Oxygen and Glucose Deprivation on Human Müller Cells (MIO-M1) and Human Organotypic Retinal Cultures (HORCs) in Relation to Glaucoma

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A THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY AT THE UNIVERSITY OF EAST ANGLIA, NORWICH, UK

SCHOOL OF PHARMACY

2015

Abstract

Purpose:

The purpose of this research was to investigate the effect of glaucoma-related insults, specifically oxygen and/or glucose deprivation (OGD) on the survival and genes expression of human Müller cells (MIO-M1), and retinal ganglion cells (RGCs) using the human organotypic retinal culture (HORC) model.

Methods:

MIO-M1 cells and HORCs were exposed to different levels of OGD using a custom-built chamber to control oxygen levels. Cell survival was evaluated using MTS and LDH assays while RGC death in HORCs was investigated using NeuN immunohistochemistry and TUNEL-labelling. Expression of genes of interest was assessed using QRT-PCR.

Results:

Reduced levels of oxygen and glucose (1.11mMglucose/4%O_2) caused proliferation of MIO-M1 cells. Full deprivation of glucose caused cell death, but full hypoxia did not affect survival. In HORCs, glucose deprivation and OGD, but not oxygen deprivation alone, caused loss of RGCs. Different levels of OGD regulated expression of genes associated with angiogenesis, glial activation, excitotoxicity and neuroprotection in MIO-M1 cells and HORCs. VEGF expression significantly increased in MIO-M1 cells and HORCs treated with full OGD, and VEGF protein was secreted under reduced levels (1.11mMglucose/4%O_2). Secretion of VEGF in MIO-M1 cells and HORCs was also increased under conditions of raised glucose. The PKCβ inhibitor LY333531 decreased VEGF secretion under conditions of raised glucose and hypoxia. Co-culture of MIO-M1 cells resulted in more damage/apoptosis to HORCs and reduced RGCs survival.

Conclusions:

Use MIO-M1 cells and the HORC model were effective in studying the effect of OGD in relation to glaucoma. Glucose rather than oxygen was the key survival factor for RGCs and MIO-M1 cells. The secreted factors by Müller cells could have protective and detrimental effects on RGC survival. Investigation of mechanisms using these models may be of benefit in development of potential therapeutic interventions for retinal neurodegenerative diseases including glaucoma.

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Acknowledgements

It is one of lifetime events to study a Ph.D. in the University of East Anglia, and I have enjoyed my time in the Norwich Eye Research Group laboratories. I would like to express gratitude to Dr. Julie Sanderson, my supervisor, for her guidance and support throughout my research period. I would also like to thank Dr. Jeremy Rhodes, my secondary supervisor, for help and support specially with the pressure chamber. It is not enough to thank my sponsor King Saud University and the tremendous effort of the Saudi Cultural Bureau in London is always appreciated.

Special thanks also to my predecessor, Dr. Andrew Osborne for teaching me all the laboratory techniques. It is not enough to thank Mary Tottman of the East Anglian Eye Bank for her dedication and hard work trying to provide us with human donor eyes. Many thanks to my colleagues, Marina Hopes for sharing her experience with me and Philip Wrights who deserves credit for coding hundreds of immunoimages for me. A special gratitude and appreciation to all members of the Norwich Eye Research Group: Dr. Michael Wormstrone, Dr. Julie Eldred, Dr. Andrew Smith and Dr. Sarah Russell, for making my Ph.D. experience enjoyable.

It is not easy to summarize four years in few words, as it is never easy to live as fibromyalgia patient. Being a patient taught me how to fight, and a Ph.D. degree taught me how to be patient. Finally, I would like to thank my family for believing in me and always supporting my choices. My friends who have supported me throughout my research especially when I was down and during difficult times. They always remind me "No winter lasts forever; no spring skips its turn, Hal Borland".

Declaration by Candidate

I hereby declare that this thesis is my own work and includes nothing, which is the outcome of work done in collaboration. Where other sources of information have been used, they have been acknowledged. This thesis has not been submitted for another degree or qualification in this or any other university or institution and does not exceed the prescribed word limit.

Signature:

Chapter 1

Introduction

1. Anatomy of the Eye

The eye is a complex photosensitive organ that takes in information from the environment in the form of light and analyses light intensity and colour reflection from objects through changing them into neuronal signals. The eyes are located in the protective bony structure of the skull called the orbits. Each orbit is pyramidal in shape. Its base is the orbital margin and its apex directed towards the optic canal at the posterior of the orbit (Grant-Kels and Kels, 1992; Kels et al., 2015). There are four rectus muscles forming the 'muscle cone', situated within each orbit, two oblique muscles and a levator palpebrae superioris muscle (Al-lens et al., 2008). The size of the normal human eye is approximately 22 to 27 mm and 69 to 85 mm anteroposteriorly and in circumference respectively (Kels et al., 2015).

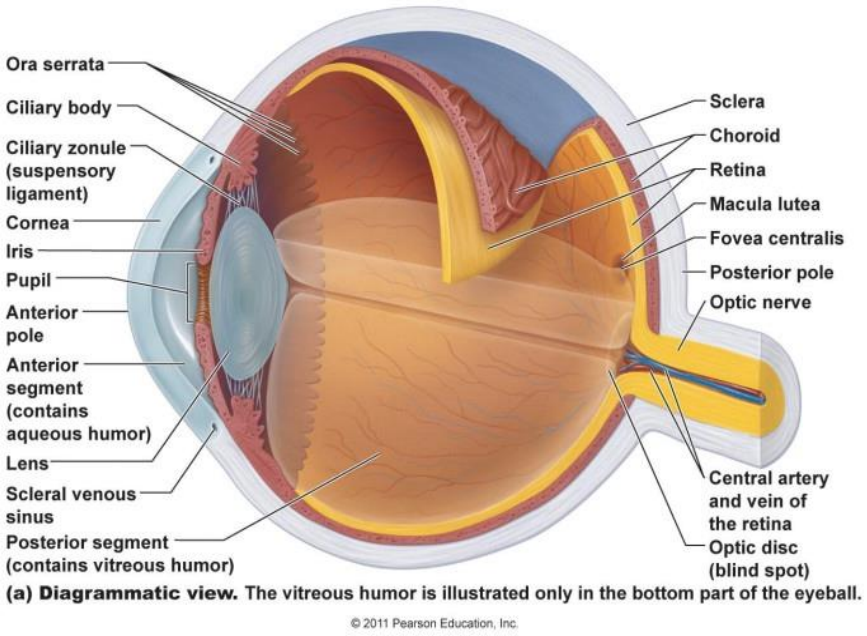


Figure 1.1: Gross anatomy of the globe. Source: http://biology-forums.com/index.php?action=gallery;sa=view;id=11303

The main blood supply to the eye is via the ophthalmic artery, which is a major branch of the internal carotid artery. It reaches the orbit through the optic canal and branches to supply the eye and extra-ocular muscles (Presland, 2007). The ciliary body receives blood from the posterior and anterior ciliary arteries. Avascular structures like the cornea and lens receive their nutritional supply via the tear film and aqueous humour. The palpebral artery, which originates from the ophthalmic artery, supplies the conjunctiva and anterior episclera together with the anterior ciliary arteries (AL-lense et al., 2008). A major branch of the ophthalmic artery is the central retinal artery, which supplies the innermost layer of the retina (Presland, 2007). The outer retinal layers receive nutrition from the choroidal capillary bed. Metabolites diffuse through Bruch's membrane and the retinal pigment epithelium (RPE) into the neural retina (Remington, 2005).

The eye is divided anatomically into three layers: the sclera, which is the fibrous exterior and supporting layer; the vascular middle uveal layer, which encompasses the iris, ciliary body, and the choroid; and a third neural internal layer, the retina (Born et al., 1998; Grant-Kels et al., 1992). The lens and its suspensory ligament separate the anterior and posterior chambers and are located between two 'fluid' media: the aqueous humour and vitreous body (Figure 1.1) (Presland, 2007).

1.1 The Fibrous Coat: The Cornea and Sclera

The fibrous coat is the exterior of the eye comprising the cornea and sclera. Both consist of collagen fibers. These fibers are arranged in highly regular laminae with cornea enabling it to be transparent. The cornea is the primary refractive structure in the visual pathway. Its transparency and avascularity are two important characteristics for optimal light transmission. The cornea is coated anteriorly by the tear film and posteriorly facing the aqueous humour (Remington, 2005).

The collagen fibers in the sclera are exteriorly interwoven and extend in all directions making it an opaque structure (Jayaram and Calder, 2004). The sclera forms the posterior five-sixths of the connective tissue coat of the globe. It is relatively inactive metabolically and nourished by the small vessel branches from episclera and choroid (Watson and Young, 2004). The sclera's unique structure helps to determine the shape of the eye (Watson and Young, 2004).

1.2 Uvea

The uveal tract is the middle layer of the eye and is composed of three parts: the iris, ciliary body, and the choroid. The iris is a thin, circular and colored structure composed predominantly of smooth muscle and located anterior to the lens and attached peripherally to the ciliary body. The pupil is the central aperture and is located inferior to the iris centre. The iris has the primary function of regulating the size of the pupil and to prevent excessive light from entering the retina and providing an optimal light condition for a good image (Al lens et al., 2008).

The ciliary body lies between the iris and choroid. The ciliary muscles within the ciliary body are involved in the accommodation process by altering the shape of the lens. The ciliary processes are small, finger-like projections located posterior to the lens. The main function of these processes is to produce aqueous humour, which circulates through the pupil into the anterior chamber and exits the eye crossing the trabecular meshwork, a sieve-like structure, and flow out into Schlemm's canal at the iridocorneal angle (Figure 1.2) (Karpinich and Caron, 2014). Drainage through the trabecular meshwork and Schlemm's canal is referred to as the conventional pathway. A minor pathway for outflow of the aqueous humour is by diffusion through intercellular spaces between the ciliary muscle fibers (Alm, 2000; Alm and Nilsson; 2009). The juxtacanalicular or cribriform meshwork layer of trabecular meshwork is composed of cells embedded in a dense extracellular matrix and characterized by narrow intercellular spaces. This layer is in direct contact with the inner wall of endothelial cells

from Schlemm's canal and tissue resistance to aqueous humour flow arise from this joint layer (Llobet et al., 2003). The flow of aqueous humour against resistance generates an average intraocular pressure (IOP) of approximately 15mmHg (Goel et al., 2010; Tamm, 2009). This pressure is important to inflate the eye maintaining the shape and optical properties of the globe (Goel et al., 2010). The impairment of aqueous humour outflow results in elevation of IOP, which is the main risk factor for of glaucoma (Braunger et al., 2015). This will be discussed in a later section of this introduction.

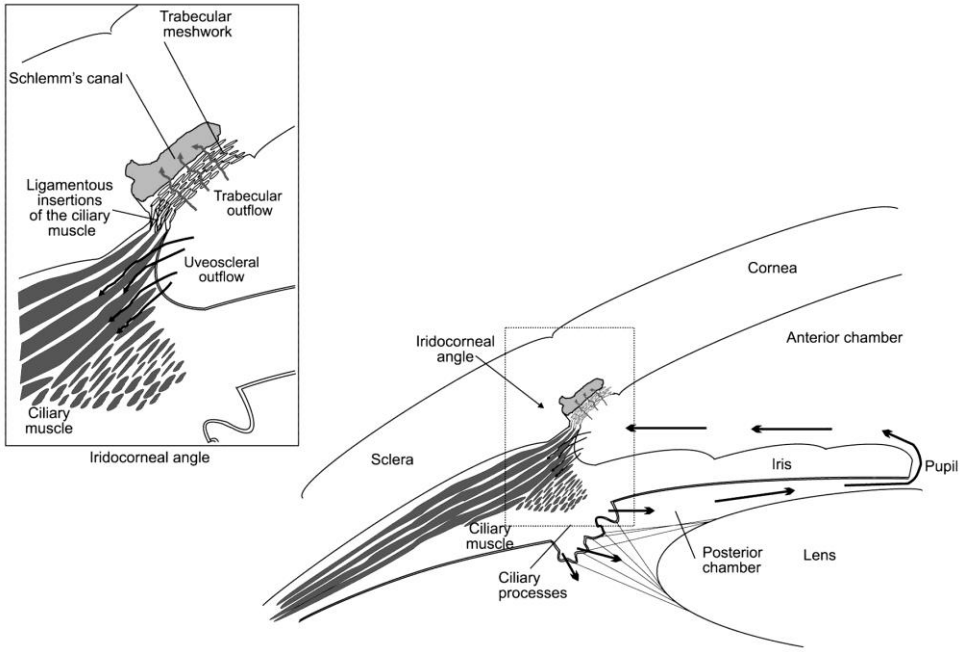


Figure 1.2: Pathway of aqueous humor outflow. Source: adapted from (Llobet et al., 2003)

The ciliary body has both sympathetic and parasympathetic nerve endings. Stimulation of sympathetic receptors increases aqueous humour secretion by the ciliary epithelium, while parasympathetic innervation to ciliary muscle causes contraction and accommodation by changing the shape of the lens. It also reduces resistance to conventional aqueous humour trabecular outflow (Al lens et al., 2008). The suspensory ligaments (zonules) of the ciliary body are attached to the lens. The lens is avascular, clear, and biconvex in shape and is

contained within an elastic capsule. It receives its nutrients from the aqueous humor (Al lens et al., 2008; Presland, 2007).

The choroid is the posterior part of the uvea. It is highly vascular tissue supplying oxygen and nutrients to the outer retina. In addition, the choroid acts as a thermo-regulator of the eye through heat dissipation.

1.3 The Vitreous Humor

The vitreous is the largest cavity of the eye, constituting two-thirds of the eye volume. It is avascular and transparent, which enables the transmission of light (Al lens et al., 2008). The composition of the vitreous is 99% water with small amount of collagen giving it the consistency of a gel (Remington, 2005). Due to its viscoelastic properties it protects the retina during rapid eye movements and physical trauma (Remington, 2005).

1.4 Retina

The retina is the neuronal tissue lining of the posterior two-thirds of the eye, located between the choroid and vitreous. The retina is embryologically derived from the neural tube and is classed as part of the CNS (Ryan et al., 2006). It consists of two parts: the neural retina layer and outer retinal pigment epithelium layer. The neural retina is composed of five major classes of neuronal cells: photoreceptors, horizontal cells, bipolar cells, amacrine cells, ganglion cells, and also Müller cells, the predominant macroglial cells. Anatomically, the retina is described as having ten layers. As shown in Figure 1.3 the retinal pigment epithelium (1), is the outermost retinal layer; the photoreceptor outer segment layer (2) contains the rod and cones. The external limiting membrane (3) is not a true membrane, and it contains intercellular junctions between photoreceptors and Müller cells (Remington, 2005). The outer nuclear layer (4) containing the rod and cone cell bodies. The outer plexiform layer (5) contains the synapses between the photoreceptors and the cells of the inner nuclear layer. The inner nuclear layer (6) consists of the cell bodies of horizontal cells, bipolar cells,

amacrine cells, Müller cells and some displaced ganglion cells. The inner plexiform layer (7) consists of synaptic connections between the bipolar cell and amacrine cells of the INL and the dendrites of ganglion cells. The ganglion cell layer (8) is a single cell layer in the peripheral regions of the retina (as shown in Figure 1.3), and about 8 to 10 cells near the macula, which is the central region of the retina. The nerve fiber layer (9) is simply ganglion cell axons, which exit the eye at the optic nerve head through the lamina cribrosa to become the optic nerve. The internal limiting membrane (10) is the innermost boundary of the retina composed of extensively expanded terminations of Müller cell (Forrester et al., 2002).

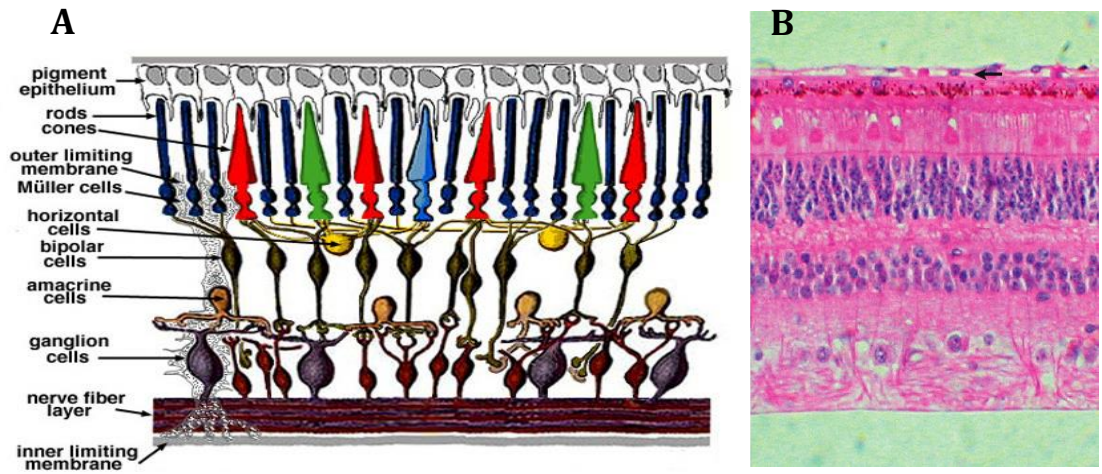


Figure 1.3: The retina (A) Layers of the retina; (B) microscopic image of the same area: (1) retinal pigment epithelial layer; (2 photoreceptor outer segment layer; (3) external limiting membrane; (4) outer nuclear layer; 95) outer plexiform layer; (6) inner nuclear layer; (7) inner plexiform layer; (8) ganglion cell layer; (9) nerve fiber layer; (10) internal limiting membrane. Source: from Leeson CR, Leeson S: Histology, Philadephia, 1976, Sauders and **http://webvision.med.utah.edu/book/part-i-foundations/simple-anatomy-of-the-retina/**

A central region of the retina is called the macula lutea, which appears as a darkened region, and is 3 mm lateral to the optic disc. It appears as a yellow area after dissection because of the presence of xanthophyll pigments (lutin and zaexanthin). These pigments have antioxidant properties and thus prevent damage from ultraviolet radiation (Remington, 2005). The *fovea (fovea centralis)* appears as a shallow depression at the center of the macula has a

central diameter of 1.5 mm. There the retinal neurons are displaced, leaving only the photoreceptors in the center. This depression is known as the foveal pit. The concentration of cones in this area is around 300,000 cones per square millimeter, and there are no blood vessels (Forrester et al., 2002, Remington, 2005). The optic disc is also known as the blind spot as there are no normal retinal layers in this area and appears as a pale pink/whitish area. This is the site where ganglion cell axons meet and leave the eye (Forrester et al., 2002). The peripheral retina is rich in rods and considered as the remainder of the retina outside of the macula (Forrester et al., 2002). The ora serrata is located 5 mm anterior to the margin of the sensory retina and is the area where the retina and ciliary epithelium meet.

