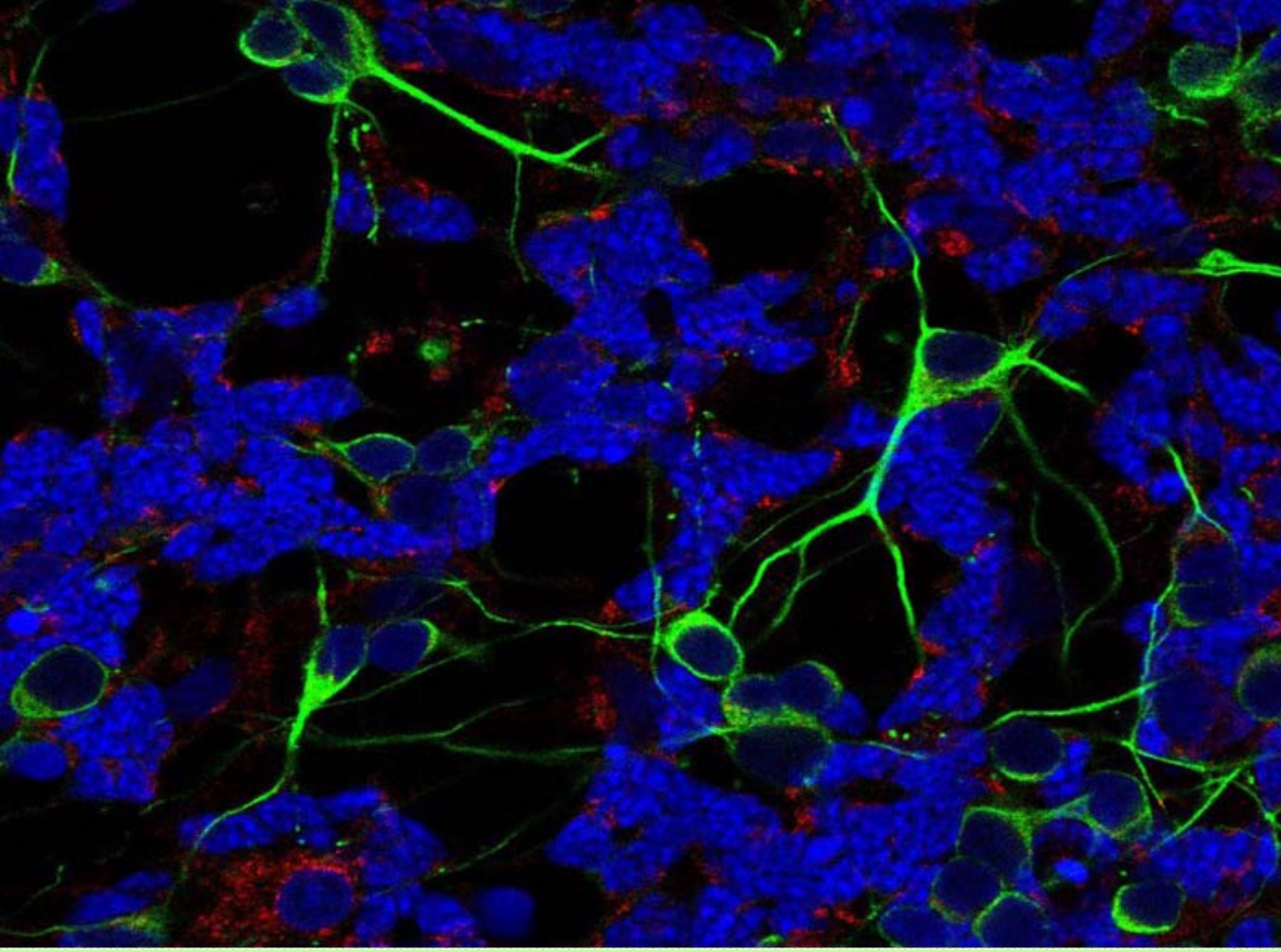

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Tauroursodeoxycholic acid: a novel neuroprotective drug in diabetic retinopathy?

Faculdade de Medicina

2011

· U C ·

UNIVERSIDADE DE COIMBRA

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Investigação Biomédica. O trabalho foi realizado sob a orientação científica da Investigadora Doutora Ana Raquel Sarabando Santiago e supervisão do Investigador Doutor António Francisco Rosa Gomes Ambrósio (Instituto Biomédico de Investigação da Luz e Imagem, Faculdade de Medicina da Universidade de Coimbra)

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Agradecimentos

Um profundo e sincero agradecimento a todos aqueles que durante este ano me aconselharam, motivaram e orientaram.

Agradeço à Doutora Ana Raquel Sarabando Santiago, pela confiança em mim depositada na oportunidade de integrar o seu grupo de trabalho. Obrigada pela orientação deste último ano, pelo incentivo, chamadas de atenção, enorme paciência e disponibilidade que demonstrou. Agradeço, ainda, por me ter proporcionado as condições necessárias para a realização deste trabalho.

Ao Doutor António Francisco Ambrósio, pela amabilidade e disponibilidade que demonstrou em co-orientar este trabalho, pela atenção e pelas sugestões que concedeu. Também agradeço por me ter proporcionado as condições necessárias para a realização deste trabalho.

Ao Professor Doutor Paulo Pereira, coordenador deste mestrado, agradeço o facto de me ter dado a oportunidade de frequentar o mestrado e proporcionando-me assim estes dois anos de contínua aprendizagem.

Aos meus colegas de laboratório, Raquel, Filipa, Áurea, Maria, Joana, Filipe, Dan, Camila, Renato, Sandra e Rosana, aqui fica o meu mais profundo agradecimento. Obrigada pela companhia, pelo ambiente descontraído, pelos jantares e happy-hours, pela ajuda prestada, pela disponibilidade, pelos conselhos e pela amizade que me proporcionaram ao longo deste ano, quer em momentos bons e menos bons.

Um especial agradecimento às tias Filipa e Áurea, sem vocês não seria a mesma coisa. Obrigada pela imensa ajuda, paciência, por esclarecerem dúvidas momentâneas, pelos conselhos e pela companhia ao longo deste ano. Fi, obrigada por me teres recebido tão bem no gabinete!

À Maria, pelo companheirismo nesta jornada, amizade, ajuda, disponibilidade e compreensão. Pela diversão, pelas visitas aos fantasmas, por me integrar junto dos seus amigos.

Agradeço também à Rosana, que me acompanhou durante algum tempo neste projecto, pela sua simpatia, disponibilidade e preciosíssima ajuda em tempos tão difíceis. E à Joana pela companhia nas pausas, boa onda e disponibilidade para ajudar.

Agradeço à Joana Gaspar pela sua contribuição, que foi sem dúvida fundamental!

Agradeço também à Joana Liberal, Joana Gonçalves, Carla Marques, Rosa Fernandes, Henrique, Steve e Hugo por qualquer esclarecimento, qualquer ajuda prestada, por de algum modo terem contribuído para a minha aprendizagem e pelo bom ambiente.

Todos contribuíram para o meu crescimento, enquanto profissional e mesmo a nível pessoal. Aprendi a dar mais valor a certas coisas e a certas pessoas.

Obrigada a todos pela simpatia, bom ambiente e por todos os bons momentos. Gostei de fazer parte do grupo IBILI!

Agradeço aos meus amigos limianos, principalmente ao Tiago, por todo o incentivo.

Um especialíssimo agradecimento às minhas meninas de longa data, Ana e Jajão pela compreensão nos momentos mais agrestes, incansável apoio, companhia, amizade. Pelas vossas críticas construtivas, pelos sorrisos e desabafos. Por toda a diversão e serões ao sabor de um bom vinho. Já lá vão 6 anos e por tudo o que já passamos juntas, obrigada.

Por último e mais importante, agradeço do fundo do meu coração à minha família. Aos meus pais pelo enorme esforço durante estes dois anos para que eu pudesse prosseguir os estudos e chegar a esta etapa, mesmo em tempos difíceis. Obrigada pela paciência, pelos desabafos, por aturar as minhas birras e teimosia, pelo apoio, pelas merecidas repreensões, pelo carinho, pelo amor, por acreditarem em mim. Obrigada pela vossa presença na minha vida e carreira. Apesar das constantes birras, também agradeço à minha irmã Vanessa, não pelo facto de me chatear e testar a minha grande paciência, mas pelo apoio dado para que eu possa atingir os meus objectivos, pelo carinho e por acreditar em mim. Adoro-vos.

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Abbreviations

AGE – Advanced glycation-end products

AIF – Apoptosis inducing factor

AMPA – α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

ANOVA – Analysis of variance

AP-1 – activating protein-1

APAF-1 – Apoptotic peptidase activating factor

BCA – Bicinchoninic acid

BRB – Blood-retinal barrier

BSA – Albumin bovine serum

Ca^{2+} – Calcium

CARD – Caspase activation and recruitment domain

CNS – Central nervous system

CTZ – Cyclothiazide

Cyt c – Cytochrome c

DAG – Dyacylglicerol

DAPI – 4`, 6-Diamidino-2- Diethyl phenylindole

DED – Death effector domain

DHAP – Dihydroxyacetone phosphate

DISC – Death-inducing signaling complex

DM – Diabetes *mellitus*

DNA – Deoxyribonucleic acid

DNP – 2,4-Dinitrophenol

DNPH – 2,4-Dinitrophenylhydrazine

dNTP – Deoxyribonucleotide triphosphate

DR – Diabetic retinopathy

DTT – Dithiothreitol

ECF – Enhanced chemifluorescence

EGTA – Ethylene glycol tetraacetic acid

ERG – Electroretinogram

ERK – Extracellular regulated kinase

ET-1 – Endothelin-1

FADD – Fas-associated death domain

FGF – Fibroblast growth factor

Fruc-6-P – Fructose-6-phosphate

GABA – Gamma-aminobutyric acid

GADPH – Glyceraldehyde -3-phosphate dehydrogenase

GCL – Ganglion cell layer

GFAP – Glial Fibrillary A Protein

GFAT – Glutamine:fructose-6 phosphate amidotransferase

H_2DCF-DA – 2', 7'-dichlorodihydrofluorescein diacetate

ILM – Inner limiting membrane

INL – Inner nuclear layer

IPL – Inner plexiform layer

KA – Kainate

MAPK – Mitogen-activated protein kinase

Mg^{2+} – Magnesium

MnSOD – Manganese superoxide dismutase

MPT – Mitochondria

mRNA – Messenger ribonucleotide acid

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD^+ – Nicotinamide adenine dinucleotide

NADH – Nicotinamide adenine dinucleotide hydrogenase

NADP – Nicotinamide adenine dinucleotide phosphate, oxidized form

NADPH – Nicotinamide adenine dinucleotide phosphate, reduced form

NFL – Nerve fiber layer

NF-κB – Nuclear factor kappa B

NGF – Neural growth factor

NPDR – Non-proliferative diabetic retinopathy

ONL – Outer nuclear layer

OPL – Outer plexiform layer

PAI-1 – Plasminogen activator inhibitor-1

PARP – Poly (ADP-ribose) polymerase

PBS – Phosphate-buffered saline

PCD – Programmed cell death

PDGF – Platelet-derived growth factor

PI3K – Phosphoinositide-3 kinase

PKC – Protein kinase C

PLC – Phospholipase C

PS – Phosphatidylserine

PVDF – Polyvinylidene fluoride

RIP – Receptor-interacting protein

ROS – Reactive oxygen species

RP – Retinitis pigmentosa

RPE – Retinal pigmented epithelium

SDH – Sorbitol dehydrogenase

SDS – Sodium dodecyl sulfate

SEM – Standard error of mean

STZ – Streptozotocin

TBS-T – Tris-buffered saline -Tween

TCA – Trichloroacetic acid

TFA – Trifluoroacetic acid

TGF-α/TGF-β1 – Tumoral growth factor-α/-β1

TRAIL – TNF-related apoptosis-inducing ligand

TUDCA – Tauroursodeoxycholic acid

TUNEL – terminal transferase dUTP nick end labeling

UDCA – Ursodeoxycholic acid

UDP-GlnNAC – UDP-N-acetylglucosamine

VEGF – Vascular endothelial growth factor

Resumo

A retinopatia diabética (RD) é uma das principais causas de cegueira em adultos entre os 20 e 74 anos, nos países ocidentais. Várias evidências demonstraram que a RD tem características de doença neurodegenerativa. A excitotoxicidade induzida pelo glutamato está envolvida em diversas doenças neurodegenerativas, incluindo na retina.

O ácido tauroursodesoxicólico (TUDCA) é um ácido biliar produzido endogenamente, em pequenas quantidades no humano. Tem sido demonstrado que o TUDCA tem propriedades anti-apoptóticas e antioxidantes em diversos modelos animais de doenças neurodegenerativas.

O objectivo principal deste projecto foi investigar o potencial efeito neuroprotector do TUDCA na morte celular induzida por um insulto agudo (estímulo excitotóxico) e por um insulto crónico (condições de elevada glicose) em culturas primárias de células neurais da retina.

Neste trabalho, mostrámos que o TUDCA preveniu a morte celular induzida por kainato (KA) na presença de ciclotiazida (CTZ). Além disso, a exposição das células ao TUDCA preveniu a morte celular induzida por elevada glicose. A translocação mito-nuclear do factor inductor de apoptose (AIF) induzida por concentração elevada de glicose foi parcialmente prevenida pela incubação com TUDCA. Além disso, o aumento do conteúdo em proteínas oxidadas e o aumento da produção de espécies reactivas de oxigénio (ROS) induzida por concentração elevada de glicose foram prevenidos pelo TUDCA.

Em conclusão, o TUDCA preveniu a morte celular induzida por excitotoxicidade e por elevada concentração de glicose em culturas primárias de células neurais da retina. No modelo de elevada concentração de glicose, o efeito protector deveu-se principalmente à inibição parcial da translocação mito-nuclear do AIF. Com base nestas evidências, TUDCA poderá ser usado como um potencial agente terapêutico para o tratamento de doenças neurodegenerativas, como a RD.

Abstract

Diabetic retinopathy (DR) is a leading cause of blindness among working-age adults in the western countries. Burden of evidence demonstrate that DR has features of a neurodegenerative disease. Glutamate-induced excitotoxicity has been implicated in several neurodegenerative disorders, including retinal degenerative diseases. Tauroursodeoxycholic acid (TUDCA) is a bile acid produced endogenously. It has been demonstrated that TUDCA has anti-apoptotic and antioxidant properties in several animal models of neurodegenerative disorders.

The main aim of this project was to investigate the potential neuroprotective effect against cell death induced by acute (excitotoxic stimulus) and chronic (high glucose conditions) insults in primary retinal neural cell cultures.

In this work, we found that TUDCA prevented cell death induced by kainate (KA) in the presence of cyclothiazide (CTZ), and also high glucose-induced cell death. The mito-nuclear translocation of apoptosis inducing factor (AIF) induced by elevated glucose concentration was partially prevented by TUDCA incubation. Also, the increase in protein-bound carbonyl content and increased production of reactive oxygen species (ROS) induced by elevated glucose concentration were prevented by TUDCA.

In conclusion, TUDCA prevented cell death induced by excitotoxicity and elevated glucose concentration in primary retinal neural cell cultures. In elevated glucose concentration model, the protective effect was mainly due to the partial inhibition of AIF mito-nuclear translocation. It is also likely that the protective effects of TUDCA are due to its anti-apoptotic and antioxidant properties. These evidences further suggest that TUDCA can be used as a potential therapeutic agent for the treatment of neurodegenerative disorders, such as DR.

CHAPTER 1 - Introduction

1.1. The retina

The retina is a thin structure that covers most of the inner part of the eye (Figures 1A and 1B), between the retinal pigmented epithelium (RPE) and the vitreous body. It is a light-sensitive nerve tissue, fundamental to vision, responsible for the perception of the visual information through the conversion of electrical impulses transmitted by neurons. Histologically, it is an organized multi-layer with cell-cell communication constituted essentially by three major classes of cells: neurons (photoreceptors, ganglion cells, bipolar cells, horizontal cells and amacrine cells), glial cells (Müller cells, astrocytes, and microglia) and cells that constitute the retinal vessels (pericytes and endothelial cells) (Fischbarg, 2006; Rogers, 2011).

