DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Evaluation of a Dipeptidyl Peptidase IV Inhibitor as a Microvascular Protector in Diabetes

Maria Luísa Morais de Almeida

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Rosa Fernandes (investigador auxiliar da Universidade de Coimbra) e do Professor Doutor Armando Cristóvão (Universidade de Coimbra)

Maria Luísa Morais de Almeida

2013

TRABALHO REALIZADO NO:

Serviço de Farmacologia e Terapêutica Experimental do Instituto

Gostaria de dedicar esta tese a mais do que uma pessoa, mais precisamente a cinco. São sem dúvida os meus heróis: a minha Família.

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

Sir William Lawrence Bragg

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CONTENTS

ABBREVIATIONS & ACRONYMS

AGEs	Advanced Glycation End-Products
AR	Adenosine Receptor
BRB	Blood Retinal Barrier
BSA	Bovine Serum Albumin
DAG	Diacylglycerol
DAPI	4',6-Diamidino-2-Phenylindole
DM	Diabetes Mellitus
DR	Diabetic Retinopathy
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
RT	Room Temperature
DMSO	Dimethylsulfoxide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DPP-IV	Dipeptidyl Peptidase IV
TNF	Tumor Necrosis Factor
BREC	Bovine Retinal
T1DM	Type 1 Diabetes Mellius
T2DM	Type 2 Diabetes Mellitus
PKC	Protein Kinase C
EC	Endothelial Cells
EDTA	Ethylenediamine tetraacetic acid
IL-1β	Interleukin-1 beta
CCL2	Chemokine (C-C motif) ligand 2
CCL5	Chemokine (C-C motif) ligand 5
CXCL8	Chemokine (C-X-C motif) ligand 8 or Interleukin-8 (IL-8)

CXCL10	chemokine (C-X-C motif) ligand 10
CXCL12	chemokine (C-X-C motif) ligand 12
ICAM	Intercellular Adhesion Molecule
VCAM	Vascular Cell Adhesion Molecule
BAEC	Bovina Aortic Endothelial Cell
CD18	Integrin beta-2
T2D	Type 2 Diabetes
BRB	Blood Retinal Barrier
TZD	Thiazolidinediones
STZ	Streptozotocin
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
ECGF	Endothelial Cell Growth Factor
PFA	Paraformaldehyde
PBS	Phosphate Buffer Saline
VEGF	Vascular Endothelial Growth Factor
DNA	Deoxyribonucleic acid
GFAP	*Glial fibrillary Acidic Protein*
BrdU	5-bromo-2'-deoxyuridine
DMSO	Dimethylsulfoxide
vWf	von Willebrand factor
AMC	Aminomethylcoumarin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PI	Propidium Iodide
RAoSMCs	Rat Aortic Smooth Muscle Cells

RESUMO

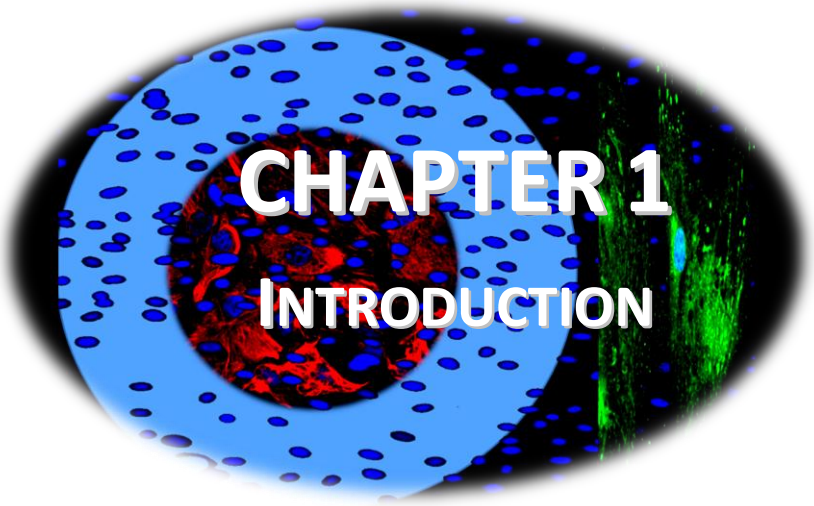
À perturbação metabólica provocada pela incapacidade ou pela diminuição da capacidade de produção de insulina no pâncreas, designamos comummente por Diabetes. Esta patologia de aparente simples definição, é de facto um processo bastante complexo e de difícil controlo, será talvez por tal facto que actualmente a apelidamos de "Epidemia do Mundo Moderno". Não obstante à medida que esta patologia progride e se adensa a severidade da sintomatologia associada aumenta proporcionalmente, estando inerente um maior risco de complicações quer microvasculares, tais como retinopatia, nefropatia, neuropatia como macrovasculares. Consequentemente, as diabetes induzem alterações fisiológicas e metabólicas na retina nas quais a inflamação parece desempenhar um importante papel, em particular no desenvolvimento da retinopatia diabética. Neste contexto o presente estudo pretendeu aferir a putativa capacidade de um antidiabético, inibidor da DPP-IV, – Sitagliptina – de aliviar ou mesmo reverter esta sintomatologia microvascular. Posto isto, células endoteliais da retina de bovino - BREC – foram em concomitância tratadas durante 6 horas com duas concentrações de uma citocina pró-inflamatória – TNF – e de Sitagliptina, respectivamente, 5 ng/ml e 10 ng/ml ou 100 nM e 100 µM. Seguidamente, a formação de estruturas semelhantes a tubos, migração e proliferação de células endoteliais foram avaliadas, tendo tais funções sido preservadas ou, quando diminuídas devido ao TNF, reestabelecidas pela Sitagliptina. Em suma, resultados preliminares obtidos sugerem que a Sitagliptina exerce efeitos citoprotectores na microvasculatura da retina, quando instalada uma condição inflamatória, o que é indicativo dos benefícios anti-inflamatórios deste inibidor da DPP-IV na retina. No entanto, estudos adicionais são necessários tanto para confirmar estes resultados como para entender melhor os adjacentes mecanismos celulares e moleculares envolvidos neste efeito protector mediado pela Sitagliptina. Por conseguinte, tais dados podem contribuir para o desenvolvimento de novas e mais eficazes estratégias terapêuticas dirigidas para a prevenção ou centradas na minimização dos efeitos nefastos induzidos pela hiperglicemia crónica na microvasculatura da retina. Assim, um conhecimento mais aprofundado e detalhado destes inibidores da DPP-IV irão contribuir para um prognóstico mais aferido e eficaz e quiçá a almejada cura completa da Diabetes.

Palavras-Chave: Retinopatia Diabética, Dipeptidil-peptidase IV, Sitagliptina, Inflamação, Células Endoteliais da Retina de Bovino (BREC)

ABSTRACT

Diabetes is essentially a metabolic disruption caused either by the inability or by the decrease in pancreas insulin's production capacity. On the other hand this apparently, in-a-few-words simple definition is in fact a process that could be as complex as hard to control. More importantly, it might be considered as the "Modern World Epidemic" becoming more severe as it extends and progresses. Thus the association between intensity and the overall duration of hyperglycemia periods increases the risk of microvascular complications such as, retinopathy, nephropathy, neuropathy and macrovascular ones. More so, Diabetes induces metabolic and physiological abnormalities in the retina in which inflammation seems to play a major role particularly in the development of diabetic retinopathy. In this context, the aim of this study was to verify if an antidiabetic DPP-IV inhibitor, Sitagliptin, could alleviate some of these symptoms. Therefore bovine retinal endothelial cells – BREC - where co-treated with two concentrations of an inflammatory cytokine TNF and Sitagliptin, respectively 5 ng/ml and 10 ng/ml or 100 nM and 100 µM for 6 hours. EC tube-like structures formation, migration and proliferation abilities seem as a result to be conserved by Sitagliptin and when impaired due to TNF reestablished by the first. In conclusion preliminary results obtained suggest that Sitagliptin exerts cytoprotective effects to the microvasculature of the retina, when an inflammatory condition is settled. What indicates that this DPP-IV inhibitor can have anti-inflammatory benefits to the retina. However additional studies are mandatory to both confirm these results and to further understand the underlined molecular and cellular mechanisms involved in this Sitagliptin-mediated-protective-action. This may contribute to the development of new and more effective therapeutic strategies directed to the prevention or focused in the minimization of the damaging-hyperglycemia-induced effects in the retina's microvasculature. Nevertheless obtaining clear information about these mechanisms could contribute to an elevated knowledge, thorough diagnosis and in general putative cure for diabetes.

Key Words: Diabetic Retinopathy, Dipeptidyl peptidase IV Sitagliptin, Inflammation, Bovine Retinal Endothelial Cells (BREC)



CHAPTER 1

INTRODUCTION

1. DIABETES MELLITUS

1.1. BRIEF STORY ABOUT DIABETES HISTORY

An Egyptian manuscript from c. 1500 BCE mentioning "*passing of too much urine*" is thought to be one of the first diseases description. In addition the first cases reported are believed to be of type 1 diabetes. Around the same time, Indian physicians noting the urine would attract ants, also identified the disease and classified it as *madhumeha* or "honey urine".

The term "*diabetes*" or "*to pass through*" was only used in 230 BCE by the Greek Appollonius of Memphis. During the Roman Empire the disease was considered rare with Galen commenting he had only seen two cases during his entire career. (DiabeWorld, 2012) This is possibly due to ancient people dieting and life-style, or even because clinical symptoms were only observed during a more advanced stage of the disease, Galen named the disease "*diarrhea of the urine*" (*diarrhea urinosa*) (MiYDiabetes, 2012).

The earliest surviving work with a detailed reference to diabetes is that of Aretaeus of Cappadocia, reflecting the beliefs of the "*Pneumatic School*" he described the symptoms and the disease course, which he attributed to the moisture and coldness. He hypothesized a correlation of diabetes with other diseases, discussing differential diagnosis from the snakebite which also provokes excessive thirst. His work remained unknown until middle of the 16th century when, in 1552, the first Latin edition was published in Venice (Diabetes India, 2012).

Moreover type 1 and type 2 diabetes where identified as separate conditions for the first time by the Indian physicians Sushruta and Charaka in 400-500 CE with type 1 associated with youth and type 2 with being overweight.

The term "*mellitus*" or "*from honey*" however was added by the Briton John Rolle in the late 1700s, when he noticed the urine of a diabetic had a sweet taste (*glycosuria*) and to separate the condition from *diabetes insipidus*, which is also associated with frequent urination. When Canadians Frederick Banting and Charles

Herbert Best isolated and purified insulin, in 1921 and 1922, the effective treatment was developed. This important finding was followed by the development of the long-acting insulin NPH in the 1940s. (Ahmed A.M. *et al*., 2002)

1.2. DEFINITION AND CHARACTERIZATION

Diabetes Mellitus was considered by the World Health Organization (WHO) as a complex and heterogeneous metabolic disorder, which is characterized through a chronic hyperglycemia with atypical carbohydrates, lipids and proteins metabolism, leading to insulin secretion and/or insulin action deficiency (WHO, 2012).This pathology may present characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss. In its most severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and, in absence of effective treatment, death. (WHO, 2012, IDF, 2012))

Moreover the pathogenetic processes involved in the development of diabetes include pancreatic beta cells destruction with consequent insulin deficiency, and others that result in resistance to insulin action (Eizirik *et al*., 2008). This leads to the progressive development of specific complications, namely retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation and Charcot joints. People with diabetes are also at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease (IDF, 2012, Medical News Today, 2012)

1.3. PREVALENCE

Worldwide, we have been assisting to an increase in Diabetes prevalence which is inevitably overwhelming to our already overburdened health system. Hence type 2 Diabetes is the one to be blamed when it comes to this escalating aggravation, not only due to ever growing world population but also the continuously aging demography. Nevertheless a massive increase in overweight and obese people due to sedentary and overall lack of a healthy life style, might as well contribute to a bigger risk of suffering from this disease (Zimmet *et al*., 2001)

Diabetes Mellitus prevalence rates have critically risen in recent years and in accordance to the *International Diabetes Federation* (IDF), *Diabetes Mellitus* is frequently a non-communicable disease, with high morbidity and mortality rates, just in 2012 alone were registered about 4.8 million casualties. (IDF, 2012)

In the present, Diabetes affects about 371 million people (**Figure 1.1**) and with an estimated increase to 552 million by 2030, thus representing 8.3% of adult population. However, these numbers are underestimated due to the undiagnosed patients, who represent almost half the people with Diabetes (IDF, 2012). Regarding Portugal, the *Sociedade Portuguesa de Diabetologia* (Portuguese Diabetes Society) estimates that 12.7% of the Portuguese adult population will be diabetic, from which 5.4% might still remain undiagnosed (SPD, 2012).

