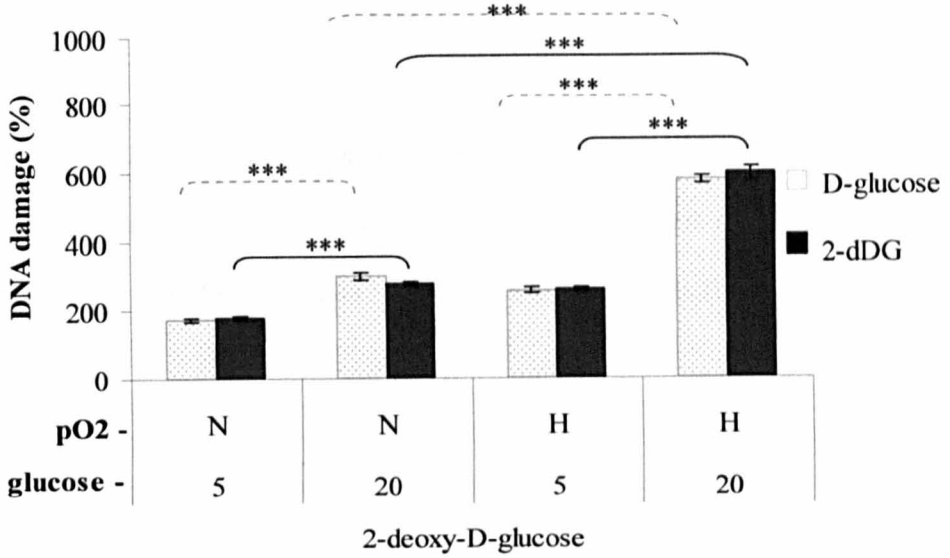
A comparison of the DNA damage profile of D-glucose to its analogues 2-deoxy-D-glucose and 3-ortho-methyl-glucose was carried out using the comet assay (Fig. 6.1). In HUVECs treated with D-glucose alone hyperglycaemia increases DNA damage levels significantly when compared to normoglycaemia (5N: 172±4; 20N: 301±11 – $p < 0.001$), (5H: 258±9; 20H: 577±12 - $p < 0.001$); (Fig. 6.1). DNA damage was further significantly increased in hyperglycaemic samples simultaneously exposed to hypoxia (20N: 301±11; 20H: 577±12 - $p < 0.001$). These observations are in agreement with earlier findings (Fig. 3.5).

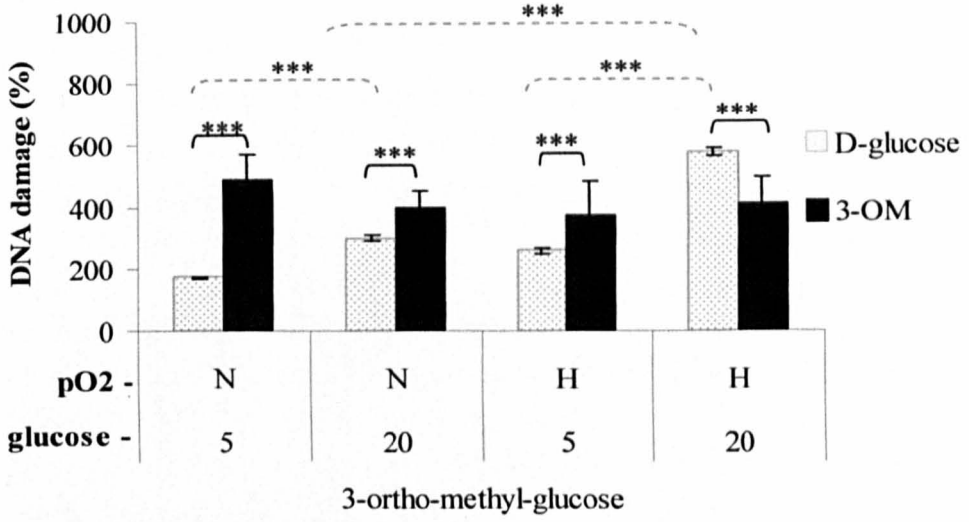
An almost identical DNA damage pattern can be seen with the glucose analogue 2-deoxy-D-glucose (5N: 178±8; 20N: 278±6 - $p<0.001$), (5H: 261±5; 20H: 599±19 - $p<0.001$), (20N: 278±6 vs 20H: 599±19 - $p < 0.001$) (Fig. 6.1).

Loss of DNA integrity by 3-ortho-methyl is significantly higher in all samples compared to D-glucose (5N: 489±81; 20N: 396±58; 5H: 376±109; 20H: 412±82 – $p > 0.05$) apart from 20H. No significant difference in DNA integrity was seen between 3-OM treated samples ($p > 0.05$).

Fig.: 6.1: Comparison of the effect of D-glucose (5mM & 20mM) and equivalent concentrations of the glucose analogues 2-deoxy-D-glucose and 3-ortho-methyl-glucose on HUVEC DNA damage (N = normoxia and H = hypoxia). Cells were stepped down overnight in glucose free/ serum free media before incubation. Cells were analysed with the comet assay. Results are expressed as mean ± SEM from three separate experiments (n = 3) (ANOVA plus post-hoc Tukey's test : *** $p<0.001$).


DNA damage (%)
1000
800
600
400
200
0
***
***
**
pO2 -
N
N
H
H
glucose -
(mM)
5
20
5
20
D-glucose


DNA damage (%)
1000
800
600
400
200
0
***
***
***
***
***
***
D-glucose
2-dDG
pO2 -
N
N
H
H
glucose -
5
20
5
20
2-deoxy-D-glucose

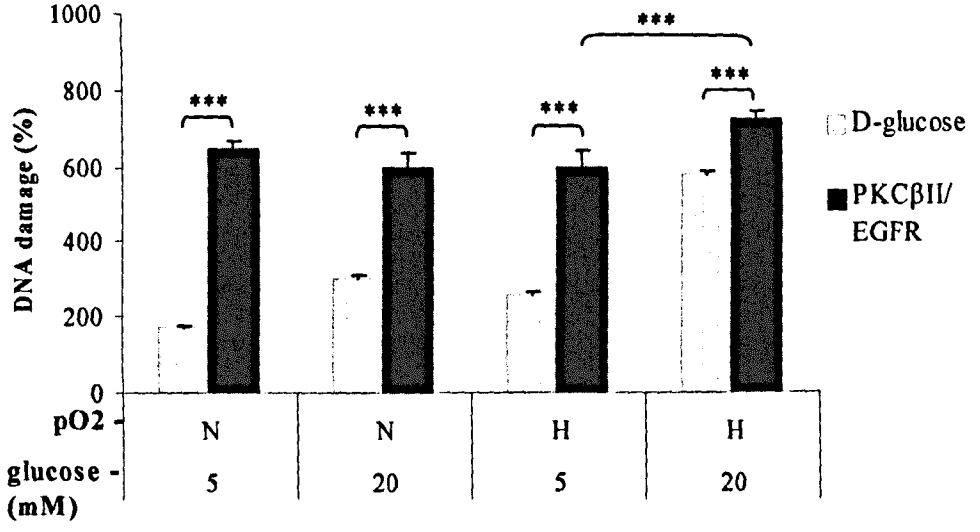

DNA damage (%)
1000
800
600
400
200
0
***
***
***
***
***
***
***
D-glucose
3-OM
pO2 -
N
N
H
H
glucose -
5
20
5
20
3-ortho-methyl-glucose

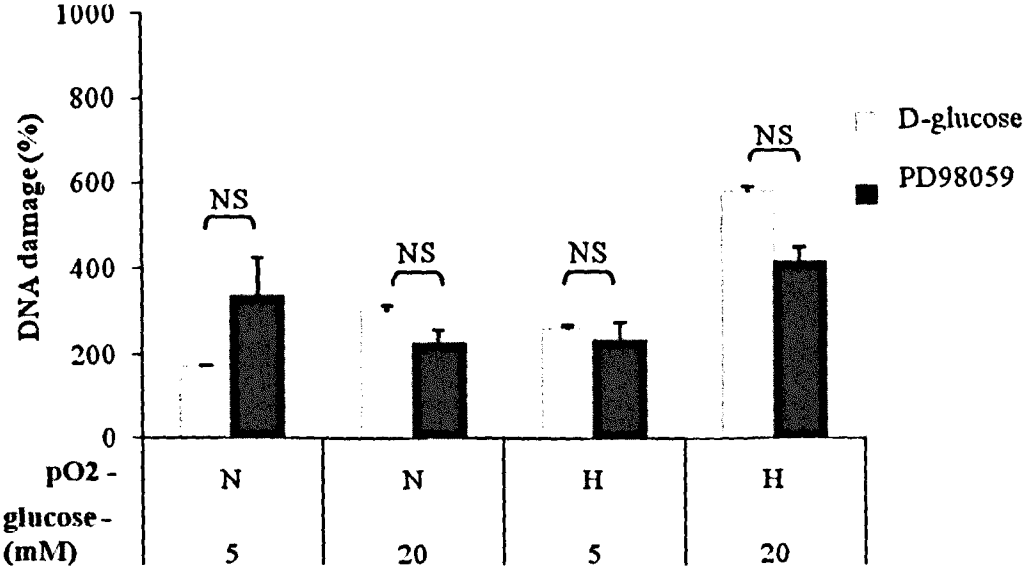
6.4.2. Effect of specific signalling inhibitors on DNA damage

In order to elucidate contributing mechanisms which lead to the observed DNA damage in the different culture conditions, various inhibitors were used; namely $PKC_{\beta II}$/EGFR inhibitor and PD98095 (p42/44 inhibitor) (Fig. 6.2). Samples incubated with a PKC inhibitor (1μM) showed a significant increase in DNA damage compared to control in all samples almost appearing to be toxic to the cells (5N: 650±4; 20N: 600±40; 5H: 599±44; 20H: 725±16 - $p < 0.001$). While there was no significant difference between most PKC treated samples incubated under different culture conditions ($p > 0.05$), there is a significant increase in hypoxic conditions with an increase in glucose concentration from 5mM (577 ± 12) to 20mM glucose (725 ± 16) ($p > 0.001$).

The addition of PD98059 (2μM) showed no difference in DNA damage when compared to D-glucose control ($p > 0.05$). There is also no significant difference between PD98059 samples (5N: 334±89; 20N: 224±31; 5H: 231±43; 20H: 414±31 - $p > 0.05$).

Fig.: 6.2: Comparison of the effects of $PKC_{\beta II}$/EGFR inhibitor (1μM) and PD98059 (2μM) on HUVEC DNA damage (N = normoxia and H = hypoxia). Cells were stepped down overnight in glucose free/ serum free media before incubation. Cells were analysed with the comet assay. The results are expressed as mean ± SEM from three separate experiments (n = 3) (ANOVA plus post-hoc Tukey's test: $p < 0.05$).


1000
800
600
400
200
0
DNA damage (%)
***
***
***
***
***
D-glucose
PKCβII/
EGFR
pO2
N
N
H
H
glucose -
(mM)
5
20
5
20


1000
800
600
400
200
0
DNA damage (%)
NS
NS
NS
NS
D-glucose
PD98059
pO2 -
N
N
H
H
glucose -
(mM)
5
20
5
20

6.5. Discussion

The analysis of DNA integrity of HUVECs in response to D-glucose and its analogues (2DG and 3OM) demonstrated that 2DG gives a similar DNA damage pattern when compared to D-glucose, suggesting that the loss of DNA integrity in response to high glucose concentration and low oxygen after 24h is not linked to glycolysis or any of its downstream events beyond the first step of breakdown of glucose into glucose-6-phosphate.

This is surprising, as glycolysis is essential in cellular respiration and energy production, feeding into the citric acid cycle (mitochondria) and subsequent mitochdonrial oxidative phosphorylation. The excessive formation of oxygen radicals is a well-established mediator of hyperglycaemic damage to a wide range of tissues in diabetes, including neurons (Vincent *et al.* 2005), retinal cells (Robinson *et al.* 2000), and vascular endothelium (Zurova-Nedelcevova *et al.* 2006). Metabolic oxidative stress is also regarded as crucial mediator of beta cell dysfunction and apoptosis under hyperglycemic conditions (Martens *et al.* 2007).

Although surprising, the observation is not entirely unreasonable as some oxidative stress mediated pathways such as NO have a reported inhibitory effect on glycolysis. NO induced cellular toxicity has been reported to prevent both mitochondrial oxidative phosphorylation and glycolysis (Mohr *et al.* 1999), affecting the blood brain barrier (Hurst *et al.* 2001). More recently, it was demonstrated that hyperglycaemia-induced overproduction of superoxide inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) another major enzyme of the glycolysis pathway. This is thought to trigger several pathways of injury involved in the pathogenesis of diabetic complications (Du *et al.* 2003).

As 2DG is phosphorylated by hexokinases giving rise to 2-D-glucose-6-phosphate (2-DG-6P) during the first step of the glycolysis reaction after which it accumulates in the cell failing to be metabolised any further, the resultant DNA damage observed must be

associated with either glucose in its unmetabolised form, the hexokinase enzyme, the phosphorylated form of glucose or the accumulation of this product within the cell. The most likely explanation falls with the activation of the polyol pathway. As described in Chapter One (Section 1.6.1), the polyol pathway is activated as a result of the hexokinase enzyme becoming saturated in the presence of high glucose concentrations. It converts glucose into sorbitol and onwards into fructose utilising NADPH and NAD^+, thereby altering the redox state of the cell, which in turn leads to depressed glutathione levels and the resulting loss of intrinsic protection against oxidative stress (Williams *et al.* 1999). It has been associated with the activation of PKC and NF-κB (Ramana *et al.* 2002), which are involved in mediating biochemical and functional changes in diabetic vessels (Sheetz *et al.* 2002).

As mentioned above, a study carried out by Flores *et al.* in 2003 showed that hyperglycaemia (25mmol) stimulates the L-arginine/NO pathway in HUVECs within the first 2min of incubation, which is thought to occur via the activation of PKC (Flores *et al.* 2003). It is possible that conditions of high glucose concentration result in high intracellular glucose, thus saturating the glycolytic pathway. ROS, such as NO will be generated which in turn inhibits glycolysis and the glucose is shunted into the polyol pathway, where it is metabolised into sorbitol and fructose, leading to changes in the cellular redox state, depression of glutathione reductase levels and the activation of PKC, all of which are known to contribute to the development of DNA damage (Williams *et al.* 1999; De Mattia *et al.* 1994; Ramana *et al.* 2002).

In contrast, 3-OM, a glucose derivative which is not metabolised, but simply diffuses in and out of the cell, had no significant effect onDNA damage. The overall level of DNA damage, however, is significantly increased compared to that seen with D-glucose (Fig. 6.1), suggesting that damage is caused by the entry of glucose into the cell even before it is metabolised. Alternatively the higher levels of DNA damage could be caused by a lack of

cellular "nutrition" as glucose enters and leaves the cell without being utilised inducing a high degree of cellular stress.

Using cellular signaling inhibitors, the contribution of certain stress pathway to the observed DNA damage was assessed. The use of a PKC inhibitor on hyperglycaemia/ hypoxia induced DNA damage shows a significant rise in DNA damage levels compared to D-glucose alone (Fig. 6.2), suggesting that a loss of PKC activity is detrimental rather than beneficial to the DNA integrity of the HUVEC. Furthermore, DNA damage levels in cells containing a PKC inhibitor did not vary significantly with a change in culture conditions, suggesting that PKC levels remain unaffected by high glucose and hypoxic culture conditions in our experimental setup with the exeption of an increase between 5mM and 20mM glucose samples cultured in low oxygen tension. These results seem to exclude PKC as a possible source or contributing factor of hyperglycaemia/ hypoxia-induced DNA damage, despite a huge body of literature observing the contrary effect. Further studies would be required to look at PKC activity and the effect of its inhibition at different time points throughout this 24h incubation period. Attention should also be paid to the method of choice since the use of the $PKC_{\beta II}$/EGFR inhibitor almost appears toxic as HUVECs DNA damage levels are very high throughout.

The use of the p42/44 MAPK inhibitor, PD98059, did not result in any significant changes to DNA damage levels obtained with D-glucose (Fig. 6.2). From these results, it appears to be unlikely that p42/44 plays a significant role in the development of endothelial DNA damage in response to glucose or hypoxia after 24h. It does, however, show a trend of reducing DNA damage when HUVECs are exposed to the simultaneous exposure of hyperglycaemia and hypoxia ($p = 0.05$) adding weight to our observations from Chapter Three (Fig.3.5). A study looking at the activation of p42/44 (ERK) in response to D-glucose (25mM) in endothelial cells also failed to observe a rise in its activation (Liu *et al.* 2000), confirming our observations. The main body of evidence surrounding p42/44 (ERK) is centred around its

activation in hypoxia, where it has been shown to phosphorylate HIF-1α and enhance the transcriptional activity of HIF-1 (Richard *et al.* 1999). Taking our result from Chapter Five into account, where we were able to discern that HIF-1α activation in response to hypoxia contributes to the development of endothelial DNA damage, this extrapolation to the involvement of p42/44 in this process seems to go someway towards explaining the observed DNA damage. Especially since PD98059 results in an inhibition of HIF-1α trans-activation, where it reduces the hypoxia-induced transcription of both the target gene and the hypoxia-responsive reporter gene (Hur *et al.* 2001). However, the observed change in DNA damage was not statistically significant ($p > 0.05$) and we did not see any effect of PD98059 on hypoxia induced DNA damage alone.

Since the results from the signalling inhibitor studies are inconclusive, more reliable and established methods should be used to analyse which signaling pathways respond to changes in glucose concentration and oxygen tension and how this impacts on the development of endothelial DNA damage. These include protein and enzyme assays such as enzyme-linked immunosorbent assays (ELISAs) which can be used to quantitate kinase levels and evaluate their activities. By incorporating a recognize protein regardless of phosphorylation state and a phospho-specific antibody, ELISA-based assays can be used to measure total protein and activated protein levels. Molecular cloning techniques may also be used to identify the role PKC and p42/44 play in response to changed in glucose and oxygen tension within the endothelium.

6.6. Conclusion

Results presented in this Chapter suggest a possible involvement of the polyol pathway as well as p42/44 signaling in the development of endothelial DNA damage observed in Chapter Three. A common denominator may be the involvement of HIF-1α which is in agreement with the observations from Chapter Five.