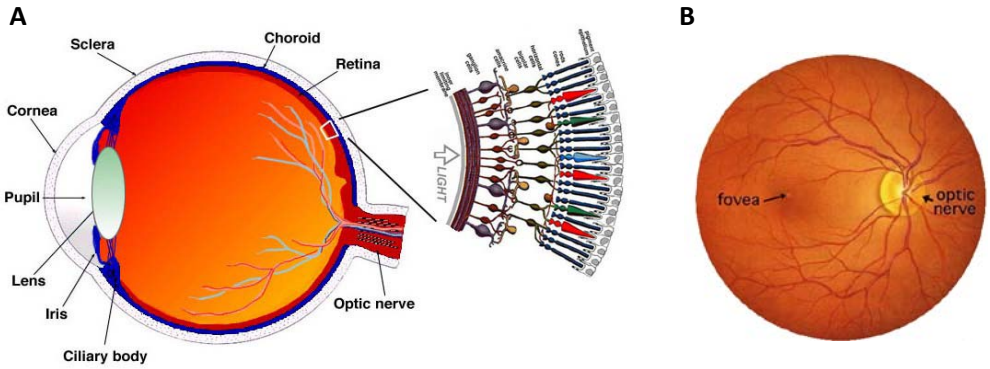


Figure 1. (A) a representative section of the human eye showing its several components, including a enlarged scheme of the multilayers that form the retina; (B) a photography of a healthy eye fundus (Adapted from Kolb, 1995).

In general, between retinal pigmented epithelium (RPE) and the vitreous body, retina is composed by three layers of nerve cells, the outer nuclear layer (ONL), the inner nuclear layer (INL), the ganglion cell layer (GCL) and two regions of synapses separating these layers, the outer plexiform layer (OPL) and the inner plexiform layer (IPL). The cell layers are organized in a network to potentiate the cell-cell communication between the different types of cells, important for normal vision (Kolb, 2003). The ONL contains cell bodies of the rods and cones, the OPL is the area where synapses occur between photoreceptors with bipolar and horizontal cell dendrites and the INL contains several types of bipolar, horizontal and amacrine cells. Next, lays

the IPL, also a synaptic region, connecting bipolar and amacrine cells with ganglion cells and the GCL, above the inner limiting membrane (ILM), containing ganglion cells and displaced amacrine cells (Figures 2A and 2B).

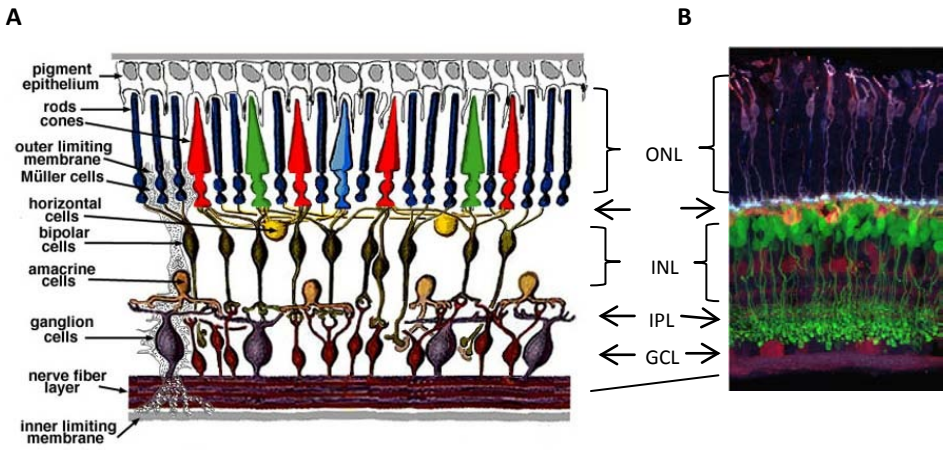


Figure 2. (A) Schematic illustration of retinal cell layers. At the top, photoreceptor layer is close to retinal pigment epithelium. Müller cells are present in different layers, from the outer limiting membrane to inner limiting membrane against the nerve fibre layer. Between them, lie the bipolar cells, the horizontal cells and the amacrine cells including their neural connections in the plexiform layers; ONL, outer nuclear layer, OPL, outer plexiform layer, INL, inner nuclear layer, IPL, inner plexiform layer, GCL, ganglion cell layer (Adapted from Kolb, 1995); **(B) Cross-section of mature mouse retina exhibiting cell layers by labeling the different neural cell types**. Photography from Confocal Microscopy (Adapted from Morgan and Wong, 1995).

Neurons mediating phototransduction include photoreceptors (rods and cones) that receive impulses and transmit the information through dendrites to bipolar cells, which synapse with ganglion cells. Retinal ganglion cells connect with the brain through its axons which forms the optic nerve after leaving the eye. Microglial cells, normally quiescent, are linked to the immune system (when activated they have phagocytic functions), modulating the local environment and they also react to stress (Fischbarg, 2006; Madsen-Bouterse and Kowluru, 2008; Rogers, 2011). Macroglial cells regulate retinal metabolism, providing nutritional and vascular support.

Müller cells are the most numerous glial cell, present along the thickness of the retina from RPE to internal limiting membrane (Gardner et al., 2002). Their main functions are the regulation of glutamate metabolism (Romano et al., 1995). In addition, these cells are also

responsible for the maintenance of the retinal blood flow and the blood-retinal barrier (Kowluru and Odenbach, 2004b) and act as scavengers of neurotransmitters, such glutamate, from the synaptic cleft (Puro and Stuenkel, 1995). Under different conditions, Müller cells respond to injuries producing certain molecules and proteins, like cytokines, heat-shock proteins, growth factor and neurotransmitters.

Astrocytes are located in the GCL, IPL and INL, mainly found around retinal blood vessels. These cells are involved in the functional composition of the blood-retinal barrier. Their presence is only observed in vascular retinas (Stone and Dreher, 1987).

Endothelial cells in microvessels are the primary physical barrier between blood and retinal tissue (Cines et al., 1998). In addition, pericytes are smooth muscle-like cells that envelope capillaries, since they surround endothelial cells, complementing vascular function. In addition, contractile functions were also established (Shepro and Morel, 1993). Both perycites and endothelial cells provide nutritional support and waste product removal for the inner retina (Gardner et al., 2002; Shepro and Morel, 1993). Disruption of any interaction and function of these cells can lead to vision impairment (Gardner et al., 2002).

1.2. Diabetes *mellitus*

Diabetes *Mellitus* (DM) is the most common metabolic disorder in the Western countries, affecting people from all age. It is characterized by elevated blood glucose levels (hyperglycemia) caused by defects in insulin secretion, in β-pancreatic Langerhans cells, or in insulin action, or both. This hormone is responsible for the control of glucose blood levels (glycemia) and thus, in hyperglycemia condition, insulin is released from pancreatic cells to restore normal glucose levels. Diabetes leads to abnormalities in carbohydrates, lipid and protein metabolisms, induced by chronic hyperglycemia, associated with other complications, such retinopathy, cardiovascular disease, nephropathy and neuropathy, reason why DM is often

described as a group of diseases (Donnelly and Davis, 2000). There is an effort to find a cure for this disorder like drug treatments for hyperlipidemia and hyperglycemia and gene therapies.

Etiologically, DM is classified in four categories: type 1, type 2, gestational and other due to specific causes. It can also be described, depending of the effect caused by insulin deficits as insulin-dependent and non-insulin-dependent (Kuzuya et al., 2002; Zimmet et al., 2001).

Type 1 diabetes is one of the main types, most common in childhood, although it may also appear at any age, and is not associated with obesity. It is the result of pancreatic β-cell loss, leading to deficiency in insulin secretion (Kobayashi, 1994; Zimmet et al., 2001). Therefore, it may also be considered an immune-mediated disease or idiopathic diabetes.

Type 2 diabetes affects 90% of the diabetic population, mostly obese adults, and has major incidence in older people. It is characterized by a time-dependent regression of pancreatic function associated with a resistance and/or abnormal secretion of insulin (Zimmet et al., 2001).

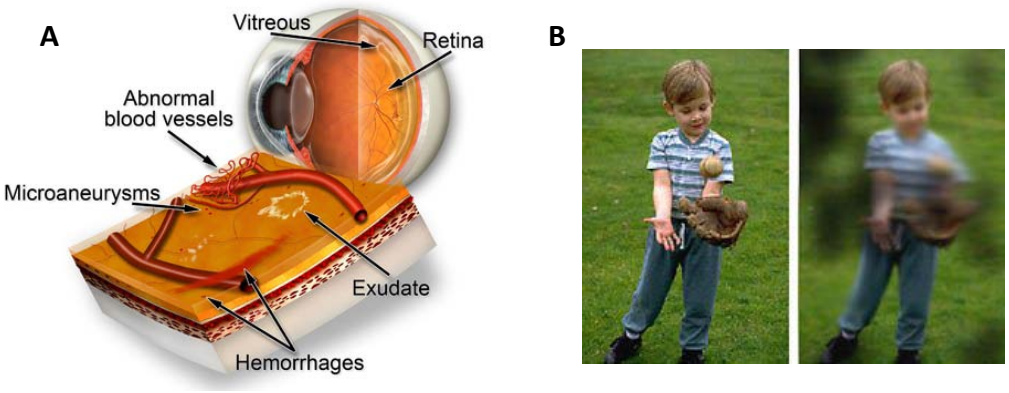
Gestational DM appears during pregnancy, due to glucose intolerance and usually patients do not develop diabetes later (Kuzuya et al., 2002; Zimmet et al., 2001).

Other types of DM, as diabetes caused by genetic mutations that increase susceptibility or by association to other diseases as Maturity-onset diabetes of the young.

1.3. Diabetic retinopathy

Diabetic Retinopathy (DR) is a long-term, microvascular, complication of diabetes, regarded as a main cause of vision loss and blindness (Figure 3B) among adults at working-age, in developed countries (Antonetti, 2006; Barber, 2003; Kowluru and Chan, 2007; Santiago, 2006; Stitt, 2003).

Hyperglycemia is considered the primary pathogenic factor for the development of DR. The risk of DR increase with the duration and degree of diabetes (Aiello, 2002; Kern et al., 2010; Rogers, 2011).



Figures 3. (A) Representation of a diabetic eye (http://www.istanbulretina.com); **(B) Normal vision vs. Vision impaired by diabetic retinopathy** (http://www.stlukeseye.com/conditions/DiabeticRetinopathy.html).

DR has been first described as a microvascular disease (Cunha-Vaz, 2001), although many studies have shown that diabetes also affects retinal neurons, taking into account certain features of neuronal dysfunction including apoptosis and glial reactivity, loss of color and contrast sensitivity, in addition to alterations in electroretinograms (ERG) of diabetic patients (Sakai et al., 1995). Therefore retinal neurodegeneration was considered as the early event of DR, before any vascular changes (Antonetti, 2006; Barber, 2003; Villarroel, 2010) (Figure 3A).

Hyperglycemia induces biochemical changes in the retina through several pathways (Armstrong et al., 1992; Kowluru et al., 2001; Kowluru et al., 1997; Kowluru et al., 1996; Villarroel, 2010).

Blood retinal barrier breakdown

The blood-retinal barrier is a selective barrier located at the endothelial and epithelial cells, separating the neural components of the retina from the circulation (Figure 4A). It is responsible for maintaining the homeostasis of retinal environment, allowing oxygen diffusion, regulating ionic composition and nutrient transport and protecting retinal neurons from cytotoxicity induced by circulating inflammatory cells (Frey and Antonetti, 2011; Gardner et al., 2002). Evidences indicated that tight junctions are closely related to BRB formation. Tight junctions are cell-cell interactions forming a paracellular barrier selective to water and solutes,

composed by structural proteins. It includes isoforms of zonula occludens, occludins and claudins and is also associated with regulatory proteins implicated in cell signaling like PKC (Harhaj and Antonetti, 2004; Schneeberger and Lynch, 2004; Stevenson and Keon, 1998). These cellular junctions and related proteins contribute for the regulation of the BRB vascular permeability.

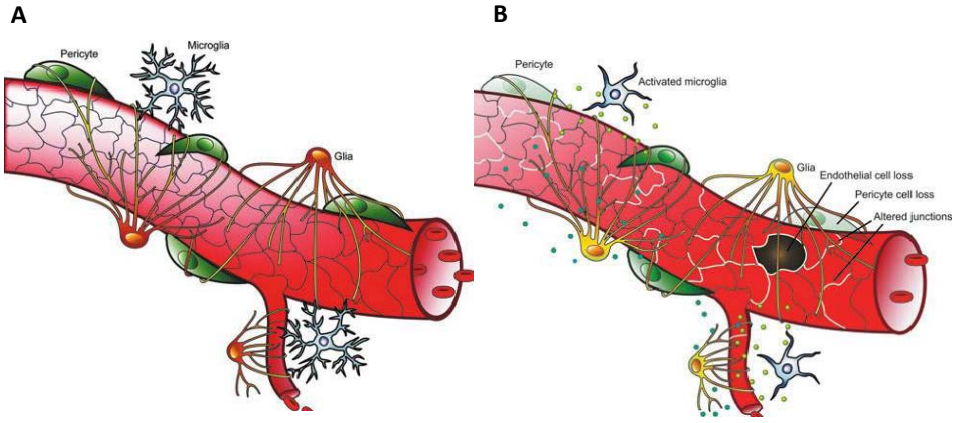


Figure 4. Representative alterations of BRB and its constituents during its breakdown; (A) Healthy BRB; (B) breakdown BRB in Diabetes (Adapted from Frey and Antonetti, 2011).

The BRB breakdown (Figure 4B) remains one of the most important hallmarks of early stages of diabetic retinopathy (Cunha-Vaz, 1976). It may be triggered by oxidative stress (Frey and Antonetti, 2011), inflammatory response (Miyamoto et al., 1999) and increase in permeabilizing growth factors expression and its binding-protein, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and mostly vascular endothelial growth factor (VEGF) (Miller et al., 1997). These factors were suggested by several studies to play a preponderant role in retinal vascular permeability (Aiello et al., 1995; Amin et al., 1997; Lutty et al., 1996), moreover reported in proliferative diabetic retinopathy, leading to angiogenesis and further to vision loss (Antonetti et al., 1999).

1.3.1. Classification of DR

DR is clinically classified in five stages (Cunha-Vaz, 1972), based on the severity of vascular changes, and taking into account its progression to vision loss.

Preclinical diabetic retinopathy

This earliest stage of DR is characterized by non-ophthalmologic alterations in the retina, despite some studies already reported the detection of histopathological and electrophysiologic changes in the retina, through sensible diagnostic technique (Cunha-Vaz and Maurice, 1967). These alterations include microaneurysms, edema, endothelial degeneration, vasodilatation and changes in BRB (Cunha-Vaz, 2006).

Nonproliferative DR (NPDR)

This stage include capillary occlusion and degeneration associated to reduction in retinal perfusion, microaneurysms leading to proliferation of endothelial cells, selective loss of pericytes and increase vascular permeability (Ashton, 1963, 1974; Cunha-Vaz, 1978). Those microaneurysms and small intraretinal hemorrhages are described as red dots (Figure 5A), appearing in retinal capillaries. These red dots are early alterations detectable by ophthalmoscopic examination and ocular fundus photography in diabetic patients and thus, suggested as marker of retinopathy progression (Klein et al., 1995). Hemorrhages are due to the breakdown of microaneurysms and microvessels. This stage is also characterized by the presence of lipid and lipoproteins exudates in the nerve fiber layer (NFL) and a significant level of retinal edema, which is caused by damaged capillaries (Cunha-Vaz and Bernardes, 2005). Visual loss may be absent or may result from retinal swelling or ischemia (Rogers, 2011). Increasing capillary nonperfusions are related to progression to advanced neovascular stages of

retinopathy in patients. Additionally, appearance of central macular edema is also a sign of advanced NPDR.

Diabetic macular edema

Macular edema is an intermediate stage characterized by retinal edema, the result of plasma leakage from microvessels and consequent increase in the content of fluids, involving or threatening the macula. This is the main cause for disturbed visual acuity in diabetic patients and, in severe cases, of vision loss (Aiello et al., 1998; Cunha-Vaz, 2006). It is a retinal region fundamental to detailed central vision. This feature can occur in both NPDR and proliferative diabetic retinopathy stages, but might have a different incidence reflected in its distribution and cystoid appearance (Kern et al., 2010).

Preproliferative diabetic retinopathy

Known as an advanced NPDR stage, preproliferative diabetic retinopathy is a subtype of DR, characterized by an increase in the number and size of the intraretinal hemorrhages due to microvascular abnormalities. Also accompanied by retinal venous dilation and increase in retinal ischemic signs, such as soft exsudates described as cotton-wool spots (Figure 5B), it can help to indicate the progression of retinopathy (Kohner and Oakley, 1975). Appearance of several regions of capillary nonperfusion, venous beading and loops are also observed (Kern et al., 2010).