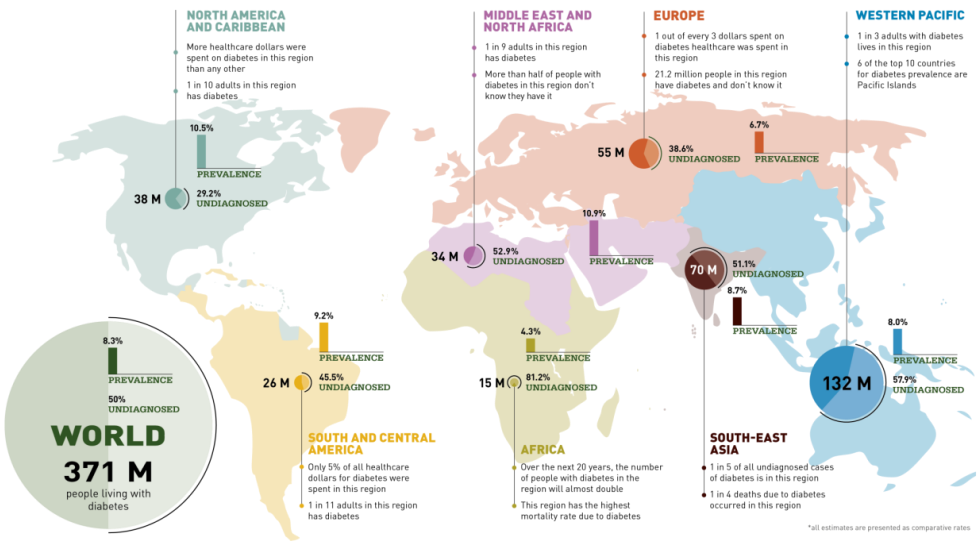


Figure 1.1 - Global prevalence (%) of *diabetes mellitus* in adult population (20-79 years) in 2012, shown by geographic region (Image taken from IDF Diabetes Atlas, 5th Edition (IDF, 2012)

1.4. MOST COMMON TYPES

In accordance to WHO and IDF there are three main types of diabetes, namely: type 1 diabetes mellitus (T1DM) also called insulin-dependent, type 2 diabetes mellitus (T2DM) known as insulin-resistance and gestational diabetes

1.4.1. TYPE 1 DIABETES MELLITUS

Type 1 diabetes also called as insulin-dependent diabetes, or juvenile-onset diabetes, is characterized by the presence of anti-GAD, islet cell or insulin antibodies which identify the autoimmune processes that mediate the destruction of pancreatic beta cells. Albeit these patients not being necessarily obese they may instead have other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis, and Addison's disease (Betterle *et al*., 1983).

The first manifestation in some patients, particularly in children might be ketoacidosis or modest fasting hyperglycaemia. A few adults however may retain residual beta-cell function, sufficient to prevent ketoacidosis, for many years (Japan and Pittsburgh Childhood Diabetes Research Groups, 1985). Eventually, individuals with this form of Type 1 diabetes often become dependent on insulin for survival in order to prevent the development of ketoacidosis (Zimmet *et al*.,1995, Willis *et al*., 1996). At this stage of the disease, there is little or no insulin secretion (**Figure 1.2**) as manifested by low or undetectable levels of plasma C-peptide (Hother *et al*., 1988) There is however both a genetic predisposition to autoimmune destruction of beta cells as a relation between environmental factors, which remains yet known.

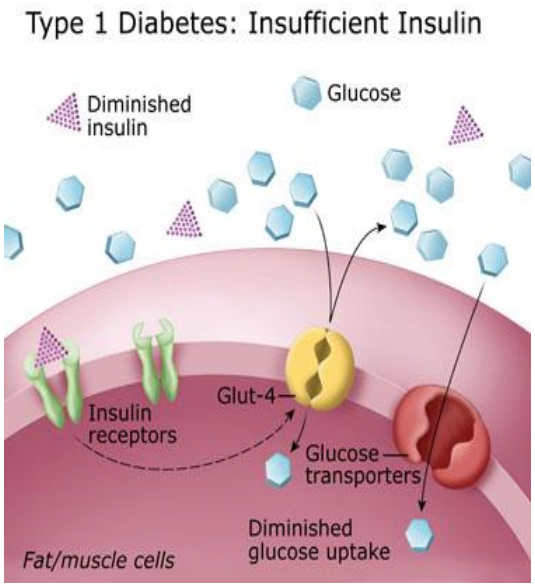


Figure 1.2 - Carrier proteins involved in type 1 Diabetes (Diabetes Education Online, 2012)

1.4.2. TYPE 2 DIABETES MELLITUS

Type 2 diabetes previously encompassed non-insulin dependent diabetes or adult-onset diabetes, and accounts for at least 90% of all cases of diabetes. It is characterised by insulin resistance and relative insulin deficiency (**Figure 1.3**), either or both of which may be present at the time diabetes is diagnosed.

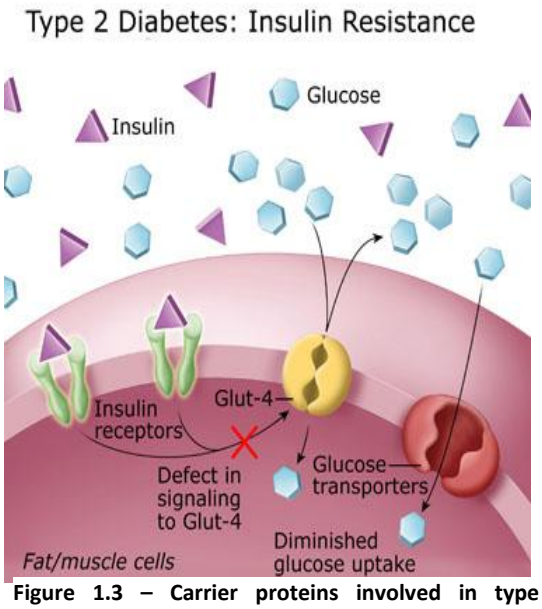


Figure 1.3 – Carrier proteins involved in type 12Diabetes (Diabetes Education Online, 2012)

Type 2 diabetes risk increases with age, hypertension, dyslipidaemia and overall lack of physical activity (Zimmet *et al*., 1992, Harris *et al*., 1995). It might be probably associated with strong familial, likely genetic, predisposition (Valle *et al*., 1997, Knowler *et al.*, 1993). Despite the genetics of this diabetic form it is still rather complex and not clearly defined.

It is often, but not always, associated with overweight, obesity or even predominantly body fat in the abdominal areas, which itself can cause insulin resistance and lead to high blood glucose levels. People with type 2 diabetes can often initially manage their condition through exercise and diet. However, over time most people will require oral drugs and or insulin (Kissebah *et al*., 1982).

2. DIABETIC RETINOPATHY

2.1. DEFINITION AND CHARACTERIZATION

Diabetic Retinopathy (DR) is a microvascular complication of diabetes (Aielo *et al*., 1998, Davidson *et al*., 2007), in early stages of the disease are characterized by vascular alterations in blood flow, death of retinal pericytes (perivascular contractile cells), basement membrane thickening and subtle increases in vascular permeability.(Aveleira *et al*., 2010, Zhang *et al.*, 2011, Sheetz *et al*., 2002) As the disease progresses, clear alterations in the vascular structure, for instance non-perfused vessels, microaneurysms, dot/blot hemorrhages,

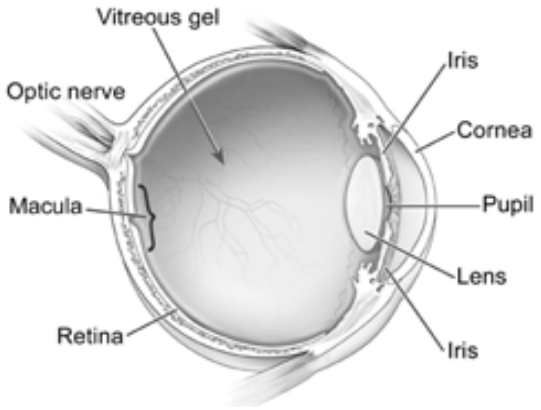


Figure 1.4 - Normal eye anatomy (*National Eye Institute*, 2012)

cotton-wool spots, venous beading, vascular loops and significant vascular leakage can be seen under ophthalmologic examination (Cockburn *et al*., 1999).

2.2. DR PROGRESSION

The vascular changes stated above occur in an early phase – the non-proliferative stage – of DR, where, as a consequence of increased vascular permeability, the macular edema formed can eventually lead to vision loss. Later on, in the proliferative stage of DR, neovascularization takes place on the retinal surface, after that vascular function becomes impaired by capillary occlusion, non-perfusion and degeneration. In this stage, severe vision loss or even blindness may as well be caused by bleeding, hemorrhage and subsequent retinal detachment due to the newly formed but rather fragile vessels (Davidson *et al*., 2007).

Diabetic retinopathy progression can be identified into four stages, which were conveniently enumerated down below:

I. Mild Nonproliferative Retinopathy: At this earliest stage, microaneurysms occur. They are small areas of balloon-like swelling in the retina's tiny blood vessels.

II. Moderate Nonproliferative Retinopathy: As the disease progresses, some blood vessels that nourish the retina are blocked.

III. Severe Nonproliferative Retinopathy: Many more blood vessels are blocked, depriving several areas of the retina with their blood supply. These areas of the retina send signals to the body to grow new blood vessels for nourishment

IV. Proliferative Retinopathy: At this advanced stage, the signals sent by the retina for nourishment trigger the growth of new blood vessels. This condition is called proliferative retinopathy. These new blood vessels are abnormal and fragile. They grow along the retina and along the surface of the clear, vitreous gel that fills the inside of the eye.

The mechanisms by which diabetes causes microvascular complications and disease progression in the retina are not yet fully understood. However, further studies

in patient samples and animal models have shown that DR could be definitely characterized as a chronic, hence subclinical inflammation.

Figure 1.5 - Normal Vision and the same scene viewed by a person with Diabetic Retinopathy (*National Eye Institute*, 2012)

2.3. MAJOR PATHOGENIC PATHWAYS IN DIABETIC RETINOPATHY

The increase in polyol pathway, non-enzymatic glycosylation of proteins, oxidative stress and protein-c kinase activation by generation of diacylglycerol, are retinal biochemical and cellular abnormalities induced by chronic hyperglycemia that lead to vascular impairments. Albeit this, the true mechanism behind hyperglycemia-induced-DR remains yet known.

2.3.1. POLYOL PATHWAY

Glucose uptake by retinal tissue is insulin-independent existing therefore an equilibrium between retinal and plasmatic glucose levels. When hyperglycemia is present it activates the polyol pathway, converting, through aldose reductase, glucose in sorbitol and then in fructose (by sorbitol dehydrogenase) (Miwa *et al*., 2003)

Subsequently, in the retina, the intracellular increase in sorbitol concentration causes osmotic breakdown, damages retinal pericytes, via apoptosis, and thickens retinal vascular endothelial cell basement membranes leading to closure of retinal capillaries. These changes in membrane permeability and integrity have been identified as the key to early cellular pathology (Lorenzi *et al*., 2007).

2.3.2. AGEs Pathway

The hyperglycemia also leads to lipid and protein glycosylation, whose oxidation produces glycotoxins and Advanced Glicosilated End-products (AGEs) (Schleicher *et al*., 1997). These AGEs can be correlated with Diabetes duration and severity, being found in the plasma, tissue and vessels walls, exerting their action through endothelium-expressed receptors, promoting an increase in vascular permeability and thrombogenicity (Kowluru and Odenbach et al., 2004, Libby *et al*., 2007). Nevertheless numerous are the mechanism that control vascular tonicity, that when Diabetes-activated can lead to vascular hemodynamic regulation loss.

2.3.3. Oxidative Stress

There is evidence that oxidative stress, defined as a persistent imbalance between the production of highly reactive molecular species (chiefly oxygen and nitrogen) and antioxidant defenses, can lead to tissue damage (Rosen *et al*., 2001). Oxidative stress results from increased content of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). Examples of ROS include charged species such as superoxide($O_2^{-\bullet}$) and the hydroxyl radical ($OH^{\bullet}$), and uncharged species such as hydrogen peroxide (Rosen *et al*., 2001, Irani *et al*, 2000). There are also data indicating that ROS formation is a direct consequence of hyperglycemia (Brownlee *et al*., 2001); more recent studies have suggested that increased free fatty acids levels may also result in ROS formation.

Because of their ability to directly oxidize and damage DNA, protein, and lipid, ROS are believed to play a key direct role in the pathogenesis of late diabetic complications (Rosen *et al*., 2001, Nishikawa *et al*., 2000). In addition, ROS can function as signaling molecules to activate a number of cellular stress-sensitive pathways that cause cellular damage, and are ultimately responsible for the late complications of diabetes. Furthermore, these same pathways are linked to insulin resistance and decreased insulin secretion (Wolff *et al*., 1991).

2.3.4. PROTEIN KINASE C PATHWAY

Studies indicate that enhanced generation of diacylglycerol – a physiologic activator of PKC – , hence a PKC pathway activation might play a major role in hyperglycaemia-induced microvascular dysfunction in diabetes by promoting increased flux through the polyol pathway (Keogh *et al*., 1997) and the generation of AGEs (Portilla *et al*., 2000) and oxidative species result in.

PKC which functions as signaling components for a variety of growth factors, hormones, neurotransmitters and cytokines, when activated results in numerous cellular changes, leading to basement membrane thickening and increased production of vasodilatory prostaglandins as well as vascular endothelial growth factor (VEGF), which in turn affect vessel permeability and/or blood flow, leading to vascular impairment (Koya and King *et al*., 1998, Das Evcimen and King *et al*.,2007).

3. INFLAMMATION

The role of inflammation in the development and progression of diabetic retinopathy has been studied for a long time but it was in the last years that it started to get some major attention. Powell and Field in 1960 observed that diabetics treated with anti-inflammatory agents like “salicylate” had a lower incidence rate of diabetic retinopathy (Powell *et al*., 1964). Earlier studies by Lutty and his group identified the important role of leukocytes in the development of diabetic retinopathy (Lutty *et al*., 1997, McLeod *et al*., 1995) and a subsequent study has established diabetic retinopathy as an “inflammatory disease” (Adamis *et al*., 2002).

This inflammatory state starts very early within one week of experimental diabetes, due to leukocytes accumulation in the vasculature of the retina (Adamis *et al*., 2002). Widely observed in diabetic retinopathy the major components of the inflammatory phenotype progress towards the increase in diabetic macular edema (retinal vascular permeability) and neovascularization (proliferative diabetic retinopathy) (**Table 1.6**).