CHAPTER SEVEN: Effect of silymarin (*Silibum marianum)* on high glucose and low oxygen mediated DNA damage in HUVECs

7.1. Hypothesis

The flavonoid antioxidant silymarin (*Silibum marianmum)* has a beneficial effect on high glucose and oxygen mediated DNA damage in HUVECs.

7.2. Introduction

Amelioration of the effect of high glucose concentration and hypoxia on DNA damage in endothelial cells was examined using a known antioxidant, silymarin (*Silibum marianum*) (SM). Silymarin, also known as milk thistle, has been used as a therapeutic agent for over 2000 years. It has been reported to have a protective effect against ROS-induced DNA damage (Anderson *et al.* 1994) and to prevent a reduction in the enzymes superoxide dismutase (Nencini *et al.* 2006), catalase (CAT) and glutathione peroxidase (GSHPx) (Aldioost *et al.* 2006), all of which are responsible for the removal of catalase and the protection against ROS induced cell damage. Further, silymarin has been shown to improve nerve conduction velocity in diabetic neuropathy (Zhang *et al.* 1993), act as a membrane stabiliser (Fraschini *et al.* 2002) and prevent ROS-induced DNA damage (Yu *et al.* 1997). It is therefore perceivable that an antioxidant such as silymarin could be beneficial in counteracting oxidative stress-mediated DNA damage in the endothelial models, as previously described in this thesis.

The cytoprotective effect of the flavonoid antioxidant, silymarin, on high glucose and low oxygen-induced DNA damage and the potential mechanisms that are involved in silymarin mediated effects were investigated.

7.3. Materials and Methods

The effect of silymarin treatment (50-5000μM) (Wen *et al.* 2007), in combination with glucose concentration and oxygen tension on cell growth was determined using the Alamar blue proliferation assay (Fields *et al.* 1993); (Section 2.2.5). Any effect of these conditions on DNA integrity in combination with HIF-1α antisense (2μM) was determined using the comet assay (Section 2.4) and the effect that silymarin treatment (50μM) has on the cellular uptake of HIF-1α antisense was assessed using a FITC labelled control oligonucleotide (Section 2.7). The effect of these conditions on cellular mitochondrial numbers, HUVEC size and cellular redox potential was determined using both MitoTracker Green FM and RedoxSensor Red CC-1 fluorescent dye (Section 2.5) and analysed using ImageJ software.

Each experiment was performed in triplicate and repeated on three separate occasions. The test distribution was assumed not to be normal to account for the limitations in sample size. Statistical significance was determined using a Kruskal-Wallis test or Mann-Whitney U test against the test hypothesis (H_0) that there are no differences between the means of the samples. Were $p < 0.05$ the H_0 must be rejected and the H_A (there is a difference between the means of the samples) accepted. Where data was normalised (100%) a Student's t-test (Mean – 100%/ SEM) and/or ANOVA (with Tukey's post-hoc test) was carried out. The level of significance is expressed as $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$. No significant difference was assigned to samples where $p > 0.05$.

7.4. Results

7.4.1. Effect of silymarin on DNA damage

Silymarin was added to HUVECs at concentrations of 50, 500 and 5000μM (in ≤0.7% (v/v) DMSO) for 24h. The level of DNA damage was measured compared to DMSO control alone

in order to ensure that the effect of silymarin (50μM) used in subsequent experiments was not due to an effect of the DMSO alone. Results indicate that silyamrin shows a pronounced DNA damaging effect at concentrations above 500μM (5000μM: 232±29; $p < 0.001$) (Fig. 7.1). There is no significant difference in the level of DNA damage seen with 50μM (68±13) & 500μM (55±8) silymarin treatment for 24h. A vehicle control (DMSO 0.7% total) was carried out and found to be within acceptable levels (49±8 i.e. vehicle control shows minimal levels of DNA damage) confirming that the potent hydroxyl radical scavenging effects of DMSO do not have any effect on the results obtained by the actions of silymarin. All subsequent experiments were carried out at a concentration of 50μM silymarin.

7.4.2. Comparison of silymarin to alpha-lipoic acid

In order to compare the ability of silymarin to reduce DNA damage a comparison to α-lipoic acid (ala) (100μM) was carried out. In normoxic conditions, α-lipoic acid treatment resulted in significantly less DNA damage compared to silymarin when compared to a normalised control (20mM Normox: 100%) (20NSM (118±7) vs 20Nala (88±7): $p = 0.024$) (Table 7.1). In hypoxic culture conditions there was no significant difference between α-lipoic acid and silymarin treated samples (20HSM (103±4) vs 20Hala (97±6): $p = 0.494$). Results suggest that silymarin appears to be a cytoprotective antioxidant but is not as potent as alpha-lipoic acid.

7.4.3. Effect of silymarin on cell proliferation

As described previously, an alamar blue assay was carried out to investigate the effect silymarin (50μM) has on cell proliferation over 24h (Fig. 7.2). As before (Fig. 3.1), HUVEC proliferation rate is significantly increased in cells incubated in high glucose and low oxygen conditions for 24h (20N: 1.2±0.01; 20H: 1.7±0.01). The addition of silymarin does not appear to have any significant effect on cell proliferation over this 24h time frame although there seems to be a slight tendency for silymarin to reduce proliferation in all samples. This

tendency is however not statistically significant (5N: 1.1±0.02; 20N: 1.1±0.01; 5H: 1.4±0.01; 20H: 1.4±0.04).

7.4.4. Comet Assay – Effect of silymarin on glucose and oxygen mediated DNA damage

In a blinded analysis, the effect of silymarin (50μM) on DNA integrity in HUVECs cultured in varying concentrations of glucose (5mM & 20mM) and oxygen (normoxia & hypoxia) for 24h was assessed. Results confirmed earlier findings in untreated cells (Fig. 3.5 and 5.6) showing that hyperglycaemia leads to a significant rise in DNA damage in cells cultured under both normoxia (5N: 49±8; 20N: 79±3) and hypoxia (5H: 84±3; 20H: 156±5) (Fig. 7.3). The greatest increase in DNA damage can be seen in HUVECs cultured in both high glucose (20mM) and hypoxic conditions for 24h (20N: 79±3; 20H: 156±5).

Addition of silymarin (50μM) had no significant effect on DNA damage levels in HUVECs cultured in normoxic conditions for 24h (5NSM: 51±4; 20NSM: 67±5). It did, however, significantly reduce DNA damage levels in HUVECs cultured in hypoxic conditions for 24h (5H: 84±3 vs 5HSM: 54±3; 20H: 156±5 vs 20HSM: 67±5). Since DNA damage levels in silymarin-treated cells seem to remain constant between all culture conditions, the results suggest that silymarin treatment prevents a loss of DNA integrity with a change in culture conditions rather than repairing existing damage.

In order to determine how quickly silymarin exerts its protective effect, DNA damage levels in HUVECs cultured in 20mM glucose at 1, 6 and 24h in both normoxia and hypoxia were determined (Fig. 7.4). As seen previously (Fig. 3.5, 5.6 and 7.3) there is a significant increase in DNA damage in control samples cultured in both high glucose and low oxygen compared to high glucose alone (20N: 61±5 vs 20H: 95±9). This effect is evident after only 6h and increases over time.

The effect of silymarin on the level of DNA damage could only be observed after 24h (Fig. 7.4). Interestingly, there was a significant rise in DNA damage in normoxia with the addition of silymarin after 24h (20N: 62±6 vs 20NSM: 104±7) whereas silymarin significantly reduced DNA damage in cell cultured in hypoxia for 24h (20H: 137±13 vs 20HSM: 72±3). These results suggest that loss of DNA integrity in endothelial cells is present after only six hours exposure to high glucose and low oxygen tension. Silymarin treatment appears to prevent DNA integrity loss in hypoxic culture conditions.

Extending the incubation time to 48h leads to a further rise in DNA damage in both normoxic (20N24: 100±0 vs 20N48: 172±11) and hypoxic (20H24: 164±6 vs 20H48: 206±5) conditions (Fig.7.5). The re-oxygenation of hypoxic cells for 24h did not cause any significant change to the level of DNA damage (20H+20N: 233±2). The addition of silymarin (50μM) to the culture medium caused no significant change to the level of DNA damage in cells cultured in normoxic conditions (20N24SM: 121±14; 20N48SM: 147±4). However HUVECs cultured in hypoxic conditions, showed a significant reduction in DNA damage with the addition of silymarin compared to control (20H24: 164±6; 20H24SM: 104±2; 20H48: 206±5; 20H48SM: 86±1). This was also true for re-oxygenated samples (20re-ox: 233±2: 20re-ox SM: 91±5) (Fig.7.5).

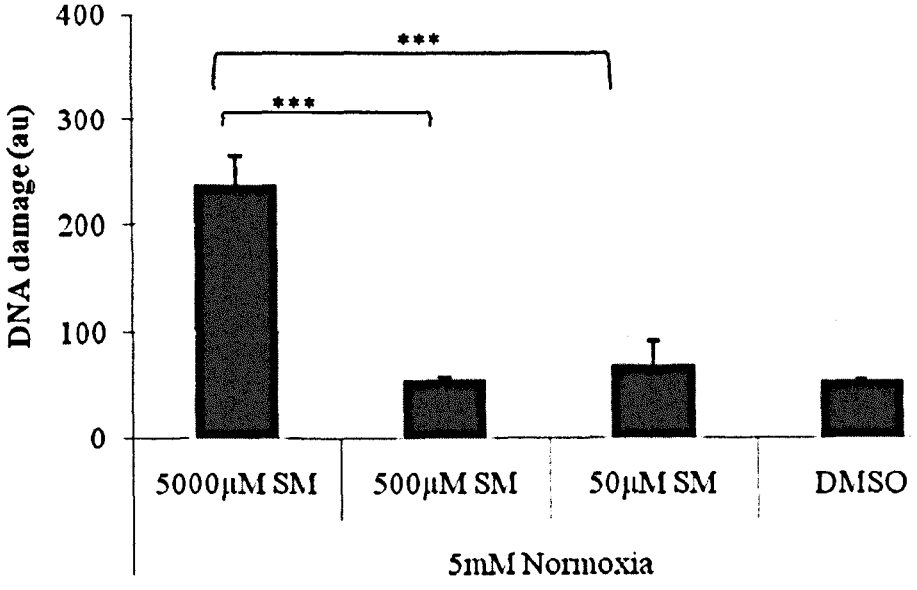


Fig. 7.1: Effect of silymarin (SM) on the level of DNA damage induced in human umbilical vein endothelial cells cultured in 5mM glucose normoxia for 24h. Results are expressed as mean ± SEM from three different batches of cells (n = 3) (Kruskal-Wallis test: *** $p < 0.001$).

Table 7.1: Effect of α-lipoic acid (100μM) compared to silymarin (50μM) on HUVEC cell DNA damage (normoxia and hypoxia). Cells were stepped down overnight in glucose free/serum free media before incubation and analysis with the comet assay. Results are expressed as relative percentage change compared to control (Mean – 100%/ SEM) ± SEM from four separate experiments (n = 4) (Student's t-test: *p<0.05, **p<0.005, ***p<0.001).

Treatment	Relative percentage change in DNA damage (%)	Student's t-test $p < 0.05$ (normalised to 20mM Normox)	Student's t-test $p < 0.05$ (compared to silymarin treatment)
20mM glucose Normox	100		
20mM glucose Normox + 50μM silymarin	118 ± 7	0.081	
20mM glucose Normox + 100μM α – lipoic acid	88 ± 7	0.191	0.024 *
20mM glucose Hypox	170 ± 7	0.002 *	0.001 ***
20mM glucose Hypox + 50μM silymarin	103 ± 4	0.536	
20mM glucose Hypox + 100μM α – lipoic acid	97 ± 6	0.710	0.494

* ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$)

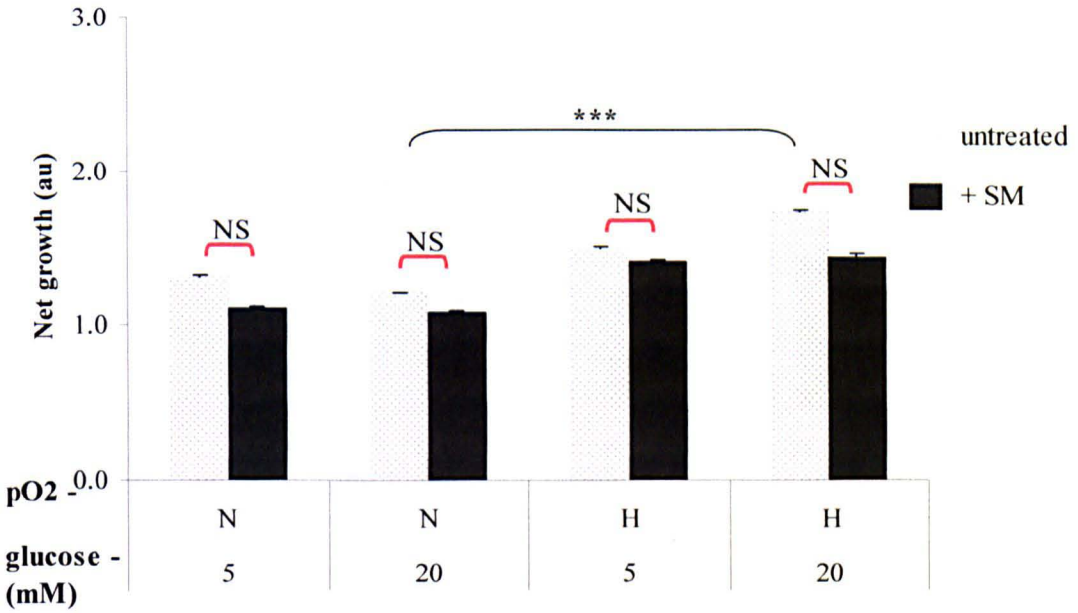


Fig. 7.2: Histogram showing the effect of silymarin (SM) treatment (50μM), on glucose (5mM & 20mM) and oxygen tension (N = normoxia and H = hypoxia) -induced changes in cell proliferation in a HUVEC line after 24h incubation. Each bar represents the mean ± SEM from three different batches of cells (n = 3) (Kruskal-Wallis test: ***$p < 0.001$).

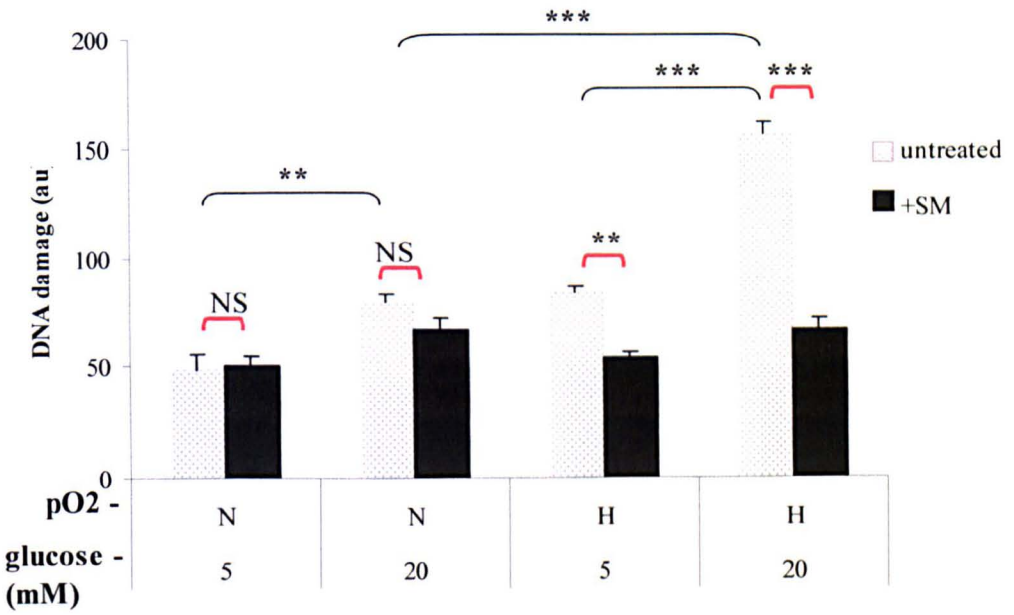


Fig. 7.3: Histogram illustrating the effect of the addition of silymarin (SM); (50μM) on endothelial DNA damage in response to 5 or 20mM glucose and oxygen concentrations (N = normoxia and H = hypoxia) on HUVECs for 24h. Cells were analysed with the comet assay. The results are expressed as mean ± SEM from three separate experiments (n = 3) (Kruskal-Wallis test: **$p < 0.01$; ***$p < 0.001$).

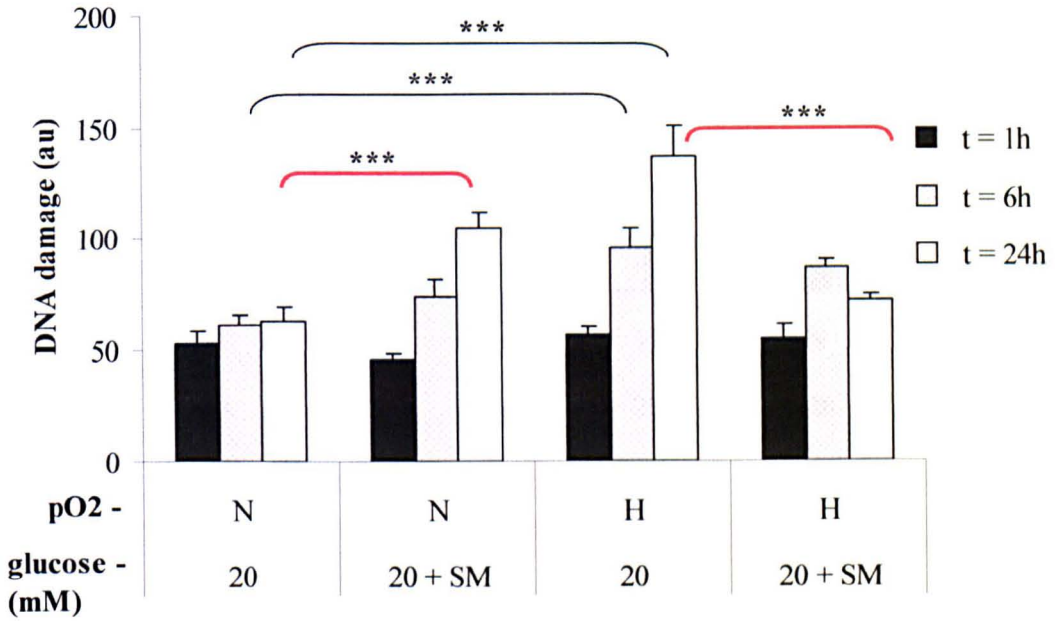


Fig. 7.4: Histogram illustrating the effect of addition of silymarin (50μM) to HUVECs incubated in high glucose (20mM) and in Normoxia and Hypoxia at 1h, 6h and 24h. The results are expressed as mean ± SEM from three separate experiments (n = 3) (Kruskal-Wallis test: ***$p < 0.001$).