Proliferative diabetic retinopathy

Finally, proliferative diabetic retinopathy (PDR), the most severe stage of the disease, is associated with neovascularization (Rogers, 2011) (Figure 5C), induced by hypoxia and ischemia through vascular nonperfusion. In fact, the formation of those abnormal microvessels, which may develop hemorrhages and proliferate in the retina, initiate in the ILM and can extended to

the vitreous (Cunha-Vaz, 2006; Kern et al., 2010). Some studies suggested that PDR not only affects the retina, but also may damage the optic disk and even iris (Frank, 1995; Rogers, 2011). Associated with fibrous tissue proliferation in the retina, the alterations described in vitreous could also result in vision deficits and perhaps in an advanced form, leading to retinal detachment and subsequent blindness (Frank, 2004; Kern et al., 2010; Klein and Klein, 1997).

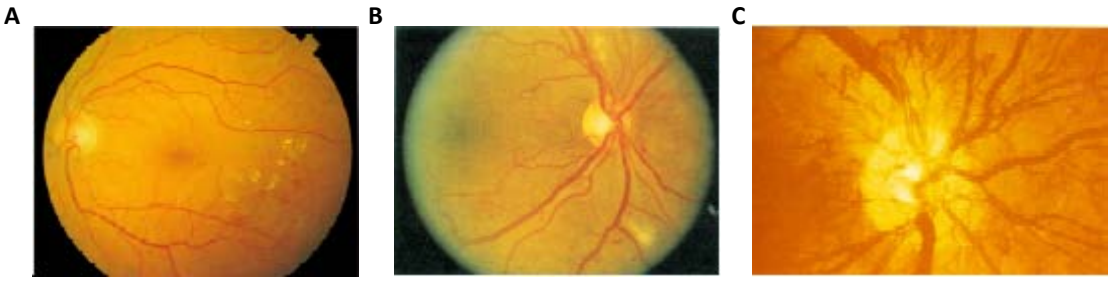


Figure 5. Eye fundus photographs from diabetic patients: view of the progression of diabetic retinopathy. (A) Non-proliferative diabetic retinopathy; (B) Pre-proliferative diabetic retinopathy; (C) Proliferative diabetic retinopathy (Adapted from Cunha-Vaz, 1972).

1.3.2. Biochemical changes induced by hyperglycemia

Several mechanisms have been proposed to explain the adverse effects of elevated glucose levels, including the polyol pathway flux, oxidative stress, non-enzymatic glycation and diacylglycerol–protein kinase C (DAG–PKC) activation (Figure 6). High glucose levels or its metabolites can affect vascular cell function directly via multiple pathways (Das Evcimen and King, 2007).

Polyol pathway

The flux of the polyol pathway begins with the activity of Aldose reductase, an oxyreductase enzyme, catalyzing carbonyl compounds, such as glucose, dependent from the co-factor nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH). In hyperglycemia, glucose is first reduced to sorbitol and then oxidized to fructose by sorbitol dehydrogenase (SDH), accompanied by nicotinamide adenine dinucleotide (NAD^+) reduction (Dvornik et al.,

1973). Increases in NADH/NAD^+ ratio, likely to mimic hypoxia (Lassegue and Clempus, 2003; Williamson et al., 1993) and NADPH depletion by NADPH oxidase lead to oxidative stress (Ola et al., 2006).

Hexosamine pathway

Hyperglycemia is known to induce an increase in mitochondrial superoxide production also through the activation of the hexosamine pathway. Fructose-6-phospate (Fruc-6-P), a glycolytic intermediate, is converted to Glucosamine-6-phosphate by glutamine: fructose-6-phosphate amidotransferase (GFAT) and further to UDP-*N*-acetyl glucosamine (UDP-GlcNAc), which is important to the formation of O-linked glycoproteins. An increase in hexosamine pathway flux results in increased transcription of plasminogen activator inhibitor-1 (PAI-1) (Kolm-Litty et al., 1998) and growth factors such as tumoral growth factor (TGF)-α and TGF-β1 (Du et al., 2000). Inhibition of superoxide overproduction induced by hyperglycemia prevents alterations in gene expression, as NF-κB activation and protein function (Nishikawa et al., 2000).

Activation of protein kinase C (PKC)

Protein kinase C family constitute a group of eleven isoforms of serine/threonine kinases (Jaken, 1996), activated by DAG and/or phosphatidylserine and/or Ca^{2+} and/or fatty acids, besides Mg^{2+}. It was shown that DAG–PKC pathway is an important mechanism by which hyperglycemia cause microvascular and macrovascular abnormalities during diabetes. In fact, hyperglycemia induce an increase in lipid second messenger DAG content, which is derived from the hydrolysis of phosphatidylinositide, from the metabolism of phosphatidylcholine by phospholipase C (PLC) (Das Evcimen and King, 2007). It also occur the *de novo* DAG synthesis by glycolytic intermediate hydroxyacetone phosphate and glycerol-3-phosphate. Increased DAG levels activate PKC isoforms that are consequently associated with diabetic complications. The increase in DAG leads to blood-flow abnormalities (by decreasing endothelial nitric oxide

synthase and increasing ET-1) (Brownlee, 2001), vascular permeability and angiogenesis through the increase in VEGF expression (Gardner et al., 2002). In addition, capillary and vascular occlusion occurs induced by increased transforming growth factor-β and plasminogen activator inhibitor-1. PKC activation also affects pro-inflammatory gene expression by NF-κB activation and other effects associated with ROS production (Giacco and Brownlee, 2010).

Advanced glycation end-products pathway

Advanced glycation end-products (AGEs), found in diabetic retinal vessels, were thought to be formed by a nonenzymatic reaction, known as Maillard reaction, between reducing sugars like glucose and free amino groups from proteins (Brownlee, 2001), lipids and DNA. This reaction is first characterized by the formation of a Shiff base which, after rearrangement to form Amadori adducts (more stable glycation products), suffers oxidation and dehydration, to further generates AGEs (Stitt, 2010). Nowadays, it is known that AGEs are also generated intracelullarly from glucose-derived dicarbonyl precursors, as glyoxal, metylglyoxal and 3-deoxyglucosone that reacts with proteins. Those AGEs precursors damage cells by modifying protein functions, altering properties of several matrix molecules and consequently their matrix-matrix and cell-matrix interactions, as well as inducing production of ROS mediated by modified plasma proteins binding to AGE receptors (RAGE). This last mechanism triggers changes in gene expression in endothelial cells, mesanglial cells and macrophages, since it activates the transcription factor NF-κB (Yan et al., 1994) and also the proto-oncogene $p21^{Ras}$ (Lander et al., 1997). Over the years, AGEs amount increase in the retina, although it was described that, in diabetic patients, those levels are higher in vascular and neural cells, comparing to control patients (Stitt, 2010).

Oxidative stress

Oxidative stress and subsequent reactive oxygen species (ROS) occur in diabetic retina. Some authors define ROS as the simple production of reactive oxygen species by oxidizing

macromolecules. Among several diabetic complications, hyperglycemia was suggested to induce ROS production (Giardino et al., 1996), which leads to activation of signal transduction pathways, increasing the expression of growth factors and cytokines. Reduced antioxidant activities can also be responsible for oxidative stress, since overexpression of manganese superoxide dismutase (MnSOD) induce a blockade in ROS generation. Thus, it was considered as a mitochondrial form of ROS scavenger (Du et al., 2000; Nishikawa et al., 2000).

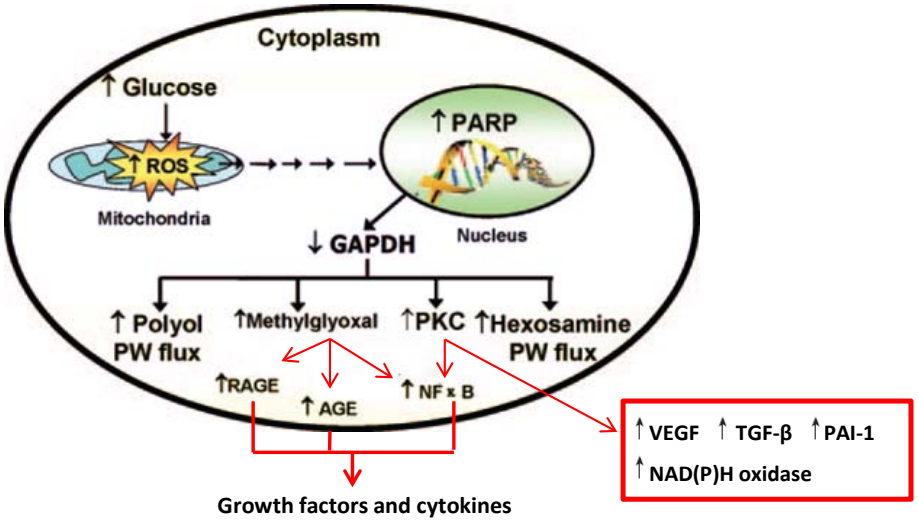


Figure 6. Hyperglyceamia induces biochemical changes leading with celular damage. Increased glucose flux triggers mechanisms of glucose auto-oxidation, increasing flux of the polyol pathway, hexosamine pathway, PKC activation pathway and AGEs formation pathway. Increased flux of these pathways lead to vascular permeability, angiosenesis and other multiple effects. GADPH, glyceraldehyde-3-phosphate dehydrogenase (Adapted from Giacco and Brownlee, 2010).

Also, reduced MnSOD mRNA expression and activity in the retinas of diabetic rats, which were further prevented by lipoic acid long-term treatment (Kowluru and Odenbach, 2004a). In fact, evidences showed that superoxide overproduction induced by elevated glucose can be a target for antioxidant, anti-inflammatory and/or anti-angiogenic therapies, preventing alterations in cell function or viability and thus, the development of DR.

1.3.3. Cellular mechanisms leading with retinal dysfunction in diabetes

Recent studies reported that major retinal cell types are implicated in vascular and neural changes in the pathogenesis of diabetes (Antonetti, 2006).

Understanding the cellular mechanisms underlying retinal cell abnormalities induced by hyperglycemia that undergoes DR is fundamental to study and develop preventive therapies.

Glial cells and DR

Several evidences described that diabetes impairs changes in macroglial cells, such as astrocytes and Müller cells. Astrocytes have migratory properties during development and are abundant in the ILM (Gardner et al., 2002). Moreover, Müller cells are recognized as the predominant macroglia subtype, existing only in the retina. Also, studies reported that Müller cell impairment occurs before any clinical evidence of NPDR (Lieth et al., 1998).

Increased expression of glial fibrillary acidic protein (GFAP) leads with glial reactivity and changes in vascular permeability and blood flow (Mizutani et al., 1998), due to the fact that, through their interactions with blood vessels, astrocytes are implicated in tight junction expression as well as BRB maintenance (Rungger-Brandle et al., 2000). Different GFAP expression in both cell subtypes demonstrated in many studies reflected their different response to hyperglycemia (Barber et al., 2000).

Another feature of increased cell permeability is the release of cytokines, as VEGF, produced by both glial (Amin et al., 1997) and neuronal cells. VEGF is thought to increase its production where tight junctions are compromised. Thus suggests that retinal cells have an important role in modulating retinal vascular permeability. Although VEGF function in vascular cells is correlated with permeability, its increased expression in neurons leads to neuroprotection (Gardner et al., 2002).

Microvascular cell injury in Diabetes

Under normal conditions, microglias are quiescent cells, while in diabetes they become activated. It was described in 1 month STZ-induced diabetic rat retinas an increase in the number, density and activity of reactive microglial cells (Barber et al., 2000; Rungger-Brandle et al., 2000). During their reactive state, microglia release cytokines and chemokines, such as tumoral necrosis factor (TNF) and interleukin-1β (IL-1β) (Krady et al., 2005), and also VEGF (as well as other retinal cells), leading with changes in permeability (Gardner et al., 2002) preceding neuronal cell death in diabetic retinas. Hyperglycemia increases the mRNA expression and consequently the release of pro-inflammatory molecules.

Among previous studies, it was shown that Minocycline appears to be a potential anti-inflammatory and anti-apoptotic therapeutic agent, reducing microglial activity and thus, also the release of cytotoxic molecules that may promote cell death (Krady et al., 2005).

Although a few is known about those microvascular cells, evidences suggested ATP as a possible stimulus that induce activation of microglial cells, since it was observed in those cells increased expression of ATP receptors subtypes (Farber and Kettenmann, 2006). It was also established that microglial cells play an important role in inflammation and phagocytosis, removing apoptotic cells.

Retinal neurons changes in DR

Loss of retinal neurons undergoing DR was first reported in the 1960's (Bloodworth, 1962; Wolter, 1961). Apoptosis was observed in GCL and INL cells in diabetic rat retinas, promoting reduced thickness of the inner retina and ganglion cell number, assessed by TUNEL staining (Barber et al., 1998; Hammes et al., 1995). Programmed cell death was prevented with nerve growth factor (NGF) treatment (Hammes et al., 1995).

Fragmented cone photoreceptors, apoptotic photoreceptors in respective layer (Aizu et al., 2002) as well as necrotic features in other retinal neural cells were also detected, consistent with diabetes-induced neurodegeneration.

Associated with certain macroglial changes, neural dysfunction leads with alterations in the oscillatory potentials of ERG, as observed in DR patients (Simonsen, 1980), color vision and contrast sensitivity. It was already observed a reduction in pattern ERGs before the clinical onset of non-proliferative retinopathy, in some patients with diabetes (Prager et al., 1990).

Other non-retinal cells implicated in DR

Leukocytes and platelets are involved in DR pathogenesis, since they have been found in retinal circulation, responding to increased leukostasis induced by diabetes (Miyamoto et al., 1999).

Leukostasis is mediated by several types of molecules with different function. Thus mainly include adhesion molecules as P-selectin, E-selectin and L-selectin, responsible for leukocyte-endothelial interactions, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 that promote cell migration (Cronstein and Weissmann, 1993; Mackay and Imhof, 1993).

Leading to leukocyte extravasation, expression of P-selectin, ICAM-1 and VCAM-1 was increased in vitreous and retina of patients with PDR (Nozaki et al., 2002). Therefore, vascular cells are affected by leukostasis and BRB alterations. Observations of a diabetic rat retina may allow authors to consider increased leukocyte adhesion, a feature of endothelial cell death (Joussen et al., 2001).

Platelets are also involved in DR, since it was showed their presence in diabetic rat retinas (Ishibashi et al., 1981) and their relation with retinal vascular endothelial cells (Boeri et al., 2001).

1.4. Glutamatergic neurotransmission

As it is clearly known, cell-cell communication occurs electrically, through gap junctions, and by neurotransmitters, chemical messengers containing the signal, in synapses. Stimulation of the presynaptic neuron leads to calcium (Ca^{2+}) channels opening and influx of Ca^{2+} ions into the axon terminal. This influx triggers neurotransmitters release to extracellular space termed synaptic fend. Neurotransmitters interact with post-synaptic cell dendrites, binding to specific membrane receptors, which allow the propagation of the signal (Connaughton, 1995; Rogers, 2011) (Figure 7).

Glutamate is a small molecule, defined as the major excitatory neurotransmitter in the central nervous system (CNS), common in the retina. It is synthesized from ammonium and α-ketoglutarate (a component of the Krebs cycle) and used in the synthesis of proteins, other amino acids, and even other neurotransmitters as GABA (Connaughton, 1995). Glutamate is present in all neurons. Nevertheless, only a few are glutamatergic.

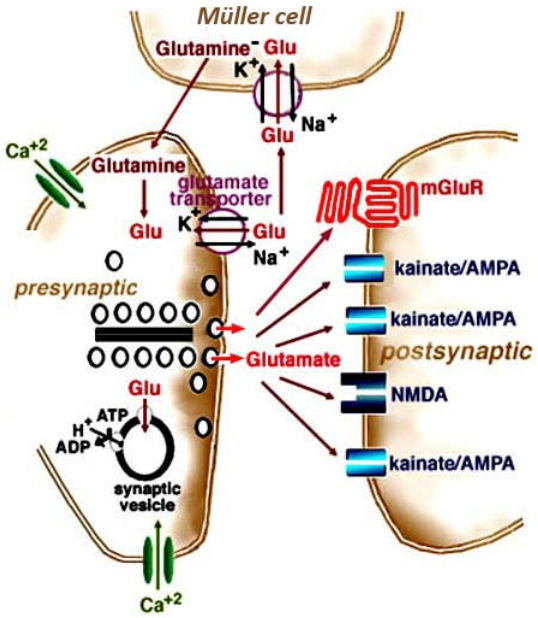


Figure 7. Representative scheme of glutamatergic neurotransmission. Involvement of several types of glutamate receptor, pre-synaptic and post-synaptic cells and also glial cells, such Müller cell, responsible for the uptake and transport of glutamate and its metabolites, such as glutamine (Adapted from Connaughton, 1995).