Increased Expression of Inflammatory Adhesion Molecules ICAM-1 and VCAM-1 in the Endothelium
Adhesion Leukocytes to Retinal vessels
Increased Expression of Cytokines and Growth factors
Microglial Cell Activation
Infiltration of Monocytes and Neutrophils
Increased Vascular permeability (Macular Edema) and Neovascularization (Proliferative Retinopathy)
Retinal Cell Death

Table 1.6 - Components of Diabetic Retinal Inflammation (Adapted from Antonetti *et al*., 2006 and Adamis *et al*.,2002)

3.1. INFLAMMATORY BIOMARKERS IN DIABETIC RETINOPATHY

The main risk factors for diabetic retinopathy include diabetes duration, hyperglycemia, hypertension, and dyslipidemia, which only can explain some degree of variance in the risk of diabetic retinopathy (Nguyen *et al*., 2009). Studies have therefore shown the association of multiple systemic inflammatory factors in the progression of diabetic retinopathy, and a further analysis of diabetic vitreous samples has also provided insights into novel proinflammatory markers in this process (Schmidt *et al*., 1995, Schram *et al*., 2003).

Furthermore inflammation is a complex biological response of vascular tissues against harmful stimuli, including damaged cells, irritants, or pathogens being also a critical step in wound healing (Wagener *et al*., 2013). This process involves multiple mediators such as pro-inflammatory cytokines, chemokines and adhesion molecules that initiate the interaction between leukocytes and the endothelium, guiding directional leukocyte migration toward infected or injured tissue (Zhang *et al*., 2011).

First, the pro-inflammatory cytokines (such as tumor necrosis factor (TNF) and interleukins) and the chemokines (such as CCL2 and CCL5) released from infected/injured tissue, activate the endothelium to increase expression of adhesion molecules (such as E-selectin, intercellular adhesion molecule (ICAM)-1, vascular cell

adhesion molecule (VCAM)-1) and chemokines (Zhang *et al*., 2011). Then leukocytes, mediated by chemokines and adhesion molecules, attach to the vessel wall, transmigrate through the endothelium and linger in the infected or injured tissue until complete healing (Barreiro *et al*., 2010). While normal inflammation is beneficial, excessive or uncontrolled one, like the one seen in DR, can cause tissue injury and ultimately result in diseases (Barreiro *et al*., 2010).

Although there are no known pathogens in DR, analysis of inflammatory molecules in vitreous, serum and retina form diabetic patients or experimental animals indicate that DR is associated with significant increases in pro-inflammatory cytokines, chemokines and adhesion molecules (Zhang *et al*., 2011). High levels of interleukin-1β (IL-1β) and caspase 1 – its downstream signaling molecule – as well as TNF are significantly increased in vitreous, retinas and serum from diabetic patients and rats (Demircan *et al*., 2006, Vincent *et al*., 2007, Kowluru *et al*., 2004). Nevertheless TNF also appears to be augmented in ocular fibrovascular membranes from patients with DR and in retinas from rodent model of diabetes mellitus (Joussen *et al*., 2006, Limb *et al*., 1996).

On the other hand, vitreous samples from DR patients also show that certain chemokines such as CCL2, CCL5, CXCL8, CXCL10 and CXCL12 are also upregulated (Murugeswari *et al*., 2008, Meleth *et al*., 2005). Furthermore, Increases in IL-6, ICAM-1 and VCAM-1 have been shown to be related to the progression of DR (Meleth *et al*., 2005, Adamiec-Mroczek, 2008) (**Figure 1.7**).

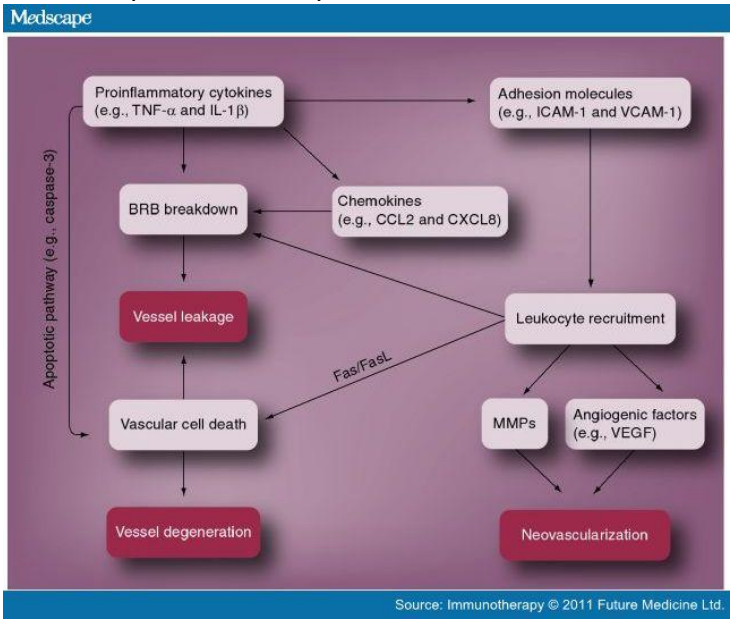


Figure 1.7 - Inflammatory pathway in Diabetic Retinopathy (Medscape 2011)

This correlation between increases in inflammatory molecules can also be associated with recruitment of leukocytes, for example the quantity of neutrophils is significantly elevated in both retinal and choroidal vessels from diabetic patients and rhesus monkeys (Kim et al., 2005). Whereas its accumulation can be either correlated with upregulation of ICAM-1 immunoreactivity in the vessels or associated with capillary closure (McLeod *et al*., 1995).

In diabetic rodent models, along with the progression of DR, there is a cumulative and sustained increase in leukocyte adherence to the retinal vasculature – leukostasis (Miyamoto *et al*., 1999). This augment however may be linked to diabetes-induced increases in ICAM-1 and integrins in endothelial cells and leukocytes, respectively, since ICAM-1 block or deletion of CD18 (ICAM-1 receptor subunit on leukocytes) seem to act as a preventive in diabetes-induced leukostasis (Miyamoto *et al*., 1999, Joussen *et al*., 2000, Barouch *et al*., 2004).

In addition to inflammation-triggered circulatory leukocytes, retinal microglial cells – resident ocular macrophages – (Fischer *et al*., 2001) are also most likely to be involved in DR (Chen *et al*., 2002), being rapidly activated to release inflammatory cytokines such as TNF (Yang *et al*., 2009). On the other hand, studies in wild-type mice revealed that the treatment with the A2A adenosine receptor agonist resulted in marked decreases either in tumor necrosis factor (TNF) release or in hyperglycemia-induced retinal apoptosis (Ibrahim *et al.,* 2011).

3.2. Role of Inflammation in Blood Retinal Barrier Alteration

Briefly, the blood-ocular barrier system is formed by two main barriers: the blood-aqueous barrier and the blood-retinal barrier (BRB). This barrier, particularly tight and restrictive, is a physiologic-type, because it regulates ion, protein, and water flux into and out of the retina. The BRB consists of inner, being formed of tight junctions between retinal capillary endothelial cells and outer components, and outer BRB which has tight junctions between retinal pigment epithelial cells. Furthermore, BRB is as

essential to maintaining the eye as a privileged site as it is indispensable for normal visual function; in fact it is exactly why alterations of the BRB play a crucial role in the development of retinal diseases. The 2 most frequent and relevant retinal diseases, diabetic retinopathy and age-related macular degeneration (AMD), are directly associated with alterations of the BRB. The first is initiated by an alteration of the inner BRB, whereas neovascular AMD is a result of an alteration of the outer BRB, being therefore the macular edema a direct result of alterations in the BRB (Cunha-Vaz *et al*., 2010).

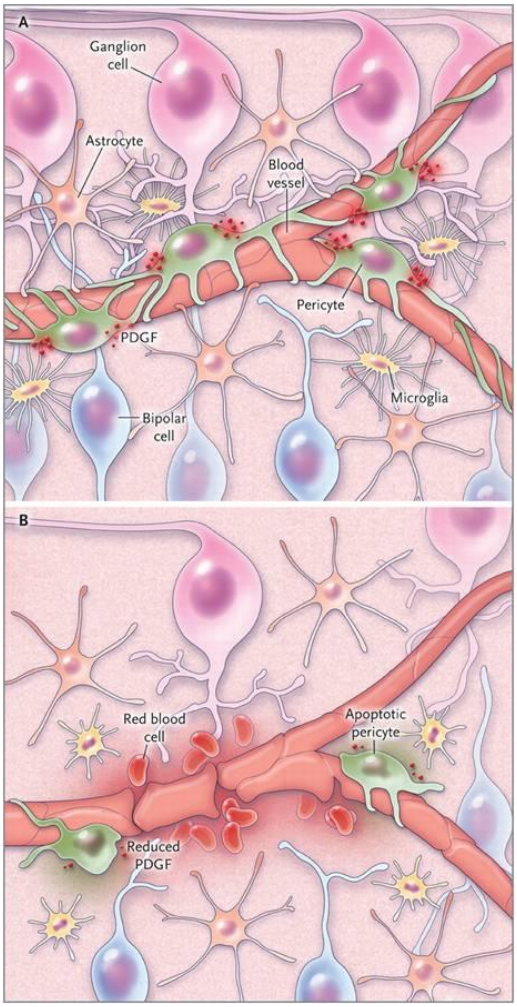


Figure 1.8 - Disruption of the Neurovascular Unit of the Retina by Diabetes (Antonetti et al., 2012).

Alteration of the inner blood retinal barrier (BRB) is the hallmark of diabetic retinopathy (Aveleira *et al*., 2010) (**Figure 1.8**). And, inflammation is one of the earlier events in diabetic retinopathy, which there is increased adhesion of leukocytes to the microvasculature, resulting in the loss of endothelial cells and breakdown of the blood-retinal barrier (Yuuki *et al*., 2001, Joussen *et al*., 2001). Hence the major stages for BRB alteration are enumerated next: increased expression of adhesion molecules, such as ICAM1, VCAM1, P-Selectin, followed by leukocyte adhesion in the diabetic retina endothelium (McLeod *et al*., 1995, Adamis *et al.*, 2008); next, release of inflammatory cytokines, vascular permeability factors, and growth cytokines, what culminates into modifications in adherens and tight junctional proteins (for example, VE-Cadherin, Occludin, ZO-1 and Claudin) and also infiltration by diapedesis of the leukocytes into the retina. Nonetheless all of these steps contribute to the Blood-Retinal Barrier breakdown.

Moreover, Yuuki *et al*., in animal models of diabetic retinopathy showed that the inhibition of leukocyte adhesion prevents the loss of pericytes and the formation of acellular capillaries, leading therefore to the suppression of the blood-retinal barrier breakdown. Another study also suggested a possible mechanism involving proteolytic degradation of VE-cadherin which could be possibly linked to diabetes contribution to BRB (Navaratma *et al*., 2001).

These recently identified inflammatory risk factors not only serve as potential biomarkers, but also give insights into the development of potential molecular targets for treating diabetic retinopathy.

4. ANTI-DIABETIC THERAPY

4.1. EXISTING NON-INSULIN ANTI-DIABETIC THERAPY

At the present time, to treat/control diabetes medicine relies on either the classic insulin therapy or on the non-insulin antidiabetic one, this is, metformin, sulphonylureas, meglitinides, thialidonediones, alpha-glucosidase inhibitors, amilin analogs, GLP-1 receptor agonists and DPP-IV inhibitors. These agents act in different sites of the organism to amelliorate insulin secretion and/or even its action. (Weiner, L. *et al*., 2010).

4.1.1. INCRETINS

In the last decade incretins have become object of attention due to its potential as a new type 2 Diabetes therapy. Nonetheless this concept was already known almost half a century ago, once it was observed that orally administered glucose stimulated a bigger insulin release then the same amount of the injected subtance (Eirick *et al*., 1964). From this investigation two hormones, namely *glucagon-like-peptide-1* (GLP-1) and *glucose-dependent insulinotropic peptide* (GIP), this is, incretin hormones were found responsible for the signal which led to gastrointestinal tract release of insulin whenever food was being consumed.

However it is now known that after being secreted by the gastrointestinal tract while food is eaten, incretin hormones bind to beta cells receptors in the pancreas, stimulating insulin secretion as a response to glucose absorption. (Ahrén, 2003). In healthy individuals it is also thought that incretin effect is responsible for approximately 50 – 70% of insulinic-response to oral glucose, but a smaller secretion in type 2 Diabetes patients, might suggest that this could be linked to the disease pathogenesis (Nauck *et al.*, 1986; Vilsbøl *et al*., 2001). Drucker *et al*., later corroborated with this by showing that an increase in GLP-1 reduces hyperglycemia (Drucker, 2003).

Consequently, and since GIP is inactive in type 2 Diabetes, a new incretin-like therapy based on GLP-1 endogenous levels was developed, in which two different approaches were pursued. The incretin mimetics, for example exenatide, and one Sitagliptin belongs to, hence the incretin secretors or DPP-IV inhibitors.

4.1.1.1. INCRETIN MIMETICS

Deacon *et al*, stated that these endogenous peptides (GLP-1 and GIP) were rapidly removed from circulation by DPP-IV enzyme and renal clearance. So it was necessary for the production of incretin-based therapies that these molecules had expand half-lifes and therefore exenatide and liraglutide were produced.

4.1.1.1.1. EXENATIDE

Exenatide was the first agent to be commercialized in 2005 and in the USA. It is a synthetic form of exenatide 4, a molecule originally isolated from the venon of the *Heloderma suspectum*. The exenatide however is not that analogous to human GLP-1, only sharing 53% of its molecular sequence. Despite the remaining structural similarity is enough for it to bind to GLP-1 receptor in order to mimic a significant number of glucoregulatory actions, not being able however to proceed as a DPP-IV susbstract.

Exenatide decreases fasting and postprandial glucose concentrations through several mechanisms, also shared with GLP-1. In the pancreas, exenatide all together stimulates beta cells insulin secretion and suppresses alpha cells glucagon secretion

reducing for that reason, in a post-prandial state, hepatic glucose production. It also intervenes in gastric emptiness by slowing it down, promoting a satiety sensation, what eventually might lead to weight loss in overweight individuals (Linnebjerg *et al*., 2008). Nonetheless these events only need to occur in the presence of high circulatory glucose concentrations, in order to minimize hypoglycemic risk.