1.4.1 Retinal Pigment Epithelium (RPE)

The retinal pigment epithelium consists of a continuous monolayer of pigmented hexagonal cells, which form the outermost retinal layer and are a component of the blood-brain barrier (Boulton and Dayhaw-Barker, 2001). It is essential in the visual process through recycling of the visual pigment maintaining adhesion of the neurosensory retina; providing a selectively permeable barrier between the choroid and neurosensory retina; phagocytosis of rod outer segment; absorption of light; and transport of metabolites and vitamins (Forrester et al., 2002). Cells contain numerous melanocytes and pigment granules, which extend to the apical area. The extended microvilli from the apical region envelop the outer segments of photoreceptors. The melanin in RPE helps in absorbing the light that passes through photoreceptors and preventing scattering the light, which would degrade the visual image. In addition, the RPE helps in scavenging reactive oxygen species (ROS) and phagocytosis, transport of nutrients, retinoids and waste products (Boulton and Dayhaw-Barker, 2001).

1.4.2 Photoreceptors: Rods and Cones

Photoreceptors are the photosensitive cells in the retina, which contain light-absorbing pigment and convert it into neuronal signals or impulses. The retina contains two types of

photoreceptors; rods account for 95% of all photoreceptors while cones make up only 5% (Luo et al., 2009). Photoreceptors are composed of the outer segment, containing the visual pigment, the cilium which connects the outer segment with the inner segment and acts as a channel between the two segments and the inner segment which contains the organelles and metabolic apparatus such as mitochondria, the endoplasmic reticulum, Golgi apparatus and the nucleus (Al lens et al., 2008, Remington, 2005).

There are approximately 130 million rods in the human retina. They are specialized for high sensitivity and are responsible for night vision. They are also responsible for peripheral vision as the majority of the rods are in the periphery of the retina, with lower levels within the fovea (Remington, 2005). The visual pigment rhodopsin contained within the rod outer segments enables them to convert light into impulses. This pigment is regenerated in the dark and is capable of absorbing wavelengths of visible light with high sensitivity to blue light of a wavelength of 500 nm (Ryan et al., 2006). Cones provide high acuity and colour vision in daylight condition. There are approximately six million cones in the retina, predominantly in the macula. The cone outer segment is shorter than that of the rod. Three different types of cone are present in the retina: red, green and blue with three different pigments, which allow them to absorb the specific wavelength of light, required. Photoreceptor axons start in the Outer Nuclear Layer (ONL) and terminate in the outer plexiform layer (OPL) (Al lens et al., 2008, Remington, 2005).

1.4.3 Horizontal Cells

Horizontal cells are interneurons located in the outer rows of INL and adjacent to the OPL. They have one long axon and several short dendrites with branching terminals ending in the OPL and they transfer information in a horizontal direction parallel to the retinal surface. They synapse with photoreceptors, bipolar cells, and other horizontal cells in the OPL (Ryan et al., 2006, Remington, 2005).

1.4.4 Bipolar Cells

Bipolar cells are interneurons, which receive input from the photoreceptors and conduct the signals into the inner retina. There are many kinds of bipolar cells, and they terminate deeply in the IPL (Ryan et al., 2006). The bipolar cells have a large nucleus and minimal cell body cytoplasm. Their dendrites synapse with photoreceptors and horizontal cells in the OPL, and the axons synapse with ganglion and amacrine cells in the IPL (Remington, 2005).

1.4.5 Amacrine Cells

Amacrine cells are inhibitory interneurons using either GABA or glycine as the neurotransmitter. Their synapses are located predominantly in the inner part of the IPL. The cell body of amacrine cells lie mainly within the proximal part of the INL, and many subtypes are displaced and can be found in the ganglion cell layer or lie within the IPL (Masland, 2005).

1.4.6 Retinal Ganglion Cells (RGCs)

Retinal ganglion cells (RGCs) are the final pathway output neurons in the retina, carrying the visual information to the brain. They receive signals from bipolar cells, which convey inputs from photoreceptors, and amacrine cells in the IPL (Marshak, 2009). The cell bodies of the ganglion cells are located in the ganglion cell layer situated between the nerve fiber layer and the IPL, but displaced ganglion cell cells can also be found in the INL. The axons of the ganglion cells form the nerve fiber layer and form bundles that are ensheathed by glial cells that leave the eye in the form of the optic nerve (Remington, 2005, Forrester et al., 2002).

1.4.7 Optic Disc

The optic disc is the site where the axons of the ganglion cells leave the eye. It is approximately 1.7 mm in diameter horizontally, and the vertical diameter is 1.9 mm with a depression in the surface, which is referred to as the cup (Jonas et al., 1992). It is pale-yellow

in colour and the two layers appearing in the optic disc are the nerve fiber layer and an internal limiting membrane only (Quigley et al., 1998).

1.4.8 Neuroglial Cells

The neuroglial cells found in the retina include microglial cells and two types of macroglial cells, astrocytes and Müller cells. They play very important roles in the support, metabolism and nutrition of the neuronal cells in the retina and in responding to infection or injury.

1.4.8.1 Microglial Cells

Microglial cells are resident of the CNS and the retina. Microglia precursors originate in the yolk sac and migrate through blood vessels into the retinal tissue during late embryonic development and in the early postnatal period (Barron, 1995). They are abundant in human retina where they are found in every layer (Chan-Ling, 1994). They are blood-derived phagocytes and play a role in retinal immunity via phagocytosis of cellular debris (Dheen et al., 2007). Activation of microglia cells in retinal injuries involves their proliferation, migration, and production of several cell-signalling factors (Dheen et al., 2007; Beynon and Walker, 2012).

1.4.8.2 Astrocytes

Astrocytes do not originate from the retinal embryonic epithelium and are thought to enter the developing retina from the brain along the developing optic nerve (Chan-Ling, 1994). They are restricted to the nerve fiber layer of the retina (Schitzer, 1987). They are flattened cells with radiating processes, which are filled with intermediate filament, GFAP. These processes envelop blood vessels in the conjunction with Müller cell processes in the superficial plexus and the basal lamina of the vitreal surface (Hollander et al., 1991).

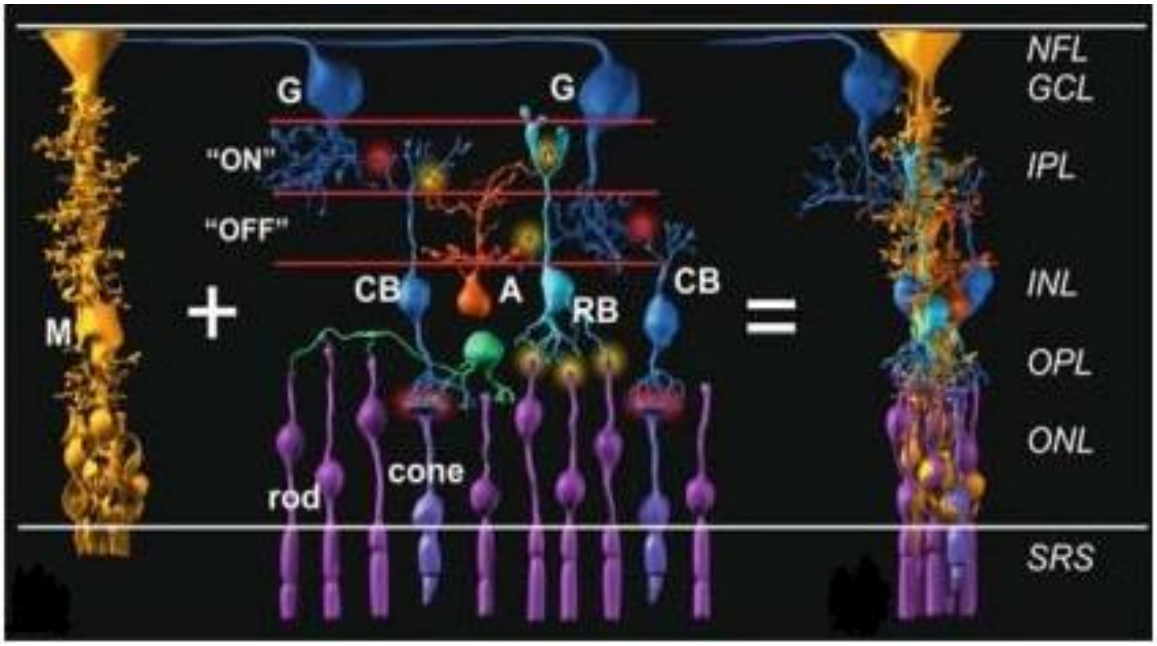


Figure 1.4: Müller cells interact with all retinal neuronal cells forming columnar units. Source: Reichenbach and Bringmann, 2010. M, Muller cells ensheathing: G, retinal ganglion cell; CB, cone-specific bipolar cells; A, amacrine cell; RB, rod bipolar cell; NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL; inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; SRS, sub-retinal space.

1.4.8.3 Müller Cells

Müller cells are radial macroglial cells, which provide structure and support. There are ten million Müller cells making up 10% of the total retinal volume. Their processes are in contact with all the neuronal elements with as shown in Figure 1.4 (Bringmann and Reichenbach, 2009; Sarthy and Ripps, 2001). In the following section, Müller cell function in healthy and disease will be discussed in greater detail.

1.4.8.3.1 Characteristics of Müller Cells

Müller cells span the entire thickness of the retina (Remington, 2005, Bringmann and Reichenbach, 2009). They terminate at their outer end in the photoreceptors layer while at the basal end they terminate in the inner retina. Müller cells ensheath most ganglion cell axons providing support and insulation. The horizontal fibers appear in the inner and outer limiting plexiform layers. The honeycomb meshwork can be found in ganglion cell layer, internal nuclear layer and the outer limiting membrane (Remington, 2005). The Müller cell and the retinal neurons that it interacts with is called a columnar unit (Reichenbach and Robinson,

1995). The human eye consists of 10,000,000 repetitive columnar units (Reichenbach and Bringmann, 2010).

1.4.8.3.2 Müller Cells Energy Metabolism

Phototransduction, maintenance of ionic gradients, and synaptic activity are complex processes that the retina performs and have a high-energy demand (Winkler, 1981; Ames et al., 1992). Normally, the retina derives its major source of energy, glucose, and oxygen from the choroidal circulation. Coles, (1996) suggested different routes by which the retina may take up glucose from the capillaries to be used by the neurons. The first is the direct route without the involvement of Müller cells where the glucose is directly transferred to neurons from the capillaries by diffusion. In the second pathway, Müller cells take up the glucose and act as a channel to feed the neurons. In the third pathway, Müller cells have a more active role in which they convert glucose to a metabolite such as lactate and release it for use by the neurons. In the final route, Müller cells take up the glucose and store it as glycogen and then break it down to glucose when needed. Glucose is transported into retinal cells through the glucose transporters, $GLUT_{1-4}$ (Hosoya et al., 2008). Endogenous breakdown of glycogen deposits that are stored in the Müller cells with the aid of glycogen phosphorylase generates more glucose and fuel for retinal neurons (Pfeiffer et al., 1994).

It is well known that one mole of lactate can generate 18 moles of ATP via oxidative phosphorylation. Early studies have suggested that metabolites such as lactate and pyruvate rather than glucose are the preferred metabolic substrates to be exchanged between Müller cells and neurons (Larrabee, 1983 and 1992). This is supported by research in the brain, which has suggested that lactate production is not limited to anaerobic metabolism, but that it also occurs in the brain under aerobic conditions (Prichard et al., 1991; Raichle, 1991; Sappey-Marinier et al., 1992). In rat hippocampal slices, a challenge with glutamate treatment was performed after perfusion with low versus high glucose medium (Schurr et al.,

1999). Results showed that glia would produce more lactate under higher glucose concentrations, and subsequently this would protect against glutamate excitotoxicity (Schurr et al., 1999). Moreover, mammalian retina and cell culture studies showed that lactate produced by Müller cells is a fuel for photoreceptors, bipolar, and ganglion cells (Poitry-Yamate et al., 1995; Tsacopoulos and Magistretti, 1996; Tsacopoulos et al., 1998). Winkler (1995) found that 90% of the glucose metabolized by the retina was converted into lactate under aerobic conditions. This is also supported by the findings that cultured human and transformed rat Müller cells consume oxygen at a lower rate in the presence of glucose and obtain ATP through glycolysis, which accounts for 99% of the produced lactate (Winkler et al., 2000; Winkler et al., 2003). Cultures of photoreceptor cells, Müller cells, ganglion cells, and retinal pigment epithelial cells incubated in 5mM glucose produced lactate aerobically and the production rate increased 2-3 fold under anaerobic conditions (Winkler et al., 2004). In another study, Winkler et al., (2003) found that hexokinase is present in the mitochondria and cytosol of rat retina and suggested that in the presence of enough glucose the retinal neurons use glucose rather than the lactate produced by Müller cells.

It is now accepted that the resistance of Müller cells to a variety of injuries including ischemia, hypoxia and hypoglycemia is related to the dominant glycolysis pathway that functions under the aerobic and anaerobic conditions in these cells (Poitry-Yamate et al., 1995; Winkler et al., 2000). Maintenance of stable physiological levels of ATP is important for cellular defense mechanism including the maintainance of high levels of glutathione (Reichelt et al., 1997; Paasche et al., 1998; Schuette and Werner, 1998).

1.4.8.3.3 Müller Cells in Healthy Retina

Müller cells express different types of neurotransmitter receptors and transporters including for gamma-aminobutyric acid (GABA), glycine and glutamate (Biedermann et al., 2004; Keirstead and Miller, 1997; Newman and Reichenbach, 1996; Stevens et al., 2003). GABA

is the major inhibitory transmitter in the retina. Müller cells take up GABA through sodium- and chloride-dependent high-affinity GABA transporters (GATs) (Yazulla, 1986; Biedermann et al., 2002). Active uptake and release of GABA is found to increase in injured conditions such as diabetes and ischemia (Ishikawa et al., 1996; Napper et al., 2001). In these conditions, Müller cells remove GABA by converting it into glutamate by the GABA transaminase enzyme (Ishikawa et al., 1996; Napper et al., 2001).

Glutamate is the major excitatory neurotransmitter in the retina that is utilized by different cells in the neuronal retina such as photoreceptors, bipolar and ganglion cells to transmit the visual signal (Massey and Miller 1987; Bringmann et al., 2013). There are five types of glutamate transporters, called excitatory amino acid transporters ($EAAT_{1-5}$). $EAAT_5$ appears to be predominant isoform in the retina (Fairman et al., 1995; Arriza et al., 1997; Eliasof et al., 1998). In astrocytes, GLAST ($EAAT_1$) and GLT-1 ($EAAT_2$) are the predominant glutamate transporters (Seal and Amara, 1999). Müller cells express, GLAST, which is required for normal function and prevention of neurotoxicity (Rauen et al., 1998; Barnett and Pow, 2000). It is a sodium-dependent transporter, which requires a negative membrane potential. A malfunction in the transport process by Müller cells causes accumulation of glutamate, which is associated with neurotoxicity (Barnett and Pow, 2000). Intracellularly, Müller cells convert glutamate into non-neuroactive glutamine by the enzyme glutamine synthetase (GS) in the presence of energy in the form of two molecules of adenosine 5′-triphosphate (ATP). This is also the route to detoxify glutamate produced through GABA metabolism (Biedermann et al., 2002). The released glutamine from Müller cells is taken up by neurons as precursor for the neurotransmitters glutamate and GABA (Pow and Crook, 1996). However, according to Umapathy et al., (2005), most of the glutamine is taken up again by Müller cells by the glutamine transporter SN1 and SN2 and used to synthesize glutamate in the Müller cells. In an alternative pathway, glutamine is transported into Müller

cell mitochondria, and it is further hydrolyzed to glutamate and ammonia by the phosphate-activated glutaminase enzyme (Takatsuna et al., 1994).

Regarding retinal homeostasis, Müller cells are responsible for regulating the potassium ion (K^+) balance in the retina through a process termed 'spatial potassium buffering' (Reichenbach et al., 1992; Newman and Reichenbach, 1996). During neuronal activity, rapid shifts of ions occur between intra and extracellular spaces in which sodium and calcium ions flow into the neurons, and K^+ efflux is increased (Karwoski et al., 1989). To correct the depolarization and excitability that results from extracellular K^+, glial cells take up excess K^+ and release it into the blood and vitreous fluid (Karwoski et al., 1989, Reichenbach et al., 1992). This mechanism will limit the spread of un-wanted excitation and thus maintain visual function (Reichenbach et al., 1993). The high permeability of Müller cells to K^+ can be explained by the expression of a variety of potassium channels especially the inwardly rectifying K^+ channel of the Kir4.1 subtype (Ishii et al., 1997). These channels are capable of mediating K^+ influx rather than efflux and with, the assistance of the glial Na, K-ATPase, Müller cells maintain potassium homeostasis in the retina (Nilius and Reichenbach, 1988).