Involvement of glutamate is essential for the neurotransmission of the visual signal from photoreceptors to bipolar cells and from these to ganglion cells (Fang et al., 2010; Villarroel, 2010) (Figure 8).

After released by the presynaptic cells, this neurotransmitter acts on both Glutamate receptors (Fang et al., 2010).

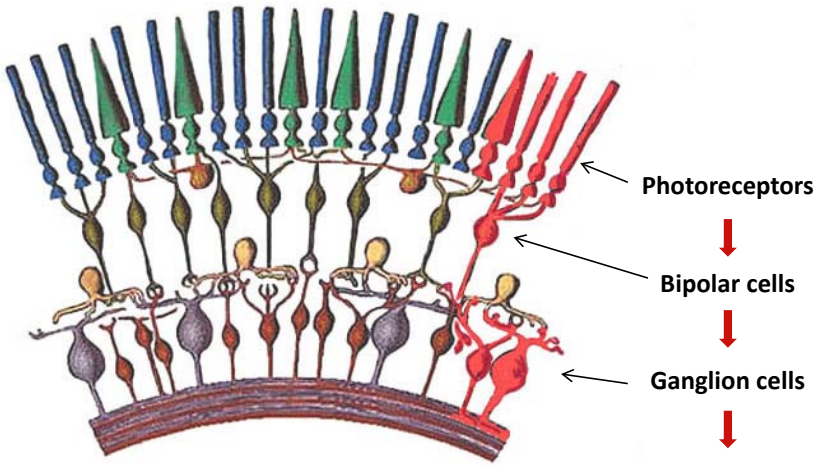


Figure 8. View of glutamate neurotransmission pathway in the retina. (Adapted from Connaughton, 1995).

1.4.1. Glutamate receptors

Glutamate receptors are transmembrane proteins that bind to their specific ligand, glutamate, in the extracellular portion (Fang et al., 2010). These receptors can be divided into two distinct groups: the multimeric fast-acting ligand-gated ion channels, ionotropic receptors, and the slower acting metabotropic receptors, which define single polypeptides linked to G-proteins that may be coupled to an ion channel or other cellular functions via an intracellular second messenger cascade (Connaughton, 1995; Meldrum, 2000).

Several studies allowed the subdivision of ionotropic glutamate receptors, based on their electrophysiological, pharmacologic, and molecular properties like agonist selectivity into two types. Thus, N-methyl-D-aspartate (NMDA) receptors are highly permeable to Ca^{2+} (Connaughton, 1995; Santiago, 2006), when binding to glutamate and have NMDA as their

selective agonist and mainly the non-NMDA receptors, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors and kainate (KA) receptors (Connaughton, 1995; Meldrum, 2000; Santiago, 2006), with respective agonists AMPA and Kainate. Glutamate binding onto a non-NMDA receptor, such AMPAR and KAR, opens cation channels more permeable to sodium (Na^+) and potassium (K^+) ions than Ca^{2+} (Connaughton, 1995). In addition, glutamate receptor agonists and antagonists are allowed to bind onto glutamate receptors, since they are structurally similar to glutamate.

For instance, cyclothiazide (CTZ) is known as positive allosteric modulators of non-NMDA-type glutamate receptors, that delays potentiation of current amplitude and increases agonist affinity**,** blocking AMPA receptor desensitization. CTZ also induces a marked lengthening of channel openings (Fucile, 2006). A recent study showed that, in the presence of CTZ, AMPARs have higher probabilities in saturating and sub-saturating glutamate (Prieto and Wollmuth, 2010).

1.4.2. Glutamate excitotoxicity in DR

Glutamate excitotoxicity leading to neurodegeneration is triggered by elevated release of glutamate levels or insufficient glutamate clearance. This altered clearance may be due to overactivation of NMDAR, with subsequent increase of cytosolic calcium, overexpressed in DM-STZ rat receptors (Fang et al., 2010; Kroemer et al., 2005; Villarroel, 2010).

Glutamate excitotoxicity is thought to contribute to several neurodegenerative disorders, including Alzheimer's disease, glaucoma and, recently described, diabetic retinopathy (Fang et al., 2010; Santiago, 2006; Villarroel, 2010).

While evidences of elevated levels of glutamate have been described in the retinas and vitreous of experimental models of diabetes and diabetic patients with PDR, there is no information in the earlier stages of DR (Villarroel, 2010).

Abnormalities in glutamate metabolism in the retinas of diabetic rats were described, namely alterations in the conversion of glutamate to glutamine and transamination to alpha-ketoglutarate, which reduced both glutamate pathway flux and caused glutamate accumulation. Therefore, high levels of glutamate in DR are also related with macroglial dysfunction, since diabetes-induced oxidative stress lead to impairment in the glutamate transporter of Müller cells (Santiago, 2006; Villarroel, 2010).

It was recently shown in diabetic rat retinas and in retinal cell cultures that high glucose induced membrane depolarization, triggering the release of D-aspartate, a marker of glutamate transmission (Santiago et al., 2006).

Glutamate excitotoxicity induce neural apoptosis by triggering a caspase-3-dependent pathway and a caspases-independent pathway involving calpain and mitochondrial apoptosis inducing factor (Zhang and Bhavnani, 2006).

1.5. Programmed cell death

Programmed cell death (PCD) defines an important physiological and polymorphic phenomenon of cell death in the multicellular organisms and tissues, such as retina (Golstein et al., 2003), eliminating unneeded cells during development, like continuous removal of cancer cells and removal of self-recognizing cells of immune system (Sloviter, 2002). In excess, it may also be cause certain pathologies (Golstein et al., 2003).

Programmed cell death (PCD) is characterized by stereotypical series of morphological and biochemical changes (Leist and Jaattela, 2001). It can be apoptotic, vacuolar or necrotic (Golstein et al., 2003).

It has also been shown that PCD can diversify into other several pathways, depending of the role played by caspases and the necessity of them.

1.5.1. Apoptosis

The main pattern of PCD was observed in different types of retinal cells, appearing prominently in ganglion cells (Fischbarg, 2006; O'Leary et al., 1986). Based on its morphology, apoptosis is characterized by rounding-up the cell, retraction of pseudopodes, pyknosis, condensed chromatin, DNA fragmentation and plasma membrane blebbing. This features may be accompanied or not by caspases activation (Kroemer et al., 2005). Although considered as well characterized, apoptosis continue to be complex and not completely understood (Golstein et al., 2003).

Before any description of this concept, it was reported the presence of pyknotic bodies in retinal neural cells from diabetic patients, through experiments with histological sections of retinas (Bloodworth, 1962; Wolter, 1961). Several studies observed cell death in a variety of conditions, where they exhibited morphological features suggestive of process of cellular destruction (Sloviter, 2002).

Since neurons are unable to proliferate, apoptosis lead to cell loss and further, chronic neurodegeneration (Barber et al., 2011), described in DR (Barber, 2003). Recently, apoptosis was also found by TUNEL assay in retinal neurons from STZ-treated rats and retinal endothelial cells (Hammes et al., 1995), suggesting an apoptotic increase induced by diabetes. Anti-apoptotic therapies are being developed for neuroprotection (Sloviter, 2002).

1.5.1.1. Caspases

Caspases, cysteine aspartate-specific proteases, have the ability to specifically cleave their substrates after aspartic acid residues (Fiers et al., 1999).

Based on their function, structure and substrate specificity, human caspases have been implicated in cell processes, namely apoptosis, inflammation, cell cycle progression and cell survival. According to several evidences, 14 mammalian caspases (Friedlander, 2003) have been

identified and further, mainly classified in three groups: inflammatory caspases (caspase-1, -4, -5, -12), characterized by the presence of a pro-domain at the N-terminus, the caspase recruitment domain (CARD); initiator caspases, characterized by the presence of either CARD (caspases-2 and -9) or death effector domains (DED) (caspases-8 and -10), and effector caspases (caspase-3, -6 and -7), responsible for the downstream execution phase associated with the cleavage of cellular components during apoptosis (Alenzi et al., 2010; Degterev et al., 2003; Friedlander, 2003).

Caspases exist as inactive pro-enzymes that can be activated by various mechanisms, under stress condition. Caspases play a central role in mediating apoptotic cell death in a variety of cell, including neurons (Leal et al., 2009; Zou et al., 1999), since they are involved in a proteolytic cascade (El-Asrar, 2004).

It is known that apoptosis can occur through two major pathways, an extrinsic pathway, also termed the death receptor pathway, or an intrinsic pathway (the mitochondrial pathway).

1.5.1.2. Extrinsic pathway

One of the caspases cascades, the death receptor-signaling pathway, is initiated by activation of death receptors, such as TNF receptor-1 (TNFR1), Fas (also known as CD95 or Apo-1), and TNF-related apoptosis-inducing ligand (TRAIL) receptor, at the plasma membrane. Through its ligand-induced receptor trimerization, Fas, belonging to the TNF-α receptor superfamily, recruits several proteins with a common death domain, including the receptor-associated death domain protein FADD, receptor-interacting protein (RIP) and/or Daxx, promoting the formation of a death-inducing signaling complex (DISC) (El-Asrar, 2004; Leist and Jaattela, 2001). This complex triggers the activation of apical caspases-8, through its interaction with DED, leading to the activation of downstream effectors, such as caspase-3, -6 and -7 (Friedlander, 2003). It has also been described in recent studies the ability of TNF and Fas to induce another type of PCD, necrosis-like PCD (Leist and Jaattela, 2001).

1.5.1.3. Classical pathway

The initiation of this caspase-dependent pathway is triggered by the release of cytochrome *c* from the mitochondrial intermembrane space, which binds to APAF-1 and is essential to recruit ATP and procaspase-9 to form the apoptosome complex. Furthermore, this DISC-like complex activates caspases-9 and subsequent effector caspases, such as caspase-3 (Zou et al., 1999).

For the past decade, some authors described a complement mechanism in which released mitochondrial proteins, such as DIABLO/Smac, play an important role by removing caspase-inhibitory factors (IAPs) before the execution caspases become completely active leading to the final apoptotic phase, where the exposure of phosphatidylserine, degradation of DNA repair enzymes and structural elements, activation of chromossomal endonucleases, chromatin condensation and viability loss occur (Leist and Jaattela, 2001).

1.5.1.4. Intrinsic pathway

Stimulated by intrinsic signals like DNA damage, mitochondria can also trigger apoptosis. Initiation of this pathway leads to membrane permeabilization changes and translocation of proapoptotic Bcl-2-related proteins, such as Bax, Bak, Bid and Bim. This Bcl-2 family also includes anti-apoptotic proteins Bcl-2 and Bcl-xL, which block many signals, regulating the ratio of death and survival signals (Leist and Jaattela, 2001).

For Intrinsic pathway, three death pathways are triggered downstream of mitochondrial changes.

1.5.2. Apoptosis-inducing factor (AIF)

Apoptosis-inducing factor (AIF), a 62 kDa flavoprotein, is first synthesized as a nonapoptogenic precursor in cytoplasm and then imported to mitochondrial intermembrane space, where is normally located (Leist and Jaattela, 2001). AIF is mainly required to cell survival,

proliferation and mitochondrial integrity. It regulates the mitochondrial respiratory chain complex I, through its NADH-oxidase domain, and also participates in redox metabolism (Hangen et al., 2010). AIF contribution may vary, depending on the apoptotic insult and/or the cell type.

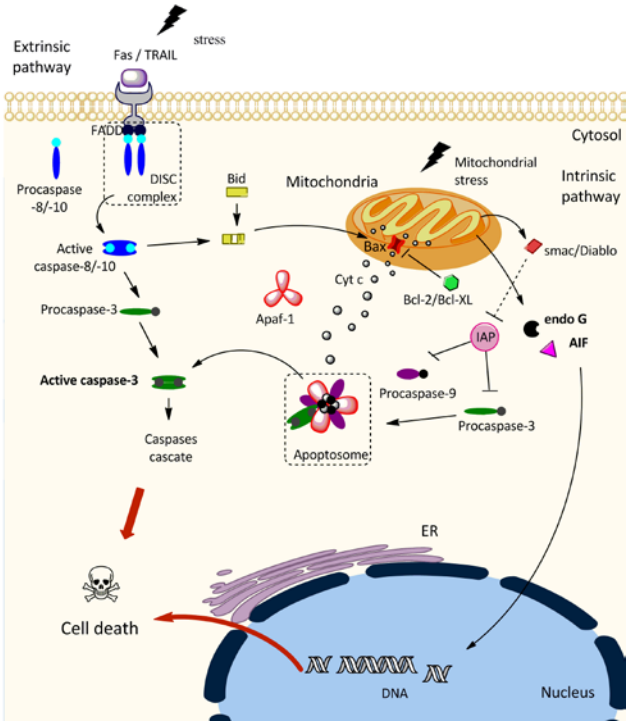


Figure 10. View of the caspases-dependent and -independent PCD pathways. Caspases-dependent pathway is triggered by cell-death receptors, such Fas, which connect with its ligand FADD, activating apical caspases-8 (extrinsic pathway), although these caspases may also be involved in intrinsic pathway, triggered by mitochondrial stress, contributing to the release of cytochrome c and caspase-9 activation. Both extrinsic and intrinsic pathways converge in activation of effector caspases, such caspases-3/-7 and subsequent downstream caspases cascade leading with apoptosis. Most death signals can be inhibited by anti-apoptotic member of the Bcl-2 family and/or by survival kinases. Mitochondria may also be responsible for a caspases-independent pathway that involve AIF and endonucleases release (apoptotic-like PCD) and for ROS/Ca^{2+}-induced necrotic-like PCD. Fas, death-receptor family protein; FADD, Fas associated death domain; DISC, death-inducing signaling complex; PCD, programmed cell death; AIF, apoptosis-inducing factor; Apaf-1, apoptotic protease-activating factor-1; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-associated X-protein; Bcl-2, B-cell lymphoma-2; Bcl-XL, Bcl-2-like protein; Bid, BH3-interacting-domain death agonist; endo G, endonuclease G; IAP, inhibitor of apoptosis; ROS, reactive oxygen species; Smac, second mitochondria-derived activator of caspases.

When apoptosis induced by cytotoxic stimuli is not mediated by caspase activation, it was suggested that, endonucleases and AIF, are released from the intermembrane space and translocate to nucleus. At this level, these molecules triggered apoptosis-like PCD leading to nuclear changes, such as DNA fragmentation and peripheral condensation of chromatin, with

subsequent death induction (Li et al., 2001; Susin et al., 1999). It was also demonstrated that, undergoing its translocation, AIF can promote phosphatidylserine exposure at plasma membrane surface (Susin et al., 1999).

Caspases-independent mechanism mediated by AIF mito-nuclear translocation is involved in high glucose-induced apoptotic neural retinal cell death (Santiago et al., 2007).

Some genetic studies reported that AIF deletion prevented all the PCD events during early development, suggesting a control role played by this factor (Leist and Jaattela, 2001).

1.5.3. Apoptosis like-PCD

As a form of PCD, apoptosis like-PCD is characterized before cell lysis by moderate chromatin condensation, into different shapes, and displayed phagocytosis markers on the plasma membrane. That form includes the classical apoptotic pathway independent from caspases activation. Zeiosis, the dynamic plasma membrane blebbing of a dying cell, can also occur at this stage (Golstein et al., 2003; Leist and Jaattela, 2001).

1.5.4. Necrosis like-PCD

Negatively defined as a type of cell death without apoptotic signs, necrosis like-PCD refers to a form of PCD where chromatin condensation can be moderate or may not occur. Other apoptotic features, such as phosphatidylserine externalization and mitochondria and cell swelling can also vary from those observed in apoptosis like-PCD (Golstein et al., 2003; Kroemer et al., 2005; Leist and Jaattela, 2001). ROS, inhibition of poly (ADP) ribose polymerase (PARP) or some mutated signaling molecules appeared to trigger necrosis like-PCD (Leist and Jaattela, 2001).

Necrotic PCD pathway

This mitochondrial pathway can occur without necessarily activating caspases. Several studies described a TNF-induced necrosis-like PCD. This death pathway depends on the kinase

activity of the receptor-interacting protein (RIP), responsible for the initiation step and is mediated by mitochondria-derived ROS formation and Ca^{2+} (Leist and Jaattela, 2001).