In animal studies, exenatide can apparently promote pancreatic islets cells differentiation and inhibit beta cells apoptosis, altering the balance by increasing islets mass. Beta cells mass cannot be measured in humans through non-invasive procedures and in addition their function can only be determined in an indirect manner, for example, through pro-insulin:insulin ratio and beta cell function homeostasis evaluation (HOMA-b). These results seem to suggest that GLP-1 receptor agonist as well as DPP-IV inhibitors enhance beta cell function (Drucker, 2006).

Finally, in contrast with exenatide and due to a higher homology between liraglutide and endogenous GLP-1, there is a decreased risk of antibody formation (Marre *et al*., 2009; Russell-Jones *et al*., 2009). Nonetheless a clinical relevance for the antibodies issue remains yet known for both agents.

4.1.2. DPP-IV INHIBITORS

DPP-IV, or Dipeptidyl-peptidase IV, Inhibitors, can often be known as gliptins, since they increase incretin hormones levels by inhibiting the enzyme responsible for their breakdown (DPP-IV). This results in higher endogenous GLP-1 half-life and levels.

In Europe, 2007 and 2008 respectively, Sitagliptin and Vildagliptin were the first agents belonging to this class to be approved. Then Saxagliptin followed in 2009 and finally in April of the next year Alogliptin, in Japan. A couple of other molecules are still in trial stages, such as, Linagliptin and further DPP-IV inhibitors are also being developed.

Their action mechanism involves a competitive and a reversible inhibition, about 90% of the plasmatic DPP-IV activity, with a duration period of 24 hours (Deacon and Holst, 2006). Although DPP-IV resistant incretin analogues (e.g. exenatide,

liraglutide) have the same biochemical target than the DPP-IV inhibitors, these last are only able to increase GLP-1 levels up to physiological values while the others can increase it by 6-10-fold that of the physiological ones found in the postprandial state (Elbrønd *et al*., 2002, Drucker *et al*., 2008, Calara *et al*., 2005).

In addition, among DPP-IV inhibitors glucoregulatory actions are included insulin secretion stimulation, glucagon secretion inhibition, on the whole improvement in beta cells function. In spite of this DPP-IV inhibitors seem to not mediate all endogenous GLP-1 glucoregulatory actions, having therefore little or no effect in gastric emptiness or satiety for that matter, what might be implied in their neutrality regarding the weight issue. DPP-IV inhibitors on other hand can be divided into two classes which are either related to their structural form or their excretory pathway (Nauck *et al*., 2009).

First, they are divided due to the presence or absence of the cyano-pirrolidine ring, since the ones who do possess it, namely Vildagliptin and Saxgliptine are less selective (not clinically relevant though) than the others who don't, hence Sitagliptin, Alogliptin and Linagliptin.

Second, Sitagliptin and Alogliptin remain almost inaltered when kidney-excreted whereas Saxagliptin and Vildagliptin are metabolized respectively, as an active metabolite (in the kidney and liver) and an inactive one.

As a final point, Linagliptin is the only DPP-IV inhibitor that could be mainly suggested to patients with renal insufficiency since it doesn't have a known renal-excreted pathway.

5. DEMAND FOR NEW DRUGS

Diabetes is a chronic disease that affects a still-rising number of people. Although the drugs available are initially efficient when trying to achieve the recommended glycaemic control, in a long term and without constant adjustments or a combination therapy it becomes difficult to accomplish this kind of management.

Above and beyond with the exception of TZD's the available drugs have a reduced effect on Diabetes progression due to the continuous degradation in pancreatic beta cells function. Furthermore a higher risk of hypoglycemia and overweight associated to several therapies also represent huge difficulties for the optimal glycaemic control.

More importantly it must be taken into consideration both the drug´s glycaemic reduction rate and the underlined mechanisms that led to this decrease. Subsequently new drugs beyond aiming for glycaemic control must be projected to control diabetes through yet unsolvable questions: better tolerance, lingering .efficiency and overall capacity to act on the actual cause of the disease.

In a previous work from our laboratory, Gonçalves *et al*, showed that sitagliptin, prevents oxidative stress, inflammation and even apoptosis in retinal cells and exerts beneficial effects on the integrity of the blood-retinal barrier in an animal model of type 2 diabetes (Gonçalves *et al*., 2012). In addition, it also demonstrated, in a model of type 1 diabetes, that sitagliptin can inhibit blood-retinal barrier breakdown, leading to restored tight junctions organization, induced neuronal cells protection and enhanced retina inflammatory condition Nevertheless these studies seem as well to indicate that sitagliptin directly protects the retinal endothelial cells, but, until now, is not clear whether this drug could have a role in angiogenesis or vascular repair.

6. OBJECTIVES OF THIS THESIS

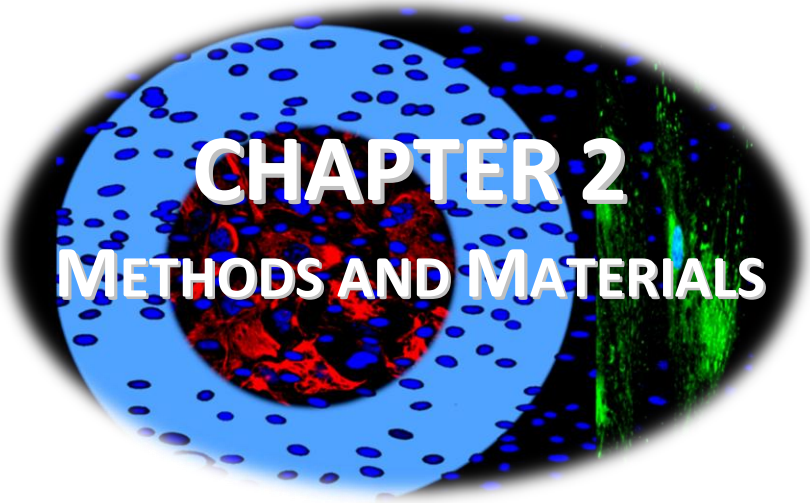
Diabetes is a metabolic disease increasingly common, with an increasing prevalence worldwide. It is expected that 220 million people have diabetes and it is estimated that this number will doubled in the next 30 years. If not controlled, diabetes can lead to an increased risk of chronic complications including diabetic retinopathy. Chronic hyperglycemia leads to endothelial cell dysfunction resulting in loss of retinal pericytes, formation of acellular capillaries, increased vascular permeability and leukocyte adhesion

The diabetes-induced vascular permeability appears to correlate with the disruption of the integrity of the tight junctions, which form a complex structure between endothelial cells, constituting the inner blood-retinal barrier. The chronic hyperglycaemia induces changes in the levels and distribution of proteins of tight junctions in the retinal vascular endothelium, which appears to directly contribute to increased vascular permeability. Diabetes induces metabolic and physiological abnormalities in the retina and it appears that inflammation plays a major role in the development of diabetic retinopathy.

Since the microvascular complications are correlated with the severity and duration of hyperglycemia, it is highly desirable to find drugs that would permit an improvement in glycemic control and may also have an important role in delaying or preventing microvascular complications.

Previous work from our laboratory showed that an inhibitor of dipeptidyl peptidase IV, sitagliptin, prevents oxidative stress, inflammation and apoptosis in retinal cells and exerts beneficial effects on the integrity of the blood-retinal barrier in an animal model of type 2 diabetes. In addition, it also showed that sitagliptin inhibits the breakdown of the blood-retinal barrier, so that tight junctions organization could be restored, inducing neuronal cells protection and enhancing the inflammatory condition of the retina in a model of type 1 diabetes. Nevertheless these studies seem to indicate that sitagliptin directly protects the endothelial cells of the retina. However, until now, is not clear whether this drug may have a role in angiogenesis or vascular repair.

For this work, we intend to use the primary cell cultures of bovine retinal endothelial cells (BREC) to evaluate the potential protective effect of sitagliptin in angiogenesis induced by a pro-inflammatory cytokine TNF (Wound-Healing, Proliferation and MTT assay and also PI staining). Finally, another objective is to assess the effect of sitagliptin on the ability of BREC to form tubular structures like vessels (Angiogenesis kit).



CHAPTER 2
METHODS AND MATERIALS

Quoting **Erwin Chargaff** – Austrian biochemist responsible for unraveling two rules that lead to the double-helix-DNA structure discovery – "Science is wonderfully equipped to answer the question '**How?**' but it gets terribly confused when you ask the question 'Why?' !"

Hence, throughout this section it is going to be briefly explained the "*how*" part by enumerating methods as well as materials and reagents used during this thesis.

BIOLOGICAL ASSAYS

A bioassay (i.e., biological assay) is a procedure for the determination of the concentration of a particular constituent of a mixture or a measurement of the effects of a substance on living organisms. Hence they can measure under controlled conditions the effects of a biologically active substance using an intermediate *in vivo* or *in vitro* tissue or cell model.

1. BREC ISOLATION

BREC were isolated as previously described (Fernandes *et al*., 2004). Briefly, BREC were isolated from retinal capillaries of fresh bovine eyes. Under sterile conditions, the retinas were separated from other ocular tissues, washed in Dulbecco's Modified Eagle Medium (DMEM) (Introgen, Carlbad, CA) and fragments of the contaminating retinal pigment epithelium were then removed.

Next the retinas were transferred to an enzyme solution containing 100 µg/ml Pronase (Roche, Mannheim, Germany), 500 µg/ml Collagenase type I (Introven, Carlsbad, CA, USA), 70 µg/ml DNase (Sigma-Aldrich, St. Louis, MO, USA) and incubated with agitation for about 20 minutes, at 37°C. After incubation, the retinal digest was passed through two nylon meshes, of 210 µm and 50 µm. The microvessels retained on top of the 50 µm nylon mesh were collected in DMEM by centrifugation. The fragments were resuspended in DMEM containing low glucose concentrations (5.5 mM), supplemented with 15% (v/v) Fetal Bovine Serum (FBS) (Introgen, Carlsbad, CA, USA), 20 µg/ml Endothelial Growth Factors (ECGF) (Roche, Mannheim, Germany), 100 µg/ml

heparin and antibiotic/antimycotic solution (100 U/ml Penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericine B, Sigma-Aldrich, St. Louis, MO, USA).

The cells were plated and grown on 50 µg/ml fibronectin-coated flasks, (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator, at 37°C, in an atmosphere with 95% of air and 5% of CO_2. BREC were divided between 7 and 10 days after isolation. BREC used in all experiences were from passages 3 to 6.

1.1. SUBCULTURING

After reaching confluency BRECs were subcultured. The DMEM medium was removed from culture flasks by aspiration and discarded. BREC were rinsed with 5 ml of warm sterile Phosphate Buffer Saline (PBS: Na_2HPO_4 10 mM, KH_2PO_4 1.8mM, NaCl 137 mM, KCl 2.7 mM, pH 7.4), in order to remove traces of serum which would inhibit the action of the trypsin. BREC cells were treated with 3 ml of trypsin 0.25% (w/v) (Life Technologies Corporation, Carlsbad, CA, USA) and incubated at 37° C for approximately 5 minutes. The tryspsinization process was monitored at an inverted microscope. DMEM medium was added to inhibit further tryptic activity and the cells were dispersed by repeated pipetting over the surface bearing the monolayer. The cell suspensions were then centrifuged at 1,000x g for 5 min and cell pellet was resuspended in DMEM medium and appropriate aliquots of cells were added to new 75 cm^2 culture flasks. The confluency and morphology of BREC were examined every day under an inverted microscope. BREC were subcultured at a ratio of 1 to 3 in the conditions described above.

1.2. FREEZING CELLS

Some BREC were stored frozen as stocks in liquid nitrogen using DMEM medium containing 10% (v/v) DMSO. Some stocks were also frozen at -80 °C for short periods of time. Cells were harvested following the same protocol used for routine subculture.The cell pellet was resuspended in DMEM medium and 900µl were aliquot into each sterile vial containing 100µl of sterile DMSO. The cells were frozen at -80 °C prior to transfer to liquid nitrogen.

1.3. DEFROSTING CELLS

The BREC were frozen in 1 ml vials that were stored at -80° C or in liquid nitrogen. The vial content was thawed, as fast as possible, in 37°C water bath and transferred to 5 ml of prewarmed regular BREC culture medium. After centrifugation at 1,000 x g for 5 minutes, the supernatant was removed and the cell pellet was resuspended in regular BREC culture medium and the cells were allowed to grow in 75 cm2 flasksat 37°C in a humidified incubator gassed with 5% carbon dioxide (CO_2) and 95% air.

1.4. TRYPAN BLUE ASSAY

The trypan blue assay was used for viability cell counting before plating for subculturing or for treatments. Trypan blue is an organic dye that is excluded by living cells with intact plasma membranes, whereas dead or dying cells with the plasma membrane compromised take up that dye. The viable cells appear brilliant under the microscope whereas the dying cells appear with a blue color, since the compromised membrane allow dye uptake. To check cell viability before plating, cells were trypsinized and resuspended in regular BREC medium. Then 20 µl of cell suspension and 20µl of trypan blue stain were mixed, and the cells counted using a haemocytometer. The percentage of viable cells and the cell density (number of cells per unit of volume) were calculated.

Calculations

The concentration of viable cells/ml was calculated considering the average of viable cells for each counted square (VCs), and their volume, plus the volume used in ressuspension:

$$\text{Concentration of Cell/ml} = \frac{\text{VCs x } dilution\ factor}{1\text{x}10^{-4}}$$

2. IMMUNOCYTOCHEMISTRY AND CONFOCAL MICROSCOPY

The evaluation of cellular distribution of the proteins in BREC was performed by immunocytochemistry. BREC culture were plated on fibronectin-coated cover slips. Twenty-four hours after plating, the medium was removed and the cells were washed with PBS and fixed in 4% (m/v) paraformaldehyde (PFA) for 10 minutes. Cells were then permeabilized for 10 minutes in 1% (v/v) Triton X-100 in PBS and blocked with 10 % normal goat serum for 20 min Primary antibodies (**Table 2.1**) were diluted in PBS containing 0.02% (m/v) of BSA and (PBS/BSA). The primary antibodies were then added, and the cells were incubated for 1 hour in a humid chamber at room temperature.