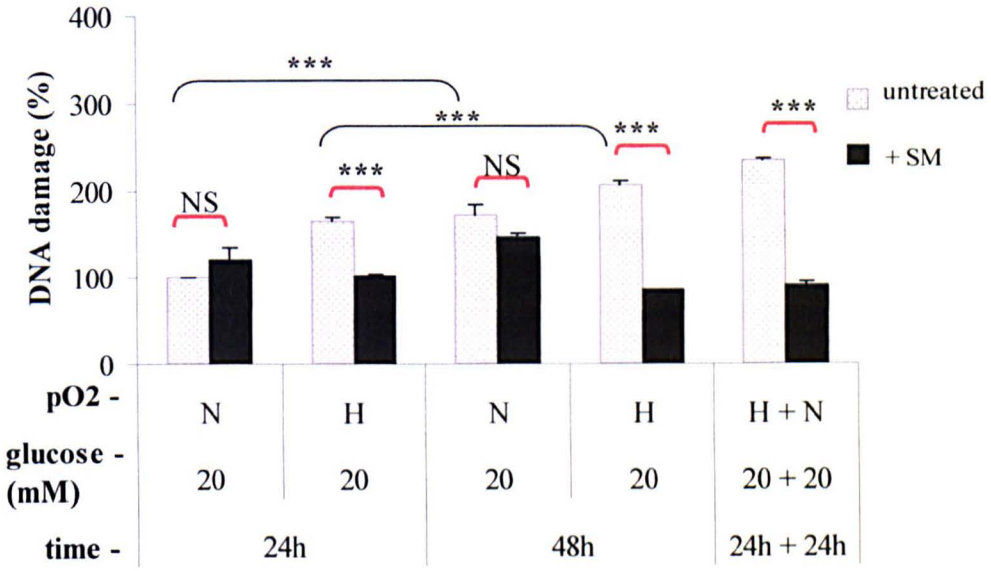


Fig. 7.5: Effect of silymarin (SM) (50μM), incubation time (24h & 48 h) and re-oxygenation (N = normoxia and H = hypoxia) on HUVEC DNA damage. Cells were analysed using the comet assay. The relative change in DNA damage is expressed as a ratio of the 20mM glucose damage in Normoxia (24h) sample (100%). The results are expressed as mean ± SEM from three separate experiments (n = 3) (Student's t-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

In order to determine the involvement of HIF-1α on the DNA protective effect of silymarin, DNA damage was analysed in HUVECs treated with silymarin ± HIF-1α antisense (2μM) (Fig. 7.6). If silymarin acts independently of HIF-1α, then a reduction in DNA damage with silymarin treatment is expected followed by a further reduction when HIF-1α activity is blocked as indicated by the results from Chapter Five (Fig. 5.6). No significant difference between silymarin treated samples alone could be observed with one exception, cells cultured in 20mM glucose normoxia which showed a significant increase in DNA damage compared to 5mM normoxia (5NSM: 41±1; 20NSM: 83±2) (Fig. 7.6). A corresponding rise in DNA damage seen in silymarin treated samples + AS was also observed (5NSMAS: 32±1; 20NSMAS: 69±1).

Antisense reduces DNA damage further when compared to SM treatment alone although in samples incubated in 5mM glucose/hypoxia (5HSM: 62±1; 5HSMAS: 32±1). Compared to untreated HUVECs (as shown in Fig. 3.5), silymarin ± HIF-1α antisense seem to prevent the loss of DNA integrity confirming the findings of Fig. 7.3.

In order to ensure that silymarin does not prevent HIF-1α antisense uptake into the cell, which would lead to the production of false negative results seen with the comet assay in Fig 7.6 silymarin treated HUVECs cultured in hyperglycaemia and normoxia were incubated with FITC labelled control oligonucleotide. Results show no impairment of oligoncleotide uptake into the cells (Fig. 7.7) confirming that the results seen in Fig. 7.6 are a true reflection of the effect HIF-1α AS has on DNA integrity in the different culture conditions.

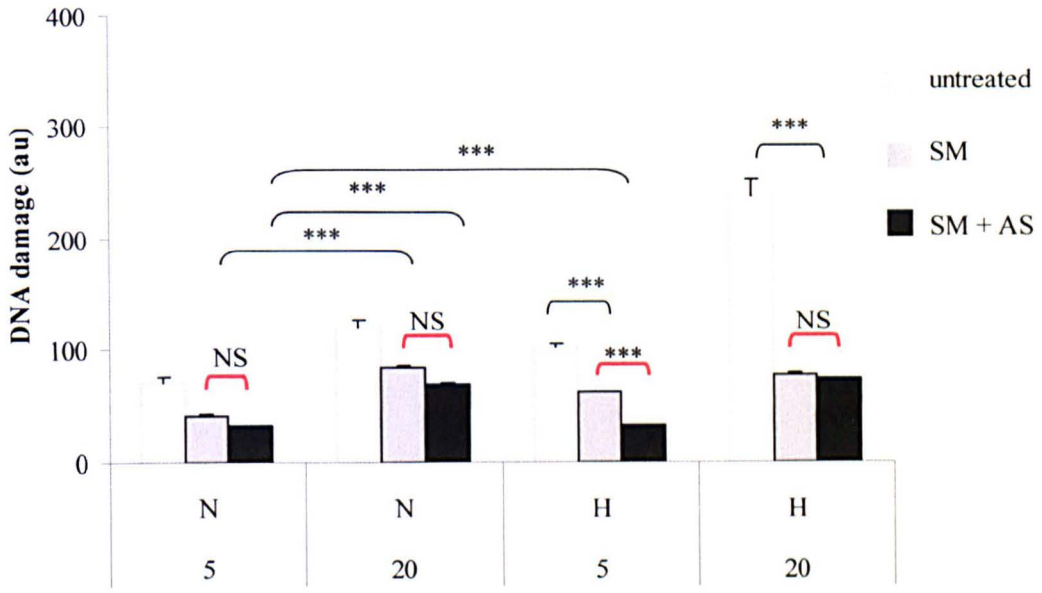
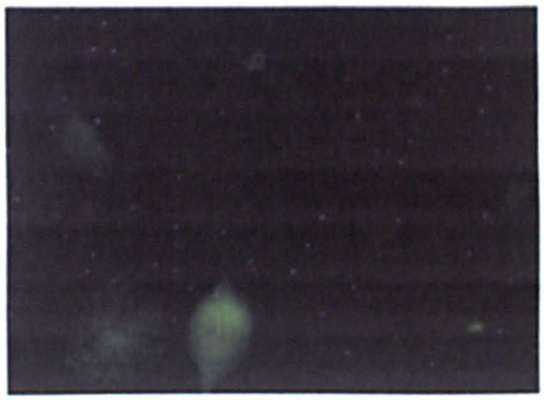


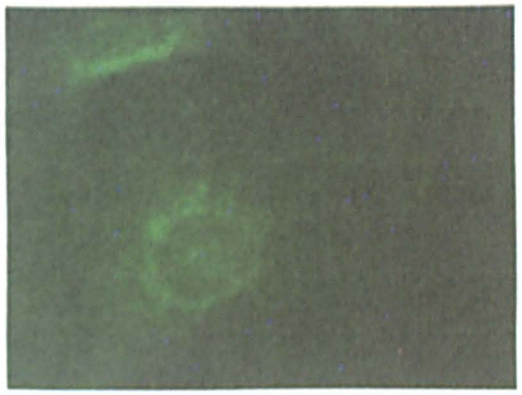
Fig. 7.6: Determination of the effect that HIF-1α antisense (AS) treatment (2μM) ± silymarin (SM) has on the level of DNA damage induced by 5mM and 20mM glucose and oxygen tension (N = normoxia and H = hypoxia) in HUVECs. Cells were analysed using the comet assay. The results are expressed as mean ± SEM from three separate experiments (n = 3) (Kruskal-Wallis test: **p < 0.01; ***p < 0.001).

Fig. 7.7: Determination of the effect silymarin treatment (50μM) has on cellular uptake of the FITC labelled control oligonucleotide (HIF-1α antisense control) in hyperglycaemic cells cultured under normoxic condition. From left to right are **(a)** 0hour, **(b)** 1hour, **(c)** 6hour, **(d)** 24hour incubation. 2μM FITC labelled control oligonucleotide was directly added to 16well chamber slides containing $0.1x10^4$ cells/well before analysis using a Leica DML Fluorescent microscope. The pictures are representative of samples from three different batches of HUVECs (n = 3). Magnification: 200x.

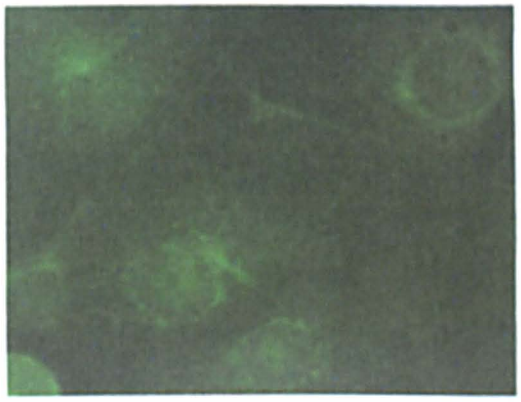
(a)



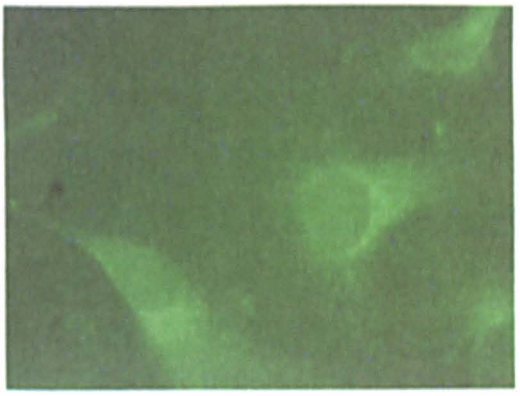
(b)



(c)



(d)



7.4.5. Effect of silymarin treatment ± HIF-1α antisense on cell size

Changes in HUVEC size over time (6h & 24h) in silymarin treated HUVECs ± HIF-1α antisense was determined using ImageJ freeware. Percentage changes in cell size were determined relative to control (t = 0h 100%) and analysed for all three experiments (Fig.7.8). Neither silymarin (50μM) treatment alone nor the addition of HIF-1α antisense (2μM) had an effect on cell size.

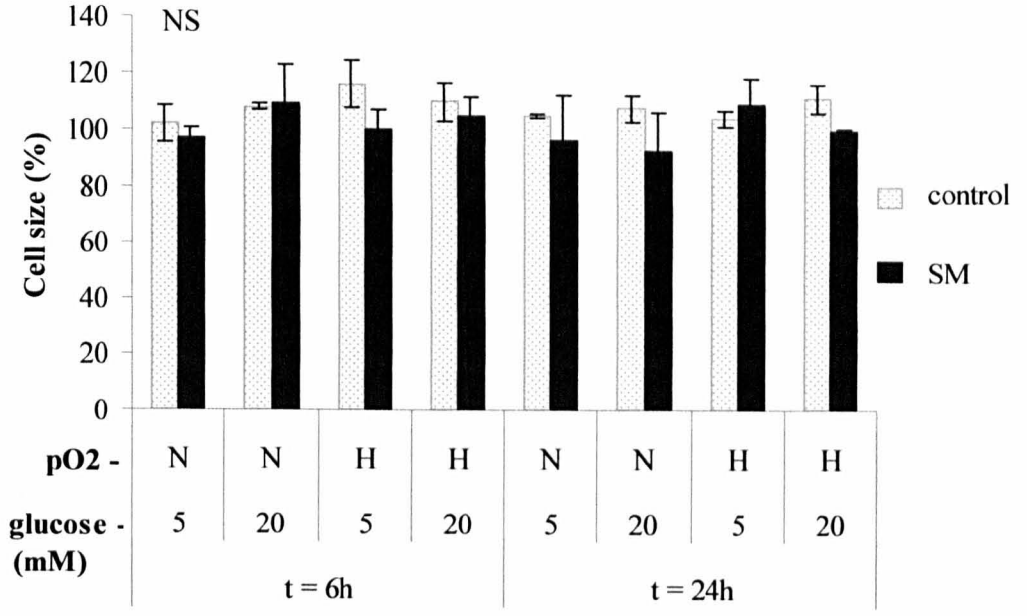
7.4.6. Effect of silymarin treatment ± HIF-1α antisense on MitoTracker Green fluorescence

Changes in MitoTracker Green FM fluorescence intensity over time (6h & 24h) in silymarin treated HUVECs ± HIF-1α antisense was determined using ImageJ freeware. The percentage changes in fluorescence intensity was determined relative to control (t = 0h 100%) (Fig.7.9). Silymarin (50μM) treatment alone reduces MitoTracker Green fluorescence intensity significantly in samples cultured in high glucose (20mM) and hypoxia after both 6h (control: 108±4; SM: 59±12 – $p < 0.05$) and 24h (control: 105±9; SM: 60±6 – $p < 0.05$). There is a reduced trend in fluorescence intensity in all samples treated with silymarin for 24h. The addition of HIF-1α antisense (2μM) on the other had had no significant effect on MitoTracker Green fluorescence intensity despite the fact that fluorescence in most samples is still reduced.

Analysis of silymarin treatment with and without HIF-1α antisense on cellular ROS production was carried out but no reliable data was obtained.

Fig. 7.8: Effect of silymarin (SM) treatment (50μM) (a) ± HIF-1α antisense (AS) (2μM) (b) on cell size in human umbilical vein endothelial cells. The change in cell size is expressed relative to control (t = 0h 100%). The above graph is representative of the mean ± SEM of three separate experiments (n = 3) with 50 observations per sample (Student's t-test and ANOVA plus post-hoc test: $p > 0.05$).

(a)


NS
Cell size (%)
140
120
100
80
60
40
20
0
control
SM
pO2 -
N N H H N N H H
glucose - (mM)
5 20 5 20 5 20 5 20
t = 6h
t = 24h

(b)

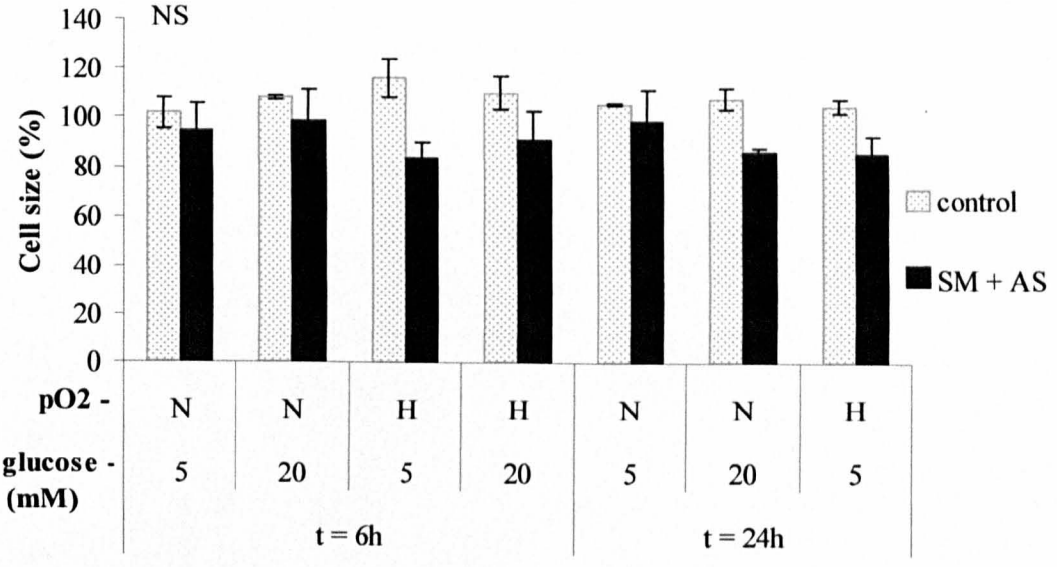
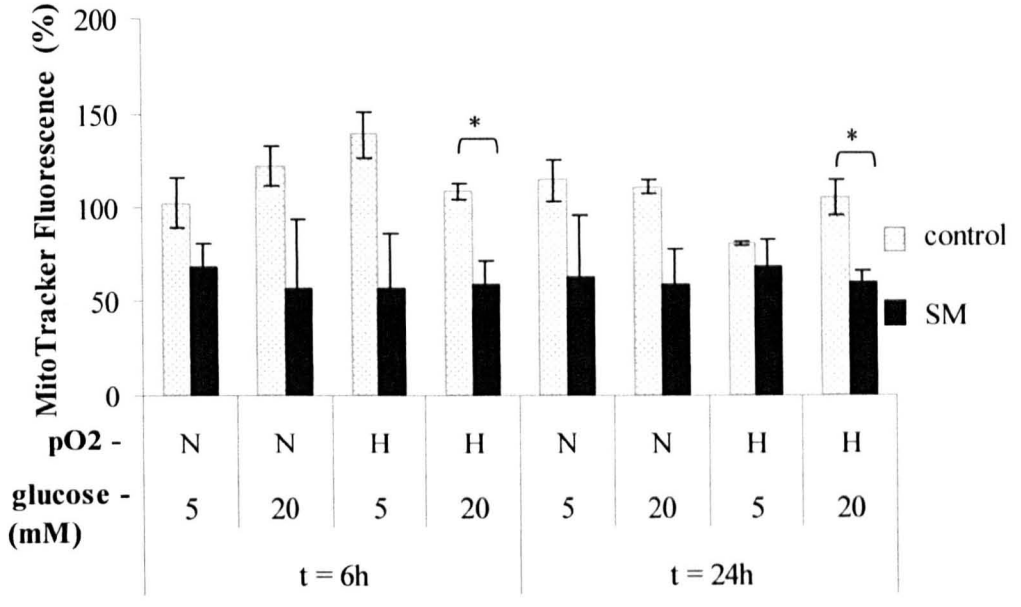
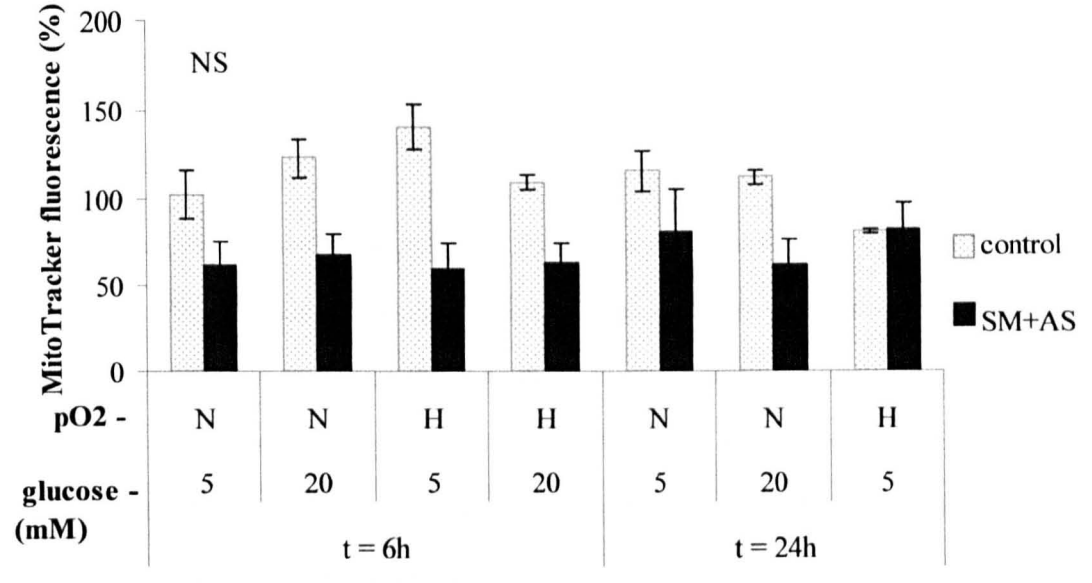

NS
Cell size (%)
140
120
100
80
60
40
20
0
control
SM + AS
pO2 -
N N H H N N H
glucose - (mM)
5 20 5 20 5 20 5
t = 6h
t = 24h

Fig. 7.9: Effect of silymarin (SM) treatment (50μM) (a) ± HIF-1α antisense (AS) (2μM) (b) on MitoTracker Green FM fluorescence intensity in human umbilical vein endothelial cells. The change in fluorescence is expressed relative to control (t = 0h 100%). The above graph is representative of the mean ± SEM of three separate experiments (n = 3) with 50 observations per sample (Student's t-test and ANOVA plus post-hoc test: $p > 0.05$).