1.6. Bile acids

Bile acids, the major constituents of bile, are molecules produced in the liver, primarily from the cholesterol metabolic pathway, and secreted into the intestine, where they play crucial biological roles, such as solubilization of lipids in the intestinal lumen, among many others (Amaral et al., 2009; Thompson, 1996).

Certain bile acids are cytotoxic molecules implicated in increased cell proliferation and cancer development in the intestinal tract and/or cell death by necrosis and apoptosis. Although mechanisms by which bile acids induce toxicity are not completely understood, studies in models of cholestasis and hepatocyte injury showed that an amount of bile acids triggers apoptosis by activating death receptors, inducing oxidative damage and also mitochondrial dysfunction. While excessive apoptosis lead to T-cell depletion, neurodegeneration, or hepatocellular degeneration, impaired apoptosis is associated with oncogenesis, autoimmune diseases and persistent infections (Amaral et al., 2009; Rodrigues et al., 1998).

1.6.1. Induction of Apoptosis by bile acid

Cytotoxic bile acids induce apoptosis in hepatocytes, which is normally characterized by swelling of hepatocytes, disruption of plasma membrane integrity, and release of intracellular constituents. Consequently, loss of hepatocellular function leads to necrosis (Amaral et al., 2009; Rodrigues et al., 1998; Thompson, 1996).

Several studies suggested that hydrophobic bile acid were responsible for cell damage, since those molecules act as detergents on cell membrane.

Bile acids also induce generation of oxidative stress through NADPH oxidase activation and/or interaction with mitochondria. Therefore, ROS formation leads to Fas activation (Amaral et al., 2009).

1.6.2. Cytoprotective bile acids

Changes in chemical structure allow bile acids to be hydrophobic or hydrophilic (Figure 11). Previous studies suggested a relation between this evidence and the different mechanism of action. Regarding this fact, hydrophobic bile acids are toxic compounds leading to liver cell death, while hydrophilic bile acids may be cytoprotective (Amaral et al., 2009; Thompson, 1996).

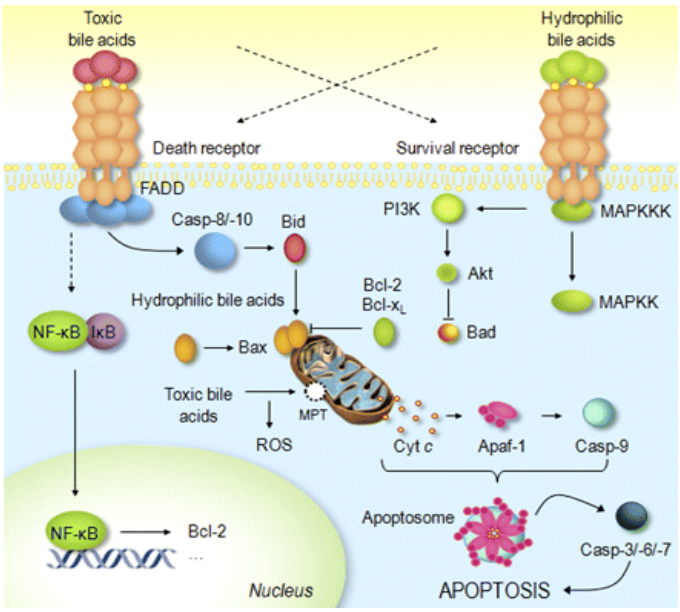


Figure 11. Apoptotic and Survival pathways induced by bile acids. Toxic bile acids induce apoptosis mediated by death receptor pathway whereas cell survival is induced by hydrophilic bile acids triggering Pi3K and MAPkinase survival pathways. FADD, fas receptor-associated death domain, Casp, caspases, Bid, BH3-interacting domain death agonist, Bax, Bcl-2-associated X-protein, Bcl-2, B-cell lymphoma-2, Bcl-XL, PI3K, phosphatidylinositol 3-phosphate kinase, Akt, serine/threonine kinase, MAPKKK, mitogen-activated protein kinase kinase kinase; MAPKK, mitogen-activated protein kinase kinase, ROS, reactive oxygen species, Cyt c, cytochrome c, Apaf-1, apoptotic protease activating factor-1, NF-κB, nuclear factor-kappa B, MPT, mitochondrial permeability transition (Adapted from Amaral et al., 2009).

These molecules are capable to trigger survival signals inhibiting any induced toxicity, reason for being considered modulator agents of intracellular molecular pathways. Protection

against apoptosis is initiated by a survival stimulus triggering transmembrane receptors activation and involves NF-κB, PI3K and MAPK pathways leading with cell survival signaling. Furthermore, extracellular regulated kinase (ERK) signaling triggered by cytoprotective bile acid has been reported as another anti-apoptotic response (Amaral et al., 2009).

1.6.2.1. Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA), constituting 4% of the bile, is a hydrophilic bile acid, thus non-toxic, in contrast of other bile acids (Amaral et al., 2009; Lazaridis et al., 2001). It is used as a drug, approved by the United States Food and Drug Administration, for treatment of certain liver diseases, such primary biliary cirrhosis. The protective role of UDCA is particularly due to its anti-apoptotic effects in the classic mitochondrial pathway, already established in human and rat liver and also observed in non-hepatic cells. Thus, evidenced as a pleiotropic agent (Amaral et al., 2009), UDCA allows membrane stabilization and/or reduces mitochondrial damage by preventing ROS formation and pro-apoptotic Bax translocation from cytosol (Amaral et al., 2009; Botla et al., 1995; Heuman and Bajaj, 1994).

For instance, the cytoprotective role of UDCA and its taurine-conjugated analog TUDCA has been reported in animal models of retinal degeneration (Boatright et al., 2009; Oveson et al., 2011; Phillips et al., 2008).

1.6.2.2. Tauroursodeoxycholic acid

Tauroursodeoxycholic acid (TUDCA) is the major component of bear bile (Fernandez-Sanchez et al., 2011). In humans, it is a hydrophilic bile acid (Boatright et al., 2009; Oveson et al., 2011) produced endogenously at very low concentrations (Colak et al., 2008; Macedo et al., 2008).

This non-toxic bile acid is a derivative formed by the conjugation of UDCA with taurine, which is normally used as a therapeutic agent for the treatment of certain hepatobiliary

disorders (Lazaridis et al., 2001; Macedo et al., 2008). Recent reports have shown that both hydrophilic bile acids, UDCA and TUDCA, can prevent hepatic cytotoxicity through several mechanisms (Macedo et al., 2008). For more than 3000 years, it has been used in Asia for visual disorders treatment (Fernandez-Sanchez et al., 2011).

Furthermore, evidences suggested that TUDCA plays an anti-apoptotic role in neurodegenerative diseases, including in retinal degeneration. Several studies confirmed the anti-apoptotic properties (preventing the translocation of the pro-apoptotic protein Bax to mitochondria) and also proved that TUDCA act as a cytoprotective agent in animal models of Huntington's (Amaral et al., 2009; Keene, 2002) Parkinson's (Duan et al., 2002) and Alzheimer's diseases (Viana et al., 2009) in addition to ischemia (Ishigami et al., 2001) and stroke (Rodrigues et al., 2002) animal models.

TUDCA is a water soluble compound with minimal toxicity, that can be administrated orally or intravenously (Amaral et al., 2009).

Tauroursodeoxycholic acid (TUDCA) appears to modulate mitochondrial membrane perturbation and toxicity, since it inhibits membrane depolarization, Bax translocation from the cytosol to mitochondria and subsequent events such cytochrome c release, activation of downstream caspases and cleavage of PARP (Amaral et al., 2009; Macedo et al., 2008; Rodrigues et al., 1998; Sola et al., 2006; Xie et al., 2002). Thereby, other authors also described that TUDCA regulates cell proliferation, transformation, and death through inhibition of the human transcription factor activating protein-1 (AP-1) formed by Jun, Fos, ATF, or MAF family proteins (Pusl et al., 2008).

Recently, a study described TUDCA as a chemical chaperone of the ER stress in an animal model of liver diseases, since it reduced calcium efflux and caspases activation (Amaral et al., 2009; Xie et al., 2002).

Further, as already reported, TUDCA is believed to act as an antioxidant agent by preventing reactive oxygen species (ROS) generation (Oveson et al., 2011; Rodrigues et al., 1998; Sokol et al., 2005) (Figure 12).

Evidences suggested that TUDCA may be a potential therapeutic agent of type 2 DM (Amaral et al., 2009).

Glutamate-induced toxicity increased cytochrome c release and caspase activation leading to apoptosis in rat cortical neurons, although, TUDCA prevented this apoptotic threshold (Castro, 2004). Also, amyloid-β-peptide-induced apoptosis is reduced by TUDCA in neuronal cell culture (Sola et al., 2006).

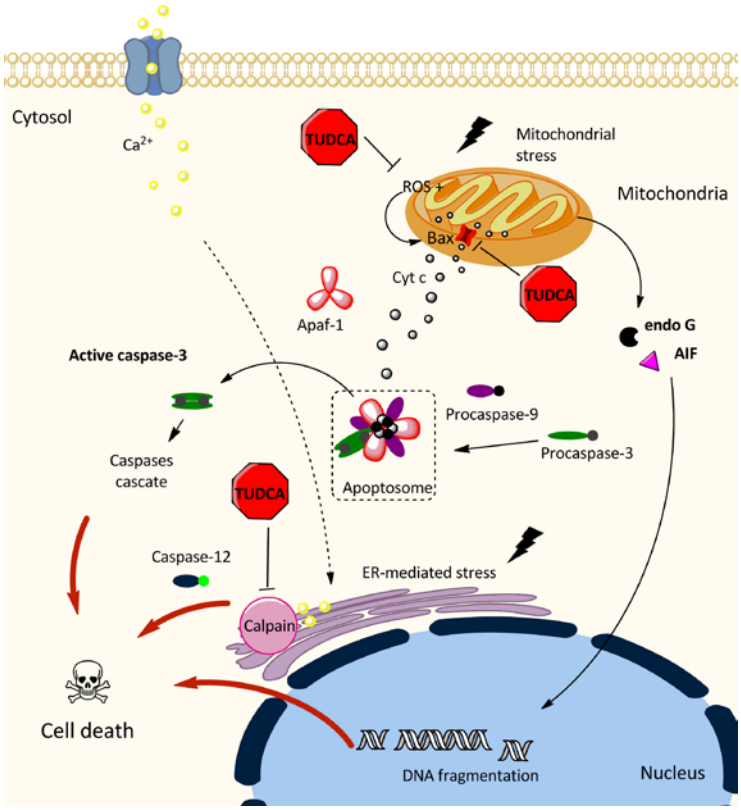


Figure 12. TUDCA protective role against ROS generation, ER-mediated stress and release of pro-apoptotic molecules from mitochondria..

The protective role of TUDCA has been investigated in animal models of neurological disorders, including Huntington's disease. Evidences showed that in cultured neurons incubated

with 3-nitropropionic acid, administration of TUDCA induced a reduction of the mitochondrial perturbations undergoing apoptosis (Rodrigues et al., 2000). Thereby, in a transgenic mouse model of Huntington's disease, Keene and colleagues demonstrated that TUDCA prevents striatal degeneration and improves locomotor and sensory-motor deficits (Keene, 2002).

Research using TUDCA administration was extended a transient focal cerebral ischemia rat model (Rodrigues et al., 2002), where TUDCA reduced apoptosis, infarct volume, and inhibited neurobehavioral impairment, while in a collagenase-induced hemorrhagic model of stroke, supporting the stability of the mitochondrial membrane and inhibiting caspases activation, TUDCA could reduce the degree of brain injury and improved neurologic performance (Amaral et al., 2009; Rodrigues, 2003).

Effects of TUDCA were also reported in experimental models of Parkinson's disease. Thus, this compound can improve the survival and function of nigral transplants in a rat model (Duan et al., 2002), may preserve dopamine neuron survival in ventral mesencephalic tissue cultures and within the transplants and prevent mitochondrial dysfunction in an *Caenorhabditis elegans* model (Ved et al., 2005).

Recently, it has been documented that in the *rd10* retinitis pigmentosa mouse model, TUDCA preserves photoreceptor structure and function at a critical time-point of degeneration (Phillips et al., 2008). Further research allowed to show reduction of photoreceptors apoptosis and preservation of the a- and b-waves of electroretinograms (ERG), in both genetic mouse model Pde6brd10 mice (*rd10*) and in wild-type mice subjected to light-induced retinal degeneration (Fernandez-Sanchez et al., 2011).

Concerning its anti-apoptotic role, TUDCA has also been used in experimental models of Alzheimer's disease. For example, in a PC12 neuronal cells study, TUDCA was shown to modulate upstream targets of the mitochondria, including the E2F-1/p53/Bax apoptotic pathway (Ramalho et al., 2004) and prevented abnormal changes in the conformation of tau protein (Amaral et al., 2009).

Since it seems to be well-tolerated, some endogenous antioxidant defense system components, like bile acids, have been widely utilized as therapeutic agents, instead of using enzymes, known to detoxify reactive oxygen species. It is important to note that those constituents of bile can either damage or protect cells.

It was established that increased serum levels of bilirubin, protect rods and cones from oxidative damage (Oveson et al., 2011).

In regard to its antioxidant properties, it was confirmed that TUDCA provides benefit from excessive light exposure in models of RP (Boatright et al., 2009; Phillips et al., 2008) by reducing oxidative stress. Although high doses of TUDCA are required, the effects are similar to those seen with bilirubin.

Some authors believe that part of the protective effects of TUDCA may be due to its conjugated taurine, which is thought to inhibit the formation of the apoptosome (Takatani et al., 2004; Udawatte et al., 2008).

The implications regarding the findings with TUDCA are also important. It is inexpensive, well tolerated, and may have a similar safety profile as the United States Food and Drug Administration approved nontaurine-conjugated analog UDCA (Oveson et al., 2011).

Therefore, its clinical application to human diseases outside of liver has been tested and authors from several studies suggested TUDCA as a good candidate for inclusion in an antioxidant test regimen (Amaral et al., 2009; Oveson et al., 2011).

1.7. Objectives of the study

The bile acid tauroursodeoxycholic acid (TUDCA) was shown to modulate caspase-dependent cell death and it was described as having antioxidant properties. Therefore, TUDCA is a potential drug for the treatment of neurodegenerative diseases such Parkinson's disease and Alzheimer's disease. Few studies in the retina reported that TUDCA prevents loss of structure and function of photoreceptors, which would lead to degeneration in both *rd10* and P23H rat models of RP.

Diabetic retinopathy has characteristics of a neurodegenerative disease and TUDCA anti-apoptotic and antioxidant properties have never been studied. Thus, the main aim of this work was to investigate the potential neuroprotective role of TUDCA in the toxicity induced by elevated glucose concentration or by overactivation of ionotropic glutamate receptors, in primary cultures of the rat retina. In the model of elevated glucose, cell death is independent of caspase activation, with mito-nuclear translocation of AIF. We studied whether TUDCA inhibits AIF translocation induced by high glucose. In addition, the antioxidant role of TUDCA was addressed.

CHAPTER 2 - Material and Methods

2.1. Material

Tauroursodeoxycholic acid (TUDCA) (Calbiochem), polyvinylidene fluoride (PVDF) membranes, nitrocellulose membranes, rabbit anti-MAP2 antibody, mouse anti-Lamin B antibody, goat anti-dinitrophenol (DNP) antibody (Chemicon) were obtained from Merck Millipore (Merck KGaA, Darmstadt, Germany). Trypsin (USP grade) was purchased from Life Technologies (Carlsbad, California, USA). Kainate and cyclothiazide were from Tocris Bioscience (Ellisvile, USA). 4',6-diamidino-2-phenylindole (DAPI), 2,4-dinitrophenylhydrazine (DNPH) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Bicinchoninic acid (BCA) Protein Assay kit was purchased from Pierce (Pierce Biotechnology, Rockford, Illinois, USA). Protease inhibitor cocktail tablets complete-mini were acquired from Roche (Basel, Switzerland). Sodium dodecyl sulfate (SDS) and polyacrilamide were acquired from Bio-Rad Laboratories (Hercules, CA, USA). Mouse monoclonal antibody anti-Apoptosis Inducing Factor (AIF) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Enhanced chemifluorescence (ECF) substrate, goat anti-mouse, goat anti-rabbit and rabbit anti-goat alkaline phosphatase-conjugated secondary antibodies were purchased from GE Healthcare (Chalfont St. Giles, UK). Secondary antibodies Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 594 goat anti-mouse and the intracellular fluoroprobe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were obtained from Molecular Probes (Life Technologies, Invitrogen, Paisley, UK).Glycergel mounting medium was from DAKO (Glostrup, Denmark). All other reagents were acquired from Sigma-Aldrich Corporation (St. Louis, MO, USA).