After incubation, the cells were extensively washed with PBS/BSA (3 washes x 5 min). Specimens were subsequently incubated with secondary antibodies produced in goat against mouse and rabbit immunoglobulins (IgG), conjugated with Alexa Fluor 568 (1:400)or Alexa Fluor 488 (1:200) fluorochromes (Molecular Probes Inc. OR, USA) and stained with 4',6-diamidino-2-phenylindole (DAPI) for 1 hour at room temperature. The coverslips were washed before mounting with Glycergel Dako mounting medium (Dako, Carpinteria, CA, USA). Negative control conditions were processed and prepared in the same manner described above with the exception of the primary antibody incubation that were omitted. Cells were visualized and images acquired on a confocal microscope (LSM 710, Carl Zeiss, Gottingen, Germany).

MARKERS	DILUTION	SUPPLIER
Monoclonal Anti-**Vimentin** antibody produced in mouse	1:400	Sigma-Aldrich, St. Louis, MO, USA
Polyclonal Rabbit Anti-Human ***Von Willebrand* Factor**	1:400	Dako, Carpinteria, CA, USA
Monoclonal Anti-**CD11b** Antibody produced in mouse	1:100	Serotec (Bio-Rad Laboratories, Inc,)
Monoclonal *Anti-Glial Fibrillary Acidic Protein* (**GFAP**) antibody produced in mouse	1:500	Sigma-Aldrich, St. Louis, MO, USA
DAPI	1:500	Life Technologies Corporation Carlsbad, CA, USA

Table 2.1 – Primary antibodies used for the immunocytochemistry

3. BREC TREATMENT

On the day of the treatment, regular BREC medium was replaced by DMEM medium (without ECGF and heparin) containing 5% FBS for 3 h. Then, BREC were non treated (only incubated in the medium of the treatments) or treated for 6 hwith: (a) 5 ng/ml or 10 ng/ml TNF (R&D Systems, Minneapolis, MN, USA); (b) 100 nM or 100 µM DPP-4 inhibitorSitagliptin (Sigma-Aldrich, St. Louis, MO, USA) (c) TNF plus sitagliptin in the concentrations above mentioned.

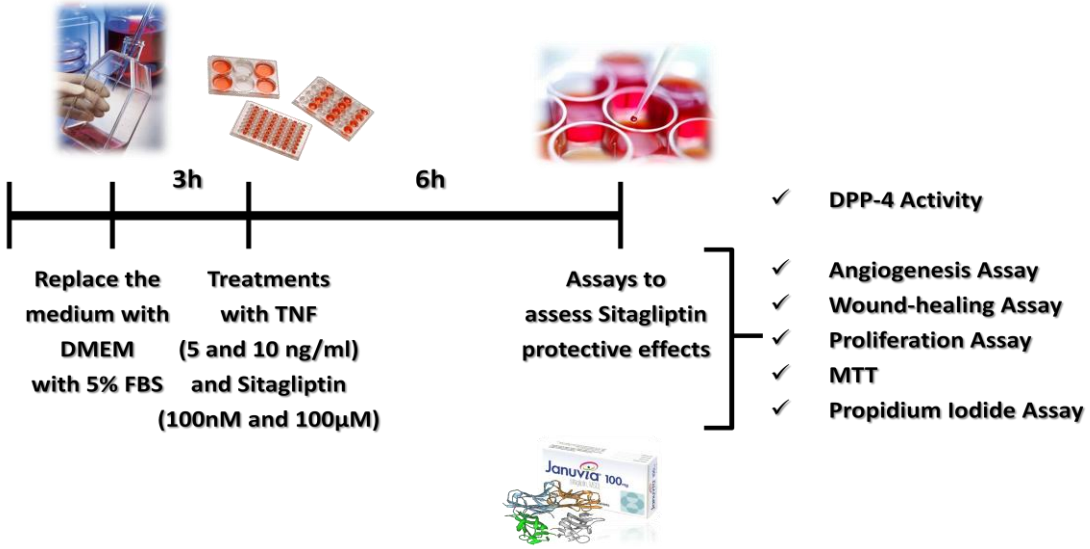


Figure 2.2 - Timeline containing sequential events to be performed in this work

4. DPP-IV ENZYMATIC ASSAY

To measure the activity of DPP-IV, a fluorometric assay was employed, using H-Gly-Pro-AMC.HBr (BACHEM, Bubendorf, Switzerland). Gly-Pro-AMC is cleaved by DPP-IV to release the fluorescent aminomethylcoumarin (AMC).

The reaction was initiated by the addition of the fluorogenic substrate to a final concentration of 50 µmol/l. The final reaction volume for each well was 150 µl. Liberation of AMC was monitored, using an excitation wavelength of 360 nm and an emission wavelength of 460 nm (microplate reader Synergy HT, BioTek, Winooski, VT, USA), every 5 min for a total of 60 min.

For comparison of DPP-IV activity between samples, data was plotted as Relative Fluorescence Units versus time for each sample. The time range over which the reaction was linear was determined. A trend line for these data points was obtained and the slopes determined.

5. ANGIOGENESIS ASSAY

In order to assess the capability of BREC to form tube-like structures the Angiogenesis Assay Kit (EMD Millipore Corporation, Billerica, MA, USA) was used according to the manufacturer's protocol. Briefly, ECMatrix gel solution was thawed at 4°C overnight, mixed with ECMatrix diluent buffer, and placed in a 96-well microplate at 37°C for 1 hour to allow the matrix solution to solidify. BRECs were trypsinized and then seeded onto the surface of the polymerized matrigel (3×10^4 cells/well). After that, BREC were treated with TNF (5 and 10ng/ml), sitagliptin (100nM and 100µM) or both. After 6 h, BREC were visualized and photographed under a phase-contrast microscope (Leica Microsystems (Model DFC350)).

6. WOUND-HEALING ASSAY

Nearly confluent cell monolayers were scraped using a sterile 200 µL micropipette tip. The medium and dislodged cells were aspirated, and plates were replenished with DMEM medium with 5% FBS for 3 hours. Treatment of BREC was performed as before described.

Images on light microscope (100x magnification; Leica Microsystems, Model DFC350) were acquired before treatment (t0) and after treatment (t6)Cells present in the denuded area represent the migration ability of the cells. Results were reported as the percentage of wound healing using the equation:

% Wound Healing = [1 − (wound length at t6 / mean wound length at t0)] × 100, where t0 is the time immediately following wounding.

7. BrdU PROLIFERATION ASSAY

BREC proliferation was measured using **Roche Cell Proliferation ELISA Kit** (Roche Diagnostics Corp., Indianapolis, IN, USA), which measures the BrdU incorporation during DNA synthesis as the cells replicate. Replacement of the traditionally used [^{3}H]-thymidine with 5-bromo-2'-deoxyuridine (BrdU) as the pyrimidine analog for DNA incorporation in dividing cells allows the incorporated BrdU to be detected by colorimetric immunoassay.

Briefly for this colorimetric assay BREC cells were plated at a density of 2x10^4 cells per well (150 µl per well), in 96-wells culture plates 24h before treatment. Next, after replacing the cells in DMEM medium with 5% FBS for 3 hours, theywere treated with TNF (5 and 10ng/ml), Sitagliptin (100nM and 100µM) or both. Cells were incubated with BrdU labeling solution during the last 4 hours of treatment. The supernatants were discarded and the cells were then fixed in 200 µL/well of FixDenat solution for 30 min. The supernatant was again thoroughly removed and anti-BrdU-POD solution (100 µL/well of 1:100 dilution) was added and incubated for 90 min at room temperature. The antibody was removed and the cells were washed in PBS and the substrate solution (TMB) was then added and incubated for 15 minutes. The absorbance of the developed color was measured at 370 nm (with reference measured at 492 nm) (microplate reader Synergy HT, BioTek, Winooski, VT, USA). The data of BrdU incorporation are presented as % of control.

8. MTT METABOLIC ACTIVITY ASSAY

MTT acronym for 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide is a yellow-colored substance added directly to the medium in the wells, which permits the evaluation of cell viability. Thus, in living cells MTT becomes reduced into an insoluble formazan – giving rise to a bluish purple color. BREC cells were plated at a density of 3x10^4 cells per well (150 µl per well), in 96-wells culture plates 24h before treatment. Next, after replacing the cells in DMEM medium with 5% FBS for 3 hours, they were treated with TNF (5 and 10ng/ml), sitagliptin (100nM and 100µM) or both.

After treatment, the medium was discarded and the cells were washed with KREBS solution. Then KREBS (50µl) with MTT at the final concentration of 0.5 $mg.ml^{-1}$ was added.

Then the plates were incubated for 4 hours at 37°C in the dark. The resulting purplish color crystals were later dissolved by adding 100 µl of acidic isopropanol (0.04 M HCl in absolute isopropanol). To solubilize completely this heterogeneous solution, repetitive pipetting was required as well as room temperature (RT) plate stirring on an automatic shaker in the dark. The positive control condition was performed by incubating the cellswith 1mM H_2O_2 for 1 hour.

The absorbance was measured at 570 nm (using 620 nm as the background wavelength), using a plate reader spectrophotometer. The percentage of absorbance for each treated sample was normalized to that of the control.

9. PROPIDIUM IODIDE (PI) VIABILITY ASSAY

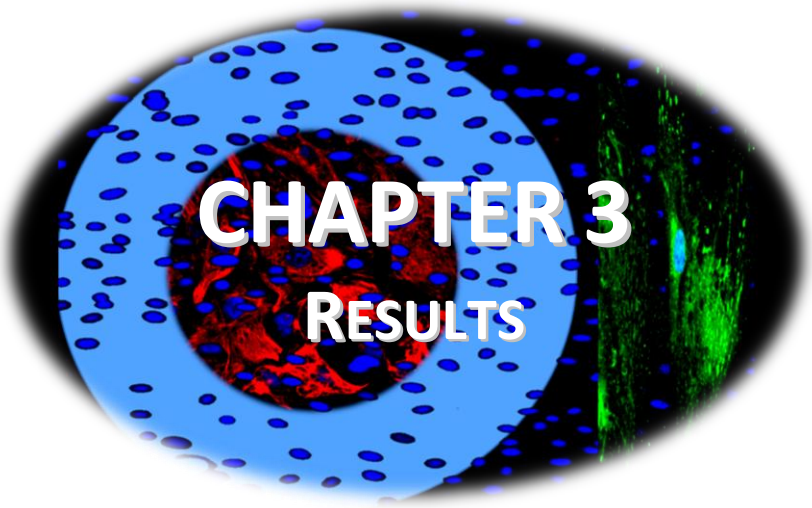
For this colorimetric assay,BREC were plated on fibronectin-coated glass coverslips 24h before treatment. Subsequently cells were placed in DMEM medium with 5% FBS for 3 hours and then treated as previously described. After treatment, cells were stained with 5µM of PI (Sigma-Aldrich Co) for 10 minutes. After several washes in PBS, cells were fixed in 4% PFA and the nuclei staining was performed by incubation with DAPI ($1\mu g.ml^{-1}$) for 10 min. The coverslips were washed before mounting with Glycergel Dako mounting medium (Dako, Carpinteria, CA, USA)The positive control condition was performed by incubating the cellswith 5mM H_2O_2 for 10 minutes. Images of the PI and DAPI staining were captured using the fluorescence microscope (Leica Microsystems, Model DFC350).

10. SOFTWARE

Image J 1.42n (Wayne Rasband, National Institute of Health, USA) was used for the quantification of the migration percentage from the control in the Angiogenesis assay.

11. STATISTIC ANALYSIS

BREC were isolated as previously described (Fernandes *et al*., 2004). Briefly, BREC were isolated from retinal capillaries of fresh bovine eyes. Under sterile conditions, the retinas were separated from other ocular tissues, washed in Dulbecco's Modified Eagle Medium (DMEM) (Introgen, Carlbad, CA) and fragments of the contaminating retinal pigment epithelium were then removed.



CHAPTER 3
RESULTS

1. *CHARACTERIZATION OF PRIMARY CULTURES OF BREC*

Retinal endothelial cells obtained from bovines were characterized. These cells have a spindle-fiber shape morphology characteristic of an endothelial cell origin and the typical phase microscopic image of BREC is shown in **Figure 3.1A**. In order to determine the purity of endothelial cell culture, the expression of vWf, GFAP, CD11b, vimentin proteins in BREC were analysed by immunocytochemistry. Strong staining was observerd following incubation with anti-vimentin and anti-vWf antibodies, indicating vWf (endothelial marker) and vimentin (cytoskeletal marker) expression in BREC (**Figure 3.1 B** and **C**) while is not the case for GFAP (astrocyte marker) and CD11b (microglia marker) (**Figure 3.1 D** and **E**). Using DAPI (nuclear dye) staining, we identified more than 95% of cells expressing vWf. This finding shows that primary cultures used in this study are almost of endothelial origin.