Any shift in ion flux during neuronal activity is associated with water flowing through aquaporin-4 (AQP4) channels (Nagelhus et al., 1999). Normally, water is removed from the sub-retinal space and the inner retina by the retinal pigment epithelium and Müller cells respectively (Bringmann et al., 2004). The normal source of water in the retina is the endogenous production that is linked to ATP synthesis, glucose uptake or forced water by the intraocular pressure (Marmor, 1999). Together, K^+ (Kir4.1) and aquaporin-4 water channels control the osmolarity between the retinal tissue, blood and vitreous by K^+ and water influx and efflux from Müller cells (Nagelhus et al., 1999).

In addition, Müller cells remove metabolic waste such as carbon dioxide (CO_2) and regulate retinal pH. Carbon dioxide results from oxidative degradation of glucose and is rapidly

converted into bicarbonate and a proton by the enzyme carbonic anhydrase II that is localized in Müller cells (Oakley and Wen, 1989). Glial cells complete their cleaning and buffering activity by transporting bicarbonate to the vitreous humor by sodium-bicarbonate co-transporters located in their endfeet (Nagelhus et al., 2005). This mechanism is responsible for maintaining the correct pH levels intracellularly.

Müller cells also play a role in the immune response in the retina (Kim et al., 1987). It has been demonstrated that Müller cells inhibit antigen presentation in lymphocytes *in vitro* (Forrester et al., 1990). They express MHC (major histocompatibility complex) class II antigen during proliferation in experimental uveitis (Romeike et al., 1998) and when exposed to supernatant from activated lymphocytes (Roberge et al., 1988). The phagocytic activity of Müller cells has been reported in animal models and cultures from *post-mortem* eyes (Algvere and Kock, 1983; Francke et al., 2001; Mano and Puro 1990; Ponsioen et al., 2007; Stolzenburg et al., 1992). The phagocytosis of a variety of substances such as carbon, copper, collagen, erythrocyte debris, subretinal hemorrhage and cell debris have been all reported (Mano and Puro, 1990; Sarthy and Ripps 2002).

In addition, Müller cells contribute to the recycling of photopigments. They express the cellular retinol binding protein (CRBP) that binds to all-*trans* retinol and cellular retinal binding protein (CRALBP) that binds 11 *cis* retinol and 11-*cis* retinal (Crabb et al., 1998). Opsin and 11-*cis*-retinal are the visual pigments of photoreceptors. Conversion of light into an electrical signal by photoreceptors (phototransduction) starts with the conversion of 11-*cis*-retinal to all-*trans* retinal, which subsequently reduced, in the outer segment of photoreceptors to all-*trans* retinol (Tsacopoulos et al., 1998). Müller cells convert all-*trans* retinol to all-*cis*-retinol, which is subsequently oxidized to 11-*cis* retinal and released into the extracellular space, and taken up by photoreceptors (Das et al., 1992; Muniz et al., 2007). To

transfer the fat-soluble retinoid into the aqueous cytosolic and extracellular location, they need to bind to retinol binding proteins (Tsacopoulos et al., 1998).

New functions of Müller cells have also been recently discovered such as light guidance (Reichenbach and Bringmann, 2013). As the light passes through the retina, it is scattered by retinal cells and their processes. Non-foveal Müller cells minimize light scattering by guiding light through the inner retinal layers towards the photoreceptors (Franze et al., 2007). In addition, Müller cells have been shown to concentrate the green-red wavelengths of the visible spectrum onto cones, to allow the blue-purple wavelengths to leak onto nearby rods (Labin et al., 2014).

1.4.8.3.4 Müller Cells in Injured Retina

In response to severe retinal injuries such as retinal detachment, trauma, ischemia, and chronic conditions such as glaucoma and diabetic retinopathy glial cells become activated (Bringmann and Reichenbach, 2001; Bringmann et al., 2004; Bringmann et al., 2006; Francke et al., 2005). This activation is termed gliosis and is accompanied by morphological, biochemical and physiological changes to Müller cells with release of inflammatory mediators and growth factors (Bringmann and Reichenbach, 2001). It has been found that Müller cells do not all respond in the same way to an injury. For example, Fischer and Reh, (2003) reported that in response to NMDA-induced retinal damage, approximately 65% of the Müller cells re-entered the cell cycle but failed to increase their expression of GFAP, while the remaining 35% increased their expression of GFAP in response to the damage but did not proliferate. Based on this principle, gliosis is described as either conservative non-proliferative or massive proliferative gliosis. The former type is characterized by up-regulation of GFAP, cellular hypertrophy, a moderate or no decrease in potassium currents, a decrease in the expression of glutamine synthetase, cellular retinaldehyde-binding protein (CRALBP) and carbonic anhydrase (Lewis et al., 1992; Lieth et al., 1998; Joly et al., 2008;

Kacza et al., 2000). In proliferative gliosis, the cells form a "gliotic scar" within the retina, at subretinal and epiretinal surfaces to fill in spaces that resulted from any loss in neurons, pigment epithelium, blood vessels or photoreceptors (Bringmann et al., 2000). It has been also reported that Müller cells can trans-differentiate into contractile myo-fibrocytes in the epiretinal region (Guidry, 2005).

Müller gliosis is associated with dependent and non-dependent responses to pathogenic stimuli. Three non-specific gliotic responses could take place including cellular hypertrophy, proliferation and up-regulation of the intermediate filaments nestin, vimentin, and GFAP (Bringmann and Reichenbach, 2001; Lewis and Fisher, 2000; Geller et al., 2001; Takeda et al., 2002; Tezel et al., 2003). On the other hand, glutamine synthetase is a Müller cell-specific gliotic enzyme normally involved in glutamate recycling.

Gliosis is considered as an attempt by Müller cells to protect the retina in response to an injury (Burke and Smith, 1981; Bringmann et al., 2006). However, gliosis can result in harmful effects to the tissue. In gliosis, Müller cells secrete mediators that have both cytoprotective and cytotoxic effects on retinal neurons (Bringmann and Reichenbach, 2001). These include growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) and transforming growth factor beta (TGF-β) (Eichler et al., 2004a; Geller et al., 2001; Volpert et al. 2002). Angiogenic factors are balanced by inhibitors of angiogenesis such as pigment epithelium-derived factor (PEDF) (Duh et al., 2002; Eichler et al., 2004b; Gao et al., 2001), thrombospondin-1 (TSP-1) and prolactin (Behzadian et al., 1995; Rivera et al., 2008). Platelet-derived growth factor (PDGF) stimulates Müller cell growth and proliferation and is known to play a role in proliferative retinopathies. Many studies have reported that Müller cells produce pro-inflammatory cytokines in response to an injury in addition to their

phagocytosis properties (Caspi and Roberge, 1989; Roberge et al., 1991; Mano and Puro, 1990; Francke et al., 2001).

In glaucoma, Müller cells have been found to express GFAP, increase glutamate uptake and proliferate. These responses have been linked to the pathophysiology of retinal ganglion cell death (Kanamori et al., 2005; Kawasaki et al., 2000; Lam et al., 2003; Tezel et al., 2003; Woldemussie et al., 2004). In the following section, the role of Müller cells in the pathogenesis of glaucoma will be discussed.

1.5 Glaucoma

Glaucoma is defined as a neurodegenerative disease with a loss of visual field caused by retinal ganglion cell (RGC) degeneration and optical nerve head damage (Cherecheanu et al., 2013). It is therefore described as an optic neuropathy that is associated clinically with a high cup to disc ratio as more ganglion cells, and their axons are affected and finally results in visual field loss (Foster et al., 2002). According to the World Health Organization (WHO), glaucoma is the second leading cause of blindness globally after cataract (Cook and Foster, 2012; Resnikoff et al., 2004) with estimated 80 million people worldwide predicted to be suffering from glaucoma in 2020 and 111.8 million by 2040 (Cook and Foster, 2012; Tham et al., 2014). In the United Kingdom, the NHS report more than one million glaucoma visits per year, which account for 23% of attendance at UK hospital eye services. Therefore, glaucoma imposes a significant social and economic burden, which is increasing as the aged population rises (Burr et al., 2007; Coleman and Miglio; 2008; Spry et al., 1999).

Increased intra-ocular pressure (IOP) is considered the most important risk factor in the pathophysiology of the disease and the reduction of IOP is the basis of glaucoma therapy (Coleman and Miglior, 2008). IOP is regulated through the balance of aqueous humor production by the epithelium of the ciliary body and outflow via the trabecular meshwork into Schlemm's canal. This generates an IOP of approximately 15 mmHg (Goel et al., 2010). A small volume of the aqueous humor also leaves the eye through the uveoscleral outflow pathway, which is independent of the IOP (Alm and Nilsson; 2009). The aqueous humor is a clear fluid containing the nutrients as well as electrolytes, proteins, cytokines, organic solutes, and growth factors necessary for the maintenance of the avascular structures of the eye specifically the lens and cornea (Chowdhury et al., 2010). The rate of aqueous humor turnover is estimated to be 1.0% to 1.5% of the anterior chamber volume per minute, which is equal to 2.4 ± 0.6µl/min (Gabelt and Kaufman; 2003). In the normal human aging eye, a

reduction in the production of aqueous humor is accompanied with a reduction in its drainage enabling a stable IOP level (Toris et al., 1999). However, changes in the structure of the endothelium lining Schlemm's canal or trabecular meshwork can cause resistance to drainage while the inflow remains normal and thus IOP can become elevated (Goel et al., 2010).

When associated with an increase in IOP, glaucoma is classified into two major categories according to the obstruction of the drainage pathway at the iridocorneal angle: Primary Angle-Closure Glaucoma (PACG) and Primary Open Angle Glaucoma (POAG). The term ocular hypertension refers to high IOP above 21mmHg with no apparent cause but with normal optic nerve head and no visual field damage. In some patients, despite the presence of open and normal-appearing anterior chamber angles and normal IOP, there is optic nerve head damage and visual field defects, these patients are said to have normal tension glaucoma (NTG) (Lee et al., 1998; Shields, 2008). Thus, it should be noted that the level of IOP in open-angle glaucoma is not a real definition criterion of the disease as the IOP levels could fall within the normal range. Angle-closure glaucoma occurs when there is physical obstruction of the trabecular meshwork caused by the peripheral iris, which prevents the drainage of aqueous humor. The accumulation of fluid inside the eye results in a sudden symptomatic increase in IOP of more than 21mmHg and results in symptoms including blurred vision, ocular pain, and corneal epithelial edema. The condition could be unilateral or bilateral and results in visual field loss if not treated rapidly (Vetrugno et al., 2008). The Shaffer system is one of the common systems to diagnosis angle closure glaucoma and is based on the assessment of the angle between the iris and the cornea. According to this system, there are five stages of angle glaucoma ranging from zero to five, where five indicates total occlusion of the angle (Amerasinghe and Aung, 2008).

The aim of the treatment is to control IOP and to decrease the complications concerning visual loss. Two different approaches are used to manage angle closure glaucoma; the first is

medication while the latter is the surgery. Drugs that control IOP such carbonic anhydrase inhibitors, beta-blockers or alpha2 adrenergic agonists are used. Miotics are sometimes used to open the angle by pulling the peripheral iris, but must be used with caution as they might cause further angle closure and exaggerate the condition (Vetrugno et al., 2008; Amerasinghe and Aung, 2008). Surgical intervention such as laser peripheral iridotomy, lens extraction, and trabeculectomy can also be used (Amerasinghe and Aung, 2008; Vetrugno et al., 2008).

1.5.1 Primary Open Angle Glaucoma (POAG)

This is also known as chronic open angle glaucoma (COAG). It is characterized by an open, normal-appearing anterior chamber angle, optic nerve head damage and increased intraocular pressure (IOP) without any apparent ocular or systemic abnormality (Allingham et al., 2009). Several mechanisms have been proposed to explain POAG including the obstruction of aqueous humour outflow due to histological changes in the structure of the juxtacanalicular tissue of the trabecular meshwork and the inner wall of Schlemm's canal. These changes include an increase in the extracellular matrix and an accumulation of plaque material in trabecular meshwork (Tamm and Fuchshofer, 2007). An early study by Zatulina et al., (1978) revealed that narrowed Schlemm's canal with adhesions between the inner and outer walls leads to the collapse of the Schlemm's canal, which will increase resistance to aqueous outflow. Genetic factors may play a role in the development of POAG, and some studies have found a link between a mutation in the myocilin gene and development of the disease (Tomarev et al., 1998). Myocilin is a protein present in the trabecular meshwork, cornea, retina, optic nerve and ciliary nerves (Fingert et al., 1998; Tomarev et al., 1998). It is produced in high amounts during stress conditions such as dexamethasone treatment, oxidative stress, and treatment with transforming growth factor β (TGF- β) (Polansky et al., 1997; Tamm et al., 1999). It has been also found that the aqueous humour of patients with POAG has a significant concentration of TGF-β, which may be responsible for increasing the

resistance through decreasing the cellularity of the trabecular meshwork and promoting a build-up of plaques (Tamm and Fuchshofer, 2007). There is evidence that patients under corticosteroid treatment develop POAG with abnormal resistance to aqueous outflow and variable degrees of elevated IOP (Lewis et al., 1988; Klemetti, 1990). Suppression of phagocytic activity of endothelial cells lining the trabecular meshwork, which removes debris in aqueous humour and prevents blocking of the Schlemm's canal (Bill, 1975).

Changes have been also observed in the optic nerve head. Astrocytes and microglia in the region of ONH have been shown to be activated with detrimental consequences (Yuan and Neufeld, 2000). Activated glial cells were found to increase secretion of matrix metalloproteinase (MMPs), the proteolytic enzymes that degrade components of extracellular matrix (ECM) (Okada et al., 1990). Increased immunostaining for MMP-1, -2 & -3 in glaucomatous ONH sections from post-mortem eyes has also been observed (Yan et al., 2000). Characteristics of a glaucomatous ONH include generalized/focal enlargement of the cup, disc hemorrhage, thinning of neuroretinal rim, loss of the nerve fiber layer and parapapillary atrophy (Bourne, 2006).

1.5.2 Management of Primary Open Angle Glaucoma (POAG)

Initiating a medical treatment is required when a patient presents with high IOP and glaucomatous damage. The treatment for open angle glaucoma includes the use of mainly topically administered drugs to lower IOP by enhancing aqueous outflow, reducing aqueous production, or with a combined mechanism. Currently, five different classes of glaucoma medications are available for the treatment of glaucoma: prostaglandin analogues, β-adrenergic receptor antagonists, adrenergic receptor agonists, carbonic anhydrase inhibitors, cholinergic, or miotic agents. A brief overview of currently available glaucoma drugs is provided below.

1.5.2.1 Prostaglandin Analogues

Prostaglandin Analogues are the most commonly prescribed drugs for the treatment of POAG. Prostaglandin $F_{2\alpha}$ analogues such as latanoprost, unoprostone, travoprost, and bimatoprost have been found to reduce intraocular pressure by increasing the uveoscleral outflow through the iris and ciliary body (Toris et al., 1997). This has been explained by either decreasing the extracellular matrix that surrounds the muscle bundles or by relaxing the ciliary musculature (Crawford and Kaufman, 1987; Lutjen-Drecoll and Tamm 1988). The most commonly reported side effects of prostaglandin analogues are muscle and joint pain, skin allergy and colour discoloration of the iris (Alm and Stjernschantz; 1995).

1.5.2.2 Beta-adrenergic Blockers

Beta-blockers were introduced for glaucoma treatment in 1979 and were rapidly considered the first line therapy (Obstbaum et al., 1978). They work by decreasing the production of aqueous humour. Neufeld (1979) showed that ß-blockers inhibit the endogenous adrenergic stimulation that stimulates the formation of aqueous humour by the ciliary processes. The non-selective β-blocker timolol was recognized as an effective drug for the management, of raised IOP. It remained the gold standard therapy for POAG for a long time due to its efficacy in decreasing the pressure, tolerability and convenient administration (Demailly et al., 1978; Obstbaum et al., 1978). Other selective $ß_1$-blockers such as betaxolol were added to glaucoma management, but they were less effective compared to timolol (Caprioli and Garway-Heath, 2007). Generally, β-blockers are associated with well-known systemic side effects that may exacerbate other conditions such as asthma, chronic obstructive pulmonary disease, and heart conditions. Moreover, they should be used with caution in diabetic patients as they mask the symptoms of hypoglycemia (Schwarzt and Budenz, 2004).

1.5.2.3 Alpha-agonists

The α-adrenergic agonists act by decreasing aqueous humour production through constricting blood vessels supplying the ciliary body and by decreasing aqueous humour production (Sears and Neufeld, 1975). Apraclonidine and brimonidine are α_2-adrenergic–agonist drugs that can be used in the treatment of glaucoma, although they are not widely prescribed. The major complaints reported with these medications are dry mouth and dry nose (Schuman, 1996).

1.5.2.4 Carbonic Anhydrase Inhibitors (CAIs)

Acetazolamide, brinzolamide, and dorzolamide are carbonic anhydrase inhibitors, which work by blocking the formation of aqueous humour by inhibiting the key enzyme responsible for bicarbonate production. As the level of bicarbonate drops, sodium and water flow to posterior chamber decreases and thus a reduction in intraocular pressure through a reduction in aqueous humour production is achieved (Becker, 1954). This class of medications is associated with a series of side effects such gastrointestinal disturbance, a metallic taste in the mouth and cramps (Epstein and Grant, 1977). More serious side effects are blood dyscrasias including aplastic anaemia, agranulocytosis, thrombocytopenia, neutropenia, and haemolytic anaemia (Werblin et al., 1980).