(a)


MitoTracker Fluorescence (%)
200
150
100
50
0
*
*
control
SM
pO2 -
N N H H N N H H
glucose - (mM)
5 20 5 20 5 20 5 20
t = 6h
t = 24h

(b)


MitoTracker fluorescence (%)
200
150
100
50
0
NS
control
SM+AS
pO2 -
N N H H N N H
glucose - (mM)
5 20 5 20 5 20 5
t = 6h
t = 24h

7.5. Discussion

The primary aim of this research was to assess the potential cytoprotective effect of silymarin on hypoxia and hyperglycaemia induced endothelial cell changes after 24h incubation.

In Chapter Three, we established that both glucose and oxygen concentration have distinct yet additive effects on endothelial cells which lead to an increase in DNA damage and a rise in cell proliferation after 24h. Since antioxidants are prime candidates for the attenuation of oxidative stress mediated DNA damage, silymarin presents itself as an attractive therapeutic agent. Although a number of different variables were being analysed each variable had its respective control that was used for the purpose of the statistical analysis. Results do indeed show a reduction in the loss of DNA integrity with silymarin compared to control after 24h but this effect seems to be limited to endothelial cells cultured in hypoxia. Since most of the research emphasis to date is based on glucose-induced cellular damage, this study adds weight to the notion that there is a hypoxic component in the development of high glucose-induced endothelial damage. To our knowledge, neither the additive effect of glucose and hypoxia induced DNA damage in endothelial cells after 24h exposure nor the strong cytoprotective effect of silymarin in this context have been reported.

Although samples cultured in hyperglycaemia and/or hypoxia showed the most significant reduction in DNA damage with silymarin, the level of DNA damage found in silymarin treated samples seems to remain constant throughout all culture conditions. This suggests that silymarin prevents DNA damage from occuring when endothelial cells are exposed to changing glucose and oxygen conditions, raising the question whether silymarin acts by improving the cellular environment due to its antioxidant capacity or if it acts through a different mechanism which is independent of its antioxidant activity.

One possible explanation for the mechanism(s) involved could lie with a recently established hypothesis by Nyengaard *et al.* (2004). The paper detailed the interaction between hypoxia and hyperglycaemia in endothelial cells via the accumulation of free cytosolic NADH by different yet additive pathways (Nyengaard *et al.* 2004), strengthening the argument that silymarin could achieve its cytoprotective effect by acting as an aldose reductase inhibitor (Nyengaard *et al.* 2004). However, in 2002 the predicted structural requirements of flavonoid aldose reductase inhibitors were published in an attempt to improve the selection process for novel therapeutic agents (Matsuda *et al.* 2002). Unfortunately, neither the structure of sililibinin, silichristine, silidianin or taxifolin correspond with any of these criteria or to the structural prediction criteria for the radical-scavenging potential of flavonoids (Rajendran *et al.* 2004), although the exclusivity of the proposed structures as aldose reductase inhibitors may not be comprehensive.

If silymarin was to act as an aldose reductase inhibitor, it would improve the cellular redox potential through its action on NADH. Although we were not able to measure changes in NADH levels per se, we attempted to measure changes in the overal cellular redox state with the use of the fluorescent marker CC-1. In Chapter Five, we showed that there is an early effect on the cellular redox state of the endothelial cell in response to changing glucose and oxygen conditions. Unfortunately, the results obtained for the addition of silymarin were inconclusive, as the CC-1 results showed a very obscure fluorescence pattern making its interpretation near to impossible. The reason for this is unknown, but is most likely due to the auto-oxidation of the fluorescent marker. Literature however details numerous accounts of silymarin protecting against oxidative stress (Svobodova *et al.* 2006, Nencini *et al.* 2006) as well as reports suggesting that silymarin restores the levels of antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT) in the pancreas of alloxan-induced diabetic rats (Soto *et al.* 1998). It is therefore perceivable that silymarin reduces the cellular imbalance between radical production and antioxidant

enzymes, thereby reducing the development of oxidative stress resulting in less intense changes in the cellular redox state and a reduction in oxidative stress induced DNA damage. Although a reduction in oxidative stress by silymarin would be in keeping with our findings, it is only one perceivable contributing mechanism. As shown in Chapter Five, we also determined that HIF-1α activation in response to hypoxia contributes to the development of endothelial DNA damage. In combination with silymarin we were able to see a further reduction in endothelial DNA damage in samples cultured in 5mM hypoxia, none however when cultured in 20mM (despite a reducing trend). Therefore the cytoprotective effect of silymarin in hypoxic conditions is unlikely to be attributable to a direct effect on HIF-1α suggesting a multi-faceted cause of DNA damage. Further we suggeted a close relationship between HIF-1α activity and cellular ROS production although we were not able to establish if HIF-1α requires a functional mtETC for its accumulation in the nucleus. If this were true and silymarin reduced hypoxia mediated cellular oxidative stress levels as indicated above then this would result in a reduced activation of HIF-1α which may explain why we are seeing less of an effect on DNA damage of silymarin + HIF-1α AS as expected. Silymarin may act by improving the cellular environment due to its antioxidant capacity which may in itself influence how effective the HIF-1α antisense is reducing DNA damage.

When compared to other well established potent antioxidants (e.g. alpha-lipoic acid) (Table 7.1) silymarin appears to be almost as potent in its cytoprotective activity, than alpha lipoic acid (Ziegler *et al.* 1999). Alpha lipoic acid is a potent neuroprotector in diabetic neuropathy as shown by two large scale randomized controlled trials (ALADINI and ALDIN III) (Ziegler *et al.* 1995, Ziegler *et al.* 1999). However, it is not clear from this data if the high antioxidant property directly accounts for silymarins' cytoprotective effect.

The majority of published data using silymarin deals with hepatic diseases and cancer using silymarin as a therapeutic agent rather than focusing on mechanistic studies. One particular

problem is the lack of standardisation of the extraction process and product composition leading to huge variations between batches and manufacturers. The European Pharmacopoea states that a minimum of 1.5% of silymarin should be expressed as silibinin and that the overall sample should not contain more than 2% foreign matter although "foreign matter" is not defined (European Phamacopoeia 2004). Even a direct request for the analytical details of silymarin from the German Central Laboratory for Pharmacists (quality assurance institution for products marketed in Germany) where silymarin is produced provides no further insight into the exact composition of silymarin (personal communication, July 2004). They do however acknowledge the presence of taxifolin but do not indicate its role or state the percentage concentration in respective batches of silymarin.

7.6. Conclusion

Given the results so far we can confirm that silymarin is a potent cytoprotective flavonoid antioxidant. It protects glucose treated HUVECs under hypoxic conditions with associtated changes in the cellular redox state, DNA damage and possibly DNA repair mechanisms. Despite its strong radical scavenging potential it is not clear if silymarin acts by improving the cellular environment or if it acts through a different mechanism independent of its antioxidant activity such as an aldose reductase inhibitor for example. In order to understand the mechanisms behind this cytoprotective activity the activity of the compounds which are found in the silymarin extract were then investigated.

CHAPTER EIGHT: Effect of silbinin and taxifolin on DNA damage in HUVECs in hypoxia and high glucose concentration

8.1. Hypothesis

The flavonoid antioxidants silibinin and taxifolin are responsible for the protective effects on endothelial DNA damage seen with silymarin in response to hypoxia and hyperglycaemia.

8.2. Introduction

Silymarin contains a number of different flavonoid isomers of which silibinin is thought to be the most therapeutically active component (Zhao *et al.* 1999). Probably less well known is that taxifolin can also be found in the silymarin compound although literature refers to taxifolin as the "parent compound" of all silymarin components (Hänsel *et al.* 1993). During the extraction process of silymarin, taxifolin combines with different coniferyl alcohol structures from the plant cell wall to give rise to the different flavonoids known collectively as silymarin which suggests that taxifolin is indeed an active ingredient found in the Milk Thistle plant. Fig. 8.1 shows a proposed chemical reaction of taxifolin and coliferyl alcohol. Taxifolin is very rarely mentioned as a component of silymarin although it is speculated that silymarin contains impurities which have a much higher antioxidant activity than the identified flavonolignans (Kvasnička *et al.* 2003). It is suggested that the purer the silymarin compound the less active it would become.

Therefore, the relative contribution that silibinin and taxifolin may have on the reduction in DNA damage mediated by silymarin, and the potential contributing mechanisms which lead to the development of endothelial cell changes in response to glucose and hypoxia was determined.

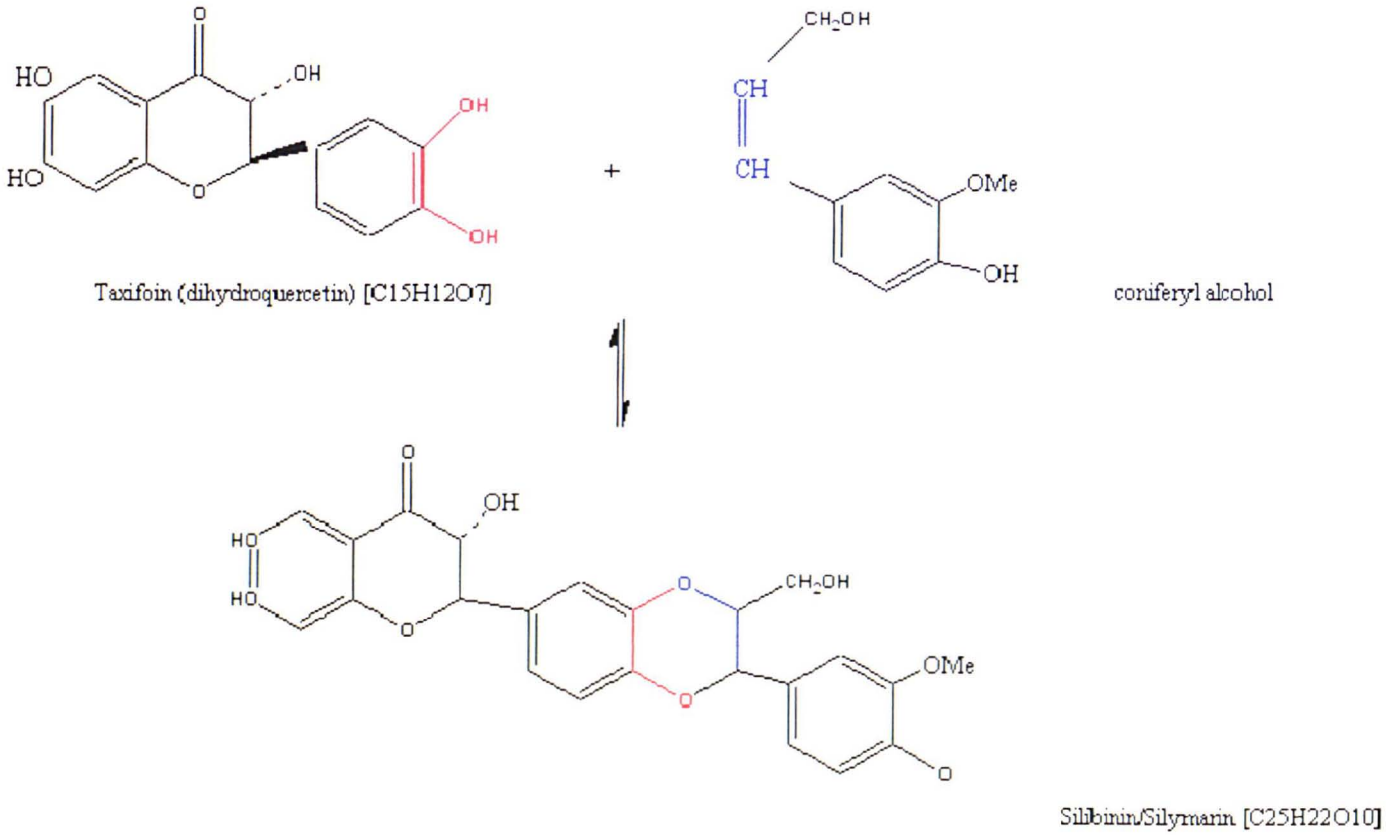


Fig. 8.1: Proposed oxidation/reduction reaction of taxifolin and coniferyl alcohol

8.3. Materials and Methods

The percentage concentration of silibinin and taxifolin within the silymarin compound was determined using Capillary Zone Electrophoresis (Section 2.9). The effect of glucose concentration, oxygen tension, silymarin (50μM), silibinin (25μM) and/or taxifolin (600μM) on DNA integrity was determined using the comet assay (Section 2.4). HUVECs were cultured as described in Section 2.2.

Each experiment was performed in triplicate and repeated on three separate occasions. The test distribution was assumed not to be normal to account for the limitations in sample size. Statistical significance was determined using a Kruskal-Wallis test or Mann-Whitney U test against the test hypothesis (H_0) that there are no differences between the means of the samples. Were $p< 0.05$ the H_0 must be rejected and the H_A (there is a difference between the means of the samples) accepted. Where data was normalised (100%) a Student's t-test (Mean – 100%/ SEM) and/or ANOVA (with Tukey's post-hoc test) was carried out. The level of significance is expressed as $*p< 0.05$, $**p< 0.01$ or $***p< 0.001$. No significant difference was assigned to samples where $p > 0.05$.

8.4. Results

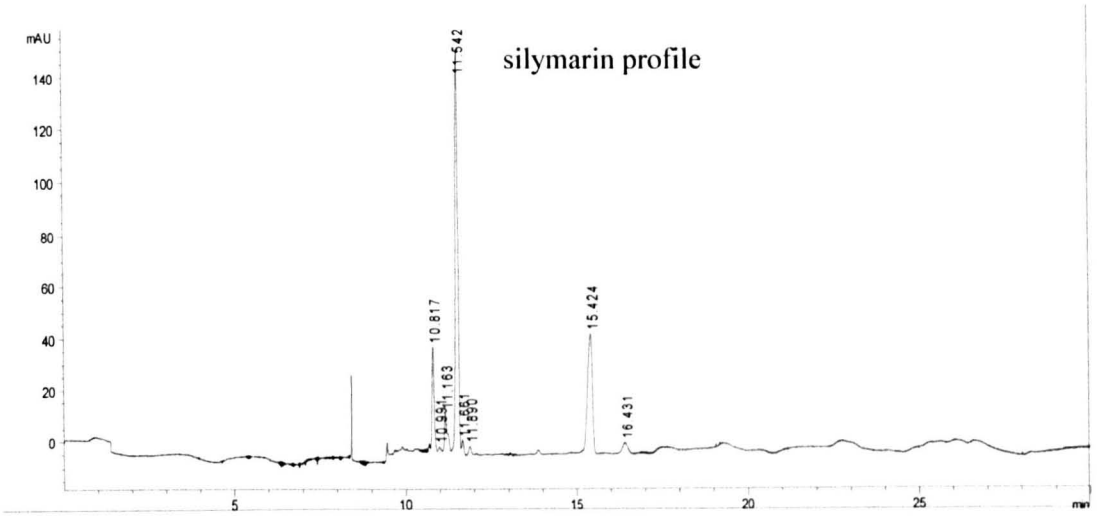
8.4.1. Relative amounts of taxifolin and silibinin found within commercially available silymarin sample

Analysis of the individual electrophoresis fractions of silibinin and taxifolin present in silymarin was carried out using capillary zone electrophoresis (CZE) analysis. Quantitative analysis indicated that taxifolin and silibinin was present at levels of 4.1% (Fig. 8.3, Table 8.1) and 46.5% (Fig. 8.4, Table 8.1) of silymarin respectively. The equivalent concentration of taxifolin (600μM) and silibinin (25μM) were then used in comparative experiments. It is

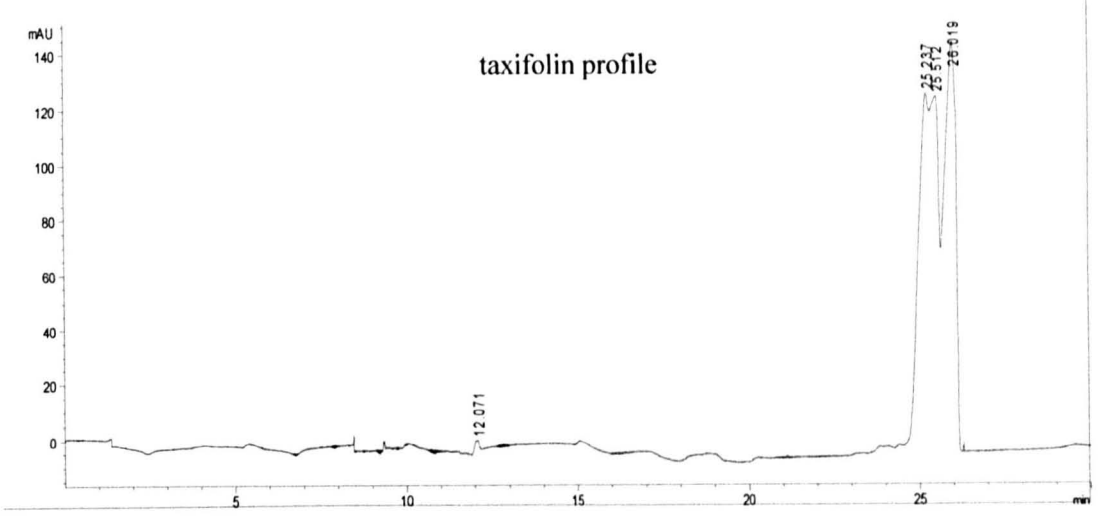
however noteworthy that the small area under the curve (AUC) associated with taxifolin may be a source of inaccuracy in the calculation of the final concentration used in all subsequent experiments.