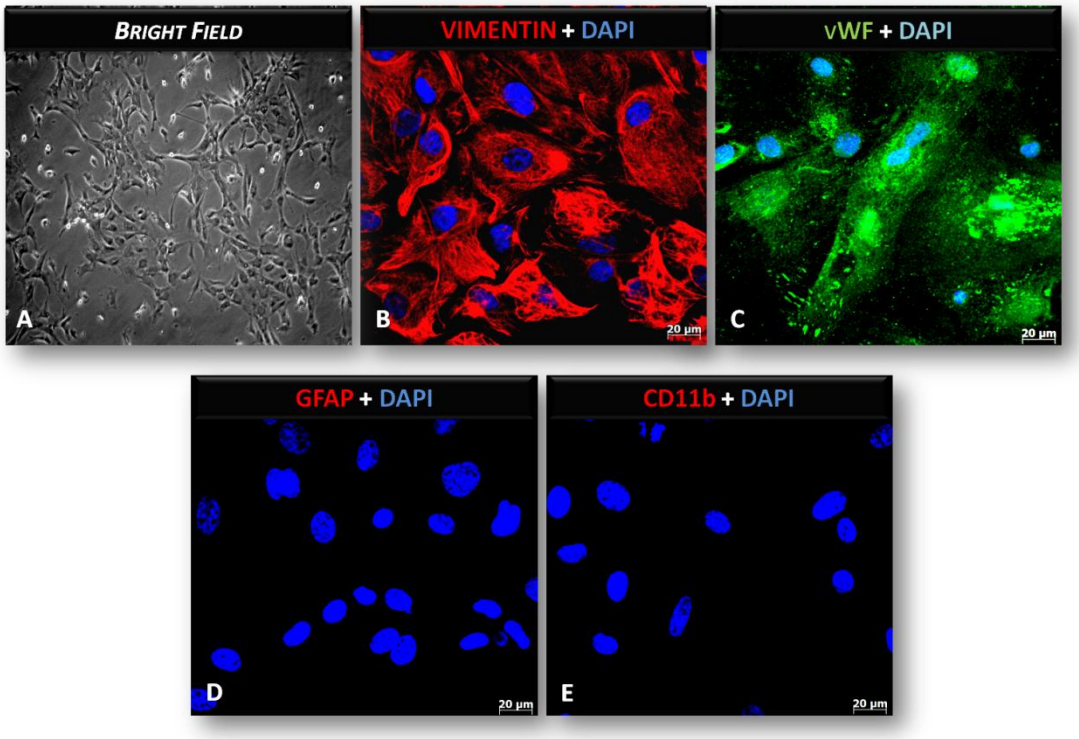


Figure 3.1 - Characterization of bovine retinal endothelial cells (BREC). (A) Phase microscopic image of BREC; (B, C, D and E) Cells were fixed and stained with antibodies directed against respectively vimentin (red), vWf (green), GFAP (red) and CD11b (red), nuclei was counterstained with DAPI (blue) and imaged by confocal microscopy Magnification 400x.

2. *SITAGLIPTIN DECREASES THE ACTIVITY OF DPP-IV IN BREC FOLLOWING EXPOSURE TO TNF*

To investigate the effect of Sitagliptin on DPP-IV its activity was evaluated in BREC previously exposed to TNF in the absence or presence of Sitagliptin. For this, a fluorescence enzymatic activity assay was performed, in which DPP-IV cleaves a substrate Gly-Pro-AMC that releases a fluoroforous – AMC. Thus, an increase in AMC fluorescence is proportional to DPP-IV activity. As a first approach, we treated BREC with increasing concentrations of Sitagliptin for 6 hours and then we measured the activity of DDP-IV. We observed an inhibition of DPP-IV activity in a concentration-dependent manner (data not shown).

We have chosen for this study two concentrations of Sitagliptin, 100 nM and 100 µM, which induce a significant decrease in DPP-IV activity, approximately about 70% (31.57% ± 1.12 % of control; $P<0.001$) and close to 90% (10.51 ± 0.82 % of control; $P<0.001$), respectively when compared to control (**Figure 3.2**). Interestingly enough, in BREC treatment alone with 5 ng/ml TNF and 10 ng/ml TNF also decreased, although less, DPP-IV activity roughly to 48% (51.91 ± 4.20 % of control; $P<0.001$) and to almost 62% (39.09 ± 4.92 % of control; $P<0.001$), respectively when compared to control (Figure 3.2). What seems to suggest some sort a relation between DPP-IV activity and TNF concentration.

Furthermore, 5 ng/ml TNF combined with each concentration of Sitagliptin also decreased DPP-IV activity, hence 100 nM Sitagliptin (29.01 ± 1.68 % of control) and 100 µM (12.97 ± 0.87 % of control) when compared to 5 ng/ml TNF addition alone. In contrast, 10 ng/ml TNF added in concomitance with 100 µM Sitagliptin reduced DPP-IV activity (11.64 ± 0.66) to a larger extent than lower concentrations of Sitagliptin (100 nM) (28.42 ± 1.81) when compared to 10 ng/ml TNF treatment alone

Finally Sitagliptin seems to decrease DPP-IV activity, when in the presence of TNF, to levels similar to those registered for the respective inhibition of DPP-IV induced by each concentration of Sitagliptin alone.

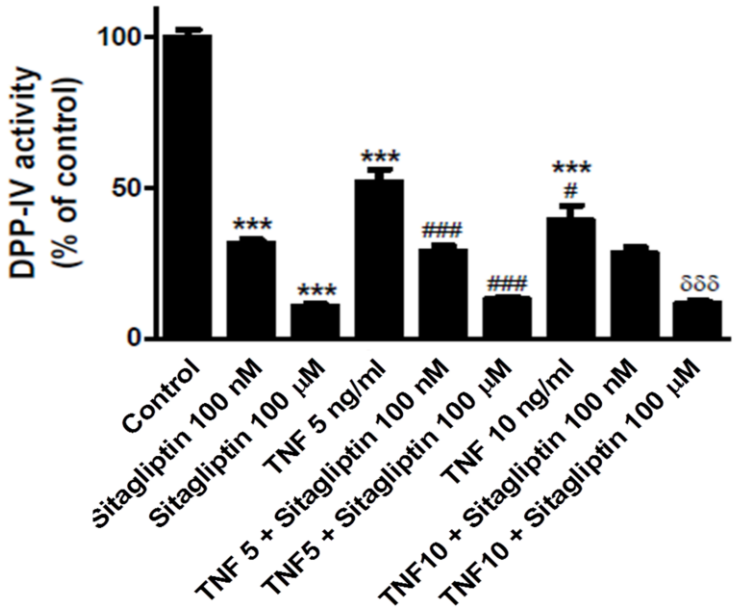


Figure 3.2 - Sitagliptin decreases the activity in BREC treated with TNF. BREC were incubated with TNF (5 or 10 ng/ml) in the absence or presence of sitagliptin (100 nM or 100 µM) for 6h. DPP-IV activity was determined using the fluorogenic substrate Gly-Pro-AMC. Data are presented as percentage of control and represent the mean ± SEM of triplicates from four independent experiments. ***P<0.001 vs. control; #P<0.05, ###P<0.001 vs. TNF 5 ng/ml; δδδP<0.001 vs. TNF 10 ng/ml; ANOVA followed by Bonferroni's *post hoc* test.

3. *EFFECT OF SITAGLIPTIN IN ENDOTHELIAL FUNCTION FOLLOWING INFLAMMATION*

Recent data from our laboratory has shown that sitagliptin prevented the increase in blood-retinal barrier permeability induced by diabetes and exerted protective effects against inflammation and pro-apoptotic state in the retina of diabetic rats, by a mechanism independent of glycemia normalization (Gonçalves *et al.,* 2012). In this work, to evaluate the potential role of DPP-IV activity in endothelial cells response to inflammation, neovasculatization, endothelial cell proliferation, migration and survival, in inflammation conditions were evaluated by using the DPP-IV inhibitor, Sitagliptin.

To investigate whether inhibition of DPP-IV protects endothelial function following an inflammatory stimulus, endothelial cells ability to form tube-like structures was evaluated using an angiogenesis assay.

The results analyzed suggest that in control condition the formed tube-like structures seem quite organized. After 6 hours of treatment with 5 or 10 ng/ml TNF, a loss of endothelial cells organization in tubular structures was observed (**Figure 3.3**). Treatment with 100 nM sitagliptin in combination with 5 or 10 ng/ml TNF was able to prevent this effect (**Figure 3.3**). Similarly, treatment with 100 µM sitagliptin, was also able to prevent the tube-like structures disorganization, but on the other hand treatment with Sitagliptin 100 µM and TNF 10 ng/ml seems not to exert the same effects under inflammatory conditions, since the structures are more brittle and less arranged (**Figure 3.3**).

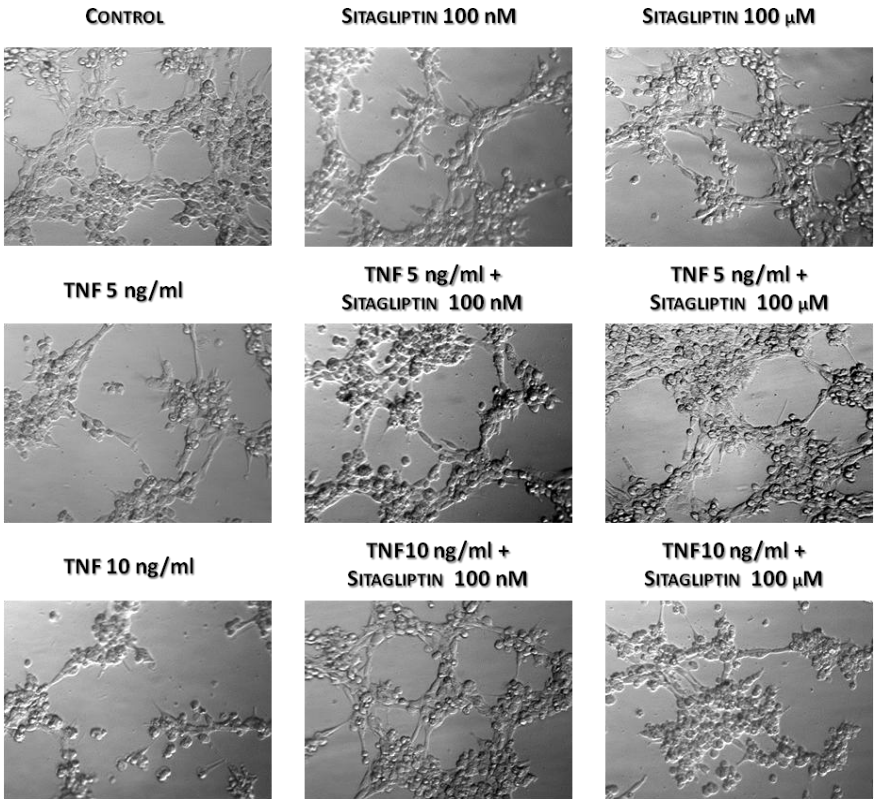


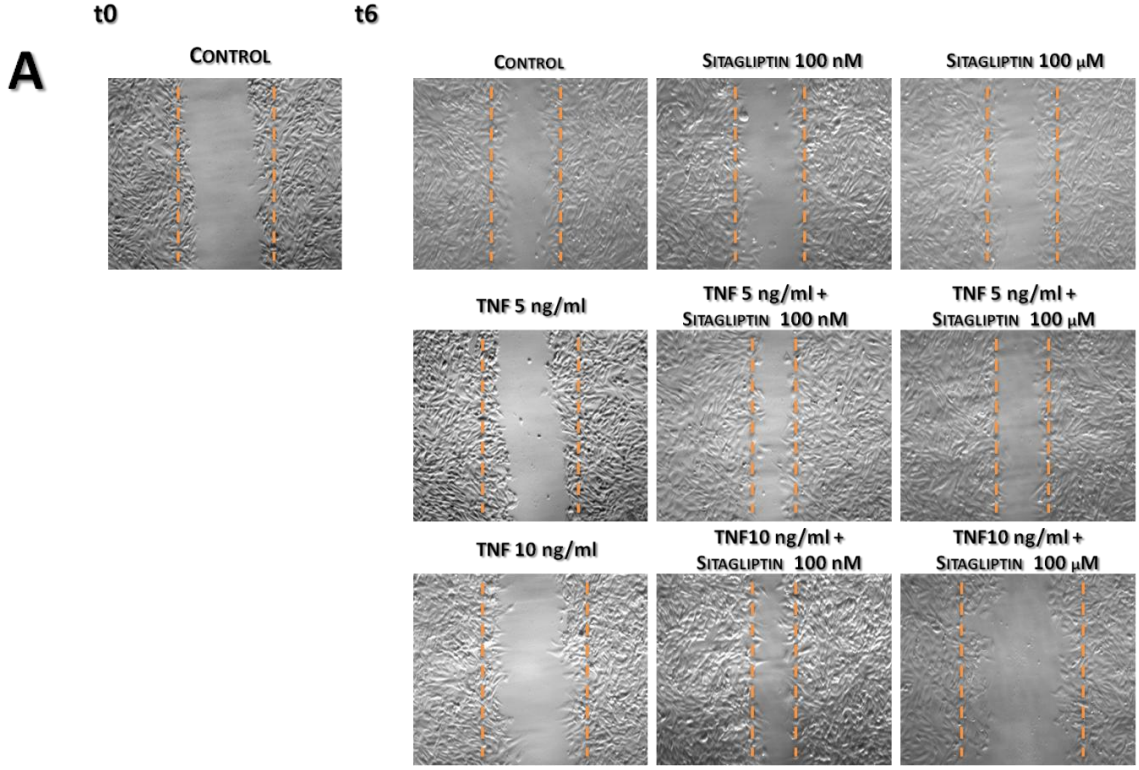
Figure 3.3 – Effect of DDP-4 inhibition on angiogenesis upon TNF treatment. BREC were incubated with TNF (5 or 10 ng/ml) in the absence or presence of sitagliptin (100 nM or 100 µM) for 6h. Representative images, from each condition, showing endothelial cells grown on matrigel matrix forming capillary network structures. Duplicates from one independent experiment. Magnification 100x.

Since our data above suggest that inhibition of DPP-IV may positively regulate endothelial cell function under inflammation, we next evaluated migration of endothelial cells, which is an important component of the cascade of events of the angiogenesis process. To evaluate endothelial cell migration, we performed a scratch-wound assay, by evaluating migration of endothelial cells into the denuded area.

By analyzing both images (**Figure 3.4 A** and **B**) we can observe that 5 ng/ml TNF can decrease BREC migration when compared to control from (32.74 ± 1.42 % from t0) to (23.31 ± 1.54 % from t0; P<0.05). More so the highest concentration used of TNF (10 ng/ml) seems to impair to a larger extent and more significantly BREC migration from (32.74 ± 1.42 % from t0) to (19.10 ± 1.28 % from t0; P<0.001) when compared to control.