1.5.2.5 Miotics

Muscarinic agonists such as pilocarpine, aceclidine and carbachol, have been used for the management of glaucoma due to their miotic effect. However, they are now obsolete because of common adverse reactions resulting from stimulation of the parasympathetic system. This may include but are not limited to intestinal cramps, bronchospasm, cardiac irregularities, ocular stinging, lacrimation, small pupil, conjunctival thickening, cataract, and retinal detachment (Vetrugno et al., 2008).

1.5.3 Pathogenesis of Primary Open Angle Glaucoma (POAG)

1.5.3.1 Increased IOP and Retinal Ganglion Cells (RGCs) Death

The death of RGCs in IOP-induced glaucoma is a multifactorial process. Mechanical injury to the axons at the optic disc as a result of high intraocular pressure, which is then transmitted to the retinal ganglion cell body in the retina, is a major proposed mechanism by which RGCs die (Anderson and Hendrickson, 1974; Mabuchi et al., 2003). Clinically, this can be recognized by ophthalmoscopy, which reflects the structural changes at the optic nerve head where ganglion cell axons exit the eye to form the optic nerve leaving a central depression called the cup. As glaucoma progresses and there is a loss of the RGC axons, the cup-to-disc ratio is increased (Quigley, 2011). The optic nerve fibers exit the eye through a mesh-like structure known as the lamina cribrosa. Structural changes such as compression or displacement of the lamina cribrosa as a consequence of high intraocular pressure has also been documented, which could mediate mechanical injury (Quigley, 2011). Several animal models have shown that raised IOP results in RGCs death. For example, in a mouse model of hereditary glaucoma (the DBA/2J mouse), IOP was elevated as a result of reduced aqueous humour outflow that caused the death of RGCs, optic nerve atrophy, and optic nerve cupping (John et al., 1998).

In addition to mechanical damage, RGCs are subjected to hypoxic (Tezel and Wax, 2004) and tissue oxidative stress (Tezel et al., 2000) as a result of elevated IOP. Although controlled or reduced IOP level with treatment has been shown to improve the prognosis of patients, it can not stop progression in all patients and therefore, elevated IOP level cannot solely account for all of the damage occurring to the RGCs. Important factors such as glial activation, vascular dysregulation induced hypoxia and ischemia as well as excitotoxicity are also proposed to be involved in the pathogenesis of POAG and will be discussed in the next sections.

1.5.3.2 Activation of Müller Cells in Glaucoma

Several studies have shown that glial cells are involved in the pathophysiology of glaucoma. Astrocyte activation at the optic nerve head has been reported in *post-mortem* eyes with open angle glaucoma (Varela and Hernandez, 1997). Reactive astrocytes in glaucomatous optic nerve heads in culture have been shown to produce an excessive nitric oxide, which exert a neurotoxic effect on the RGCs (Liu and Neufeld, 2000).

In a chronic model of elevated IOP in rats induced by episcleral vein cauterization, Müller cells gained GFAP immunoreactivity lasted even after normalization of IOP (Kanamori et al., 2005). In this model, Vidal et al. (2010) also reported hypertrophy of Müller cells with a significant increase in GFAP. Non-proliferative reactivity of glial has been reported in a chronic model of glaucoma using the DBA/2J mouse (Inman and Horner, 2007).

In addition, in ischemic rat retina, induced by high IOP, GFAP immunoreactivity was detected in the endfeet and distal processes of Müller cells immediately after reperfusion and continued to increase with a correlation to neuronal degeneration (Kim et al., 1998). Permanent occlusion of the carotid arteries in rats has also been show to cause GFAP expression in retinal Müller cells (Osborne et al., 1991).

1.5.3.3 Reduced Ocular Blood Flow and Vascular Dysregulation

The retinal blood supply is derived from the central retinal artery, which is a branch of the ophthalmic artery. It is characterized by a very small flow rate but with a high oxygen extraction level (Grant-Kels and Kels, 1992; Kels et al., 2015). The regulation of retinal blood flow depends on the relationship between perfusion pressure and local resistance. Perfusion pressure is defined as the difference between retinal arterial and retinal venous pressure while the local resistance is controlled by the size of the local vessels. In the normal eye, the venous pressure is less than or equal to IOP (Flammer et al., 2002; Stodtmeister, 2008). Local mediators control local resistance, for example endothelium-derived nitric oxide

and endothelin (ET) are very potent modulators of vascular tone (Haefliger et al., 1992; Haefliger et al., 2001).

In glaucoma, decreased optic nerve head and juxtapapillary retinal capillary blood flow was found in early and advanced stage POAG patients (Michelson et al., 1996). In addition, a reduction in blood flow was found in the retina, choroid and optic nerve head in glaucoma patients with high IOP (Arnold, 1995; Harju and Vesti, 2001; Sugiyama et al., 2000; Yamazaki et al., 1996). In patients with POAG and visual field deterioration, low blood perfusion was detected and seems to be associated with the progression of the disease (Gherghel et al., 2000).

Patients with NTG have been reported to have a greater reduction in the choroidal circulation (Geijssen and Greve, 1995; Duijm et al., 1997) and blood flow disturbances generally are reported to be more marked in NTG than patients with high IOP (Drance et al., 2001; Findl et al., 2000; Schmidt et al., 1998). Earlier studies have shown that some patients may present with a number of signs related to compromised blood flow such as changes in conjunctival capillaries (Orgul and Flammer, 1995), local vasoconstriction in the retina (Rankin and Drance, 1996), increased prevalence of ONH haemorrhage (Drance et al., 2001; Sugiyama et al., 1997; Orgul and Flammer, 1994) and increased prevalence of venous thrombosis (Malayan et al., 1999; Sonnsjo and Krakau, 1993). Flammer et al., (2002) argued that reduced ocular blood flow occurs due to primary rather than secondary causes (i.e., due to elevated IOP). The author also proposed that increased resistance to flow could be caused by structural changes such as vasculitis or arteriosclerosis, or it might be due to a functional dysregulation of the vascular diameter. Endothelial leukocyte adhesion molecule-1 (ELAM-1) is an early marker of arteriosclerosis and was found in the trabecular meshwork (TM) cells in the outflow pathways of eyes with glaucoma, which may support the role of atherosclerosis in the aetiology of reduced blood flow in glaucoma (Wang et al., 2001).

Vascular dysregulation is a phenomenon used to describe the imbalance between vasodilator and vasoconstrictor mechanisms and could be due to the release of circulating hormones or local mediator produced by the vascular endothelium such as endothelin-1 (ET-1), nitric oxide or prostacyclin, which regulates local blood flow. A relationship between the endothelin system and between glaucomatous optic neuropathy has been documented. Animal studies have also shown that the level of ET-1 in aqueous humour was found to be 2-4 fold higher compared to control (Kallberg et al., 2002; Prasanna et al. 2005). *In vivo* studies have shown that chronic administration of endothelin-1 to the optic nerve of rats resulted in a time and dose-dependent loss of RGCs and their axons (Chauhan et al., 2004; Cioffi et al., 1995). In patients with POAG, the level of ET-1 and nitric oxide (NO) concentrations in aqueous humour were found elevated (Ghanem et al., 2011). Few studies have shown that patients with NTG demonstrated an impaired peripheral endothelium-mediated vasodilation and peripheral abnormal contractile responses to 5-HT (5-hydroxytryptamine), ET-1 and ET-1 antagonist (Buckley et al., 2002). The plasma levels of ET-1 tended to be higher in patients with NTG compared to high-tension glaucoma (Sugiyama et al., 1995).

In these patients, the plasma ET-1 levels were higher in the initial stage of visual field loss than those in the middle phase (Sugiyama et al., 1995). To the contrary, in a study, which involved patients with POAG, endothelin levels were comparable to control (Tezel et al., 1997). Moreover, dual inhibition of endothelin receptors (ETA and ETB) increases ocular blood flow in patients with glaucoma and also healthy subjects (Resch et al., 2009).

Moreover, ET-1 has also been found to play a role in the development of visual impairment in non-glaucoma related conditions. High levels of ET-1 were found in cerebrospinal fluid and circulating blood in patients with multiple sclerosis (Speciale et al., 2000). Interestingly, in this subgroup of patients, the raised ET-1 was associated with a decrease in the macular thickness (Gugleta et al., 2008), sub-clinical visual defect (Mienberg et al., 1982), thinner

arterioles and subclinical swelling of the optic nerve (Gugleta et al., 2009). Similarly, ET-1 was found in high levels in optic neuritis (Haufschild et al., 2003), and retinal vein occlusion (Haufschild et al., 2004). In addition to its role as a vasoconstrictor, ET-1 was found to be a potent mitogenic factor for different cells including the astrocytes (Baba, 1998). Nicolela, (2008) suggested that vascular dysregulation is unlikely to lead to ischemic damage at the level of the optic nerve head. Instead, it is suggested that vascular dysregulation impairs auto-regulation in the retina, which causes relative ischemia and damage as a result of subsequent reperfusion. The reperfusion injury results from inflammatory mediators and oxidative stress generated by restoring the blood flow (Flammer, 2002). Elevated IOP and induction of ischemia in rats followed by reperfusion has been reported to cause damage to the retinal ganglion cell layer and the optic nerve (Adachi et al., 1996).

Other conditions that may be associated with the development of glaucoma include low blood pressure, which leads to low ocular perfusion (Pache et al., 2002). Also, ischemia due to occlusion of the retinal artery, retinal hypoperfusion secondary to systemic diseases such as diabetes and Alzheimer's disease (Kaur et al., 2008). Furthermore, several authors have shown that patients with optic nerve damage tend to have several characteristics such as high prevalence of a migraine, cold hands, and a vasospastic response to cold in the finger circulation (Broadway and Drance, 1998; Nicolela and Drance, 1996). Grieshaber et al. (2007), found that activated retinal astrocytes and Müller cells were associated with vascular dysregulation in patients with POAG and glial cells activation was found to be unilateral or bilateral. Dysregulation of perfusion will therefore deprive the tissue of the necessary supply of oxygen and nutrients and possibly activate glial cells, which may, in turn underlie pathogenic events in POAG and NTG. The next section will focus on important mechanisms that follow vascular dysregulation and reduction of ocular blood flow specifically oxygen-glucose deprivation (OGD) and their consequences to RGC survival in glaucoma.

1.5.4 Hypoxia-Ischemia

The retina is a highly metabolically active tissue with the highest oxygen consumption rate in the body (Ames, 2000). It absolutely depends on blood to supply oxygen and glucose in order to maintain the structural and functional integrity. The choroidal circulation provides oxygen mainly to the outer retina while the retinal vasculature nourishes the inner retina. The retina is less vascularized compared to the choroid to allow passage of light to the photoreceptors and is characterized by a high arteriovenous oxygen difference and well developed auto-regulatory mechanisms (Pournaras et al., 2008). In the rat retina, the highest level of oxygen is in the choroid, the inner segments of the photoreceptors, the outer plexiform layer, and the inner plexiform layer (Cringle and Yu, 2002; Yu and Cringle, 2001). It has been reported that photoreceptor oxygen consumption increased in dark, but it decreases in the inner retina (Cringle et al., 2002). Systemic factors such as oxygen tension, carbon dioxide level, and blood pressure can influence the vascular structures supplying the retina. It has been found that systemic hyperoxia to increase the oxygen levels in the choroid and all layers of the rat retina (Yu et al., 2009). In hypoxemia, a reduction in oxygen tension in the choroid was observed, because of reduction in choroidal blood flow (Ahmed et al., 2001). When the IOP increases, the choroidal blood flow decreases, which leads to a reduction in oxygen tension and subsequently a decrease in photoreceptors oxygen tension (Yancey and Linsenmeier, 1989).

Hypoxia refers to a reduction in oxygen supply to a tissue. Ischemia is a combined reduction of oxygen and blood flow with a subsequent reduction in nutrients and removal of waste products. Hence, ischemia could be partial or complete as occurs in vasospasm, artery occlusion or venous thrombosis. Hypoxia-induced expression and release of VEGF regulates vascular permeability and induce proliferation and differentiation of endothelial cells as well as Müller cells (Shima et al., 1995; Stone et al., 1995). In a recent study by Huang et al.,

(2015); VEGF was detected in the aqueous humour samples of PACG patients, which could be attributed to hypoxia/ischemia observed in glaucoma patients. Oxidative stress has been acknowledged as an important mechanism of RGCs death as a result of hypoxic-ischemic insult. Reactive oxygen species (ROS) are produced in the retina in two stages; both during ischemia and the reperfusion stage (Ophir et al., 1993). During ischemia, degradation of ATP leads to the formation of hypoxanthine that increase Ca^{2+} activated enzymes such as calpain, which converts xanthine dehydrogenase into xanthine oxidase (Cazevieille et al. 1994). When reperfusion occurs, the xanthine oxidase oxidizes the accumulated hypoxanthine to uric acid resulting in the release of superoxide (Cazevieille et al. 1994).

Several mechanisms have been proposed by which hypoxia-ischemia causes neuronal death including excitotoxicity, oxidative stress, inflammation and glial cells activation (Lipton, 1999). Selective mechanisms will be discussed in detail.

1.5.4.1 Hypoxia-Ischemia Induced Glutamate Excitotoxicity

Glutamate is an excitatory amino acid and an important neurotransmitter in the CNS including the retina. It is involved in the different physiological processes and pathophysiological conditions (Ozawa et al., 1998). Glutamate is released by photoreceptors, bipolar cells, and ganglion cells and mediates the transfer of visual signals in the retina (Massey and Miller 1987; Yang, 1997). Excess glutamate is also known to cause excitotoxicity (Dallas et al., 2007) and early evidence of glutamate toxicity was obtained from Lucas and Newhouse (1957), who found that parenteral injection of glutamate selectively damaged the RGCs in mice. This has been also suggested by many subsequent studies. For example, chronic intravitreal injection of low dose glutamate in rats resulted in loss of 42% of the RGCs (Vorwerk et al., 1996). The response of rat and pig retinal cultures to glutamate was compared in a study by Luo et al., (2001). The author reported that the loss of RGCs varied between the two species with a close resemblance of pig RGCs to human

(Luo et al., 2001). These responses were found to be mediated by both NMDA and non-NMDA receptor pathways such as kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) (Luo et al., 2001). It has been found that glutamate levels were elevated in patients with glaucoma undergoing vitrectomy (Honkanen et al., 2003). In addition, elevated glutamate was found in different disease models and species including chronic elevation of IOP in rats (Levkovitch-Verbin et al., 2002), glaucomatous eyes in dog (Brooks et al., 1997; Källberg et al., 2007) and in a rabbit model of optic nerve ischemia (Kim et al., 2000). Animal studies have suggested that glutamate excitotoxicity causes RGCs death in central retinal ischemia (Dallas et al., 2007; Duker and Brown, 1988; Ffytche, 1974; Hayreh and Jonas, 2000). Elevated and toxic concentrations of glutamate to RGCs have been found in the vitreous humour in animal models of glaucoma (Brooks et al., 1997). These findings however were questioned by Kwon et al., (2005) who failed to show any significant increase in the vitreous and retinal concentrations of glutamate following acute central retinal artery occlusion in rhesus monkeys. However, local increase has been proposed to occur. For example, accumulation of glutamate as a result of decreased clearance might happen due to the inefficient uptake by the glutamate transporter (GLAST) in glial cells, which has been observed in an experimental rat model of glaucoma (Martin et al., 2002).

Hypoxia can contribute to the accumulation of glutamate in retinal tissue. A recent study by Kaur et al., (2012) reported hypoxia-induced glutamate overload in neonatal rat retina with increased intracellular calcium (Ca^{2+}), and increased expression of caspase-3 and NMDA receptor, which mediate damage to the neurons. Studies using neuronal cultures have also shown that excess glutamate is one source of reactive oxygen species (ROS) generation in the ischemic retina (Cazevieille et al. 1993; Dutrait et al. 1995; Dykens et al. 1987; Lafon-Cazal et al. 1993). In addition, Aizenman et al., (1988) showed that rat RGCs are sensitive to

glutamate and excitatory amino acid analogues such as kainite, quisqualate, and N-Methyl-D-aspartate (NMDA). Increased Ca^{2+} accumulation and ROS generation with the involvement of ionotropic AMPA glutamate receptors has been reported as a mechanism of RGC loss (Sivakumar et al., 2013). Systemic treatment with NMDA and non-NMDA receptor antagonists prevented IOP-induced RGCs loss in rats (Nucci et al., 2005). Further studies indicate that NMDA receptor antagonists, are neuroprotective against RGC loss in *in vitro* and *in vivo* animal models (Chen and Lipton, 1997; Osborne, 1999; WoldeMussie et al., 2002). Evidence therefore suggests that glutamate toxicity plays a major role in hypoxia-ischemia induced RGC loss.

1.5.4.2 Hypoxia-Ischemia Induced Purinergic Excitotoxicity

ATP and adenosine act as neurotransmitters and mediators in the retina and purine receptors are expressed throughout the eye, including in the RPE, RGCs and Müller cells (Hodges et al., 2011; Kiel et al., 2011; Mathias et al., 2007; Mitchell and Reigada, 2008; Sanderson et al., 2014; Oswald et al., 2012; Wurm et al., 2011).

In normal healthy retinal tissue, the majority of the endogenous purine exists in the form of ATP, and a small amount exists as adenosine (Perez et al., 1986). Müller cells express both adenosine and nucleotide receptors (Bringmann et al., 2001). Adenosine receptors (A_{1-3}) are G protein-coupled receptors, while the nucleotide receptors are either G protein-coupled receptors (P2Y) or ligand-gated cation channels (P2X). Both types are expressed in human Müller cells (Bringmann et al., 2001; Pannicke et al., 2000). Activation of the $P2X_7$ receptor in human Müller cells by external ATP resulted in the release of Ca^{2+} from intracellular stores, which in turns activate big conductance K^+ channels (BK). This may contribute to Müller cell proliferation as seen in proliferative vitreoretinopathy (Bringmann et al., 2001; Pannicke et al., 2000). This is also seen in retinal detachment and transient retinal ischemia (Uckermann et al., 2005; Uckermann et al., 2003). Activation of $P2X_7$ also increases sodium

ion influx resulting in cell depolarization and decreases the efficiency of sodium-dependant glutamate uptake (Pannicke et al., 2000).