2.2. Methods

2.2.1. Primary cultures of rat retinal neural cells

Procedures involving animals were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) guidelines for the use of animals in vision research. Retinal cell cultures were prepared from 3- to 4-day-old Wistar rat pups after decapitation, as previously described (Santiago et al., 2007). The retinas were dissected using a light microscope, in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS, in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, 5 glucose; pH 7.4). The retinas were digested with 0.1% trypsin (w/v) in HBSS for 12 min at 37 °C. After dissociation, the cells were pelleted by centrifugation for 1 minute at 12800 x *g* and resuspended in Eagle's minimum essential medium (MEM) supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were plated at a density of 2.0×10^6 cells/cm^2 on 24-well plates for MTT viability assay, on 12-well plates for ROS detection, on 60 mm Petri dishes and 6-well plates for protein extraction, and on glass coverslips for annexin V staining, TUNEL staining and immunocytochemistry, all coated with poly-D-lysine (0.1 mg/ml). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2/air. After 2 days in culture, the cells were incubated with 25 mM D-glucose (yielding a total 30 mM glucose) or with 25 mM D-mannitol (plus 5 mM glucose), which was used as an osmotic control, and maintained for further 7 days. The concentration of glucose in control conditions was 5 mM. TUDCA (100 µM) was added each 2 days, beginning at day 2.

For neurotoxicity experiments, cells were incubated with kainate (KA; 100 µM) and 30 µM cyclothiazide (CTZ, which prevents the desensitization of AMPA and KA receptors) (Patneau et al., 1993) 24 h before the experiment. TUDCA, in these conditions, was added 1 h before KA and CTZ incubation.

2.2.2. Assessment of cell viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used for the assessment of cell viability. MTT, when taken up by living cells, is converted from a yellow- to a water-insoluble blue-colored precipitate by cellular dehydrogenases (Mosmann, 1983). Briefly, cells were washed with Krebs buffer (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1.4 $CaCl_2$, 6 glucose, 10 HEPES; pH 7.4), and then MTT (0.5 mg/ml), previously solubilized with pre-warmed Krebs buffer, was added and cultures were incubated for 1 h at 37 °C in the incubation chamber. After incubation, the medium was removed and the precipitated dye was dissolved in 0.04 M HCl in isopropanol. The extension of the reduction was quantitated colorimetrically (absorbance at 570 nm, with a reference filter at 620 nm).

2.2.3. Terminal transferase dUTP nick-end labeling (TUNEL) assay

Apoptotic cells were detected using a TUNEL assay, which labels the fragmented DNA (Saraste, 1999). The TUNEL assay was performed in cultured retinal neural cells with horseradish peroxidase detection (TUNEL–HRP), following the instructions provided by the manufacturer (Promega). Cells were washed in phosphate-buffered saline (PBS) (in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4) and fixed in 4% paraformaldehyde with 4% of sucrose for 30 min at room temperature. After washing, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. The cells were incubated for 10 min with equilibration buffer [200 mM potassium cacodylate (pH 6.6), 25 mM Tris-HCl (pH 6.6), 0.2 mM DTT, 2.5 mM cobalt chloride, and 0.25mg/ml BSA], and then incubated with biotinylated nucleotides and Terminal deoxynucleotidyl Transferase (TdT) in equilibration buffer, for 1 h at 37 °C, in a humified chamber. Cells were rinsed with 2x saline-sodium citrate (SSC) buffer (17.54 mg/ml NaCl and 8.82 mg/ml sodium citrate, pH 7.2) to remove unincorporated nucleotides and endogenous peroxidases were blocked with 0.3% hydrogen peroxide for 5 min at room temperature. Cells were then incubated with HRP-labeled streptavidin for 30 min. The HRP was reacted with 3,3́ -

diaminobenzidine tetrahydrochloride (DAB) to develop a brown precipitate that was easily observed in cell cultures with a microscope. Cells were then washed several times with deionized water, air-dried for 24 h and mounted with Entellan (Merck). The preparations were visualized using a Zeiss Axioshop 2 microscope, and at least 10 random fields were counted in each preparation. Images were acquired with a photomicrograph system (Cool Snap HQ digital camera; Roper Scientific, Tucson, AZ, USA).

2.2.4. Annexin V staining

Annexin V, a 35.8 kDa protein, binds with high affinity to phosphatidylserine (PS), which becomes exposed on the outer leaflet of the plasma membrane during apoptosis (Martin et al., 1995). Annexin V staining, a nonenzymatic assay was performed with a kit from BD Biosciences Clontech (San Jose, CA, USA) following the instructions provided. Briefly, cells were rinsed previously in warm PBS and then incubated with annexin V (20 μg/ml in Tris-NaCl) for 10 min in the dark at room temperature. Cells were washed and mounted on a glass slide. The preparations were visualized with a Zeiss Axioshop 2 Plus microscope, and for each preparation, at least 6 random fields were counted.

2.2.5. Preparation of cellular protein extracts (total, mitochondrial and nuclear fractions)

For total extracts, cells were washed twice with ice-cold PBS and then lysed and homogenized with lysis buffer [150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS) supplemented with complete-mini protease inhibitor cocktail tablets and 1 mM DTT. The lysates were incubated on ice, sonicated (4 pulses, 2 seconds each) then centrifuged at 16100 × *g* for 10 min at 4 °C and stored at -80 °C until use.

For mitochondrial fraction, cells were washed in ice-cold sucrose buffer (in mM: 250 sucrose, 20 HEPES, 10 KCl, 1.5 $MgCl_2$, 1 EGTA; pH 7.4). Cell were lysed in ice-cold sucrose buffer

supplemented with 1 mM DTT, 100 µM PMSF, 1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antiparin, and 1 µg/ml pepstatin A. Lysates were homogenized and centrifuged (4 °C) at 500 × *g* for 12 min to pelletize the nucleus and cell debris. The supernatant (total fraction) was further centrifuged at 12000 × *g* for 20 min at 4 °C, and the resulting pellet (mitochondrial fraction) was resuspended in supplemented sucrose buffer. The samples were stored at −80 °C until use.

The preparation of nuclear extracts was performed as described previously (Santiago et al., 2007), cells were washed in ice-cold PBS and cell extracts were obtained with buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA] supplemented with protease inhibitors, 1 mM DTT and 0.4% Nonidet P-40. The lysates were placed on ice for 30 min and then centrifuged at 16100 x *g* for 5 min at 4 °C. The supernatant (cytosolic fraction) was discarded and the pellet was resuspended in buffer B [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA] supplemented with protease inhibitors, 1 mM DTT and 10% glycerol, and incubated for 60 min on ice, with vortex each 20 min. Then, the samples were sonicated and centrifuged at 16100 x *g* for 5 min at 4 °C. The supernatant containing the nuclear fraction was collected, and then stored at −80 °C until use.

2.2.6. Protein quantification

The protein concentration was measured by the colorimetric bicinchoninic acid (BCA) (Pierce, Rockford, USA) assay (Brenner and Harris, 1995). This method combines the biuret reaction (reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium) with the highly sensitive and selective colorimetric chelation of BCA with the cuprous (Cu^{+}) cation, leading with a purple colored final product. This BCA/copper complex exhibits a strong absorbance at 562 nm directly proportional to the protein concentration. Samples were diluted in deionized water and incubated with BCA reagent for 30 min at 37 °C, in a 96-well plate. The content of each well was mixed and the absorbance of each sample was read at 570 nm in a microplate reader (Synergy HT; Biotek, Winooski, USA). The protein standard curve was prepared using BSA (2.0mg/ml) in

MiliQ water, and the absorbance of BSA standard samples was used to determine protein concentration in samples.

2.2.7. Western blot analysis

Equivalent amounts of protein (40 µg or 15 µg for AIF detection from mitochondrial fraction or nuclear fraction, respectively were used for Western blot analysis, after adding 6× concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and heating the samples for 5 min at 95 °C. Proteins were separated on 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) for 60 min at 160 V in Tris-Bicine buffer (25 mM Tris, 25 mM Bicine, 1% SDS, pH 8.3) and transferred electrophoretically to PVDF membranes in CAPS-methanol buffer (10mM CAPS, pH 11, 10% methanol), for 90 min at 750 mA (4 °C). The membranes were blocked for 1 h at room temperature, in Tris-buffered saline (in mM: 137 NaCl, 20 Tris–HCl; pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% skimmed milk. The membranes were incubated with primary antibody (mouse anti-AIF, 1:1000) overnight at 4 °C. After washing for 1 h in TBS-T, the membranes were incubated for 1 h at room temperature with an alkaline phosphatase-linked secondary antibody (rabbit anti-mouse IgG, 1:10000) in TBS-T with 1% skimmed milk. The membranes were washed with TBS-T and then processed for detection of AIF using the ECF substrate. Fluorescence was detected on an imaging system (Typhoon FLA 9000, GE Healthcare, Chalfont St. Giles, UK) and quantification was performed using Image Quant 5.0 software (Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden).

2.2.8. Detection of oxidized proteins

2.2.8.1. Sample derivatization for protein carbonyl formation

Protein carbonyl formation was used as an indicator of oxidized proteins (Nakamura and Goto, 1996). The protein concentration of total cellular extracts was adjusted to 1.5 mg/ml with

lysis buffer (same used for Western blotting samples), and equal amounts of protein were mixed with equal volume of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 10% of trifluoroacetic acid (TFA) and incubated for 15 min, at room temperature. The reaction was stopped with trichloroacetic acid (TCA) during 10 min, at room temperature. After centrifugation at 16100 x *g* for 5 min, the supernatant was discarded and the pellet was washed twice with ethylacetate:ethanol (1:1) to remove free DNPH. The pellet was solubilized with Laemmli sample buffer (15.6 mM Tris-HCl pH 6.8, 2.5% glycerol, 0.5% SDS, 1.25% 2-β-mercaptoetanol, 0.01% bromophenol blue) followed by a brief sonication (4 pulses, 2 seconds each) and stored at -20 °C, for later use.

2.2.8.2. Dot blot for oxidized carbonyl in proteins

The DNP binding sites of the oxidized proteins were specifically detected using a goat anti-DNP antibody (1:2,000, in TBS-T solution with 5% low-fat milk) by dot blot. Briefly, 2 µl of the protein carbonyl-DNPH derivatives were loaded on a nitrocellulose membrane at each dot. Membranes were then incubated at 37 °C for 40 min, to fix proteins to the membrane. After blocking the membrane with TBS-T containing 5% (w/v) low-fat milk, during 1 h at room temperature, the membranes were incubated with goat anti-DNP primary antibody for 1h at room temperature. The membranes were washed, 6 times for 5 min, in TBS-T, and then incubated for 1 h at room temperature, with an alkaline phosphatase-linked rabbit anti-goat IgG secondary antibody (1:10 000), in TBS-T containing 1% (w/v) low fat milk, followed by washing in TBS-T. Protein immunoreactive dots were then visualized using ECF substrate, and fluorescence was detected on an imaging system (Typhoon FLA 9000, GE Healthcare, Chalfont St. Giles, UK). Digital quantification of the densitometry of the bands was performed using Image Quant 5.0 software (Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden).

2.2.9. Immunocytochemistry

Cells were washed twice with pre-warmed PBS and fixed with 4% paraformaldehyde with 4% sucrose for 10 min at room temperature. Cells were rinsed twice in PBS and then permeabilized with 1% Triton X-100 in PBS for 5 min. After blocking for 1 h with 3% BSA plus 0.2% Tween-20 in PBS, cells were incubated with the primary antibody (anti-TUJ1, 1:500 and anti-AIF, 1:100) for 90 min, at room temperature. Cells were rinsed three times with the blocking solution and incubated with Alexa Fluor 568-conjugated secondary antibody (goat anti-mouse IgG, 1:200) and Alexa Fluor 488-conjugated secondary antibody (goat anti-rabbit IgG, 1:500) for 1h at room temperature in the dark. Cells were washed three times with PBS, and the nuclei were stained with DAPI (1:2000) in PBS for 10 min. Upon rinsing with PBS, the coverslips were mounted on glass slides with Glycergel mounting medium. The preparations labeled were visualized with Leica DM IRE2 confocal microscope.

2.2.10. Generation of intracellular reactive oxygen species (ROS)

Production of ROS was evaluated by changes in the fluorescence intensity resulted from oxidation of the intracellular fluoroprobe 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). Briefly, retinal cultured cells were rinsed in pre-warmed Dulbecco´s PBS (DPBS, 20 X) solution, followed by incubation of 5 µM H_2DCF-DA in DPBS, for 35-45 min at 37 °C. Cells were rinsed twice with DPBS, and then incubated for 15-20 min with the pre-warmed DPBS. Cells were harvested by scraping with DPBS, and an aliquot was saved for protein quantification. The fluorescence intensity was measured in a black, solid bottom, 96 well microplate (Greiner Bio-one GmbH, Frankfurt, Germany) using a fluorescent microplate reader at the excitation and emission wavelengths of 485 nm and 528 nm, respectively.

2.2.11. Statistical analysis

Data are reported as mean ± SEM. Statistical significance was determined using analysis of variance (ANOVA), followed by Dunnett's post hoc test and Bonferroni's non parametric test, as indicated in the figure legends. Differences were considered significant for $p < 0.05$.

CHAPTER 3 - Results

Results

Taking into account several evidences about TUDCA protective role in neurodegeneration, this study allowed to investigate whether TUDCA may prevent neural cell death induced by acute (excitotoxic stimulus) and chronic (high glucose conditions) insults, including its effect in some features contributing and triggering the neurodegenerative process.

3.1. TUDCA prevents retinal neural cell death induced by excitotoxicity

Several studies already established that excitotoxicity induced by elevated levels of glutamate in the retina can lead to retinal damage and neurodegeneration (Shen et al., 2006; Villarroel, 2010). Thus, the potential neuroprotective effect of TUDCA in retinal neural cell death, in an experimental model of excitotoxicity, was further investigated.

Retinal neural primary cells were maintained in culture during nine days. Cells were stimulated with 100 μM KA and 30 μM CTZ (KA+CTZ) (which prevents AMPA receptors desensitization) for 24 h. Since the goal was to study the potential neuroprotective role of TUDCA, cells were incubated with 100 μM TUDCA 1 h prior the excitotoxic stimulus. Cell viability was assessed using the MTT reduction assay.

In the presence of KA and CTZ (Figure 13), MTT reduction decreased significantly to 73.7±6.4% of the control ($p<0.001$). In cells incubated with TUDCA before the exposure to KA and CTZ, MTT reduction was 88.4±3% of control, which was significantly different from KA+CTZ condition ($p<0.05$). TUDCA, by itself, did not cause any significant effect in cell viability.

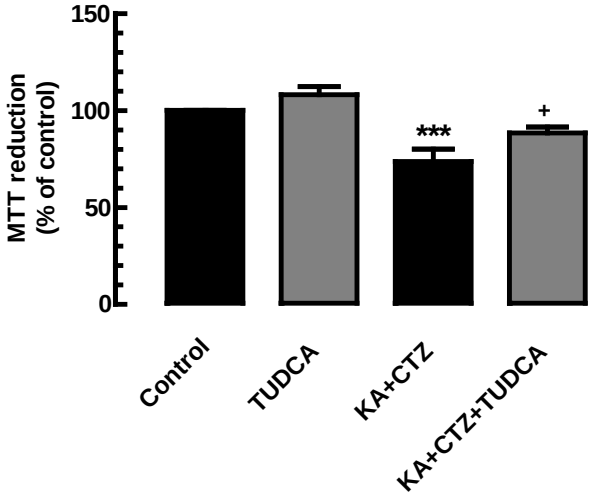


Figure 13. TUDCA reduced the changes on cell viability induced by an excitotoxic stimulus. Cell viability was assessed by MTT reduction in cultured retinal neural cells. Retinal cells were incubated with 100 µM KA and 30 µM CTZ (KA+CTZ), for 24 hours, in the presence or absence of TUDCA (added 1 h before). The results represent the mean ± SEM of four independent experiments performed in triplicate, and are presented as percentage of control. ***p<0.001 significantly different from control, one-way ANOVA followed by Dunnett's post-hoc test; +p<0.05 significantly different from KA+CTZ condition, one-way ANOVA followed by Bonferroni's post-test.