Fig. 8.2: Electropherogram showing the separation of **(a)** silymarin **(b)** taxifolin **(c)** silibinin. Capillary total length 650mm x 50μm. I.D.; running buffer (pH 2.0) containing 80mM SDS, 50mM DSHP and 25% methanol. Detection at 200nm. Injection time 300s at 50mbar.

(a)


silymarin profile
mAU
140
120
100
80
60
40
20
0
11.642
10.817
15.424
16.431
5
10
15
20
25
min

(b)


taxifolin profile
mAU
140
120
100
80
60
40
20
0
25.237
25.512
26.019
12.071
5
10
15
20
25
min

(c)

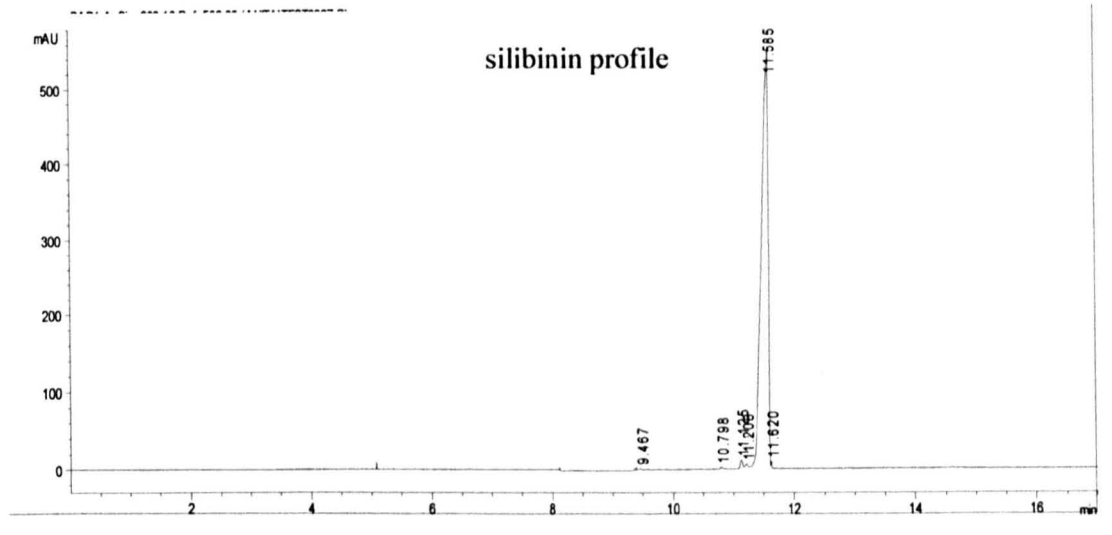
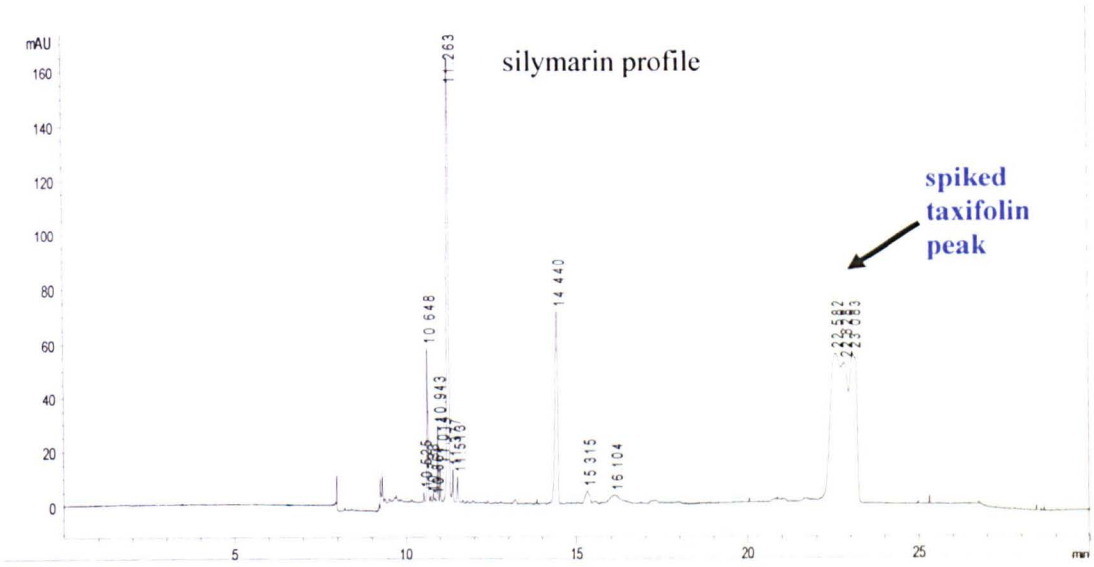

silibinin profile
mAU
500
400
300
200
100
0
11.585
9.467
10.798
11.620
2
4
6
8
10
12
14
16
min

Fig 8.3: Separation of taxifolin from silymarin. Results show respective electropherograms of the separation of silymarin **(a)** and taxifolin **(b)**. Capillary total length 650mm x 50μm. I.D.; running buffer (pH 2.0) containing 80mM SDS, 50mM DSHP and 25% methanol. Detection at 280nm. Injection time 300s at 50mbar.

(a)


mAU
160
140
120
100
80
60
40
20
0
11.263
10.648
14.440
15.315
16.104
silymarin profile
spiked
taxifolin
peak
5
10
15
20
25
min

(b)

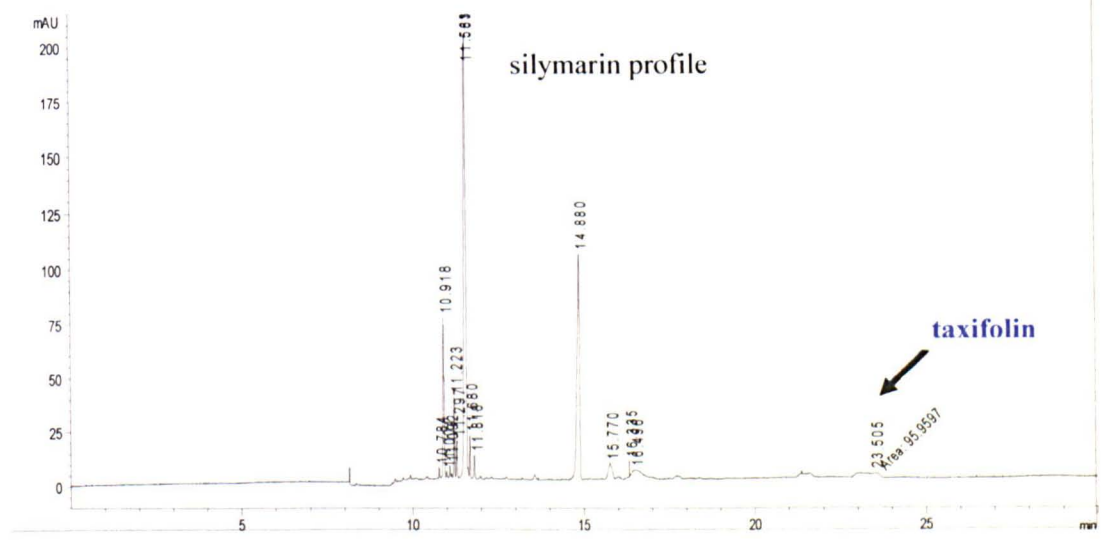
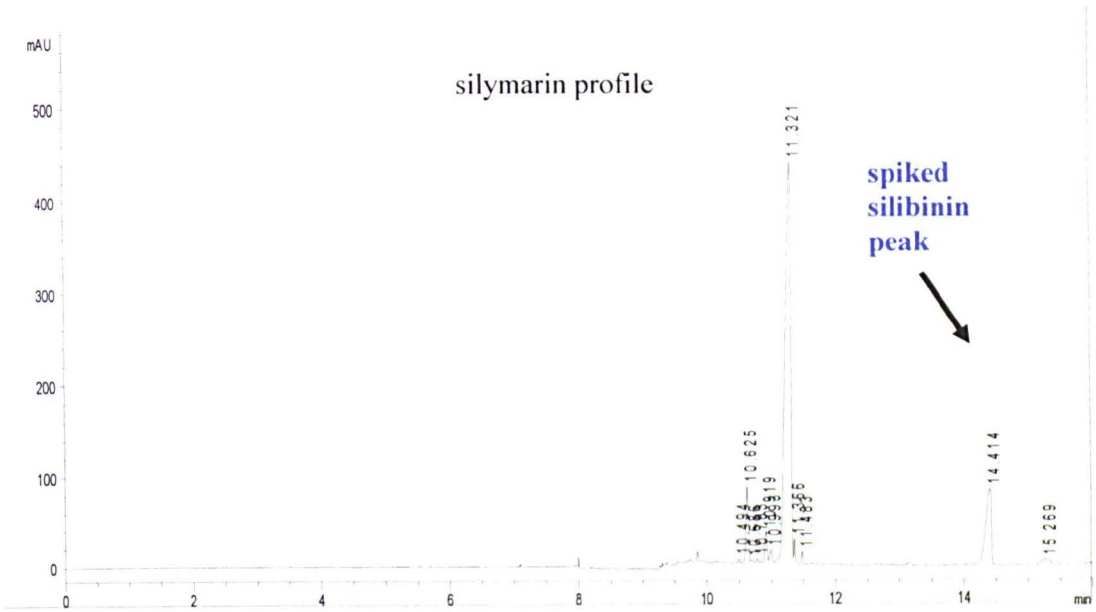
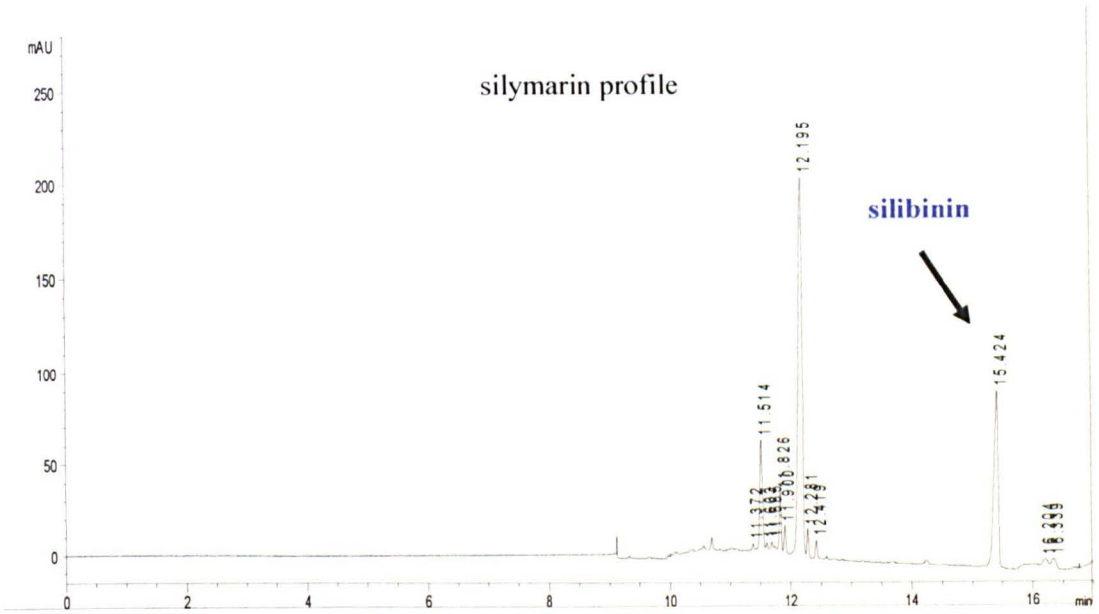

mAU
200
175
150
125
100
75
50
25
0
10.918
14.880
15.770
23.505
silymarin profile
taxifolin
5
10
15
20
25
min

Fig. 8.4: Separation of silibinin from silymarin. Results show respective electropherograms showing the separation of silymarin **(a)** and silibinin **(b)**. Capillary total length 650mm x 50μm. I.D.; running buffer (pH 2.0) containing 80mM SDS, 50mM DSHP and 25% methanol. Detection at 200nm. Injection time 300s at 50mbar.

(a)


mAU
silymarin profile
spiked
silibinin
peak
11.321
10.625
14.414
15.269
min

(b)


mAU
silymarin profile
silibinin
12.195
11.514
15.424
min

	Total area under the curve (AUC) of silymarin 1:25 [mAU*s]	AUC of identified taxifolin peak in silymarin sample 1:25 [mAU*s]	Percentage Area (%) of total	Molecular equivalent in silymarin sample (μM)
taxifolin	2372	97.3	4.1	600
silibinin	2150.7	1000.3	46.5	25

Table 8.1: Area under the curve (AUC) values for taxifolin and silibinin identified during CZE analysis of a 1M silymarin sample. Percentage analysis (%) indicates that taxifolin represents 4% of the total of the commercially available sample of silymarin while silibinin represents 46.5%.

8.4.2. Effect of taxifolin on DNA damage

Analysis of all antioxidants was limited to HUVECs cultured in high glucose conditions as Chapters Three and Seven have shown that glucose/hypoxia mediated cytotoxicity and the cytoprotective effect of silymarin are limited to these conditions. Analysis of the effect of different taxifolin concentrations (50μM - 600μM) on endothelial DNA damage indicate that all three concentrations of taxifolin (600TX: 48±6; 100TX: 53±9; 50TX: 48±5) as well as silymarin (SM: 60±11) significantly reduces DNA damage in hypoxia treated samples compared to untreated (20H: 78±18) (Fig. 8.5). No effect on DNA damage of either silymarin or taxifolin could be observed in cells cultured in normoxic conditions (20mM) (600TX: 56±8; 100TX: 56±6; 50TX: 59±4; SM: 67±9; 20N: 55±11).

8.4.3. Effect of pre-incubation of HUVECs with antioxidants on DNA damage levels

Pre-exposure of cells to antioxidants has to be distinguished from any effect on DNA integrity seen with simultaneous exposure. HUVECs (20mM) were pre-incubated with silymarin (50μM) (N: 51±9; H: 38±5), taxifolin (600μM) (N: 50±6; H: 42±9) and silibinin (25μM) (N: 64±7; H: 52±9) for 24h in hypoxic and normoxic culture conditions (Fig. 8.6). Pre-incubation did not lead to any changes in DNA damage profile compared to acute incubation (Fig. 8.6) (control (N: 60±8; H: 98±16); silymarin (N: 68±9; H:60±6), taxifolin (N:42±7; H:50±2) and silibinin (N:74±9; H:83±11)), with the only exception of silibinin. Its cytoprotective effect appeared to be significantly enhanced in hypoxic conditions when cells were pre-incubated (20H: 98±16; 20HSB: 83±11). These results suggest that silibinin could be acting through a different mechanism than taxifolin.

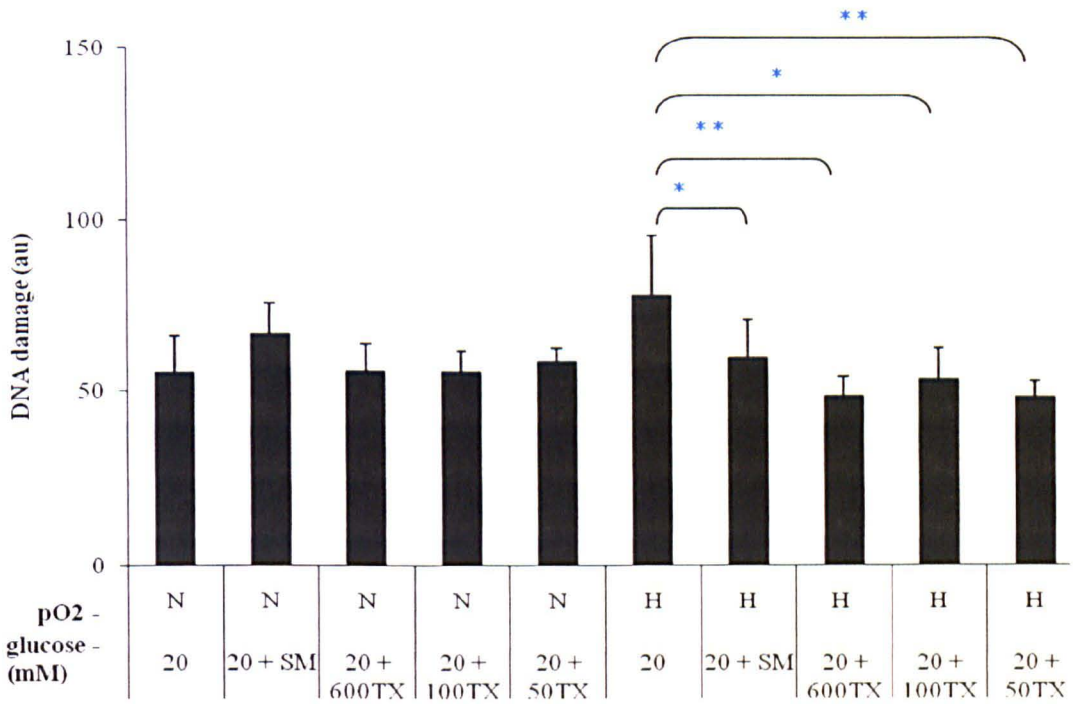


Fig. 8.5: Effect of taxifolin (TX) concentration (50μM - 600μM) on HUVEC DNA damage levels) incubated in 20mM glucose compared to silymarin (SM) (50μM) and untreated control (N = normoxia and H = hypoxia). Cells were stepped down overnight in glucose free/serum free media before incubation and subsequent analysis with the comet assay. The results are expressed as mean ± SEM from three separate experiments (n=3) (Kruskal-Wallis test: * $p < 0.05$; ** $p < 0.01$).