It is also proposed that elevated extracellular ATP can be associated with high IOP and RGCs death (Resta et al., 2007; Sanderson et al., 2014; Zhang et al., 2007). Sustained stimulation of $P2X_7$ receptor caused RGCs death *in vitro* (Zhang et al., 2005) and *in vivo* (Hu et al., 2010) and blocking of purinergic receptors or dephosphorylating ATP attenuated elevated IOP induce RGCs damage in rats (Resta et al., 2007). Simulated ischemia in human organotypic retinal cultures (HORCs), as well as $P2X_7$ receptor stimulation, caused a marked loss in RGCs, which was inhibited by $P2X_7$ receptor antagonism (Niyadurupola et al., 2013). $P2X_7$ receptor activation has been shown to be involved in hypoxia-mediated retinal neuronal cell death (Sugiyama et al., 2010).

Pannexin 1 (*PANX1*) is a high-conductance channel, which respond to different retinal insults by mediating the release of ATP (Kurtenbach et al., 2014). The released ATP is hydrolysed by autotaxin or nucleotide pyrophosphatase/phosphodiesterase 2 (NPP2), a plasma lysophospholipase D (lysoPLD), which generates lysophosphatidic acid (LPA). LPA receptors are expressed in astrocytes, microglia, and oligodendrocytes (Rao et al., 2003; Tabuchi et al., 2000; Yu et al., 2003). In addition, it has been found that LPA can inhibit gap junction channels and resulting in inhibition of ATP release (De Vuyst et al., 2007). A study by Newman, (2003) reported that LPA induces calcium responses in Müller cells, which causes ATP release from the cells. Inhibition of ecto-ATPase and ectonucleaotidase reduced glial mediated hyperpolarization in neighbouring ganglion cells indicating that this response is mediated by ATP release from Müller cells in mammalian retina (Newman, 2006). Activation of LPA receptor in primary rat brain astrocytes caused a reduction in glutamate and glucose uptake (Keller et al., 1996). Moreover, LPA induces VEGF via HIF-1α activation in cancer cells (Jeon et al., 2010; Lee et al., 2006; Park et al., 2007). In patients

with PDR, significant high levels of VEGF and LPA were detected in the vitreous samples suggesting that LPA inducing VEGF play an important role in the progression of diabetes (Abu El-Asrar et al., 2013). LPA and its receptor LPA1 were also expressed in epiretinal membranes of patients with proliferative vitreoretinopathy (Abu El-Asrar et al., 2012).

1.5.4.3 Effects of Hypoxia-Ischemia on Müller Cells

Retinal hypoxia is proposed to play a role in many retinal disease including ischemia, glaucoma, diabetic retinopathy, retinal vein occlusion and age-related macular degeneration (Kaur et al., 2008). Retinal hypoxia may affect the survival of RGCs by inducing apoptosis (Kitano et al., 1996). Several mechanisms have been proposed in the pathogenesis of hypoxia-induced RGCs death including glutamate excitotoxicity (Kaur et al., 2008), calcium overload (Sivakumar et al., 2013), and oxidative stress (Love, 1999). In addition, inflammatory markers such as tumor necrosis factor-alpha (TNF-α) have been reported to induce neuronal death in hypoxia (Martin-Villalba et al., 1999).

Moreover, hypoxia-inducible factor-1α (HIF-1α), has been found to increase in glaucomatous human eyes (Tezel and Wax 2004). Hypoxia is associated with neovascularization and expression of hypoxia-HIF-1α and its downstream gene, vascular endothelial growth factor (VEGF), is a well-known pathway of angiogenesis (Bernaudin et al., 2002). Müller cells are important producers of angiogenic and anti-angiogenic factors in the neural retina (Bringmann et al., 2009), and they have been identified as the cells in the inner nuclear layer of the retina responsible for producing VEGF (Pierce et al., 1995). It is believed that VEGF is the main growth factor that mediates retinal angiogenesis, and it is controlled by local oxygen concentrations (Shima et al., 1995). In a mouse model of proliferative retinopathy, VEGF mRNA expression increased early after hypoxia induction and remained elevated during the development of neovascularization (Aiello et al., 1994). This is also supported by high VEGF

levels in the retina and the vitreous of patients with ocular ischemic disorders (Adamis et al., 1994).

Exposure of human Müller cells (MIO-M1) to hypoxia has been shown to cause VEGF release and cell proliferation that was inhibited by TGF-β2 and PEDF (Eichler et al., 2004a). Findings by Eichler et al., (2004b) also showed that human and guinea pig Müller cells express and release the antiangiogenic factor, PEDF in response to hypoxia. Exposure of Müller cells to VEGF suppressed PEDF release in a dose-dependent manner. It has also been reported that VEGF protects primary cultures of rat cerebral cortical neurons from oxygen and glucose deprivation (Jin et al., 2000).

1.5 Aims of the Study

It is accepted that IOP is an important risk factor in glaucoma. However, despite the treatment with pressure controlling medication, the progression of disease continues to cause loss of vision. Furthermore, classification of some patients as having normal tension glaucoma indicates that the relationship between IOP and progression of the disease is not simple. The underlying mechanisms of glaucoma are diverse and poorly understood. Vascular dysregulation and reduction of ocular blood flow is one mechanism by which oxygen and glucose deprivation (OGD) occurs in ocular tissues. Simulating oxygen and glucose deprivation is one approach to study glaucoma by mimicking the restriction of metabolite and oxygen supply caused by ischemia. The aim of this study was to investigate the effects of oxygen and glucose deprivation on Müller cells (the MIO-M1 cell line) and the human retina using human organotypic retinal cultures (HORCs).

In both models, two selected levels of OGD were chosen to study the survival of MIO-M1 cells and RGCs in HORCs and the expression of key growth factors, excitotoxicity markers and Müller cells specific markers under conditions of OGD. It is hoped that understanding more about these responses in human retina will lead to new approaches in managing vision loss in glaucoma.

The specific aims of the current work are listed below:

1- To investigate the viability and cytotoxicity of human Müller cells using the MIO-M1 cell line under different levels of oxygen and glucose deprivation ranging from 70-100% deprivation for up to 72hrs.

2- To investigate the gene expression of different angiogenic and anti-angiogenic growth factors, excitotoxicity markers, glial markers and heat shock protein under two levels of deprivations (80% and full deprivation) of both oxygen and glucose in MIO-M1 cells.

3- To investigate RGC survival in HORCs and gene expression of different angiogenic and anti-angiogenic growth factors, excitotoxicity markers, glial markers and heat shock protein were also evaluated under under two levels of deprivations (80% and full deprivation) of both oxygen and glucose.

4- To investigate the effects of co-culture of MIO-M1 cells and HORC in protectection of RGC death under conditions of glucose deprivation.

5-To investigate the effects of high glucose on the survival and gene expression of MIO-M1 and HORCs.

Chapter 2

Materials and Methods

2. Tissue and Cell Culture

Human eyes for the research purposes were provided by the East Anglian Eye Bank (Norfolk and Norwich University Hospital, UK). Human donated eyes were retrieved after obtaining the informed consent from next of kin and under the tenets of the Declaration of Helsinki. Donor identity was hidden and replaced with a sequential number. Each pair of eyes was accompanied by brief information on the donor's medical history, age, gender, cause, and time of death and retrieval time. Only eyes within 24 hours *post-mortem* were used for research and those with evidence of retinal diseases such as glaucoma, age-related macular degeneration, diabetic retinopathy and haemorrhages were excluded. In total 58 human eyes were used from donors with an average age of 69.8±1.31 years in which males represented 60.34% of the total number of donors.

Corneas were removed at the Eye Bank for transplantation and the remaining globes were transported to the University of East Anglia (UEA) in Eagle's Minimum Essential Medium (EMEM) (Sigma-Aldrich, Poole, UK) supplemented with 10µl/ml antibiotic/antimycotic solution (10,000 units/ml penicillin G, 10,000µg/ml streptomycin sulphate and 25µg/ml amphotericin B; Invitrogen, Paisley, UK). The lens was extracted before retinal dissection and usually used for cataract-related research at UEA.

2.1 Human Organotypic Retinal Culture (HORC) Dissection

HORC dissection was performed as has been previously described (Niyadurupola et al., 2011). The procedure is shown in (Figure 2.1). Under sterile condition, the globe was placed in a sterile 60 mm Petri dish (Thermo Fisher Scientific, Leicestershire, UK). A 10mm cut was made, and the ciliary body and anterior sclera removed via circumferential cut (Figure 2.1B). The globe was then slowly rotated to dissociate the neuronal retina from the underlying

retinal pigment epithelium and choroid (Figure 2.1C). A single cut was performed at the optic nerve head (Figure 2.1D) and with the aid of forceps, the neural retina, with the vitreous still attached, was removed. Warmed serum-free (SF) Dulbecco's Minimum Essential Medium (DMEM; 500µl), was added (Gibco Invitrogen, Paisley, UK), supplemented with 50mg/l Penicillin/Streptomycin (Gibco Invitrogen, Paisley, UK). The vitreous was dissociated carefully, and the retina edges were cut to allow the retina to lie flat (Figure 2.1E). A micro-dissecting trephine (Biomedical Research Instruments, Rockville, Maryland, USA), of 4 mm in diameter, was used to take sections of the retina. Initially, the central macula then five para-macular retinal explants were removed using a template (Osborne et al., 2015). Niyadurupola et al., (2010) have shown there to be comparable RGCs in each paramacular explant (Figure 2.2). Explants were placed into a 35 mm dish containing 1.5 ml of serum-free DMEM (Figure 2.1F) and randomized to different treatments.

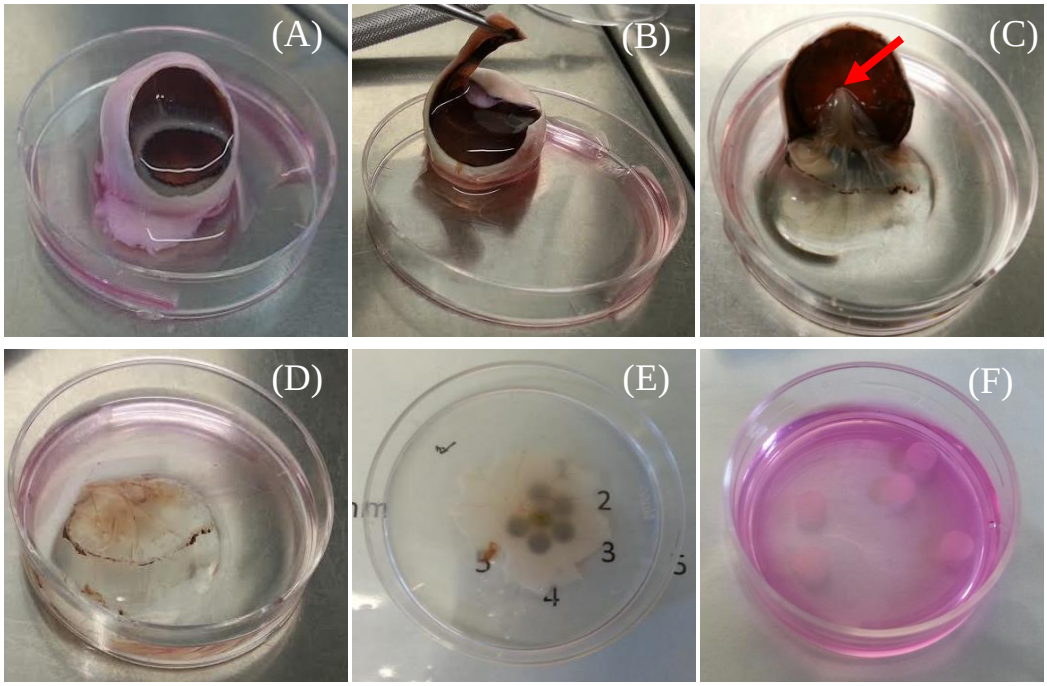


Figure 2.1: Dissection of the human retina. (A) The globe with anterior section, including lens removed. (B) Circular ring of tissue was removed approximately 10mm below the ciliary body. (C) The globe was rotated to dissociate the retina from the underlying RPE and choroid. A single cut was performed at the optic nerve head (arrow) to detach the retina. (D) The vitreous attached to the retina. (E) The vitreous was removed and the retina flattened, ganglion cell side up. The macula was removed as a reference for the para-macular sections and a template used to remove 5 paramacular sections. (F) Five paramacular samples were taken and placed into warmed culture medium.

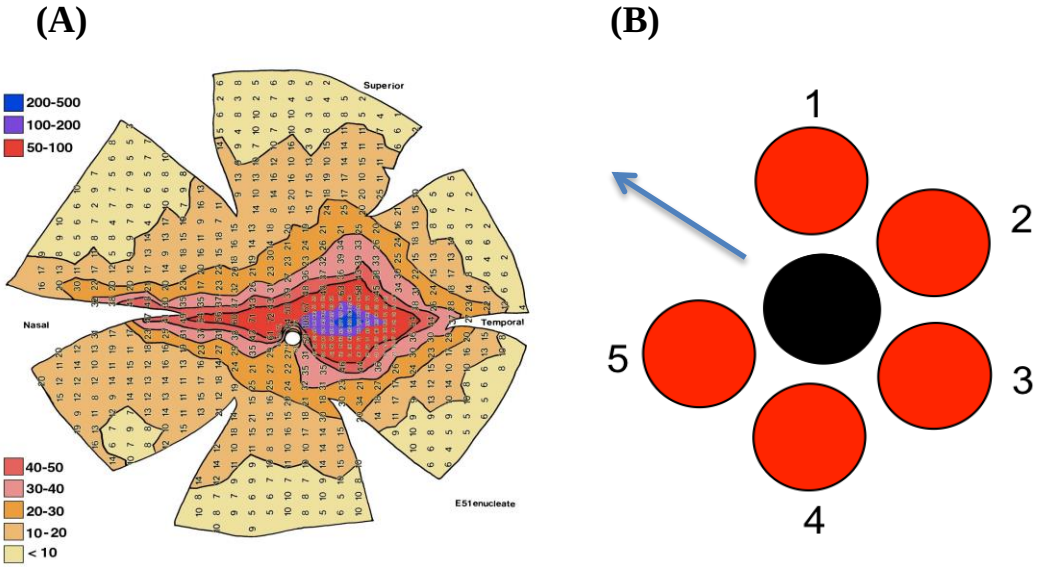


Figure 2.2: Retinal dissection template (A) Retinal Ganglion cell distribution of a mammalian retina. Source: http://www.nervenet.org/main/papers05.html (B) Template used to dissect the five-paramacular regions of a human donor retina around a central macula. Arrow shows the direction of the optic nerve

2.2 MIO-M1 (Moorfields/Institute of Ophthalmology-Müller-1) Cells

MIO-M1 cells are a spontaneously immortalized human cell line derived from an eye of a 68-year-old female corneal donor 36 hours after death (Limb et al., 2002). They were named after the institution where they were isolated, Moorfields/Institute of Ophthalmology-Müller 1. MIO-M1 have been shown to express Müller cell markers specifically glutamine synthetase, glial fibrillary acidic protein (GFAP), α-smooth muscle actin (α-SMA), vimentin, cellular retinaldehyde-binding protein (CRALBP), epidermal growth factor receptor and neuronal Thy-1 cell surface antigen (Limb et al., 2002; Hollborn et al., 2011). MIO-M1 cells were a gift from G.A. Limb, Institute of Ophthalmology and Moorfields Eye Hospital, London, UK

2.2.1 Culture of the MIO-M1 Glial Cell Line

MIO-M1 cells were used between passage numbers 28 and 38. MIO-M1 cells were cultured in Dulbecco's Minimal Essential Medium containing GLUTAMAX and physiological glucose levels of 1g/L **(**DMEM GLUTAMAX**,** Gibco-invitrogen, Paisley UK). The medium was supplemented with 10% foetal bovine serum (FBS) (Invitrogen, Paisley, UK) and 50mg/l Penicillin/Streptomycin (Invitrogen, Paisley, UK). Cells were passaged when confluent by washing in Dulbecco's Phosphate Buffered Saline solution (DPBS) and trypsinised using 0.05% (w/v) trypsin and 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) in Hank's balanced saline solution (HBSS) (Invitrogen, Paisley, UK) for 5 minutes at 37ºC, followed by gentle tapping before addition of 10ml cell culture medium supplemented with FBS to neutralise the trypsin.

Total cell number was determined using a haemocytometer (Assistant, Sondheim-Rhön, Germany) and the cell suspension transferred to a 25ml universal tube (Sterilin, Aberbargoed, UK) for 10 minutes centrifugation at 800 rpm to obtain a pellet. The supernatant was aspirated and the pellet re-suspended in 5 ml of culture medium.

Cells were re-seeded at a density of 1:5 per 75cm^2 sterile culture flask (Nunc A/S, Roskilde, Denmark) for future use and passaged every 5-7 days. For preparation for the experiments, cells were seeded at 5,000 cells per well in 96 well plates (100µl medium) (Nunc A/S, Roskilde, Denmark) and 75,000 cells per 35mm culture dish (Corning, NY, USA) (1.5ml medium). Cells were grown for three days to achieve >80% confluency and starved with DMEM without FBS (serum free DMEM) for at least 24 h before any experiment.