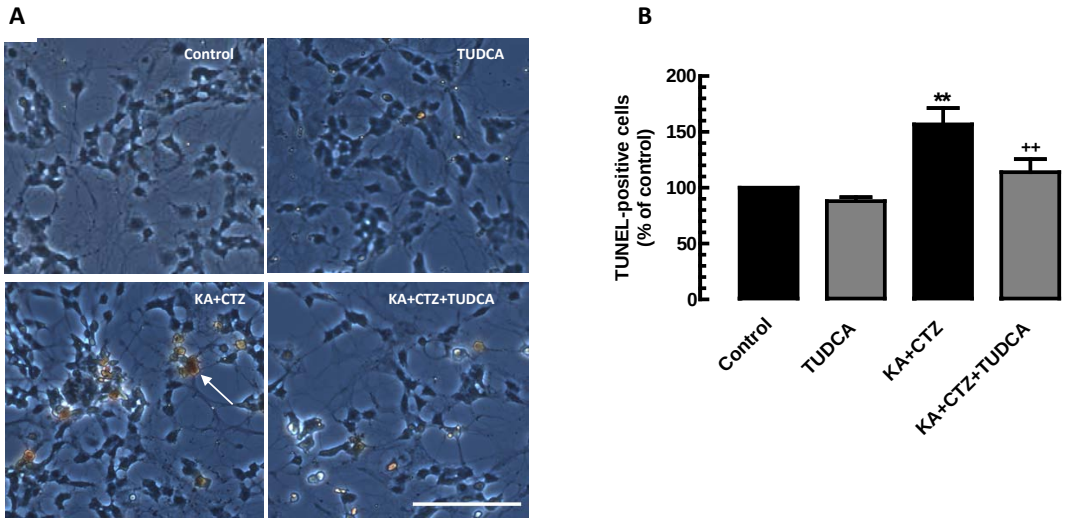


Figure 14. TUDCA prevented excitotoxicity-induced increase in TUNEL-positive cells. Retinal neural cells were stimulated with 100 µM KA and 30 µM CTZ (KA+CTZ) for 24 h, in the absence or presence of 100 µM TUDCA (added 1 h before). Cells were labeled by TUNEL assay. (a) Representative images for each condition. Arrows indicate TUNEL-positive cells. (b) From each condition, 10 random fields were acquired and the number of TUNEL-positive cells was counted. Results represent the mean ± SEM of at least four independent experiments, and are presented as percentage of control. **p<0.01 significantly different from control, one-way ANOVA followed by Dunnett's post-hoc test; ++p<0.01 significantly different from KA+CTZ condition, one-way ANOVA followed by Bonferroni's post-test. Scale bar: 50 µm.

As evidence for apoptotic-like features, TUNEL assay was performed to investigate the effect of TUDCA in cell death induced by excitotoxicity. Stimulation of cells with KA and CTZ for

24 hours significantly increased ($p<0.01$) the number of TUNEL-positive cells to 156.8±14.6% of control (Figure 14). When cells were incubated with TUDCA followed by KA and CTZ (KA+CTZ+TUDCA), there were no significant changes compared to control condition (114±11.6% from control).

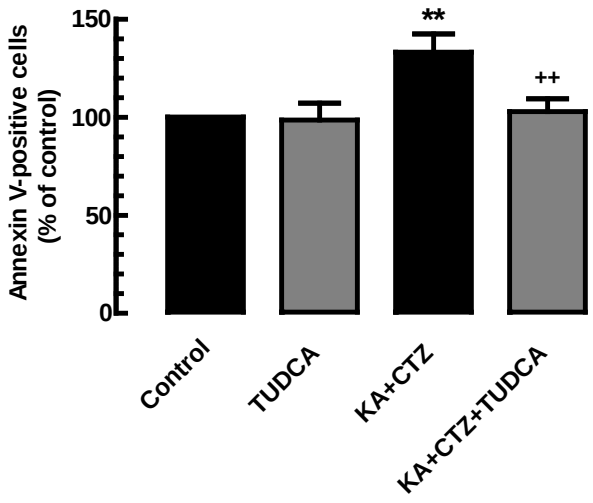


Figure 15. TUDCA reduced the number of annexin V-positive cells induced by excitotoxicity. Retinal cells were incubated with 100 µM KA and 30 µM CTZ (KA+CTZ), for 24 h, in the presence or absence of TUDCA (added 1 hour before). Cells were labeled with annexin V-FITC, and from each condition six random fields were counted. Results represent the mean ± SEM of at least four independent experiments, and are presented as percentage of control. **p<0.01 significantly different from control, one-way ANOVA followed by Dunnett's post-hoc test; ++p<0.01 significantly different from KA+CTZ condition, one-way ANOVA followed by Bonferroni's post-test.

One of the features of cell death is phosphatidylserine (PS) externalization (Brumatti et al., 2008; Fruhwirth and Hermetter, 2008). Since annexin-V binds with affinity to the negatively charged PS, cells were labeled with annexin-V conjugated to fluorescein isothiocyanate (FITC). There was a significant ($p<0.01$) increase in the percentage of annexin-V positive cells in the KA+CTZ condition, as compared to control (133.2±9.2% of control) (Figure 15). The incubation of TUDCA prior stimulation of cells with KA+CTZ completely prevented the increase of annexin-V positive cells induced by KA+CTZ (102.8±6.6% from control). Taken together, these results suggest that TUDCA can prevent cell death induced by kainate, in the presence of cyclothiazide.

3.2. TUDCA protects against retinal neural cell death induced by high glucose concentration

It was already reported that retinal neurons dye by apoptosis in both experimental and human diabetes (Barber et al., 2011). Primary mixed retinal neural cell cultures were prepared and cells were incubated with 30 mM glucose for seven days to mimic chronic hyperglycemic conditions. Since the osmolarity of the culture medium increases when cells are exposed to elevated glucose, cells were incubated with 25 mM mannitol (plus 5 mM glucose) to control for osmolarity. It was already reported that TUDCA appears to be a neuroprotective agent in degenerative diseases such as Huntington's disease and retinitis pigmentosa (Fernandez-Sanchez et al., 2011; Keene, 2002), but its role in models of diabetic retinopathy is not known. Thus, the aim of this study was to investigate the potential neuroprotective role of TUDCA in cultured retinal neural cells exposed to elevated glucose concentration.

Retinal neural primary cells were maintained in culture during seven days and incubated with 5 mM glucose (Control), 30 mM glucose (Glucose), or 25 mM mannitol (Mannitol), in the presence or absence of 100 μM TUDCA, which was added every 2 days beginning at the second day of culture.

MTT reduction assay was used to assess cell viability. High glucose concentration significantly (p<0.05) decreased MTT reduction to 80.7±2.3% of control (Figure 16). When cells were exposed to high glucose concentration in the presence of TUDCA, MTT reduction was not significantly different from control. Incubation of cells with mannitol, with or without TUDCA, did not significantly change MTT reduction, as compared to the control, indicating that the results are not due to increased osmolarity.

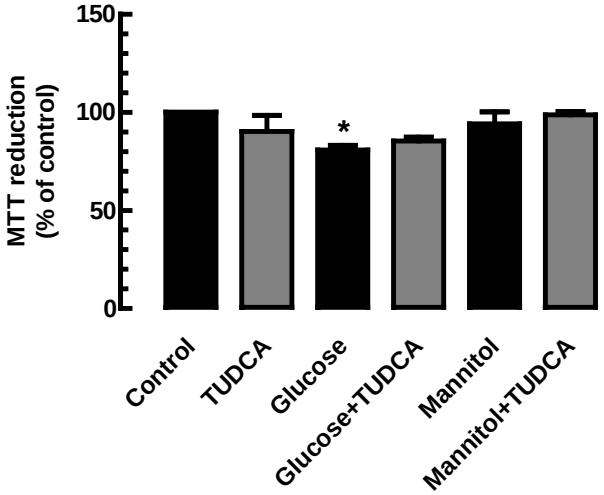


Figure 16. TUDCA reduced the changes on cell viability induced by high glucose concentration. Cell viability as assessed by MTT reduction assay. Retinal cell cultures were incubated with 5 mM glucose (Control and TUDCA), 30 mM glucose (Glucose and Glucose+TUDCA) or 25 mM mannitol (Mannitol and Mannitol+TUDCA) for 7 days, in the absence or presence of 100 µM TUDCA (added every two days). Results represent the mean ± SEM of at least four independent experiments performed in triplicate, and are presented as percentage of control.*p<0.05 significantly different from control; one-way ANOVA followed by Dunnett's post-hoc test.

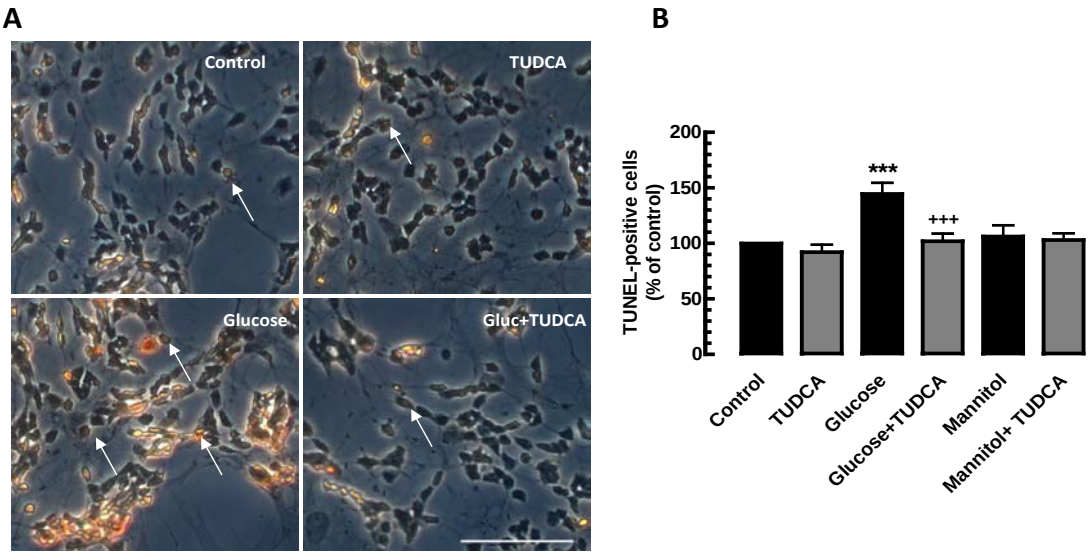


Figure 17. TUDCA prevented the TUNEL-positive cells increase induced by high glucose concentration. Retinal cells were incubated with 5 mM glucose (Control and TUDCA), 30 mM glucose (Glucose and Glucose+TUDCA) or 25 mM Mannitol (Mannitol and Mannitol+TUDCA) for 7 days, in the absence or presence of 100 µM TUDCA (added every two days). Cells were labeled by TUNEL assay. (A) Representative images for each condition. Arrows indicate TUNEL-positive cells. (B) From each condition, 10 random fields were acquired and the number of TUNEL-positive cells was counted. Results represent the mean ± SEM of at least four independent experiments, and are presented as percentage of control. ***p<0.001 significantly different from control with one-way ANOVA followed by Dunnett's post-hoc test; ^{+++}p<0.001 significantly different from glucose condition, one-way ANOVA followed by Bonferroni's post-test. Scale bar: 50 µm.

TUNEL assay was performed to study the potential neuroprotective effect of TUDCA in primary mixed neural cultures exposed to elevated glucose (Figure 17). Similarly as previously reported (Santiago et al., 2007), exposure to elevated glucose for seven days significantly increased the percentage of TUNEL-positive cells, as compared to control (144.6±9.9% of control; p<0.001). In high glucose-treated cells exposed to TUDCA, the percentage of TUNEL-positive cells decreased significantly (p<0.001) to 101.9±6.9% of the control. Cells treated with mannitol, the osmotic control, did not significantly differ from the control (106.3±10.1% of control).

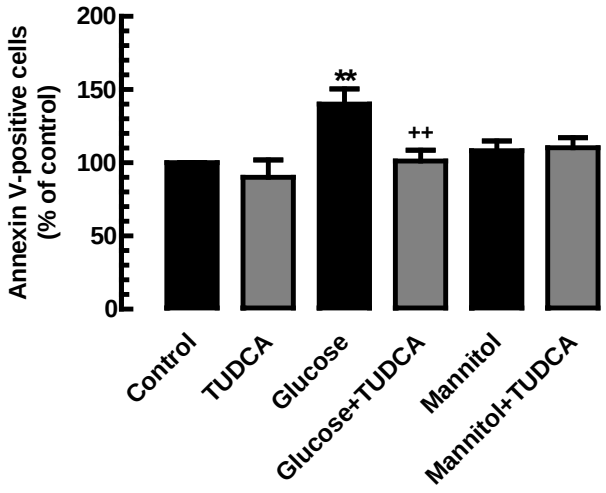


Figure 18. TUDCA reduced the number of annexin V-positive cells induced by elevated glucose concentration. Retinal cells were incubated with 5 mM glucose (Control and TUDCA), 30 mM glucose (Glucose and Glucose+TUDCA) or 25 mM mannitol (Mannitol and Mannitol+TUDCA) for 7 days, in the absence or presence of 100 µM TUDCA (added every two days). Cells were labeled with annexin V-FITC, and from each condition six random fields were counted. Results represent the mean ± SEM of at least four independent experiments, and are presented as percentage of control. ** $p<0.01$ significantly different from control, one-way ANOVA followed by Dunnett's post-hoc test; $^{++}p<0.01$ significantly different from glucose condition, one-way ANOVA followed by Bonferroni's post-test.

Another feature of cell death is the translocation of PS from the inner leaflet of cell membrane to the outer leaflet. Cells were labeled with annexin V-FITC. As described previously (Santiago et al., 2007), high glucose concentration induced a significant increase (p<0.01) in annexin V-positive cells (140.1±10.4% of control) (Figure 18). The presence of TUDCA decreased

significantly ($p<0.01$) the number of apoptotic cells to 101.2±7.3% of control. There were no significant changes in annexin-V positive cells treated with mannitol, in the presence or absence of TUDCA, as compared to control.

It was already documented that in rat retinal mixed cultures exposed to high glucose concentration the increase in cell death is not dependent from caspase activation, but due to AIF translocation to the nucleus (Santiago et al., 2007). In order to study if TUDCA prevents AIF mito-nuclear translocation induced by high glucose concentration, mitochondrial and nuclear protein fractions were prepared and AIF levels were quantified by western blotting (Figures 19A and 19B).

The protein levels of mitochondrial AIF significantly decreased in high glucose-treated cells (35.7±8.3% of the control). When the cells were exposed to elevated glucose concentration in the presence of TUDCA, mitochondrial AIF protein levels increased towards control levels (74.4±8.6% of the control). TUDCA alone did not change significantly the protein levels of AIF.

Regarding the nuclear fraction, AIF protein levels increased significantly when cultured cells were exposed to high glucose concentration (136.3±18.3% of control) (Figure 19B). In the presence of TUDCA, the protein levels of AIF in the nucleus decreased, reaching control values (100.1±4.4% of control).

By immunocytochemistry (Figure 19C), we observed that, in control condition, AIF immunoreactivity was mainly found in the cytosol region. In high glucose conditions, AIF immunoreactivity is mainly found in the nuclear region. In the presence of TUDCA in high glucose conditions, AIF immunoreactivity was similar to the control conditions, confirming previous results obtained by Western blotting.