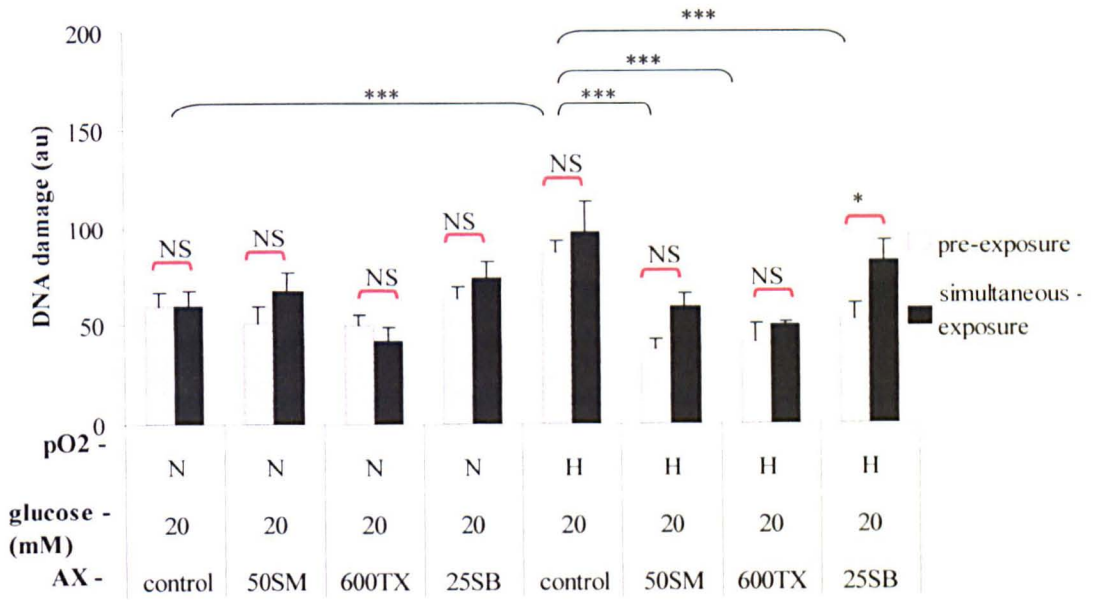


Fig. 8.6: Effect of 24h pre-exposure of HUVECs with silymarin (50μM), taxifolin (600μM) and silibinin (25μM) on hyperglycaemia induced DNA damage (N = normoxia and H = hypoxia) compared to 24h simultaneous exposure as seen in Fig. 8.7. Cells were stepped down overnight in glucose free/serum free media before incubation. Cells were analysed with the comet assay. The results are expressed as mean ± SEM from three separate experiments (n = 3) (Kruskal-Wallis test: ***$p < 0.001$).

8.4.4. Effect of HIF-1α antisense treatment in addition to silibinin and taxifolin

As with silymarin (Fig. 7.6) the effect of HIF-1α antisense in addition to treatment with taxifolin or silibinin was assessed. This was examined across the spectrum of concentrations used eg. 5mM & 20mM in both normoxia and hypoxia. A significant decrease in DNA damage was observed in samples treated with taxifolin alone in response to an increase in hypoxia as well as glucose when compared to control (5NTX: 34±1; 20NTX: 43±1, 5HTX: 58±1; 20HTX: 64±1) (Fig. 8.7a). This was accompanied by a further decrease in hypoxia and hyperglycaemia induced DNA damage in samples treated with both taxifolin and HIF-1α antisense (5NTXAS: 28±1; 20NTXAS: 43±1, 5HTXAS: 43±1; 20HTXAS: 52±1).

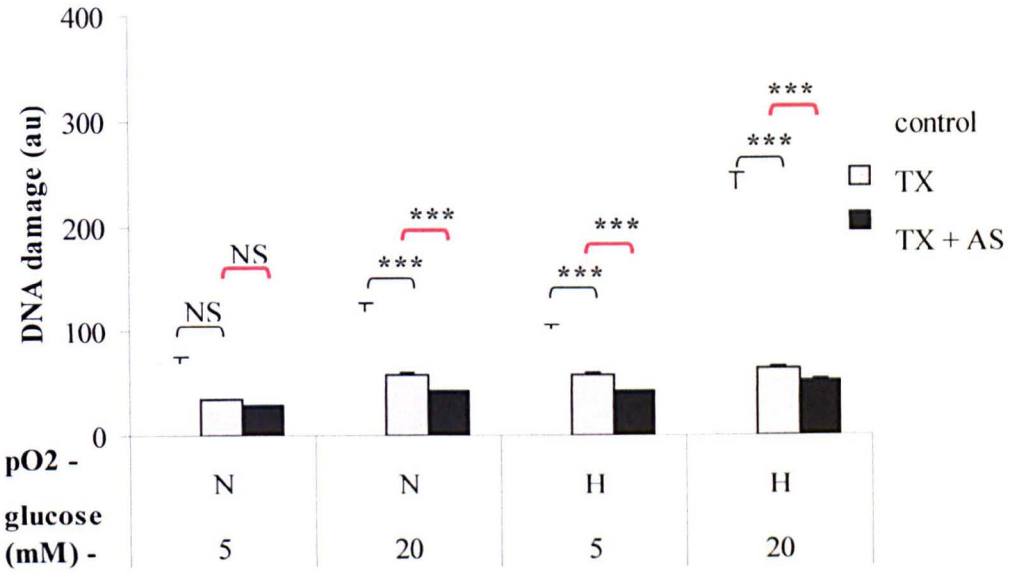
Addition of silibinin to HUVECs did not result in a significant change of DNA damage level compared to control except for samples incubated in both high glucose concentration and hypoxia (20H: 188±2; 20HSB: 81±1). Addition of HIF-1α antisense to silibinin treated cells had no effect on DNA damage levels compared to silibinin treatement alone (Fig. 8.7 b).

8.4.5. Superoxide radical production in silymarin, taxifolin and silibinin treated HUVECs

Using a commercially available kit, the total superoxide radical production in response to antioxidants, silymarin (50μM), taxifolin (600μM) and silibinin (25μM) was determined. The percentage proportion of superoxide radicals found in mitochondria and the cytoplasm was analysed. The results show no significant change in superoxide radical production in and between any of the treatment groups ($p > 0.05$) (Fig. 8.8 – 8.10). However for cells incubated with silymarin and silibinin there was a decrease in mitochondrial SOD associated with an increase in cytoplasmic SOD. In contrast no such correlation was evident for taxifolin indicating that these antioxidants do not protect HUVECs from a loss of DNA intetegrity in response to hyperglycaemia and/ or hypoxia by acting on superoxide radical production or scavenging.

Fig. 8.7: Determination of the effect HIF-1α antisense (AS) treatment (2μM) has on the level of DNA damage induced in taxifolin (600μM TX) (a) and silibinin (25μM SB) (b) treated HUVECs. Cells were stepped down overnight in glucose free/serum free media before 24h incubation. Cells were analysed using the comet assay. The results are expressed as mean ± SEM (n = 3) (Kruskal-Wallis test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

(a)


400
300
200
100
0
DNA damage (au)
NS
NS
***
***
***
***
***
***
control
TX
TX + AS
pO2 -
N
N
H
H
glucose (mM) -
5
20
5
20

(b)

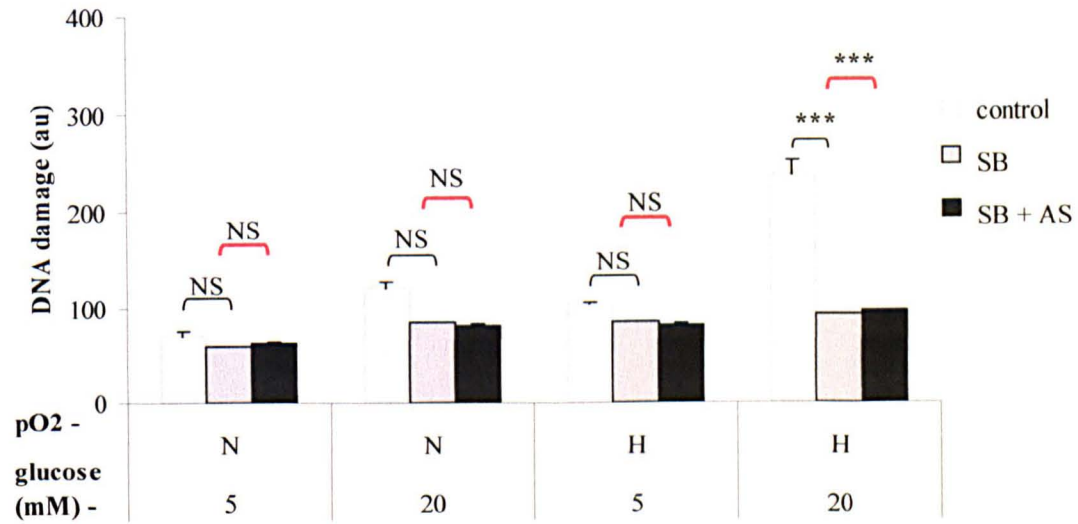
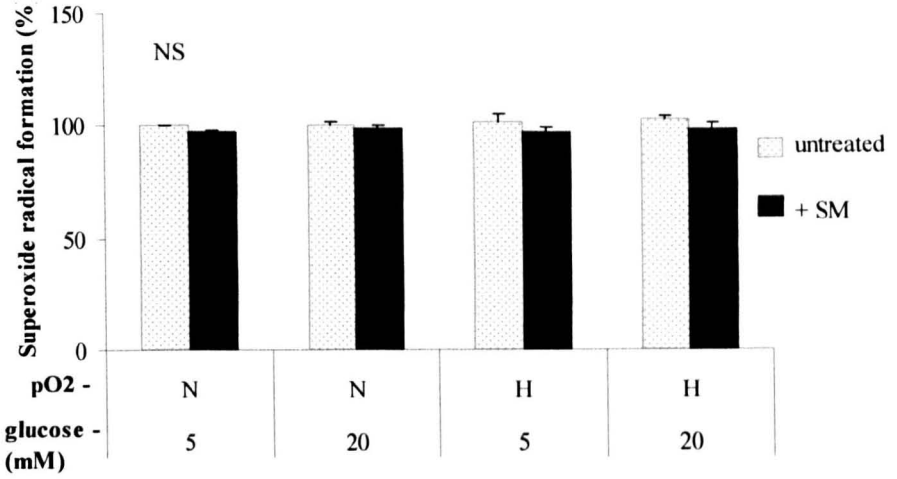
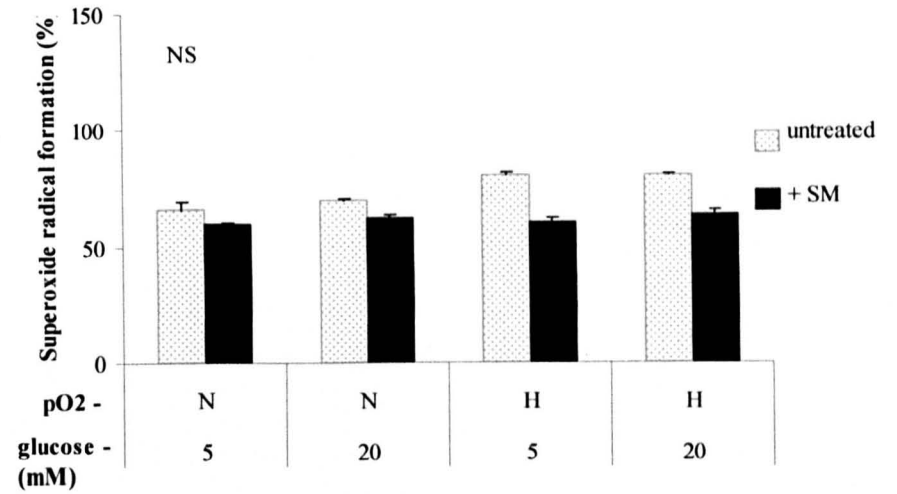

400
300
200
100
0
DNA damage (au)
NS
NS
NS
NS
NS
NS
***
***
control
SB
SB + AS
pO2 -
N
N
H
H
glucose (mM) -
5
20
5
20

Fig. 8.8: Determination of changes in cellular superoxide radical formation in response to changes in glucose (5mM and 20mM) and oxygen concentration (N = normoxia; H = hypoxia) in silymarin (50μM) treated HUVECs. The change in superoxide radical formation is compared relative to total cellular superoxide radical control (5N = 100%). **(a)** total superoxide radical formation in HUVECs **(b)** percentage proportion superoxide radical formation found in mitochondria **(c)** percentage proportion superoxide radical formation found in the cytoplasm. Samples were analysed using a SOD-WST kit. Each bar represents the mean ± SEM from three different experiments (n = 3) (Student's t-test and ANOVA plus post hoc test: $p > 0.05$).

(a) total cellular



(b) mitochondrial



(c) cytoplasmic

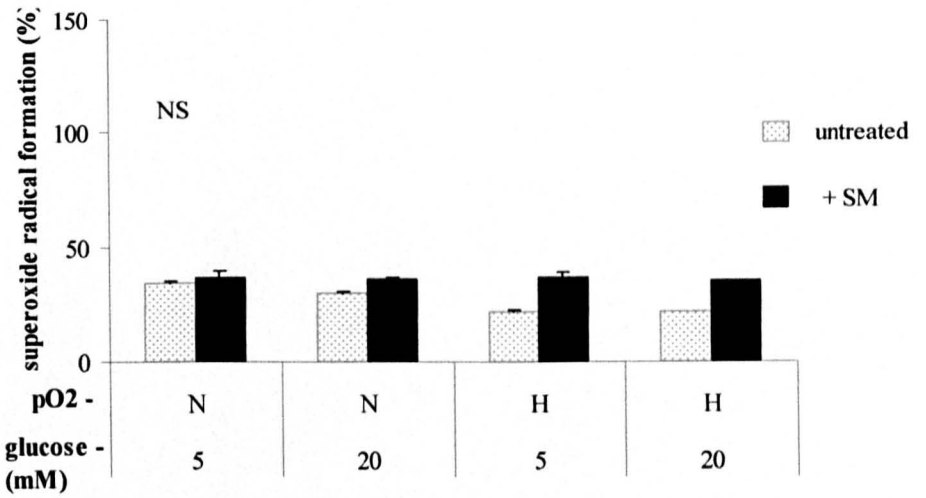
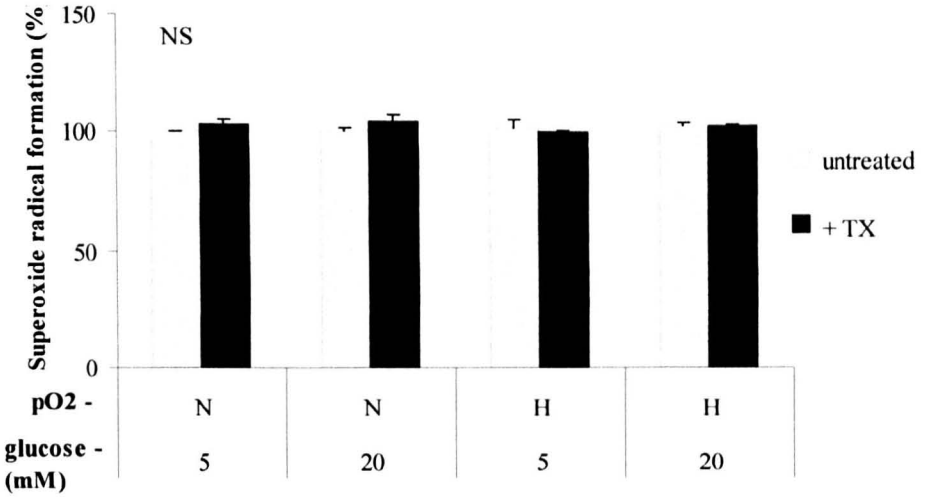
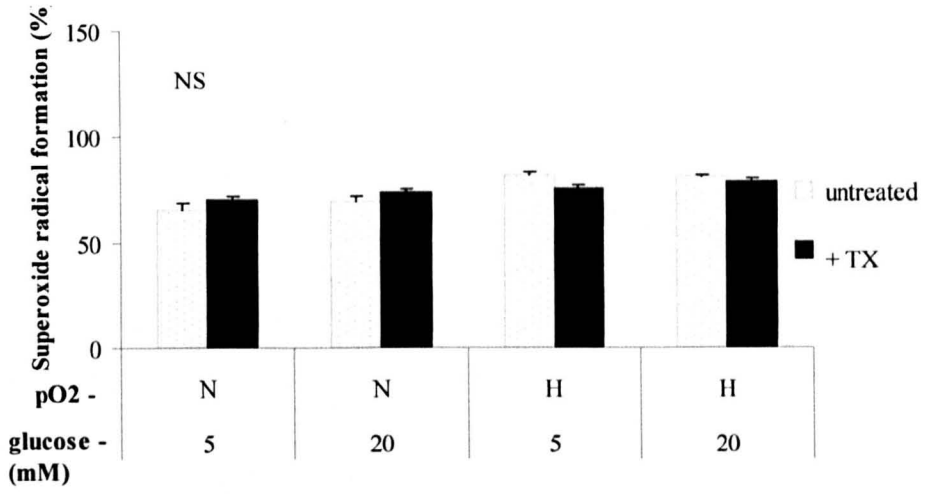


Fig. 8.9: Determination of changes in cellular superoxide radical formation in response to changes in glucose (5 & 20mM) and oxygen concentration (N = normoxia; H = hypoxia) in taxifolin (600μM) treated HUVECs. The change in superoxide radical formation is compared relative to total cellular superoxide radical control (5N = 100%). **(a)** total superoxide radical formation in HUVECs **(b)** percentage proportion superoxide radical formation found in mitochondria **(c)** percentage proportion superoxide radical formation found in the cytoplasm. Samples were analysed using a SOD-WST kit. Each bar represents the mean ± SEM from three different experiments (n = 3) (Student's t-test: $p > 0.05$).