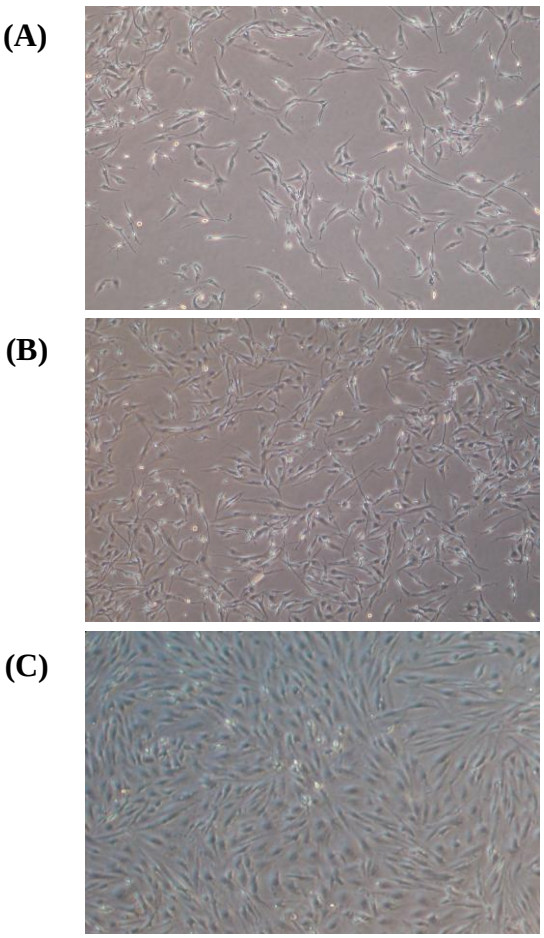


Figure 2.3: Cell morphology of (A) growing Müller cells (MIO-M1), (B) & (C) 80-100% confluence

2.3 Co-culture of Retinal Explants and Müller Cells

The retina was dissected as described earlier and retinal explants were placed into the medium of a 35 mm culture dish containing a Müller cell monolayer in a total volume of 1.5 ml with 1.11mM glucose. At the end the 24hrs experiment period, the medium was collected for LDH assay and VEGF protein. Explants were fixed as will be described later, for immunohistochemistry with TUNEL.

2.4 Oxygen and Glucose Deprivation Experiments

2.4.1 Glucose Deprivation

Cultured MIO-M1 cells were grown in 35mm dishes for 72hrs in serum-supplemented DMEM medium and then incubated in serum-free DMEM for 24hrs before being randomized to experimental conditions. All culture medium was supplemented with 50mg/l Penicillin/Streptomycin and control medium contained glucose (1g/L), which is equivalent to the physiological blood glucose level (5.55 mmol/L). In human retina experiments, HORC explants (four in total) were randomized to a control (SF-DMEM), glucose deprivation, oxygen deprivation or oxygen and glucose deprivation (OGD).

Four different levels of glucose-deprived medium were prepared to achieve 30, 20 and 10% of the glucose at the control level, equivalent to 1.67, 1.11 and 0.56mM respectively. This was achieved by mixing appropriate volume from glucose-free DMEM and DMEM containing glucose (1g/L), as shown in Table 2.1. Since glucose was not added directly to the medium, the osmolarity was not adjusted. This leads to differences in final osmolarity of the medium (Table 2.2). It is important to note here that VEGF expression has been shown to be stable with an increase in osmolarity of 20mOsmols in RPE cells (Holborn et al., 2015), which is greater than the differences in glucose deprivation experiments (Max. of 12 mOsmols) and high glucose experiments (11 mOsmols).

Level of Deprivation (%)	Glucose Concentration in DMEM medium (mmol/L)	Oxygen level (%)
Control (No deprivation)	5.55	20
70	1.668	6
80	1.112	4
90	0.556	2
Full starvation (OGD)	0	0

Table 2.1 Levels of glucose and oxygen in OGD experiments

Type of Medium	Glucose Concentration in DMEM medium (mmol/L)	Osmolarity mOsm
High Glucose	25	326±2.08
10% Cell culture	5.55	310±0.57
Serum free	5.55	315±1.32
20% glucose medium	1.112	304±3.5
Full glucose starvation	0	303±0.76

Table 2.2 Osmolarity of the different medium used in the experiments (average ±s.e.m, n=4).

2.4.2 Oxygen Deprivation (Hypoxia)

2.4.2.1 Hypoxia Chamber

A custom-made chamber was constructed from aluminum with a window at the front of the chamber (UEA mechanical workshop, Norwich, UK) to expose cultured cells or tissue to low oxygen conditions (Figure 2.3). Chamber dimensions were 260mm x 130mm x 140mm giving an overall volume within the chamber of 4732ml. A door was used to seal the chamber by tightening 6 wing-nuts against a continuous rubber O-ring. The chamber was housed

inside a standard cell culture incubator to maintain a constant temperature (35°C). This temperature was chosen since Landers et al., (2012) has found that retinal surface temperature (measured prior to vitrectomy) is 34.8-35.2ºC. Retinal explants are therefore routinely cultured at 34-35ºC (Bull et al., 2011; Johnson and Martin, 2008; Niyadurupola et al., 2013; Osborne et al., 2015a; Osborne et al., 2015b). Control experiments were performed outside the hypoxia chamber in the same incubator. Inside the chamber, humidity was maintained by a reservoir of distilled water at the base of the chamber. The chamber used mass flow controllers (MFC), positioned at the inlet and outlet ports, to simultaneously regulate the internal pressure and the rate of gas flow through the chamber. The chamber was attached to two gas sources (95% air/ 5% CO_2 and 95%N_2/ 5% CO_2), which could be rapidly pumped into the chamber using 1000ml/min MFCs, and released via a solenoid exhaust valve. The gases were mixed to give the level of O_2 required. Pressure inside the chamber was measured by a high accuracy digital pressure sensor (Omega Engineering Inc, Manchester, UK). Oxygen and pressure measurements were fed into a computer-controlled regulator unit. Control of gas flow to achieve the desired O_2 and pressure was via an analogue to digital interface. A third MFC positioned on the outflow ensured a constant flow of gas through the chamber at 100ml/min that was independent of pressure (Figure 2.3).

2.4.3 Simulated Ischemia (Oxygen-Glucose Deprivation, OGD)

Oxygen-glucose deprivation was performed by incubating cultured cells/HORCs in glucose-free DMEM medium to simulate complete deprivation of glucose or full OGD when oxygen deprivation accompanies it. Each level of ischemia was induced when oxygen and glucose were deprived to the same level as shown in Table 2.1.

The plates or dishes were exposed to experimental conditions and incubated in the hypoxia chamber for 24-72 hours where the degree of 5% Carbon dioxide/oxygen and nitrogen flow was controlled using computer software. This allowed induction of variable hypoxic

conditions between 0.0–6.0% oxygen. At the end of the experiment, 96 well plates were used for viability assays (MTS-MIO-M1 only) while medium from dishes was used for cytotoxicity assays (LDH). Cells and tissue for PCR analysis were lysed with RNeasy lysis buffer (RLT) and frozen immediately in liquid nitrogen and stored at -80°C before to RNA isolation.

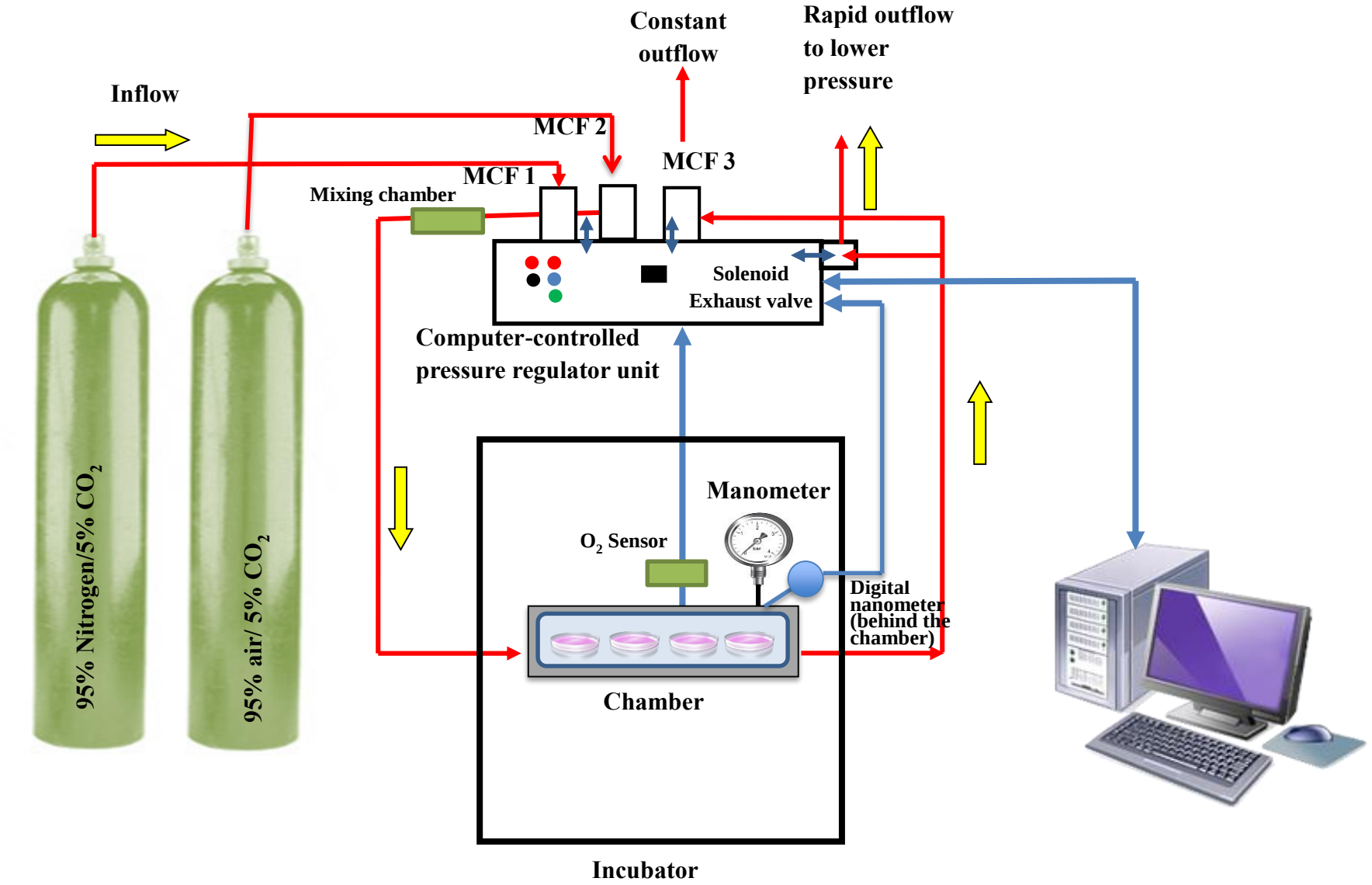


Figure 2.4 Schematic diagram of hypoxia chamber

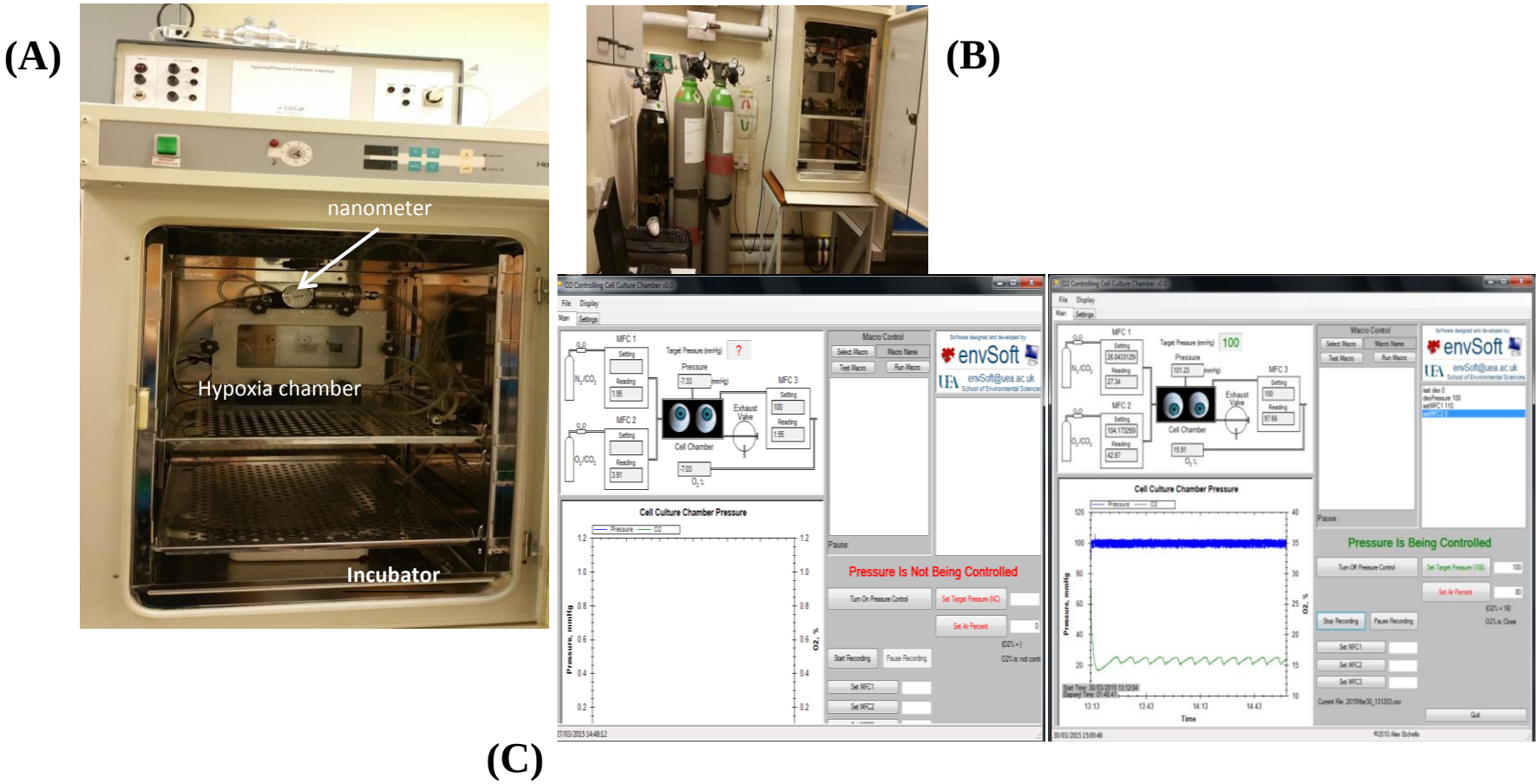


Figure 2.5: Photographic images of the hypoxia chamber (A) the hypoxia chamber inside a 35°C incubator. (B) The whole unit connected to gases and computer controlling the oxygen and pressure levels inside the chamber. (C) Images of a computer-based software controlling the pressure and oxygen levels.

2.5 Cytotoxicity Assay (Lactate Dehydrogenase, LDH)

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that is rapidly released into the culture medium when the plasma membrane is damaged (Bonfoco *et al.*, 1995).

The LDH activity is determined in an enzymatic reaction consists of two steps. In the first step, NAD^+ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. Secondly, the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to reduce the tetrazolium salt INT (2-[4-iodophenyl]-5-phenyltetrazolium chloride) to form a coloured formazan product, which can be measured by absorbance at 490 nm (Korzeniewski & Callewaert, 1983).

Cytotoxicity Detection Kit (LDH assay) was purchased from Roche (Burgess Hill, UK). TritonX-100 was purchased from Sigma-Aldrich (Dorset, UK). The assay was performed according to the manufacturer's instructions. The catalyst and dye solution were mixed in a ratio of 1:45 respectively. The cell-free culture supernatant medium (750 µl) was removed and centrifuged at 13,000 RPM for 5 min. To measure the maximum releasable LDH, Triton X-100 (concentration 2%, volume 750 µl) was added to each dish, mixed thoroughly and placed on a shaker for at least 30 min. A total volume of 350 µl was then removed and centrifuged for 5 min at 13,000 RPM. In HORCs, the released LDH was measured by removing 750 µl of the medium at the end of the experimental period and centrifuged at 13,000 RPM for 5 min.

One non-sterile, clear 96 well plate was used to load 100µl of each sample (i.e. supernatant medium from MIO-M1 or HORCs) in triplicate. Background controls were used in this assay measured using the appropriate medium. A volume of 100 µl of the mixed detection kit reagent was then added to each of the assay wells and absorbance at 490 nm was measured with a micro-plate reader (BMG LABTECH, Bucks, UK) at 5 min.

Results were expressed as a percentage of total releasable LDH using the following formula:

$$\%\ \boldsymbol{of\ Total\ Releasable\ LDH} = \frac{\boldsymbol{a}}{\boldsymbol{a+b}}\ \boldsymbol{X\ 100}$$

a= Sample absorbance-background absorbance

b= TritonX100 positive control absorbance – TritonX100 control absorbance

In other experiments, LDH release is expressed as a percentage of the control.

2.6 Cell Viability Assay (MTS)

Cell viability was assessed using the CellTiter 96 Aqueous Proliferation Assay (Promega, UK). The MTS test is a colorimetric assay used to determine the number of viable cells. It is based on the conversion of a 3- (4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) into a brown formazan product when reduced by active cells (Cory et al., 1991). The tetrazolium reduction takes place in the mitochondria and measures mitochondrial metabolic rate as a measure of cell viability (Riss et al., 2011).