Furthermore this impairment induced by both concentrations of TNF seems to be alleviated in the presence of 100 nM Sitagliptin, from (23.31 ± 1.54 % from t0; P<0.05) to (40.51 ± 1.62 % from t0; P<0.001) when compared to 5 ng/ml TNF alone and from (19.10 ± 1.28 % from t0; P<0.001) to (37.45 ± 1.45 % from t0; P<0.001) when compared to 10 ng/ml TNF alone (**Figure 3.4 A** and **B**).

In contrast the highest concentration of Sitagliptin used (100 µM) does not seem to protect BREC from 10 ng/ml TNF-induced impairment, but in co-treatment with 5 ng/ml TNF a increase is registered from (23.31 ± 1.54 % from t0; P<0.05) to (32.88 ± 1.62 % from t0; P<0.05) when compared to 5 ng/ml TNF alone (**Figure 3.4 A** and **B**).



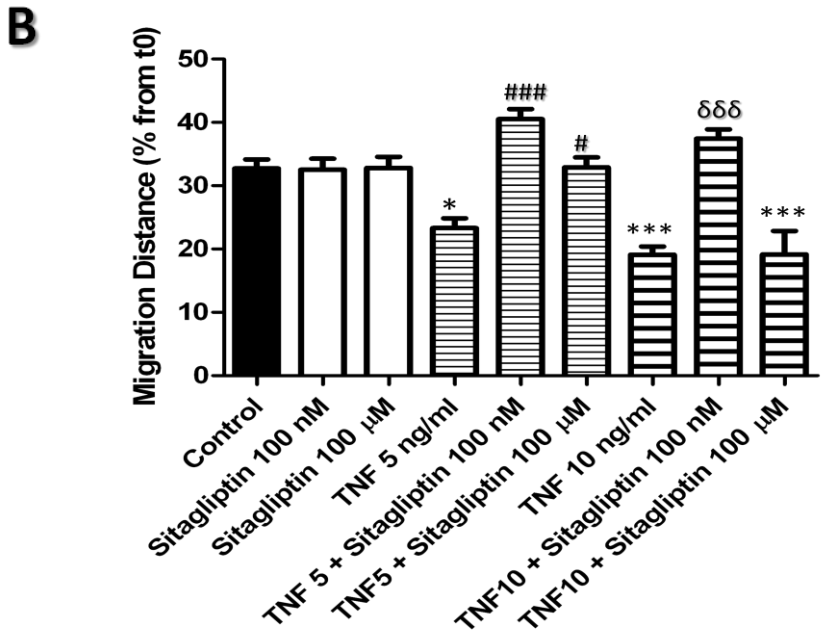


Figure 3.4 - Effect of sitagliptin on BREC migration upon TNF exposure. BREC were incubated with TNF (5 or 10 ng/ml) in the absence or presence of Sitagliptin (100 nM or 100 µM) for 6h. (A) Representative images, from each condition, showing endothelial cells migration compared to control and t0 hours. Duplicates from one independent experiment. Magnification 100x. (B) Data are presented as the percentage of migrated distance and represent the mean ± SEM of duplicates from one independent experiment. *p<0.05, ***p<0.001 vs. control; #p<0.05, ###p<0.001 vs. TNF 5 ng/ml; δδδp<0.001 vs. TNF 10 ng/ml; ANOVA followed by *Bonferroni's post hoc* test.

After performing the wound-healing evaluation, a BrdU cell proliferation ELISA (colorimetric) KIT was used so that a correlation between both analyses could be established.

The figure below shows that Sitagliptin does not seem to have any effect in BREC proliferation when compared to control. In contrast it appears that there is a tendency for increased BREC proliferation when they are treated with 100 nM Sitagliptin and both TNF concentrations (either 5 ng/ml or 10 ng/ml) when compared to control. Yet no alteration in proliferation induced by 100 µM Sitagliptin seem to exist when 5 ng/ml TNF and 10 ng/ml TNF are present, when compared to each concentration of TNF alone (**Figure 3.5**).

Nevertheless no statistical analysis was performed for this assay since it was made only one independent experiment.

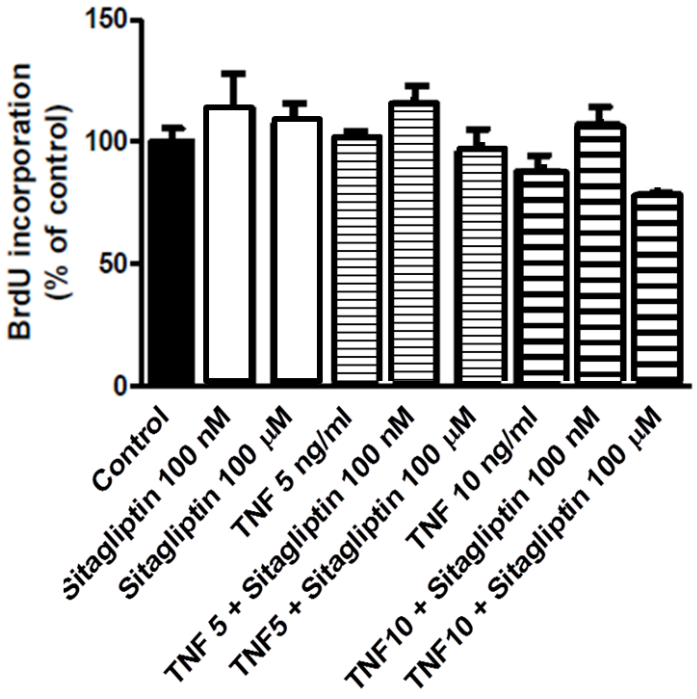


Figure 3.5 - Effect of sitagliptin on BREC proliferation upon exposure to TNF. BREC were incubated with TNF (5 or 10 ng/ml) in the absence or presence of sitagliptin (100 nM or 100 µM) for 6h. Cell proliferation was assessed by BrdU incorporation. Data are presented as percentage of control and represent the mean ± SEM of triplicates from one independent experiment.

After confirming that Sitagliptin exerts protective effects in BREC migration, proliferation and formation in tube-like structures, a cell viability assay was performed to support these results.

Primary cultures of BREC were treated during a 6 hour period with two different concentrations of Sitagliptin (100nM or 100µM) and TNF (5 or 10 ng/ml). After this period the cell viability was evaluated by MTT colorimetric assay, in which this the yellow-colored MTT is reduced, by living cells mitochondrial dehydrogenases, to a sort of purplish-colored formazan precipitate. The absorption of dissolved formazan can be then correlated with living cells number, hence, being expressed by the percentage of cell viability in BREC cells. No significant cytotoxicity was registered when BREC were treated with TNF in the absence or presence of Sitagliptin (**Figure 3.6**).However, in the positive control condition, where the cells were exposed to hydrogen peroxide (H_2O_2) for 1 hour, an increased cytotoxicity was observed (45.43 ± 6.54 % of control; $P < 0.001$) when compared to control (**Figure 3.6**).

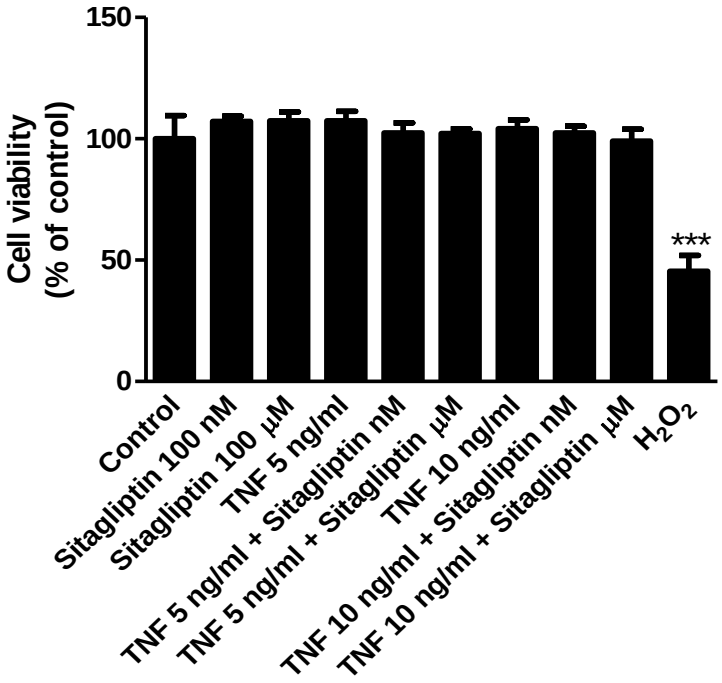


Figure 3.6 – Effecct of sitagliptin on BREC viability after incubation with TNF. BREC were incubated with TNF (5 and 10 ng/ml) and sitagliptin 100 nM or 100 µM for 6h. Cell viability was assessed by MTT assay. Data are presented as percentage of control and represent the mean ± SEM of triplicates from two independent experiments. ***p<0.001

Theoretically, in the MTT assay, formazan dye color intensity is correlated with the number of viable cells. However, this assay is dependent of cellular metabolism to reduce formazan, so in order to confirm the obtained results, additional studies using the propidium iodide (PI) were performed. Since PI is excluded from live cells intact membrane and it is retained in dead cells, it can serve as a complementary assay to MTT.

Furthermore, PI was added at the end of the co-treatments with two different concentrations of Sitagliptin (100nM e 100µM) and TNF (5 and 10 ng/ml). Qualitative evaluation of the images was performed by Fluorescence Microscopy.

The representative images below show no cytotoxicity induced either by Sitagliptin and/or TNF. In addition, a positive control was performed, where the cells were exposed to 5mM hydrogen peroxide (H_2O_2) for ten minutes and increased cytotoxicity was observed due to total positive staining for PI. (**Figure 3.7**).

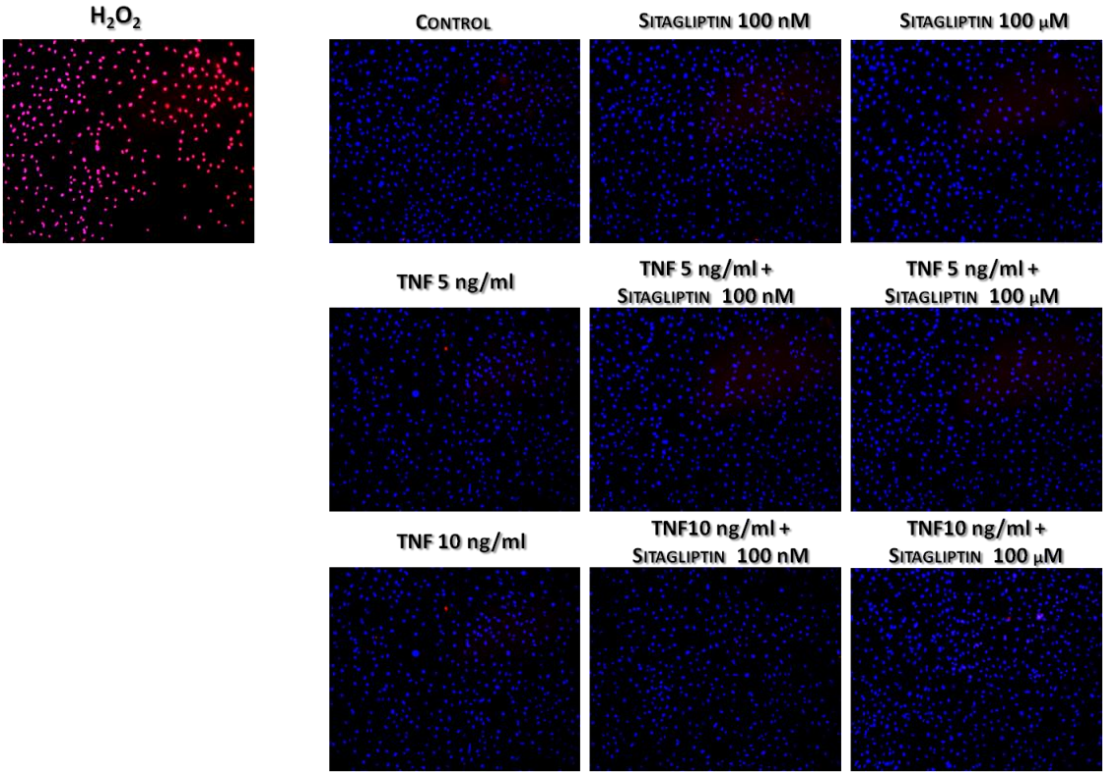
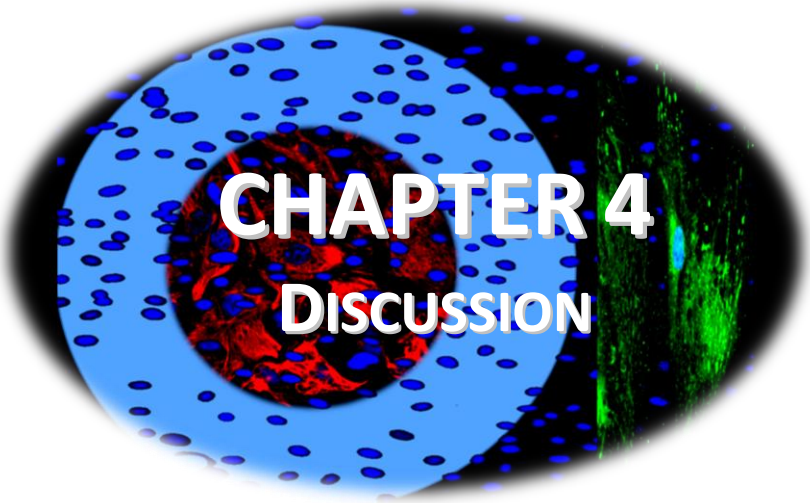


Figure 3.7 - BREC were incubated with TNF (5 or 10 ng/ml) and/or sitagliptin (100 nM or 100 µM) for 6h. Representative images, from each condition, showing cell stained with PI. Duplicates from one independent experiment. Magnification 100x



CHAPTER 4
DISCUSSION

Diabetic Retinopathy (DR) is one of the most common microvascular complications in diabetic patients and it is the major cause of blindness in the working-age population of most developed countries. It is now widely accepted that the complications of diabetes, including DR, are related to elevation of the plasma glucose concentration (*American Diabetes Association*, Implications of the diabetes control and complications trial 2003; 23: S26-S27).It is also known that lowering blood glucose concentration significantly decreases the risk of DR development (Kowluru & Chan 2007). Therefore, there is an urgent need to implement novel and more effective therapeutic strategies.