(a) total cellular



(b) mitochondrial



(c) cytoplasmic

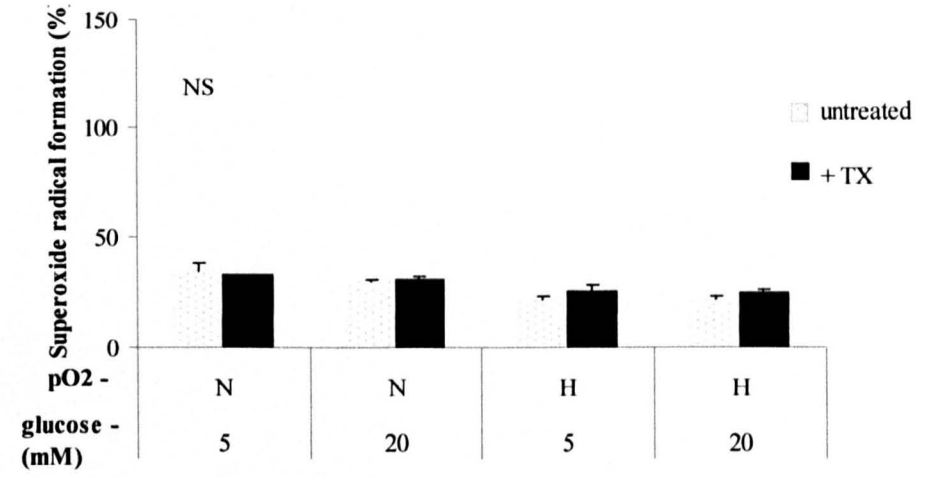
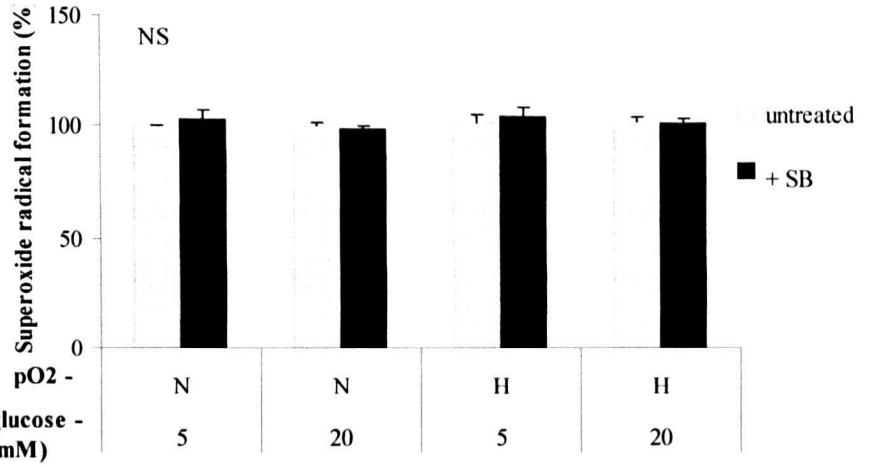
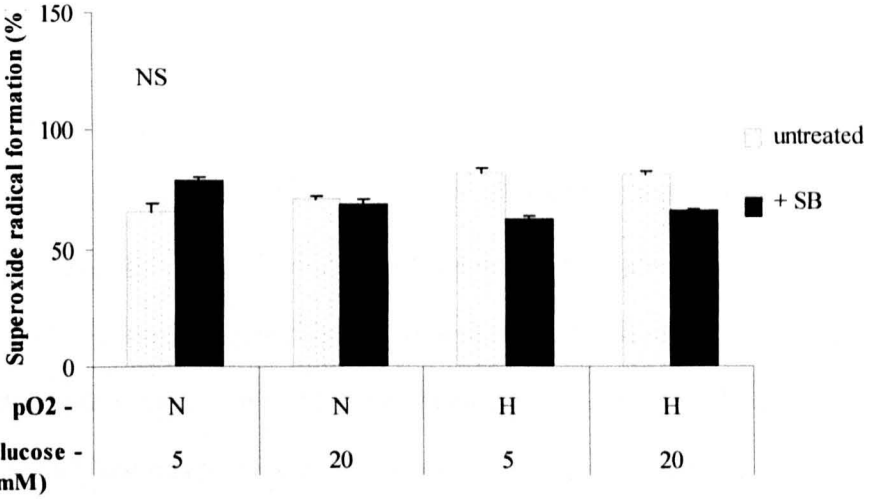


Fig. 8.10: Determination of changes in cellular superoxide radical formation in response to changes in glucose (5 & 20mM) and oxygen concentration (N = normoxia; H = hypoxia) in silibinin (25μM) treated HUVECs. The change in superoxide radical formation is compared relative to total cellular superoxide radical control (5N = 100%). **(a)** total superoxide radical formation in HUVECs **(b)** percentage proportion superoxide radical formation found in mitochondria **(c)** percentage proportion superoxide radical formation found in the cytoplasm. Samples were analysed using a SOD-WST kit. Each bar represents the mean ± SEM from three different experiments (n = 3) (Student's t-test: $p > 0.05$).

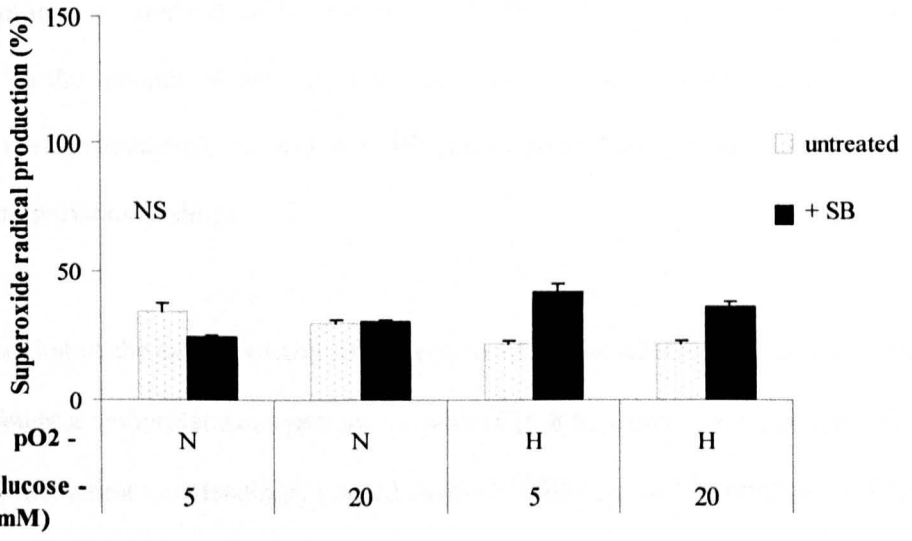
(a) total cellular



(b) mitochondrial



(c) cytoplasmic



8.5. Discussion

Results showing the beneficial effects of taxifolin and silibinin on reducing DNA damage mediated by high glucose and hypoxia facilitate our understanding of silymarin effects. Although taxifolin is not considered as an active component of silymarin, these results raise questions about its role in the protective effects of silymarin. With the lack of standardisation of the relative components of silymarin, this may contribute to variation in effectiveness.

At a concentration of 600μM – 50μM taxifolin has a strong cytoprotective property reducing the loss of DNA integrity in response to high glucose and/ or hypoxic conditions (Fig. 8.5). This effect is as potent as that of silymarin and appears to be independent of the concentration used in this study (Fig. 8.5). Since taxifolin is classed as a flavonoid antioxidant, its effect does not come as a surprise. What is surprising is that the potent effect of taxifolin does not appear to be concentration dependent, suggesting that its powerful effects can be achieved with very small quantities as low as 50μM equivalent to $1/12^{th}$ of the total taxifolin concentration found in the silymarin sample tested. At this level taxifolin would hardly be detectable within the silymarin complex and would indeed be classified as a likely "impurity". The fact that it is a very potent DNA protective agent even at this very low concentration adds weight to the hypothesis that silymarin contains impurities which are more potent than silyamrin itself (Kvasnička *et al.* 2003). As it was not possible to find any references to the amount of taxifolin commonly used in *in vitro* studies (or the amount present in the maritime bark extract), it is difficult to place these and any future work into a context with previous findings.

A study looking at the cytoprotective effectiveness of 25μM silibinin revealed that silibinin is not as potent a cytoprotective agent as taxifolin (Fig. 8.6). Since the molecular weight for silymarin is sometimes misleadingly quoted as that of silibinin, a concentration of 50μM was chosen which is equivalent to the final concentration of silymarin used. Since the CZE

anlaysis (Fig. 8.4) revealed that only approx 50% of the total amount is composed of silibinin the effects were compared to a concentrations of 25μM which is representative of the true contribution of silibinin in the silymarin complex.

Despite the fact that the potency of silibinin as a therapeutic agent has been convincingly shown (Lirussi *et al.* 2002) these results would suggest that although silibinin is a potent cytoprotective agent, it is not the sole reason for the therapeutic effectiveness of the silymarin compound. Taxifolin, the "parent compound" appears to be more potent than silibinin. In an attempt to confirm these results a direct comparison study was carried out comparing 50μM silymarin against the effect of 600μM taxifolin and 25μM silibinin. As Fig. 8.6 clearly shows taxifolin is a more potent cytoprotective agent than silibinin significantly reducing hypoxia/hyperglycaemia induced DNA damage after 24h (simultaneous exposure). At a concentration of 25μM silibinin failed to induce a significant cyto-protective effect (simultaneous exposure).

The protective effectiveness of neither silymarin nor taxifolin are affected in any way when added prior to test conditions (pre-exposure), although silibinin (25μM) was found to be significantly more effective in protecting the cell against hypoxia/hyperglycaemia-induced endothelial DNA damage (pre-exposure), indicating that its potency is dependent on the activation of the mechanisms that prepare the cell to cope with the change in glucose concentration and oxygen tension. This could help explain the many *in vivo* results for silibinin found in the literature in which studies are carried out over a period of weeks/months, with silibinin content varying from product to product (and possibly batch to batch) due to the lack of standardisation of the extraction process and composition requirements for silymarin. Taken together, these results add weight to the suggestion that silibinin is not the sole reason for the therapeutic effectiveness of silymarin, with taxifolin potentially playing a very significant but potentially distinct role.

As demonstrated in Chapters Five and Six, HIF-1α could potentially contribute to the observed hypoxia/hyperglycaemia-induced endothelial DNA damage while the cyto-protective effect of silymarin does not seem to be attributable to a direct effect on HIF-1α. Fig. 8.7 illustrates the effect of HIF-1α antisense treatment in both 600μM taxifolin (a) and 25μM silibinin (b) treated HUVECs. As with silymarin, a reduction in DNA damage can be expected if either antioxidant acts on or downstream of HIF-1α with the levels increasing when antisense is added. Taxifolin does not appear to act on HIF-1α, as DNA damage levels are further reduced with the addition of HIF-1α antisense (Fig. 8.7a). Results for silibinin are less clear, since the removal of HIF-1α has no effect on DNA damage levels. The most likely cause is a protective mechanism totally independent of HIF-1α but mirrors the findings for silymarin-treated HIF-1α antisense samples (Fig. 7.6). Although these results still do not offer an answer as to whether the effect of silymarin and its components is achieved by acting on the hypoxia inducible factor -1α it appears likely that both taxifolin and silibinin act through two very different mechanisms with taxifolin mediated protection being afforded independent of HIF-1α.

Silymarin has shown to have a potent cytoprotective effect on endothelial DNA damage (Fig. 7.3). This poses the question as to the type of radical(s) silymarin or any of its constituents acts on (if any). The literature reports evidence for silymarin acting on hydrogen peroxide (Wang *et al.* 2005), superoxide radicals (Kostyuk *et al.* 2004) and recently even phenylglyoxylic ketyl radicals (Sersen *et al.* 2006).

Figures 8.8 to 8.10 show results for the effects of silymarin, taxifolin and silibinin on superoxide radical production in the mitochondrial as well as the cytoplasmic fraction. No statistical difference can be seen between any of these compounds ($p > 0.05$). This lack of

effect is surprising, since the effect of silymarin on mitochondrial generated ROS was very prominent and given the potent nature of all three antioxidants a change in superoxide radical production was anticipated. This could have been caused by the limitations in sample size and method of analysis. Future studies should take these limitations into account and include the analysis of other radicals such as $^{\cdot}OH$ or H_2O_2.

These results indicate that endothelial cell DNA damage observed after 24h seen with an exposure of high glucose and low oxygen (Fig. 3.5) does not seem to be due to mitochondrial produced superoxide radicals (Fig. 4.9, 8.8-10) and other radicals such as hydrogen peroxide, nitric oxide and peroxynitrite need to be investigated.

8.6. Conclusion

Evidence provided in this Chapter suggests an important role for the formerly perceived impurity taxifolin as a potent cytoprotective agent in the silymarin complex. It is evident that both taxifolin and silibinin act via different mechanisms to exert their cytoprotective effect. More studies into possible mechanisms and stress pathways such as PKC, p42/44 or PI3K as well as the effects of the flavonoid antioxidants on these pathways need to be carried out.

8.7. Limitations

CZE analysis, to identify the relative amounts of silibinin and taxifolin in our commercially available silymarin sample was chosen over HPLC due to its lower cost, short analysis time and easy implementation. Silymarin samples were spiked with the compound of interest and the resulting electropherograms used to identify and measure the area(s) under the curve of silibinin and taxifolin. From that we were able to obtain the relative amount of both taxifolin and silibinin present in the silymarin sample.

It is widely documented that in CZE analysis problems arise concerning the reproducibility of the measurements (Bächmann *et al.* 1994). This is thought to be due to sample preparation, separation such as variations in electroosmotic flow, analyte velocities and the discontinuous use of high voltage (Bächmann *et al.* 1994, Li *et al.* 2006). This was evident in our CZE output where the retention time for taxifolin (Fig. 8.3) changed from 22min to 22.8min and the retention time for silibinin (Fig. 8.4) changed from 14.4min to 16.6min. This could have been caused in part, by the use of a pseudo-stationary phase and eliminated by the use of further internal standards.

An additional contributing factor is the separation of polyphenolic compounds itself, as the polarity of the compound which is associated with the number of hydroxyl groups, can affect retention times (Wang *et al.* 2007). This is important as compounds such as taxifolin are made up of different isomers which have not yet been successfully isolated. The presence of isomers and other unknown impurities may have an impact on the number of hydroxyl groups present and whence may affect retention times. This requires strict and elaborate optimization of the sample preparation such as the method of extraction and the requirement to concentrate the sample prior to use. In addition, separation factors including buffer type, concentration and pH, the presence of various additives, voltage and temperature, as well as detection method are important factors to consider (Li *et al.* 2006). However, these were out-with the scope and time frame of this thesis but should be determined prior to any further more detailed work using silibinin and taxifolin is carried out.

Despite the limitations highlighted, the CZE analysis was sufficient for the relative amounts of silibinin and taxifolin to be determined prior to their preliminary comparison with silymarin.

CHAPTER NINE: Discussion

9.1. Discussion

It is well established that diabetes mellitus is associated with an increase in oxidative stress (Baynes *et al.* 1999; Son *et al.* 2007) which is believed to play a key role in the pathogenesis of diabetic vascular dysfunction (Panus *et al.* 2003; Tesfamariam *et al.* 2007). The results presented in this thesis indicate that glucose and oxygen exhibit distinct yet potentially additive effects on endothelial cells which results in changes in cell proliferation and DNA damage.

Literature reports on the effect of high glucose in endothelial cells show a reduction in cell proliferation (Lorenzi *et al.* 1986; Varma *et al.* 2005), abnormal cell cycling (Lorenzi *et al.* 1987) and an increase in apoptosis over time (Baumgartnet-Parzer *et al.* 1995), whereas exposure of endothelial cells to hypoxia has been shown to accelerate cell proliferation as seen in tumour aetiology (Vender *et al.* 1992; Byrne *et al.* 2005). Although the exact mechanisms for the differing effects on cell proliferation remains unclear, ROS and ROS associated signalling pathways seem to play a key role. Sheu *et al.* (2005) have shown that high glucose causes ROS generation in HUVECs via a PI3K/Akt-dependent pathway. PI3K-Akt dependent signalling plays a crucial role in cell survival, proliferation, microvascular permeability and angiogenesis (Varma *et al.* 2005), while hypoxia associated high flux of NO in contrast promotes cell death via the inhibition of cytochrome c oxidase mediated loss of mitochondrial membrane potential (Lee *et al.* 2002; Walford *et al.* 2004).

Despite the observed acceleration in cell proliferation seen with high glucose concentration and/or low oxygen tension in HUVECs within the first 24h of exposure, our samples show no detectable change in cell viability. This suggests that the associated rise in DNA damage seen in these cells is not due to hyperglycaemia and/or hypoxia induced apoptosis but may indicate a pro-apoptotic state. Jiang *et al.* (2001) have shown that a threshold of DNA damage is necessary for the activation of DNA damage checkpoints and the initiation of

DNA repair. It is perceivable that the increase in proliferation rate observed leads to the potentiation of HUVEC DNA damage because the level of DNA damage incurred in response to high glucose concentration and/or low oxygen tension is too low to trigger DNA repair mechanisms prior to replication. This may eventually result in the HUVECs viability and proliferation being compromised, accounting for the frequently reported reduction in cell proliferation and apoptosis often observed with later time points (Lorenzi *et al.* 1986; Varma *et al.* 2005; Baumgartnet-Parzer *et al.* 1995).

One of the most novel and interesting findings of this thesis is the potential additive effect of hypoxia and hyperglycaemia and its resultant impact on cell proliferation, DNA damage and mitochondrial ROS production. Although it is not possible to explain these effects, it is evident that although these stimuli trigger very different signalling cascades the different mediators must converge at some common point in the signalling pathway. Such possible signalling points include mitochondrial ROS production, expression and activation of the transcription factor HIF-1α and the involvement of mitogen activated protein kinases (MAPK) such as p42/44 which links growth and differentiation signals.