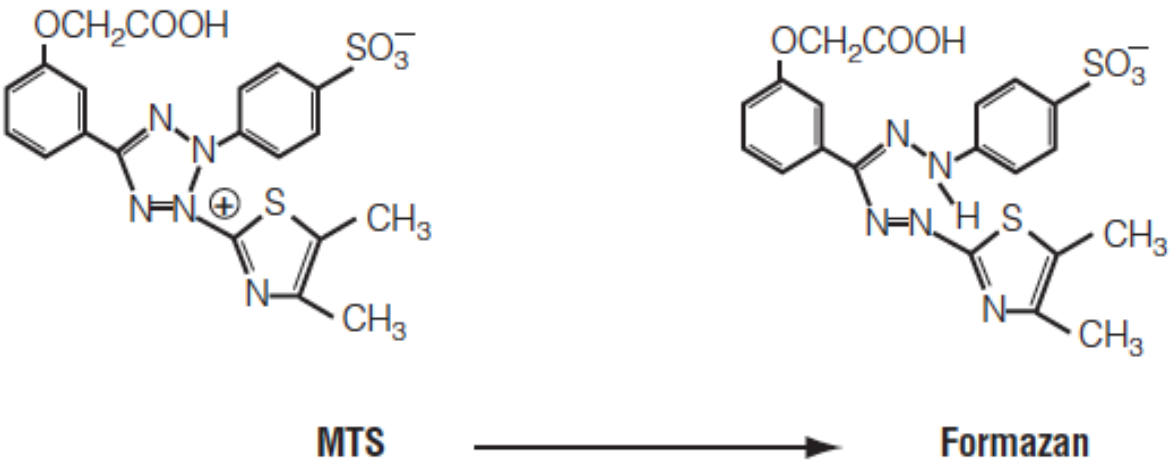


Figure 2.6: Conversion of MTS tetrazolium into Formazan by living cells. Image from CellTiter 96® AQueous One Solution Cell Proliferation Assay.

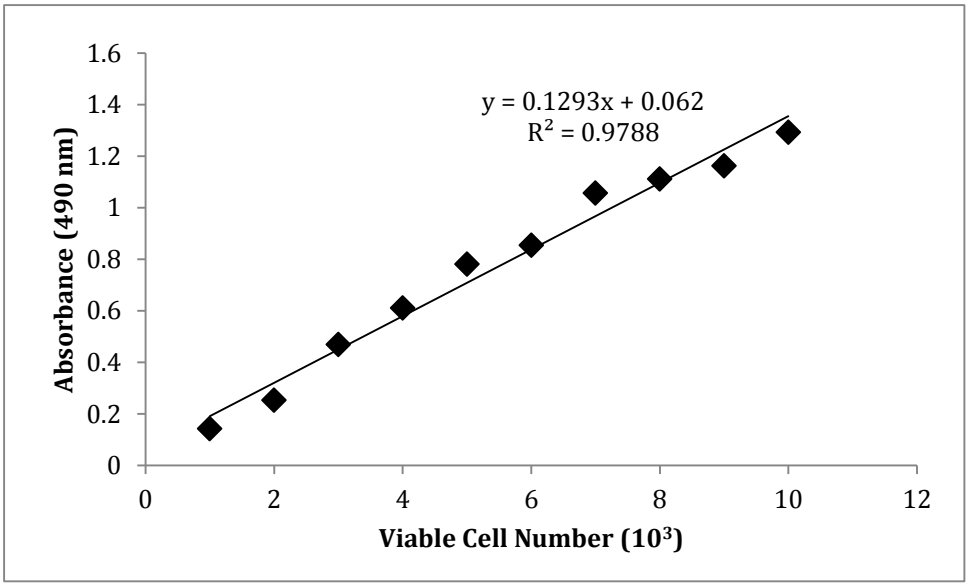


Figure 2.7: Standard curve of MTS absorbance values in relation to number of cell seeded. MIO-M1 cells (0 to 10,000 cells per well) were seeded into 96 well plates and cultured for 72 hrs and kept in in SF medium for 24hrs hours before the addition of MTS solution (n=4).

The assay was carried out according to the manufacturer's protocol. Briefly, after exposing cells to experimental conditions, the medium was removed from the 96 well plate and replaced with 100 µl MTS solution which is a mixture of 10 µl of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and 90µl DMEM+Glutamax (serum free) and incubated for 2 hours at 37ºC. Absorbance at 490nm was measured with a micro-plate reader (BMG LABTECH, Bucks, UK). The quantity of formazan product is proportional to the number of living cells in culture (Figure 2.7). Data was presented as percentage cell viability compared to control.

2.7 Glucose Assay

The glucose assay kit, GAGO-20 (Sigma-Aldrich, Poole, UK) was used to assess the rate of glucose utilization by human Müller cells (MIO-M1) under normal (5.55mM) and low glucose levels (1.11mM) for 24hrs.

The principle of the assay is based on oxidation of glucose to gluconic acid and hydrogen peroxide by glucose oxidase (step1). Hydrogen peroxide then reacts with o-dianisidine in the presence of peroxidase to form oxidized o-dianisidine, which has brown colour (step 2). This product reacts with sulphuric acid to form a more stable coloured product that is pink in colour (step 3). The intensity of the pink colour measured at 540 nm and is proportional to the original glucose concentration.

(1) D-Glucose + H_2O + O_2 $\xrightarrow{\text{Glucose Oxidase}}$ D-Gluconic Acid + H_2O_2

(2) H2O2 + Reduced o-Dianisidine (colourless $\xrightarrow{\text{Peroxidase}}$ Oxidized o-Dianisidine (brown)

(3) Oxidized o-Dianisidine (brown) $\xrightarrow{H_2SO_4}$ Oxidized o-Dianisidine (pink)

The assay was carried out according to the manufacturer's protocol, although the reaction was scaled down to enable use of a 96-well plate. Glucose oxidase/peroxidase enzyme was provided as one capsule containing 500 units of glucose oxidase (*Aspergillus niger*), 100 purpurogallin units of peroxidase (horseradish) and buffer salts. The contents of the capsule were dissolved in an amber bottle with 39.2 ml of deionized water. The o-Dianisidine Reagent was provided as preweighed vial contains 5 mg of o-dianisidine dihydrochloride. The vial was reconstituted with 1.0 ml of deionized water. The assay reagent was prepared by mixing 0.8 ml of the o-Dianisidine reagent to the amber bottle containing the 39.2 ml of glucose oxidase/peroxidase reagent. This product was stable for one month at 2-8 ºC.

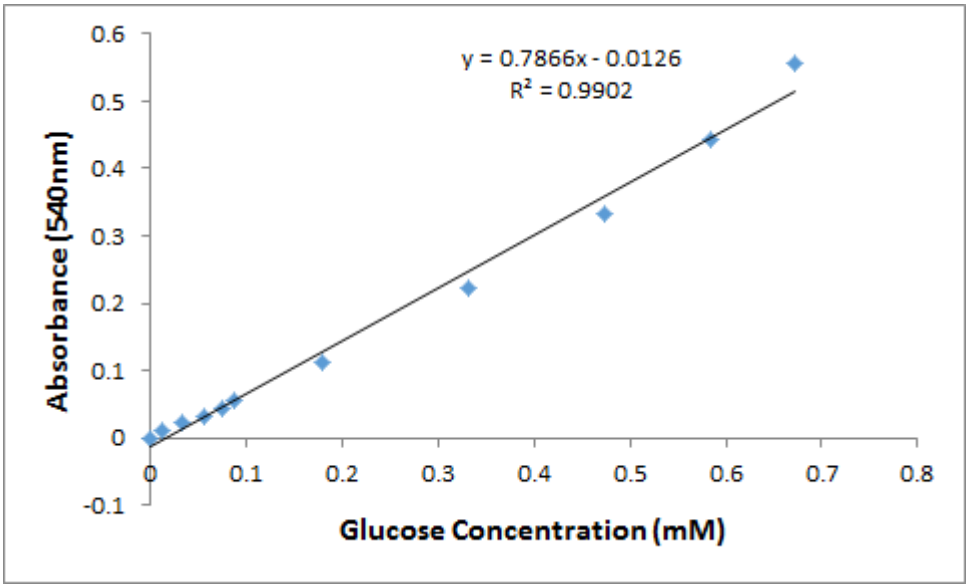


Figure 2.8: Standard curve of glucose concentration (mM) against absorbance (n=4).

Müller cells (MIO-M1) were cultured in 35 mm dishes (1.5 ml) for 24hrs under normal oxygen level and both in normal glucose medium (5.55 mM) and low glucose medium (1.11 mM). The dishes were divided into two sets, the first were sampled sampled every 2 hours for 12hrs and the second were samples to cover the next 12 hrs of the next day. A sample of 50µl medium was frozen immediately in liquid nitrogen and stored in -80 °C prior to analysis. The kit was designed to be used in cuvettes, but was adapted to work in a 96 well plate format as following: 40 µl of each sample was pipetted followed by addition of 80µl assay reagent. The plate was incubated for 30 minutes at 37ºC. The reaction was terminated by adding 80µl sulphuric acid (12 N H2SO4) and read at 540 nm. A standard curve using glucose standard solution 1.0 mg/ml in 0.1% benzoic acid provided in the kit with standard linearity in the glucose range of 0-100 µg/ml (Figure 2.8)

2.8 RNA Extraction

Total RNA was extracted using an RNeasy® mini kit according to the manufacturer's instructions (Qiagen, Crawley, UK). Following experimentation, MIO-M1 cells were lysed in 350 µl of buffer RLT containing 10% β-mercaptoethanol (Sigma-Aldrich, Poole, UK) using a plastic cell scraper and the lysate collected and placed into 1.5 ml Eppendorf tubes. The samples were immediately placed in liquid nitrogen and then stored in -80ºC until the time of processing. HORCs were homogenised in 350 µl of RLT containing 10% β-mercaptoethanol using a 20-gauge needle (Becton Dickinson, Oxford, UK) 8 times. The samples (either cell lysate or tissue homogenate) were treated with 350 µl of 70% ethanol and mixed by repeated pipetting. A total volume of 700µl of each sample was collected and transferred to RNeasy mini columns placed in 2 ml collection tubes and centrifuged for 15 seconds at 13,000 rpm, allowing the RNA to bind to the column membrane. The flow through was discarded and 700µl of buffer RW1 was directly applied to the RNeasy columns which were then centrifuged for 15 seconds at 13, 0000 rpm.

This was followed by digestion of DNA using DNase 1 (Qiagen, Crawley, UK) in RDD buffer for 15 minutes at room temperature. The columns were washed again with buffer RW1and flow through was discarded. Columns were placed into new 2 ml collection tubes and then 500 µl of buffer RPE was pippeted onto each column. Columns were centrifuged for 15 seconds at 13,000 rpm and flow through flow was discarded. An additional 500µl of buffer RPE was pipetted onto each column and centrifuged for 2 minutes at 13,000 rpm to dry the membranes and flow through was discarded. Columns were placed in 1.5 ml RNase-free Eppendorf tubes and 30µl of RNase-free water was applied directly onto the silica-gel membrane of the RNeasy columns, which were then centrifuged for 1 minute at 13,000 rpm and flow through was collected. Total RNA was quantified (ng/µl) using a Nanodrop ND-

1000 spectrophotometer (NanoDrop Technologies, Delaware, USA) and the purity of RNA was judged by A 260/280 ratio of ~2.0.

2.9 cDNA Synthesis

Extracted RNA was converted to complementary DNA (cDNA) in a reaction catalysed by Reverse Transcriptase (Invitrogen, Paisley, UK). Each sample was diluted with distilled water to get a total volume of 10µl (100ng/µl) using thin walled Eppendorf tubes. Random primers (500µg/ml) (Promega, Southampton, UK) and 10 mM dNTP (Bioline, London, UK) were mixed in a ratio of 1:1 and 2 µl of this mixture was added to each of the diluted RNA samples, and centrifuged for 15 seconds. Samples were placed in a Peltier Thermal Cycler-DNA engine (MJ Research Inc, Reno, NY) and incubated at 65 ºC for five minutes, followed by brief incubation in ice. A mixture of 5x first Strand Buffer (4µl) (Invitrogen, Paisley, UK), 0.1M DTT (2µl) (Invitrogen, Paisley, UK) and RNase inhibitor (1µl) (Promega, Southampton, UK) was prepared and 7µl of this mixture was then added to each sample. The samples were centrifuged for 15 seconds at 10,000 rpm before incubation at 25 ºC for 10 minutes in the Peltier Thermal Cycler followed by 2 minutes at 42ºC. Samples were briefly chilled and 1µl of superscript II was pipetted to each tube. Samples were returned back to into the thermal Cycler and incubated at 45ºC for 50 minutes followed by heating at 70ºC for 15 minutes. The generated cDNA samples were then stored at -20ºC till further processing.

2.10 Quantitavive Reverse Transcriptase-Polymerase Chain Reaction (QRT-PCR, TaqMan) Amplification

QRT-PCR amplification of targeted genes was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington, UK). The generated cDNA was diluted with RNA free water such that 5ng total cDNA in 10µl was added to each well of a microAmp Optical 96 well reaction plate (Applied Biosystems, Warrington, UK). Each reaction was composed of 10µl of diluted cDNA, 8.33µl Taqman Mastermix (Applied

Biosystems, UK), 1.25 µl of each probe (Table 2.2) and RNase-free water to give an overall reaction volume of 25 µl per well. Plates were sealed with clear adhesive PCR film (Thermo Scientific, Surrey, UK) and reaction was performed using the Applied Biosystems 7500 Fast Real-Time PCR System, (Applied Biosystems, UK).

Reaction conditions for the PCR amplification were initiated by heating to 50 ºC for 2 min, followed by 10 min at 95 ºC. Amplification of cDNA was through 40 cycles, each consisting of one minute at 95 ºC and 30 seconds at 60 ºC. Results were generated in an Excel file and cycle threshold (CT) value, the point at which the fluorescent signal becomes statistically significant above background, for each sample was determined. Gene expression was determined using a standard curve of gene of interest. Normalization of data was performed using the geometric mean of two housekeeping genes; CYC-1 (cytochrome c-1) and TOP1 (Topoisomerase I) and was expressed as a percentage of control. Each sample was run as a single replicate, and the average (mean±s.e.m) of four separate experiments is presented.

Gene name		Assay ID No	Company
PDGF-A	Platelet-derived growth factor alpha polypeptide-A	Hs00964426_m1	Applied Biosystem
VEGF-A	Vascular endothelia Growth Factor	Hs00900055_m1	Applied Biosystem
FGF2	Fibroblast growth factor 2 (basic)	Hs00266645_m1	Applied Biosystem
SERPINF1	Serpin peptidase inhibitor or pigment epithelium derived factor (PEDF)	Hs01106934_m1	Applied Biosystem
LIF	Leukemia inhibitory factor	Hs01055668_m1	Applied Biosystem
GLUL	Glutamate-ammonia ligase (Glutamine Synthetase)	Hs00365928_g1	Applied Biosystem
SLC1A3	Glial high affinity glutamate transporter	Hs00188193_m1	Applied Biosystem
GAD1	Glutamate decarboxylase 1	Hs01065893_m1	Applied Biosystem
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2)	Hs00905117_m1	Applied Biosystem
PANX1	Pannexin 1	Hs00209790_m1	Applied Biosystem
GFAP	Glial fibrillary acidic protein	Hs00909233_m1	Applied Biosystem
HSPA1B	Heat shock 70kDa protein 1B	Hs01040501_sH	Applied Biosystem
CYC-1	Cytochrome c1	HK-DD-hu-300	Primerdesign
TOP-1	DNA topoisomerase 1	HK-DD-hu-300	Primerdesign

Table 2.2: The Primers used for QRT-PCR Experiments showing the gene symbol, gene name, the assay ID number and the supplier.

2.11 Immunohistochemistry

2.11.1 Cryosectioning

After experiments, HORC explants were fixed in 4% formaldehyde for 24 hours at 4°C. Tissue was then dehydrated with 30% sucrose in PBS for another 24 hours at 4°C. Subsequently, each retinal explant was mounted vertically into tinfoil circular cups filled with Optimal Cutting Temperature (OCT) medium (Sakura Finetek, Zoeterwoude, Netherlands). Cups were frozen on dry ice for few minutes and stored at -80°C until use. Retinal slices of 13µm were sectioned using a Bright OTF 5000 cryostat (Bright Instruments, Huntingdon, UK) as follows. Frozen cups were sliced until a retinal piece of approximately 4mm was visible to ensured that sections were taken at the center of the circular retinal sample. This was measured using a digital vernier calliper (Clarke, Essex, UK). At least 6-8 non-consecutive slices were collected per glass slide (Sigma-Aldrich, Poole, UK). Slides were coated with 3-triethoxysilylpropylamine (TESPA) (Sigma-Aldrich, Poole, UK) to aid adhesion. The slides were kept in a slide-box in -20°C until the time of immunohistochemistry staining.

2.11.2 Staining

Retinal slices were washed three times in PBS, each for 10 minutes to remove OCT medium from the slides. Slides were then incubated in blocking solution consisting of 5% normal goat serum (Sigma-Aldrich, Poole, UK) and 0.2% TritonX-100 in PBS for 90 minutes at room temperature to block binding sites and to permeabilize cell membranes. Afterwards, retinal slices were incubated in a primary antibody (Table 2.3) made up in blocking solution, overnight at 4°C.

Following primary antibody binding, retinal slices were washed in PBS three times for 10 minutes before the addition of a secondary antibody (2mg/ml; 1:1000) diluted

in blocking solution in the dark for 2 hours at room temperature. Samples were protected from light exposure from this point onwards. Samples were then washed three times in PBS for 10 minutes to remove unbound antibody and stained with DAPI (0.5µg/ml) for 10 minutes at room temperature. Slides were washed again before the addition of a drop of Hydromount (Fisher Scientific, Leicestershire, UK) onto each retinal slice. 12mm coverslips (Warner Instruments, Kent, UK) were placed over each sample which were then allowed to set at room temperature, protected from light, for at least 12 hours before they were imaged using Widefield microscope Zeiss AxioPlan 2ie.

Target	**Source**	**Clonality/ Conjugate**	**Dilution**	**Supplier**
Primary Antibodies				
Neuronal Nuclei (NeuN)	Mouse	Monoclonal	1:200	Chemicon International, Millipore, Watford, UK
Glial Fibrillary Acidic Protein (GFAP)	Rabbit	Polyclonal	1:1000	Dako, Z0334, Glostrup, Denmark
Secondary Antibodies				
Mouse IgG (H+L)	Goat	AlexaFluor 568	1:1000	Invitrogen, Paisley, UK
Rabbit IgG (H+L)	Goat	AlexaFluor 568	1:1000	Invitrogen, Paisley, UK

Table 2.3: Immunohistochemistry primary and secondary antibodies.