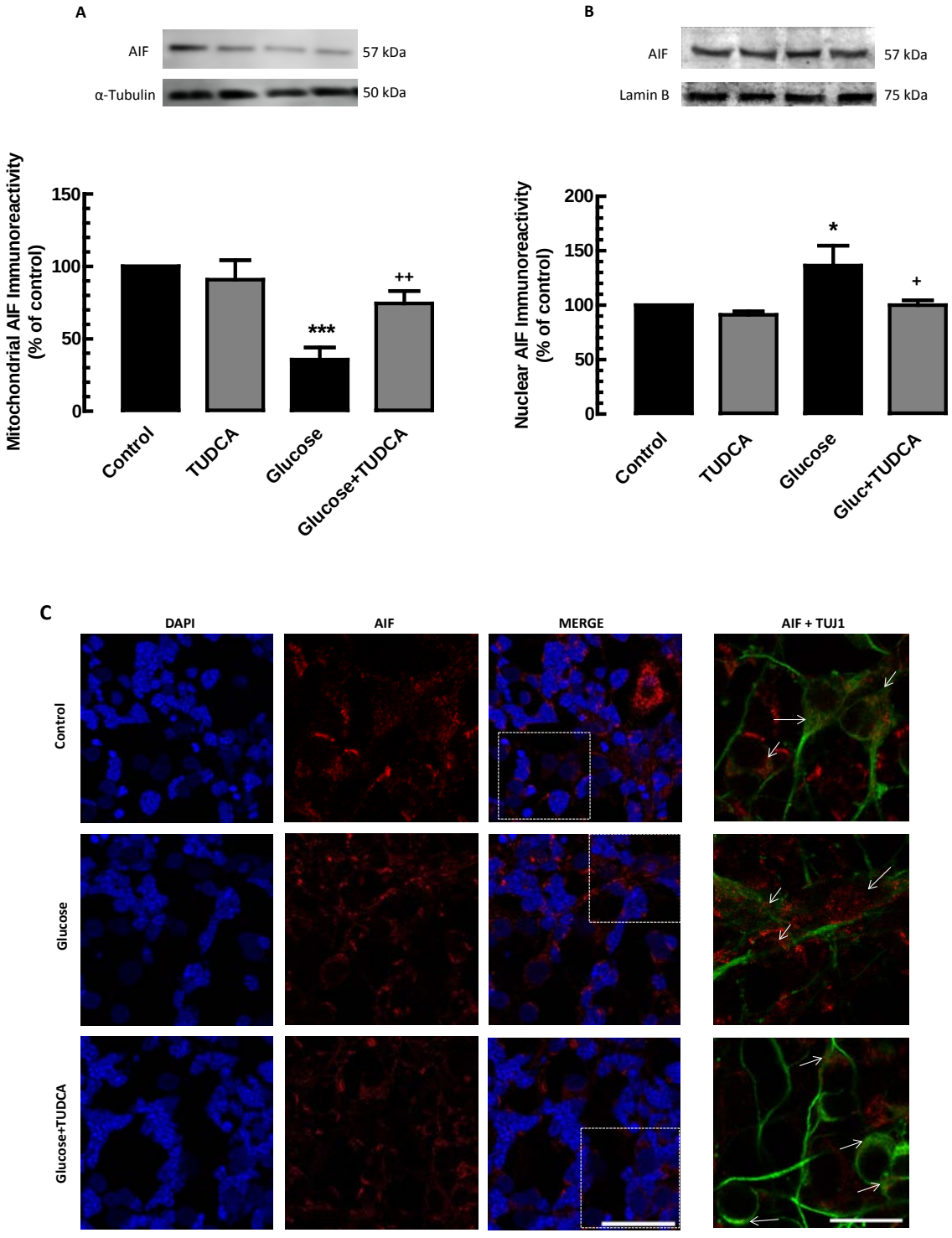


Figure 19. TUDCA prevented AIF translocation from the mitochondria to the nucleus induced by elevated glucose. Retinal neural cell cultures were incubated with 5 mM glucose (Control) or 30 mM glucose (Glucose and Glucose+TUDCA), in the absence or presence of 100 µM TUDCA (added every two days). Mitochondrial fractions (A) and nuclear fractions (B) were assayed for AIF by western blotting. Representative western blots for AIF, α-tubulin and lamin B are shown above the graphs. The results are presented as mean ± SEM from at least four (A) or three (B) independent experiments, and are expressed as percentage of control. (C) Representative images of the AIF immunoreactivity (red) assessed by immunocytochemistry. Nuclei were visualized with DAPI (blue) and neurons were stained with TUJ1. *p<0.05, ***p<0.001 significantly different from control, one-way ANOVA followed by Dunnett's post-hoc test; ^{+}p<0.05, ^{++}p<0.01 significantly different from glucose condition, one-way ANOVA followed by Bonferroni's post-test. Scale bar: 20 µm.

3.3. TUDCA prevents protein oxidation induced by high glucose exposure

Increased protein-bound carbonyl content is a biomarker for protein oxidation ((Dalle-Donne, 2003).

The effect of TUDCA in high glucose-induced protein oxidation was studied. Samples were derivatized to form protein-bound carbonyls and analysed by dot blot, using an antibody against DNP.

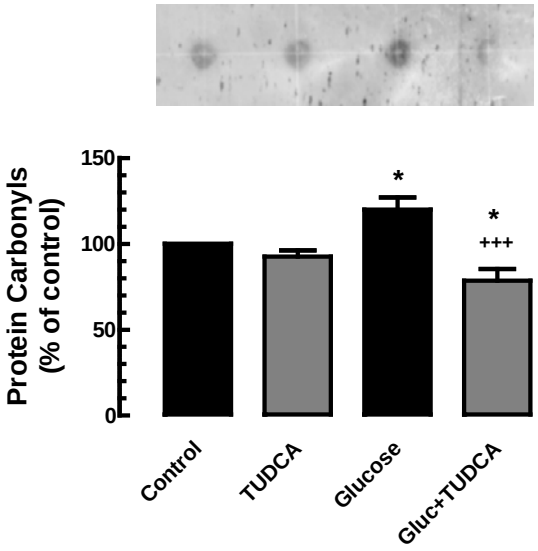


Figure 20 - TUDCA reduced the increase in protein-bound carbonyls induced by high glucose concentration. Retinal neural cell cultures were incubated with 5 mM glucose (Control) or 30 mM glucose (Glucose and Glucose+TUDCA), in the absence or presence of 100 μM TUDCA (added every two days). Cellular extracts were prepared and derivatization of carbonyl-containing proteins was performed with DNPH, as described in the Methods section. The membranes were probed with an antibody to dinitrophenylhydrazone derivatives. Results represent the mean ± SEM of at least four independent experiments, and are presented as percentage of control. *$p<0.05$ significantly different from control, one-way ANOVA followed by Dunnett's post-hoc test; $^{+++}p<0.001$ significantly different from glucose condition, one-way ANOVA followed by Bonferroni's post-test.

Cells exposed to high glucose concentration significantly ($p<0.05$) increased DNP immunoreactivity as compared to the control (119.9±7.1% of the control; Figure 20), indicating that elevated glucose concentration increased the amount of protein-bound carbonyls. Treatment with TUDCA completely abolished the increase in protein-bound carbonyl levels

induced by high glucose concentration. TUDCA alone did not cause any significant alteration compared to control.

3.4. TUDCA partially prevents elevated glucose-induced ROS production

It was investigated the effect of TUDCA in the increase of ROS production induced by elevated glucose, with a fluorescent probe (H_2DCF-DA) sensitive to intracellular oxidation.

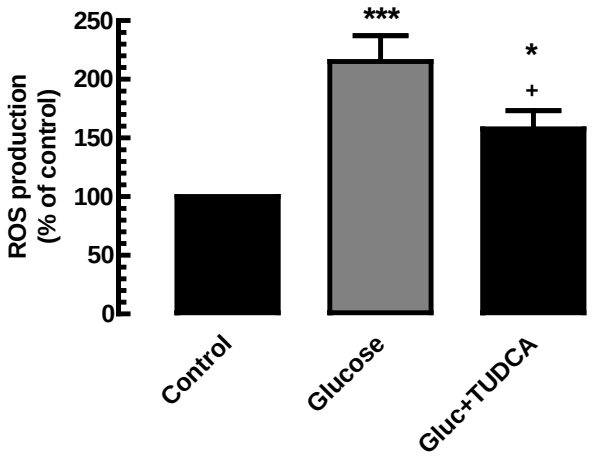


Figure 21 - TUDCA prevented the increase in ROS production induced by high glucose concentration. Retinal neural cell cultures were incubated with 5 mM glucose (Control) or 30 mM glucose (Glucose and Glucose+TUDCA), in the absence or presence of 100 µM TUDCA (added every two days). Results represent the mean ± SEM of at least four independent experiments, and are presented as percentage of control. *p<0.05, ***p<0.001 significantly different from control, one-way ANOVA followed by Dunnett's post-hoc test; $^{+}$p<0.05 significantly different from glucose condition, one-way ANOVA followed by Bonferroni's post-test.

In cells exposed to high glucose concentration, there was a significant (p<0.001) increase in H_2DCF fluorescence compared to control cells (215.1±22.2% of the control) (Figure 21), indicating that elevated glucose induced ROS production. Incubation with TUDCA in the presence of high glucose concentration significantly (p<0.05) decreased H_2DCF fluorescence to 157.6±15.7% of the control. These results indicate that TUDCA prevented ROS production induced by elevated glucose concentration.

CHAPTER 4 - Discussion

Discussion

TUDCA is a bile acid derivative which has been reported to prevent apoptosis (Amaral et al., 2009), ER-mediated stress (Malo et al., 2010) and oxidative stress (Oveson et al., 2011; Rodrigues et al., 2000) in models of stroke (Rodrigues, 2003), ischemia (Rodrigues et al., 2002) and neurodegenerative disorders such Huntington's disease (Rodrigues et al., 2000), Alzheimer's disease (Ramalho et al., 2004) and Parkinson's disease (Duan et al., 2002). Recently, it was described that TUDCA prevented photoreceptor degeneration in the rd10 mouse (Phillips et al., 2008) and in transgenic P23H rat (Fernandez-Sanchez et al., 2011), both models of RP. In addition, TUDCA improved synaptic connectivity between horizontal and bipolar cells and retinal function in P23H rats (Fernandez-Sanchez et al., 2011).

Based on these evidences, the main aim of this study was to investigate the potential protective effects of TUDCA in models of retinal cell death. Two experimental toxic stimuli were used: a model of excitotoxicity, by overstimulating ionotropic glutamate receptors and a chronic stimulus, by exposing cells to elevated glucose for seven days.

Overstimulation of ionotropic glutamate receptors leads to cell dysfunction and death (Lucas and Newhouse, 1957) and it was also reported in retinal diseases, such glaucoma (Dreyer et al., 1996) and ischemia (Neal et al., 1994; Perlman et al., 1996). In addition, glutamate excitotoxicity has been postulated as one of several mechanisms responsible for neuronal dysfunction and cell death in DR (Santiago, 2006).

Hyperstimulation of glutamate ionotropic receptors can also lead to an increase in intracellular Ca^{2+} concentration, involved in neural dysfunction and cell death (Ng et al., 2004; Santiago, 2006; Sattler and Tymianski, 2000).

In the present study, we found that TUDCA prevented cell death induced by overstimulation of AMPA/KA receptors in retinal neuronal cell cultures. TUDCA was reported to prevent cell death in several models of neurodegeneration (Colak et al., 2008; Duan et al., 2002).

In addition, it was also demonstrated that TUDCA modulates phosphorylation and translocation of Bad via phosphatidylinositol 3-kinase in glutamate-induced apoptosis of rat cortical neurons (Castro, 2004). Diabetic retinopathy is a complication of diabetes mellitus and a leading cause of legal blindness (Gardner et al., 2011). It is clinically considered a microvascular disease, but several evidences demonstrate that neuronal dysfunction and cell death occurs both in animal models (Sakai et al., 1995) and in diabetic patients (Barber et al., 1998; Sakai et al., 1995). Hyperglycemia is considered the main pathogenic factor for the development of DR. We used an *in vitro* model to mimic hyperglycemic conditions that occur in diabetes. As reported previously, we found that high glucose concentration increased cell death in retinal neuronal cultures (Santiago et al., 2007). TUDCA prevented cell death induced by elevated glucose concentration, suggesting a protective role for TUDCA. In this study, mannitol did not increase cell death, indicating that the increase in osmolarity did not caused cell death, as previously demonstrated (Santiago et al., 2007). TUDCA was shown to inhibit apoptosis in ischemia-reperfusion injury (Ishigami et al., 2001) and myocardial infarction (Rivard et al., 2007). TUDCA also appeared to partially prevent mitochondrial dysfunction in an experimental model of Parkinson's disease (Ved et al., 2005). It was reported that TUDCA modulates mitochondrial membrane perturbation, Bax translocation and subsequent cytochrome c release and caspase activation (Rodrigues et al., 1999; Rodrigues et al., 2003). Additionally, it can regulate calcium efflux and caspases-12 activation (Xie et al., 2002) and inhibit the human transcription factor activating protein-1 (AP-1) involved in cell proliferation, transformation and death (Pusl et al., 2008).

Elevated glucose induces retinal neural cell death (Barber et al., 2005; El-Asrar, 2004; Martin, 2004; Santiago et al., 2007). Although apoptotic features were previously reported in this in vitro model of DR, activation of caspases was not observed, suggesting that cell death is triggered by a pathway independent of caspases (Santiago et al., 2007). It was further shown that the mechanism underlying cell death in this model was the translocation of AIF from the mitochondria to the nucleus (Santiago et al., 2007), inducing chromatin condensation and DNA

fragmentation (Hangen et al., 2010). Thereby, AIF may also be involved in phosphatildylserine externalization (Susin et al., 1999) and is also present during redox metabolism, since it is thought to regulate the respiratory chain complex I (Hangen et al., 2010; Wang et al., 2009). Our results showed a decrease in AIF mitochondrial content, and an increase of AIF in nuclei, in cells exposed to high glucose concentration, indicating that AIF translocation occurred, corroborating previous studies (Santiago et al., 2007). In retinal endothelial cells incubated with elevated glucose, AIF mito-nuclear translocation also occurs (Leal et al., 2009). In the presence of TUDCA, AIF mito-nuclear translocation was partially inhibited, suggesting that TUDCA is able to prevent AIF translocation. TUDCA stabilizes mitochondrial membrane (Rodrigues et al., 2003), which could thus prevent AIF release.

Still, it could be interesting to further study TUDCA anti-apoptotic properties in other mechanisms involved in apoptosis, like endonucleases, since apoptotic-cell death is known to be triggered by several biochemical pathways.

Evidences described that oxidative stress has been implicated in the development of diabetic complications. Therefore, the detection of protein CO groups as biomarkers of oxidative stress in addition to the measurement of other oxidation products is advantageous, considering the relative early formation and the relative stability of carbonylated proteins (Dalle-Donne, 2003; Giacco and Brownlee, 2010). We showed that TUDCA partially prevented the increase in protein-bound carbonyls induced by high glucose concentration in retinal neural cell cultures.

Elevated ROS production and lipid peroxidation were described in healthy retina, and may lead oxidative damage (van Reyk et al., 2003). Moreover, it was demonstrated that elevated glucose induces a sustained increase in ROS production through several pathways (Brownlee, 2001). of glucose auto-oxidation (Pennathur and Heinecke, 2004), including activation of AR, PKC, NF-κB and AGEs formation (Giacco and Brownlee, 2010; Nishikawa et al., 2000), responsible for vascular damage in diabetes (van Reyk et al., 2003). Also, changes in mitochondrial

transmembrane potential results in ROS production and involvement of Bax leading to apoptotic caspases cascate and subsequent cell death (Xiang et al., 1996) observed in astrocytes (Wang et al., 2009).

Our results revealed that TUDCA partially prevented increased ROS production in cells exposed to elevated glucose concentration. Thus, in addition to previous results from oxidized protein determination, TUDCA appears to have antioxidant effects, preventing oxidative stress. These results are in agreement with previous studies showing that TUDCA protects from retinal degeneration induced by oxidative damage (Oveson et al., 2011). Since increased ROS level can be triggered by mitochondrial dysfunction leading to an energy deficit (Dauer and Przedborski, 2003; Kowluru and Chan, 2007) it is possible that the antioxidant effect of TUDCA may be due to its ability in suppressing mitochondrial membrane perturbation (Rodrigues et al., 2003).

Once observed the neuroprotective role of TUDCA in high glucose-induced retinal neuronal cell death described above, this bile acid may be considered a neuroprotective agent, targeting pro-apoptotic components to prevent apoptotic cell death. Interesting is the fact that UDCA was approved by United States Food and Drug Administration (USFDA) for the treatment of primary biliary cirrhosis (Lazaridis et al., 2001), and it can be administrated orally or intravenously (Amaral et al., 2009). Since TUDCA is structurally and functionally similar to UDCA, eventually it can be also used for the treatment of diseases related to oxidative damage. Nevertheless, in order to consolidate the idea that TUDCA could be a good candidate to include in an antioxidant test regimen for the treatment of DR, more experiments should be performed.

CHAPTER 5 - Conclusion

Conclusions

In this study, we concluded that treatment with TUDCA prevented cell death induced by glutamate excitotoxicity in retinal neuronal cell cultures.

We also concluded that TUDCA prevented cell death induced by elevated glucose concentration through the inhibition of AIF mito-nuclear translocation. In addition, TUDCA prevented the increase in oxidative stress induced by elevated glucose, which may also account for the protective effect of TUDCA in high glucose concentration model.

Therefore, TUDCA may be a novel therapeutic agent for the treatment of retinal neurodegenerative diseases, such DR.

CHAPTER 6 - References

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