Several reports have shown that a low-grade inflammation is implicated in the pathogenesis of DR (Kowluru *et al.*, 2004). Elevated levels of proinflammatory cytokines have also been detected both in the vitreous of diabetic patients with DR (Kowluru *et al.*, 2004) and in diabetic retinas (Lutty *et al.,* 1997; Joussen *et al.*, 2001). In fact, DR shares numerous characteristics of chronic inflammatory disease, such as, increased vascular permeability, edema, infiltration of inflammatory cells, presence of cytokines, tissue damage and neovascularization (Antonetti *et al.*, 2006; Kowluru *et al.*, 2004).

Recently, a new class of antihyperglycemic agents, in which is included Sitagliptin, have been used for the treatment of Type 2 Diabetes (T2D). Sitagliptin has been shown to improve glycemic control by enhancing the incretin levels of active incretin hormones, like GLP-1, and consequently increasing the insulin secretion levels in humans with T2D (Drucker *et al.*, 2006).

Moreover, it has also been shown that this DPP-IV inhibitor increases circulating progenitor endothelial cells in diabetic patients with T2D, which may have an important role in the repair of the damaged vessels (Fadini *et al.,* 2010). Recently, our group has shown that administration of Sitagliptin to diabetic animals improved microvascular permeability and ameliorated the detrimental changes associated with retinal dysfunction. In fact, sitaglitin administration to T1DM and T2DM animal models reduced tissue inflammation, improving the functionality of the inner blood-retinal barrier. Nevertheless, the putative role of Sitagliptin in protecting retinal endothelial

cells under inflammatory stress conditions remains to clarify. In this study, we used primary cultures of bovine retinal endothelial cells to evaluate whether inhibition of DPP-4 by Sitagliptin regulates retinal endothelial cell response to inflammation induced by TNF.

In both type 2 and type 1 diabetic patients and in STZ-induced diabetes in Wistar rats (after 1 month of diabetes induction), DPP-IV activity in serum was shown to be elevated (Bose *et al.,* 2005; Gaspari *et al.,* 2011). Sitagliptin treatment for two weeks was able to inhibit by 70% the activity of DPP-IV in the serum of diabetic animals, compared to untreated animals (A. Gonçalves, E. Leal, C. F. Ribeiro, F. Reis, A. F. Ambrósio and **R. Fernandes**. Sitagliptin prevents blood-retinal barrier breakdown, inflammation and neuronal cell death in the retina of type 1 diabetic rats (submitted for publication)). Other authors observed that DPP-IV activity in the plasma is increased 1 week after STZ injection and Sitagliptin treatment for a month led to a strong inhibition of DPP-IV activity and significantly increased levels of active plasma GLP-1 levels (Zhang *et al.,* 2011).

In a preliminary set of experiments we tested several concentrations of Sitagliptin, in order to choose one that inhibits by about 70% the activity of DPP-IV (in a similar way that was previously observed in diabetic animals) and another with the DPP-IV activity almost totally inhibited (90% of inhibition), corresponding to 100 nM and 100 µM Sitagliptin, respectively. When BREC were treated with 5 and 10 ng/ml TNF, we found an inhibition of about 50 and 60% in the activity of DPP-IV. The decreased DPP-IV activity could be related to a reduction in DPP-IV levels. In agreement with that, a recent study has recently described that proinflammatory cytokines such as TNF and IL-1b decrease the levels of DPP-IV protein and mRNA in microvascular cells (Takasawa *et al.,* 2010). And, when BREC were co-treated with TNF and Sitagliptin, DPP-IV activity presented similar values to BREC treated only with Sitagliptin. However, further studies evaluating the protein levels of DPP-IV would be necessary to clarify whether unchanged activity is related or not to changes in the content of the DPP-IV protein.

Previous reports in the literature have shown that exogenous recombinant DPP-IV strongly regulated the migratory ability of T cells through (Endothelial Cells) EC (Ikushima *et al.*, 2002). Lamalice *et al*., also claimed that endothelial cell migration is an essential component of angiogenesis that requires a tight regulation between proangiogenic and antiangiogenic factors. We found that TNF-induced inflammation impaired angiogenesis and that this was impeded in the presence of 100 nM Sitagliptin. This is in agreement with studies from Takasawa et al., which have shown that inhibition of CD26/DPP-IV enhances endothelial growth *in vivo* and *in vitro*, suggesting that inhibition of DPP-IV is directly associated with EC proliferation, migration and neovascularization in inflammatory settings (Takasawa *et al*., 2010).

Moreover, the target vessels for angiogenic factors are the postcapillary venules and small terminal venules. These small-caliber vessels which consist of flattened endothelial cells that lie upon a basal lamina, are surrounded by an interrupted layer of pericytes and smooth muscle cells, and are invested in an extracellular matrix (Bar and Wolf, 1972; Ausprunk *et al*., 1974, Ausprunk and Folkman, 1977; Sims *et al*., 1986; Philips *et al.*, 1991). So, one of the first steps in the angiogenic response involves therefore the disruption of focal contacts between adjacent endothelial cells, pericytes, and smooth muscle cells (Bar and Wolf, 1972; Ausprunk *et al.*, 1974, Ausprunk and Folkman, 1977; Sims *et al.*, 1986; Philips *et al.*, 1991). Suggesting that in our studies the brittleness in the tube-like structures induced by TNF could be related to an initial angiogenic response. In addition, Saison *et al*., proved that when 10 ng/ml of TNF was added to the fibrin gel assay it dramatically blocked sprouting, (Saison *et al*., 2008). We have also seen that this concentration of TNF does not seem to induce the formation of tube-like structures, since these last are so shortened and flimsy, that are almost non-existing.

Saison *et al*., also demonstrated that delayed addition of TNF resulted in a quantitative reduction in the number of sprouts generated. Interestingly, however, when cultures were treated with a pulse of TNF for 2 days, followed by fresh medium, and the number of sprouts was counted 4 to 6 days later, there were more than twice as many sprouts by day 8 in TNF-pretreated than in control cultures never receiving

TNF. Thus, removal of TNF after 2 days led to robust sprouting that was more vigorous than in cultures that had never seen TNF. A pulse of TNF between days 2 and 4 also induced sprouting, however, a later pulse had no effect, likely because most of the sprouts had already been primed and initiated. This might suggest that our single TNF addition and the 6-hours- time-window could be too small, in order to observe TNF – proangiogenic effect or perhaps the used TNF concentrations are low given the co-treatment method employed. Nevertheless, more studies are needed to elucidate TNF angiogenic role in ECs tube-like structures formation and to confirm if Sitagliptin can in fact revert this impairment seen in BREC when exposed to an inflammatory stimulus.

Furthermore, recent studies also referred that proliferation and migration of endothelial cells may play a crucial role in vascular self-repair in normal physiological as well as pathological conditions. More so, these cells are able to maintain their monolayer integrity via proliferation and migration of neighboring cells (Xiao *et al*, 2013). In our studies we found that when BREC were treated with 5 and 10 ng/ml TNF in the presence of 100 nM Sitagliptin there was an increase in BREC proliferation (tendency) and migration, but when they were only exposed to TNF alone BREC migration significantly decreased. This might be in accordance with previous studies which showed that TNF blocks EC proliferation and migration *in vitro* (Mano-Hirano *et al.*, 1987, Sato *et al*., 1986).

However Park *et al*., demonstrated that Sitagliptin (50 to 200 µM) treatment inhibited PDGF-BB-induced cell proliferation in Rat Aortic Smooth Muscle Cells (RAoSMCs) and that the same treatment also inhibited TNF directed migration of RAoSMCs. Similar finding had been seen in the thymidine incorporation assay (proliferation). So it was verified that Sitagliptin treatment also inhibited TNF-directed migration of RAoSMCs in a dose-dependent manner from 50 to 200 µg/ml (Park et al, 2012). Our findings show however that 100 nM Sitagliptin treatment seem to increase BREC migration and proliferation even in the presence of TNF. This increase could be related to the time gap between this investigation (24 hours) and ours (6 hours) as well as the cell type and Sitagliptin concentrations used for these experiments, which were different. This later is in fact in agreement to this research (Park *et al*, 2012) since they

also showed that migration with Sitagliptin 100 µM and TNF 10 ng/ml was decreased what is to some extent in accordance with our own studies.

In regard to EC growth and proliferation, it appears that Sitagliptin effects depend on concentration as well as cell source. However further studies are required to clarify Sitagliptin protective action in BREC subjected to inflammatory stimulus.

Moreover, in pathological conditions, such as inflammatory ones cell viability which is an indicator of a cell status, hence its ability for self-repair and self-preserve, might be impaired, so in order to assess the veracity of the premise we tested the experimental settings used previously to verify their effects on BREC cells viability.

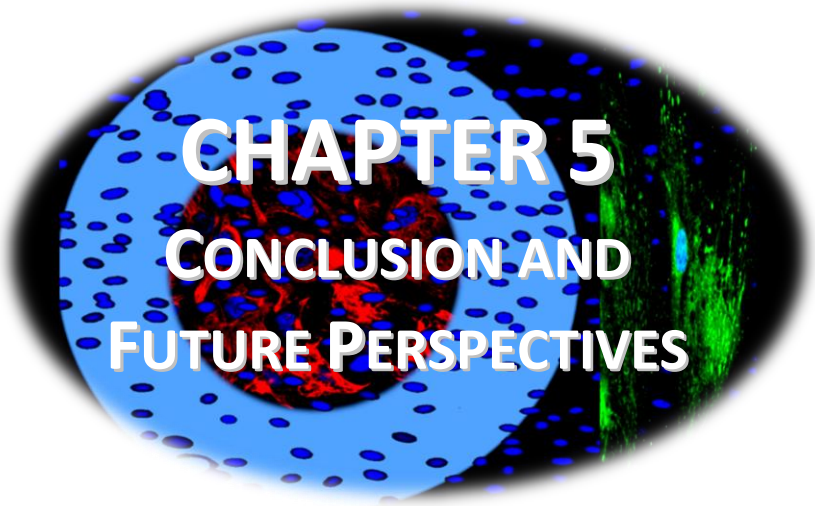
The results obtained either through MTT assay or by qualitative analysis of complementary assay – PI – reveal that the concentrations of Sitagliptin and TNF used are devoid of cytotoxicity.

Aveleira *et al*, has observed that TNF (5 ng/ml) induced a significant decrease of cell viability after 24 h treatment, but no effect was observed after 6 h treatment (Aveleira *et al*., 2009). Using lower concentrations of TNF Chen *et al*., also demonstrated that treatment of HAECs with 2 ng/ml of TNF for 6 h did not result in cytotoxicity (Chen *et al*., 2002). Accordingly our results were partially consistent with those studies. Moreover and for higher concentrations of TNF (10 ng/ml) was verified that TNF treatment alone for 24 h significantly decreased ($P < 0.01$) BAEC proliferation by comparison with controls (Xiao *et al*., 2013).

This might reinforce the fact that the chosen time-window of 6 hours could be too small in order to observe TNF-induced cytotoxic effects or that these effects seen so far are not due to an increase in cell death but just an impair in their function. All the same additional experiences are required to confirm that Sitagliptin exerts these cytoprotective effects in BREC when exposed to an inflammatory stimulus

In conclusion, another BREC isolation and further studies are mandatory to confirm if this effect seen in DPP-IV inhibition can be directly associated to an improve in EC proliferation and migration. If so, this could imply that DPP-IV inhibitors, like

Sitagliptin, which are widely used in the treatment of diabetes, may be of potential use in treating diabetic vascular complications.



CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVES

In this thesis we intended to evaluate the potential role of Sitagliptin in protecting the endothelial cells of the retina under inflammatory stress conditions. So in order to study the effect of Sitagliptin in the microvasculature of the retina, when an inflammatory state-TNF-induced is present, were used primary culture of Bovine Retinal Endothelial Cells (BREC). The fact that much is already known about the development and pathophysiology of the retinal microvasculature makes this capillary bed particularly well suited for *in vitro* studies (Bowman *et al*, 1982)

Therefore a series of assays were performed on these cells together with 6 hours in co-treatment with TNF and Sitagliptin. Hence endothelial cells ability to form tube-like structures, migrate and proliferate were analyzed and the results obtained suggest that Sitagliptin has protective and regenerative effects in these cells when exposed to an inflammatory-TNF-induced state. More so therapeutic benefits of Sitagliptin are consequently reinforced by these studies but TNF's endothelial cells impairment ability was not yet fully understood.

Nonetheless it is still important to refer that TNF role in angiogenesis continues to be highly controversial with numerous studies showing that it is either proangiogenic or antiangiogenic, with the preponderance of published studies suggesting that TNF blocks EC activation *in vitro*, but enhances angiogenesis *in vivo* (Patterson C *et al*., 1996, Frater-Schroder M *et al*., 1987, Mano-Hirano Y *et al*., 1987, Sato N *et al*, 1986, Leibovich SJ *et al*., 1987).

Therefore to confirm the scientific veracity of our findings it is imperative to do another BREC cell culture isolation in which all the assays performed throughout this thesis will be redone and revised, comparing afterwards these future results with the former ones.

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