Mitochondrial ROS generation is a key factor in oxidative stress-mediated cell changes (Nishikawa *et al.* 2000). As described in Chapter One, mitochondria produce substantial amounts of ROS which will attack molecular targets such as membrane phospholipids, transporters, enzymes, transcription factors and DNA (Thannickal *et al.* 2000). Hyperglycaemia is thought to be synonymous with an increase in superoxide radical formation from the mitochondria of endothelial cells (Du *et al.* 2003), which possibly accounts for the sharp rise in mitochondrial ROS production observed in our data after 6h exposure to high glucose concentration and low oxygen tension. Du *et al.* (2003) have reported that superoxide production induces DNA strand breaks which in turn leads to the activation of PARP (poly (ADP-ribose) polymerase) a nuclear DNA repair enzyme. PARP

initiates the poly (ADP-ribosyl)ation of GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) a catalytic enzyme involved in glycolysis and a mediator for the activation of biochemical stress pathways. Our results do, however, show a significant increase in mitochondrial ROS production in all four treatment samples, making it an unlikely event attributable specifically to high glucose concentration and/or low oxygen tension alone and is more likely to be the result of stress induced by incubation conditions. Direct measurement of superoxide radical production did also not produce any significant change, adding weight to the notion that superoxide radicals are not the sole cause for the observed rise in redox-sensor red CC-1 fluorescence intensity. Other reactive oxygen species must therefore play an important role in the observed changes. The most likely being nitric oxide (NO).

NO is a highly diffusible gas which can regulate mitochondrial ROS generation on multiple levels (Cassina *et al.* 1996). It can remove $O_2^{\cdot -}$ from peroxynitrite and via cytochrome c by stabilizing the enzyme and preventing its leakage from the mitochondria (Zhang *et al.* 2007). By acting on cytochrome c, oxidase NO can regulate cellular oxygen consumption (Moncada *et al.* 2002) instigating an event known as "metabolic hypoxia" a condition in which cells and tissues are unable to use oxygen despite it being available (Moncada *et al.* 2002). The signalling consequences of this effect are reported to include the prevention of HIF-1α stabilization and the activation of AMPK (Quintero *et al.* 2006), both of which are key mediators of cellular stress responses.

Although the measurement of NO production and its related changes in endothelial cells were out with the scope of this thesis, the effect low oxygen tension and high glucose concentration have on HIF-1α activity in endothelial cells was determined using antisense technology. Our results clearly show the contribution of HIF-1α in hypoxia-mediated mitochondrial ROS production after 6h (Fig. 5.9) which is reflected in a subsequent increase in DNA damage under the same conditions after 24h (Fig. 5.6), suggesting the involvement

of HIF-1α in the development of the observed hypoxia-mediated endothelial cell changes. Although no comparable reports exist which explore the direct effect HIF-1α has on endothelial DNA damage, many studies are devoted to the elucidation of the mechanisms leading to the activation of HIF-1α under oxygen dependent (Wenger *et al.* 2000) and independent (Semenza *et al.* 2002) pathways. It appears that the hypoxia-induced activation of HIF-1α is regulated by different mechanisms from its stabilisation and DNA binding (Hur *et al.* 2001). Many reports show that the activation of p42/44 MAPK appears to be a critical step in the transcription HIF-1α (Richards *et al.* 1999, Hofer *et al.* 2001, Hur *et al.* 2001) while endogenous NO formation seems to induce HIF-1α accumulation, HIF-1 DNA binding and downstream target expression in normoxic conditions (Brune *et al.* 2003). High glucose concentration has also been shown to increase p42/44, activity resulting in eNOS expression in HUVECs (Vasquez *et al.* 2007). If the hypothesis that the observed DNA damage in HUVECs is linked to a p42/44 dependent effect on HIF-1α transcription is correct, then the use of the p42/44 MAPK inhibitor, PD98059, should result in the protection of HUVECs against DNA damage, similar to the effect seen with HIF-1α antisense. Our results, however, show no significant effect of PD98059 on DNA damage (Fig. 6.2), although a slight reduction in DNA damage can be seen in samples cultured in both high glucose concentration and low oxygen tension simultaneously for 24h an effect which might be more pronounced with an increase in sample number. It is however unlikely that the p42/44 dependent effect on HIF-1α is a suitable explanation for our observations. This is not necessarily surprising as mechanisms of oxygen sensing and signalling during hypoxia and normoxia involve different pathways in various cell types (Lopez-Barneo *et al.* 2001). In addition, the findings from Fig. 5.9 also show a significant rise in mitochondrial ROS production in the presence of HIF-1α antisense under normoxic conditions (6h) making it altogether unlikely that we are looking at a HIF-1α dependent effect on DNA damage in

normoxia, while the HIF-1α activation appears to be involved in hypoxia induced DNA damage.

Interesting points have been highlighted in the course of these investigations, including the difference between high and low oxygen tension on endothelial cell responses and the immediate mitochondrial response present after 6h which is seemingly manifested as endothelial DNA damage after 24h. These changes in CC-1 fluorescence are possibly due to the effect of incubating cells over-night in glucose free/serum free media and the subsequent "strain" of restoring glucose levels. As the ability to restore glucose concentration is not influenced by any of the subsequent culture conditions, the DNA damage seen in 20mM hypoxia must be independent of the fluorescence changes observed after 6h which suggests that ROS production is not involved to a significant extent.

The use of flavonoid antioxidants provide further insight into events leading to DNA damage. These compounds have been explored extensively for their chemopreventative potential for many types of human cancers (Soobattree *et al.* 2006; Qin *et al.* 2007). Our results clearly show that silymarin has a strong protective effect on DNA damage of HUVECs cultured in hypoxia for 24h which results in a significant reduction in DNA damage in both low and high glucose concentrations (Fig. 7.3). At the same time, it has no effect on cells cultured in normoxia for 24h (Fig. 7.3), seemingly even leading to the induction of DNA damage between 1h, 6h and 24h (Fig. 7.4).

This distinct effect highlights once more that a change in cellular oxygen concentration triggers distinct signalling pathways, which may contribute to the development of endothelial DNA damage. The observed hypoxia-dependent DNA protective effect of silymarin is in agreement with its anti-carcinogenic properties, as tumour aetiology may be characterised by hypoxia due to the inadequate supply of oxygen triggered by cellular proliferation (Shannon *et al.* 2003). The expression of genes controlling tumour cell survival

such as hypoxia-responsive transcription factors (HIF-1α), which modulate the expression of genes that promote tumour growth and growth factors, such as VEGF, which govern the formation of new blood vessels known as angiogenesis, are regulated by hypoxia (Shannon *et al.* 2003). In 2000, Jiang *et al.* showed that silymarin led to a dose-dependent decrease in VEGF level in human prostate and breast-cancer epithelial cells (Jiang *et al.* 2000). Since VEGF expression is HIF-1α dependent a reduction in VEGF expression could be association with a reduction in HIF-1α activity. Furthermore, a reduction in VEGF expression would lead to a reduction of cell proliferation and angiogenesis.

Looking at those parameters in our study, we were unable to observe an effect of silymarin on HUVEC proliferation (Fig. 7.2) after 24h, or any effect of HIF-1α antisense in addition to silymarin on endothelial DNA damage (Fig. 7.6), suggesting that the mechanism of action through which silymarin protects endothelial DNA against hypoxia-dependent changes is possibly not VEGF dependent. However, no direct comparison can be established to the results of Jiang *et al.* (2000) or indeed any of the existing literature, as cancer cell lines behave very differently from healthy endothelial cells lines. In order to further elucidate the mechanisms through which silymarin acts, the effect of taxifolin and silibinin, two of the compounds found within the silymarin extract, on DNA damage was assessed.

Taxifolin shows a very potent DNA protective effect in hypoxia, while the use of silibinin shows no effect on the level of DNA damage incurred by hypoxia after 24h (Fig. 8.5 & 8.6). This is somewhat surprising as silibinin is thought to be the most therapeutically active component of the silymarin compound, while taxifolin has not been attributed as an important therapeutic role (Flora *et al.* 1998; Gazak *et al.* 2007). Silibinin has been extensively researched in many areas and has been found to have an anti-tumourgenic effect, act as a neuro, nephro, and cardio-protector, interact with steroid hormone receptors to exert an antiandrogenic effect, have an anti-inflammatory and a gastro-protective effect as well as

being useful against the development of multidrug resistance through the accumulation of daunomycin in P-glycoprotein-positive cells (Křen *et al.* 2005). It is therefore surprising that it does not appear to be the compound which is the most DNA protective. One possible explanation could be its reported action as an iron-chelating agent (Borsari *et al.* 2001).

Iron chelators such as desferrioxamine, bind excess iron in the blood stream and lead to its excretion. It has been suggested that iron chelators may modulate certain inflammatory mediators as they seem to be able to induce the transcription of NO synthase and the subsequent release of interleukin-1β in human alveolar macrophages (O'Brien-Ladner *et al.* 1998; Dalska *et al.* 1998). Woo *et al.* (2006) hypothesise that desferrioxamine increases HIF-1α protein stability through induction of COX-2 under hypoxic conditions in human colon cancer cells (Woo *et al.* 2006). If we work on the assumption that silibinin, as an iron chelator, enhances HIF-1α stability (as hypoxia has been shown to induce COX-2 in HUVECs (Schmedtje *et al.* 1997)) then the addition of silibinin in our experimental conditions would lead to an increase in HIF-1α activity which we believe contributes to the development of endothelial DNA damage. This could explain why silibinin in contrast to taxifolin has no DNA protective effect after 24h.

However, the addition of HIF-1α antisense to silibinin treated cells has no effect on DNA damage levels (Fig. 8.8b). If silibinin acted by stabilising HIF-1α, then we would expect to see a rise in DNA damage levels. Since this is not the case, it is unlikely that silibinin/ silymarin acts by stabilising HIF-1α. We do, however, have no evidence to corroborate this suggestion.

The role of taxifolin within the silymarin compound is clearly very important as it seems to account for a significant proportion of its DNA protective effect. However, literature reports surrounding taxifolin do not provide much insight into any possible mechanisms of action as

it is mainly used in comparison studies alongside quercetin, its close structural relative, and other flavonoid antioxidants. No studies have, to our knowledge, been carried out looking at the mechanism of taxifolin as a potential therapeutic agent. It is perceivable that taxifolin, as the parent compound found in the milk thistle plant, is an important therapeutically active compound found in the silymarin extract and not just a mere impurity.

9.2. Conclusion

The mechanisms leading to endothelial DNA damage in response to oxidative stress are clearly very complex. Glucose and oxygen exhibit distinct yet potentially additive effects on endothelial cells which results in changes in cell proliferation and DNA damage within 24h of exposure. This DNA damage appears to be mediated via the mitochondria and there seems to be a close relationship between cellular ROS production and HIF-1α activity. It appears that HIF-1α is involved in the development of endothelial DNA damage. The flavonoid antioxidant silymarin protects glucose treated HUVECs under hypoxic conditions. Many of these effects appear to be due to the taxifolin component of the silymarin compound.

9.3. Limitations of thesis and directions for future work

This study has identified a number of interesting findings that are dependent on changes in glucose and oxygen concentration which merit further investigation.

1. *To compare the effects seen in HUVECs with other micro- and macrovascular tissues in primary cell culture.*

The data collected in this thesis was observed in a well established model system for the study of endothelial function, using a reputable cell line. Although established cell lines are widely used models for preliminary studies, their significance is limited by the use of culture clones, a constraint avoided with the use of primary cells.

As the results obtained indicate early and potentially additive cellular responses to changes in glucose concentration and oxygen tension, future research would benefit greatly from the investigation of these changes in primary cultures of micro- and macrovascular tissue(s). Such as the use of bovine- or human retinal endothelial cells (McBain *et al.* 2003, MacKinnon *et al.* 2004), cell culture of peripheral neurons and Schwann cells (Sango *et al.* 2006) and primary culture of HUVECs (Piconi *et al.* 2006).

Careful consideration will need to be given to the culture conditions used as the concentration of glucose and oxygen tension reported in the literature is widely varied. Other limitations of *in vitro* cell culture models compared to *in vivo* models of diabetes include the lack of interaction with other cell/tissue types and a lack of insulin release in response to the addition of glucose. Nevertheless the identification of the responses to changes in glucose and oxygen in those tissues will provide important information about potential early cellular changes which occur and possible mechanistic events which may contribute to the process of endothelial dysfunction.

2. *To optimise the CZE analysis, carefully looking at sample preparation, method of extraction, buffer concentrations and pH, voltage, temperature and method of detection (HPLC/ CZE), in order to separate all the individual components of silymarin.*

The composition of silymarin has been determined with various degrees of success. Literature reports detail a considerable number of isomers, flavonolignans and acids (Frömming *et al.* 1999, Carrier *et al.* 2004). This discrepancy is mainly due to the lack of standardised composition of commercially available silymarin and the different extraction methods used such as hot water (Barreto et al. 2003) and organic solvent extraction (Wallace *et al.* 2003). This poses considerable problems with respect to the interpretation of results and their comparison to other reported literature findings. As evident in the electropherograms presented in this thesis (Fig.8.2) compounds such as silibinin and taxifolin can be identified but are clearly made up of many unknown isomeric forms and compounds, the presence of which can affect retention times and reproducibility of the data.

It would be of enormous benefit to this and other studies to completely separate and identify all the components found in the silymarin sample used. This requires detailed optimisation of the CZE analysis such as sample preparation and concentration, buffer- type, -concentration and pH, the discontinuous use of high voltage and the associated variations in electro-osmotic flow, presence of additives and temperature changes. A comparison between CZE and HPLC separation would also be desirable.

In order to clearly determine the therapeutic effects of any of these compounds, complete and detailed separation is necessary and would not only be beneficial for the interpretation of our data but also hugely contribute to the interpretation of other reported literature findings.

Other work which will further clarify cellular mechanisms involved include:

3. The determination of apoptosis markers seen with changes in glucose concentration and oxygen tension to distinguish between reversible endothelial DNA damage and irreversible cell death which impacts on the cellular ability to cope with repeated exposure to abnormal glucose and oxygen levels.

4. A detailed analysis of mitochondrial ROS production including superoxide and peroxynitrite to better understand the immediate cellular responses.

5. The use of molecular cloning techniques to determine HIF-1α gene expression and stability in response to abnormal glucose and oxygen levels and its subsequent effect on endothelial DNA damage and pro-apoptotic pathways.

CHAPTER TEN: References

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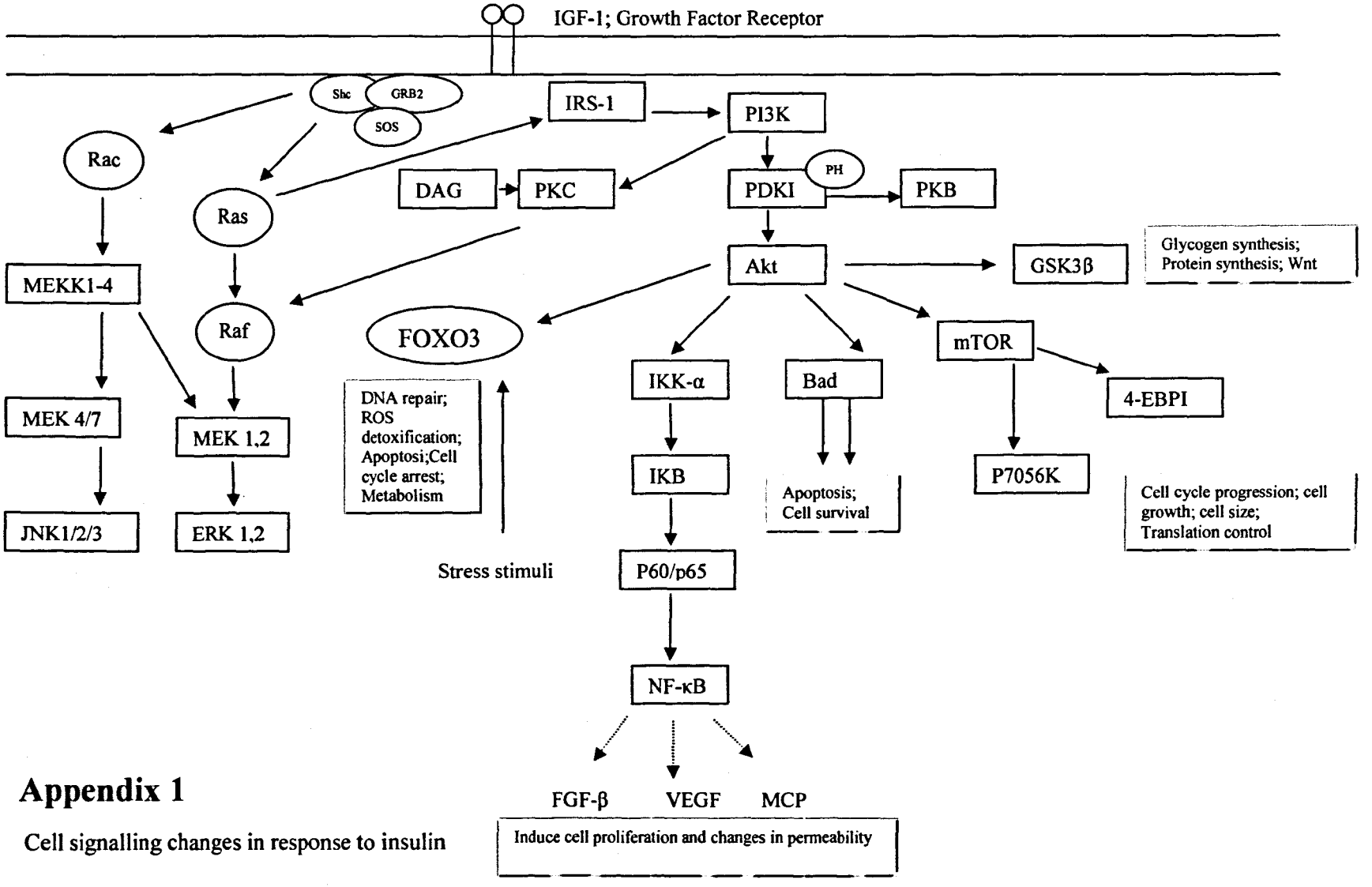

Appendix 1

Cell signalling changes in response to insulin

Appendix 2

Publications

Weidmann AE, Stewart D, Wahle K, Knott RM. Silymarin attenuates hypoxia and hyperglycaemic-induced DNA damage in a human umbilical vein endothelial cell line. *Diabetologia*. 2005; 48 Suppl 1:A23.

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