2.11.3 Immunohistochemistry with Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay

To assess apoptotic cell death within the ganglion layer, immunohistochemistry combined with TUNEL-labelling was performed. Immunohistochemistry was performed as previously described until the end of the overnight primary antibody incubation stage. Retinal slices were washed three times for 10 minutes with PBS and immersed in TUNEL equilibration buffer for 10 minutes at room temperature. The assay was carried out according to manufacturer's instructions. TUNEL reaction mixture was prepared and added to samples and incubated in dark for one hour at 37°C. The reaction was terminated by washing the slides twice with 2X SCC solution and washing three times for 10 minutes with PBS. The immunohistochemistry protocol was continued from the addition of the secondary antibodies. Retinal sections were imaged using a wide-field Zeiss Axiovert 200M fluorescence microscope and analysed with Zeiss Axiovision 4.7 software.

2.11.4 TUNEL-Positive RGC Quantification

The numbers of RGCs were counted after image coding by another member of the laboratory. Three images were taken from each section (note there were 6-8 sections from each HORC) and 2-3 areas corresponding to a distance of 200μm were selected and the number of NeuN-immunolabelled cells co-localised with DAPI stained nuclei in the RGC layer were counted. TUNEL counting was performed in the same manner except the number of TUNEL-positive cells that co-localised with NeuN was assessed per 200μm sections. The mean NeuN-positive cells in 3x 200μm section and mean TUNEL-positive NeuN-labelled cells were calculated for all images before treatments were unmasked.

2.12 High Glucose Experiments

Cell survival, expression of selected growth factors and VEGF release were investigated in MIO-M1 and HORCs after exposure to high glucose conditions. In HORCs, high glucose treatment was achieved by incubating the retinal explants in high glucose DMEM (Invitrogen, UK) of 25mM for 24hrs. Medium was collected for LDH analysis; explants were either frozen for RNA extraction or fixed for immunohistochemistry. To investigate the effect of high glucose, oxidative stress and PKC inhibition, MIO-M1 cells were utilized as described in the following sections.

2.12.1 In Vitro Oxidative Stress and High Glucose Treatment

Hydrogen peroxide (H_2O_2) (Sigma-Aldrich, UK) was utilized to induce oxidative stress in MIO-M1 cells. MIO-M1 cells were cultured in 96-well plates as described earlier and H_2O_2 was added in serum medium at a range of concentrations (100-2000μM). Cell viability (MTS assay), and LDH were measured to determine the appropriate concentration that induces stress without loss of viability. This was done in low and high glucose serum free medium for up to 72hrs.

2.12.2 Protein Kinase C beta (PKCβ) Inhibitor with High Glucose Treatment

The PKC-β inhibitor, ruboxistaurin (LY-333531), was purchased from Sigma-Aldrich, UK and dissolved in sterile dimethyl sulfoxide (DMSO) (Sigma Aldrich, UK). A dose response for PKC-β inhibitor at a arrange of 100nM-1μM was carried out in MIO-M1 cells cultured in serum free medium followed by MTS and LDH assays to determine the appropriate concentration for treatment under high glucose, oxidative stress and hypoxic conditions. Medium was collected from all conditions for the measurement of released VEGF concentration from MIO-M1 cells.

2.13 Human VEGF Enzyme Linked-Immuno-Sorbent Assay (ELISA)

A Human Vascular Endothelial Growth Factor (Hu VEGF) ELISA (Invitrogen, Paisley, UK), was used for the determination of VEGF concentration in culture medium. The assay will recognize both natural and recombinant Hu VEGF-165

The Invitrogen human VEGF kit is a solid phase sandwich ELISA. The assay was performed according to the manufacturer's instructions. A polyclonal antibody specific for human VEGF has been coated onto the wells of the microtiter strips provided. Incubation buffer (50µl) was pipetted into all wells before adding samples (50 µl) or standards of known human VEGF content (100 µl). Standard diluent buffer (50µl) was added into all wells except the standards. During the first incubation (30 minutes), the VEGF antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated monoclonal antibody (100 µl) specific for human VEGF was added. During the second incubation (30 minutes), this antibody binds to the immobilized VEGF captured during the first incubation. After removal of excess secondary antibody, Streptavidin-Peroxidase enzyme (100µl) was added. This binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation (30 minutes) and washing to remove unbound enzyme, a substrate solution (100µl) was added, which is acted upon by the bound enzyme to produce color. A stop solution was added and the intensity of this color was measured at 490 nm. This is directly proportional to the concentration of VEGF present in the original sample. A plot of the absorbance of the standards against the standard concentrations (0-1500 pg/ml) was constructed. Concentration of VEGF in the samples was presented as pg/ml using the plotted standard curve.

2.14 Data Analysis

Data is presented as mean ± standard error of the mean (s.e.m) of at least four experiments. GraphPad (Prism®, version 6) was used to analyse and present data. A one-way analysis of variance (ANOVA) was used to establish statistical significance between data sets in combination with Dunnett post-hoc test. Student's t-test was used to test for significance when only two variables were present. A *P* value equal or less than 0.05 was considered statistically significant.

Chapter 3

Effect of Oxygen and Glucose Deprivation on the Survival and Cytotoxicity of Human Müller Cells (MIO-M1)

3.1 Introduction

Müller cells are the principle macroglial cells in the retina, and they are radially oriented cells spanning the whole thickness of the retina, communicating with all type of neurons within the retina (Newman and Reichenbach, 1996; Reichenbach and Bringmann, 2013). They provide structural support and regulate neuronal cell metabolism. They control essential processes such as glucose metabolism, antioxidant production, ion and substrate exchange and vascular regulation (Bringmann et al., 2000; Eichler et al., 2000; Bringmann and Reichenbach, 2001; Winkler et al., 2000). In addition, Müller cells play a major role in the retina by responding to pathological stimuli. This includes responses to hypoxia, ischemia, glucose deprivation and inflammatory conditions (Bringmann et al., 2006). Reactivity of Müller glia during retinal detachment and other forms of retinal injury such as proliferative diabetic retinopathy (PDR), proliferative vitreoretinopathy (PVR) and glaucoma has been documented (Andjelic et al., 2014; Campochiaro et al., 1997; Flammer and Mozaffarieh, 2007; Hollborn et al., 2004).

In PVR, uncontrolled Müller cell proliferation and hypertrophy and eventual formation of a glial scar are the hallmarks of the disease (Morescalchi et al., 2013). Activation of Müller cells in experimental retinal detachment in rats led to the expression of GFAP, vimentin and nestin with evidence of Müller cell proliferation and growth within the retina and into the subretinal space (Luna et al., 2010).

In addition, immunochemical staining has identified Müller cells in diabetic retinal scars. Theses scars prevent flattening of the retina and cause macular wrinkling

leading to loss of vision (Lewis et al., 1991; Lewis et al., 2010). In a mouse model of inherited glaucoma (DBA/2J), microglia activation was an early alteration in the retina and optic nerve, contributing to disease progression (Bosco et al., 2011). In a recent study by Bosco et al., (2015); using the DBA/2J mouse model, an early microgliosis at the optic nerve head was detected. The author suggested that monitoring microglial activation is a good indicator of future neurodegeneration severity (Bosco et al., 2015). In addition, in an IOP-induced glaucoma model in rats, evidence of glial cell proliferation at the optic head region was detected by upregulation of cell proliferation associated genes: Top2a plays a role in DNA replication, whereas Prc1 regulates the mitotic spindle midzone formation and Espl1 regulates chromatid separation at anaphase. Interestingly, this was not associated with a change in the expression of GFAP (Johnson et al., 2011).

On the other hand, different experimental stressors have been shown to decrease the viability of glial cells *in vitro*. For example in one study, high dose of hydroquinone decreased the viability of the human Müller cell line (MIO-M1) to 41% of control with a 5-fold increase in LDH levels. Death under this condition was attributed to oxidative, mitochondrial and autophagic pathways with no apoptosis involved (Ramirez et al., 2013). Cell death in MIO-M1 cells was also induced by a high dose of catechol, in which viability decreased to 46% with increased caspase 3/7 activity (Mansoor et al., 2010). Moreover, exposure of rat C6 glioma cells to H_2O_2 caused a dose-dependent cell death (Ahn et al., 2015). Similarly, the MIO-M1 cell viability was decreased after exposure to H_2O_2 induced oxidative stress (Hu et al., 2014).

In addition, the effects of oxygen-glucose deprivation has not previously been investigated in Müller cells but various studies have been carried out using cultured astrocytes (Huang et al., 2013; Lee et al., 2006; Lee et al., 2009; Niu et al., 2009; Pei

and Cheung, 2003; Velly et al., 2009). As oxygen and glucose deprivation (OGD) is part of the pathophysiology of glaucoma and could occur as a result of decreased ocular blood flow, the aim of the experiments presented in this chapter was to identify whether oxygen and/or glucose deprivation would have a direct effect on cell survival of human Müller cells (MIO-M1).

3.2 Results

3.2.1 Effect of Oxygen–Glucose Deprivation (OGD) on the Viability and Cytotoxicity of Human Müller Cells (MIO-M1)

Human MIO-M1 cells were exposed for 24hrs to full oxygen-glucose deprivation (OGD) or reduced levels of oxygen and glucose of 10-30% (0.56-1.67 mM glucose/ 2-6%O_2) of control levels (5.55mM glucose/20% O_2). Full OGD caused a significant 5-fold increase (*$p \leq 0.05$; n=4) in released LDH compared to control while reduced levels to 10-30% did not change LDH release (Figure 3.1A) indicating cell death with total OGD only. This was confirmed by the viability test, which showed a significant reduction of approximately 50% in the survival rate with full OGD, but no loss of viability with reduced (10-30%) oxygen and glucose levels compared to control after 24hrs (Figure 3.1B).

Interestingly, when oxygen and glucose were reduced to 20% of control level (1.11mMglucose/4%O_2), MIO-M1 cell viability increased significantly (*$p \leq 0.05$; n=4) by approximately 20% compared to control, which indicated, possible proliferation of the cells under these conditions. These results indicate that MIO-M1 cells withstand oxygen and/or glucose deprivation levels below full deprivation but when they are completely depleted of both oxygen and glucose they lose viability. Since both oxygen and glucose were changed together in these experiments, then it was important to look at them separately to determine the main substrate affecting the proliferation and survival.

3.2.2 Effect of Oxygen Deprivation on the Survival and Cytotoxicity of Human Müller Cells (MIO-M1)

Despite the reduction of oxygen concentration to the MIO-M1 cells for 24hrs, the LDH levels were comparable to control at all levels of oxygen deprivation including full deprivation (Figure 3.2A). Furthermore, MIO-M1 cells maintained the same survival rate as control with reduced oxygen and even with complete deprivation. The viability of the MIO-M1 cells slightly, but significantly, increased (*$p \leq 0.05$; n=4) by 13% and 21% at 2% and 4% oxygen respectively as compared to control (Figure 3.2B).

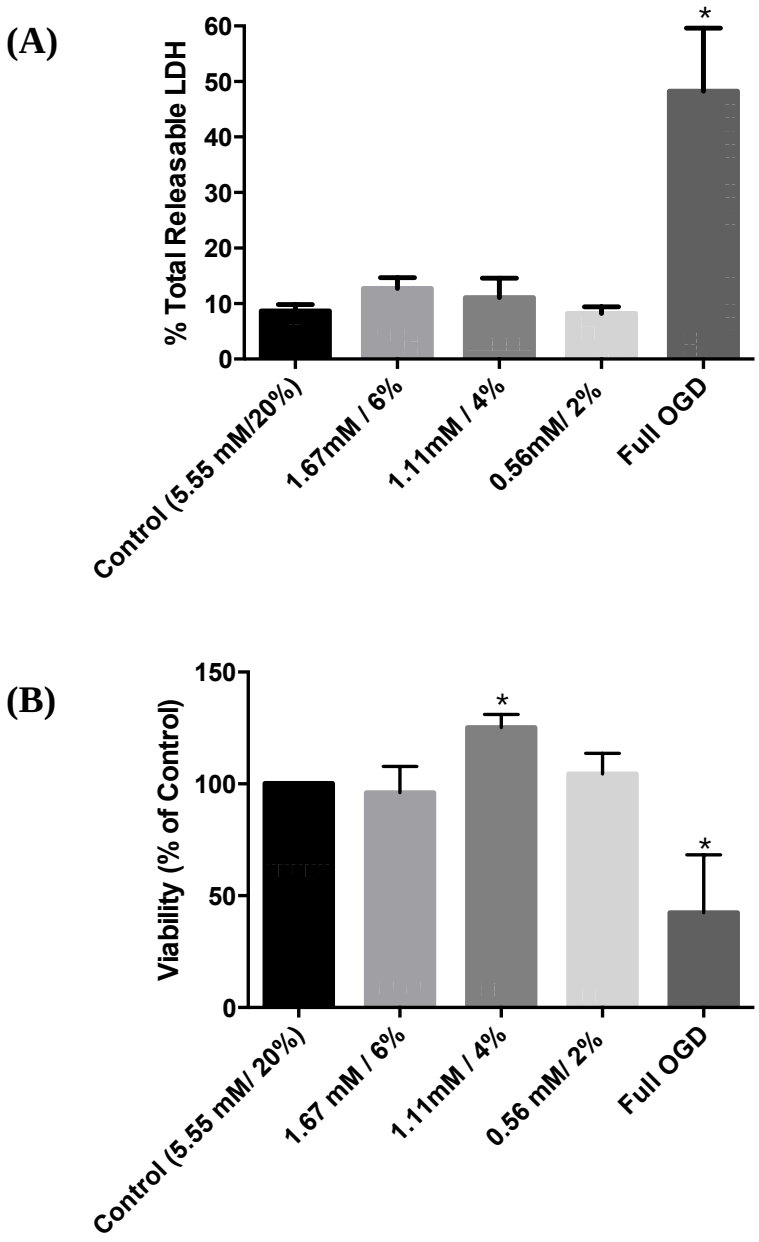


Figure 3.1: (A) Cytotoxicity (total releasable LDH) and (B) viability (MTS assay) of human Müller cells (MIO-M1) after 24hrs exposure to full oxygen-glucose deprivation (OGD) or reduced levels of glucose and O_2 (0.56-1.67mM glucose/2-6%O_2 (mean ± s.e.m, * p≤0.05; n=4).

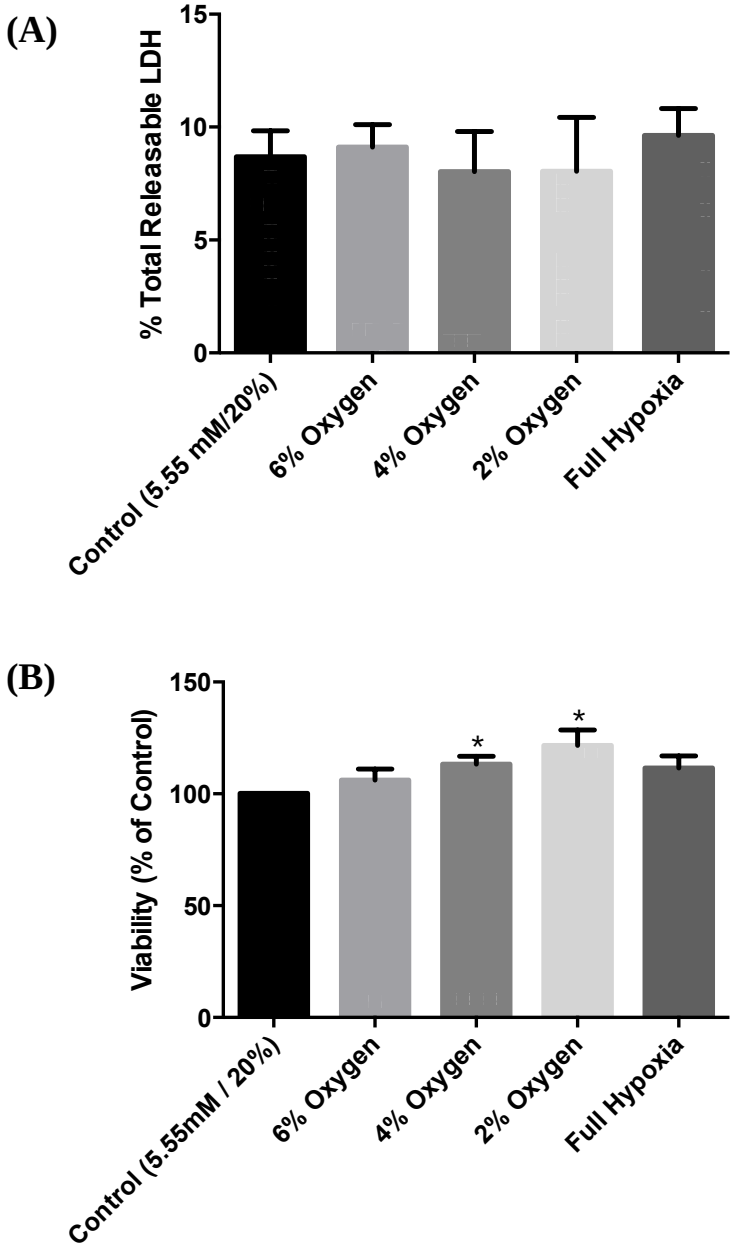


Figure 3.2: (A) Cytotoxicity (total releasable LDH) and (B) viability (MTS assay) of human Müller cells (MIO-M1) after 24hrs exposure to full oxygen deprivation or reduced levels of oxygen 2-6% (mean ± s.e.m, * $p \leq 0.05$; n=4).

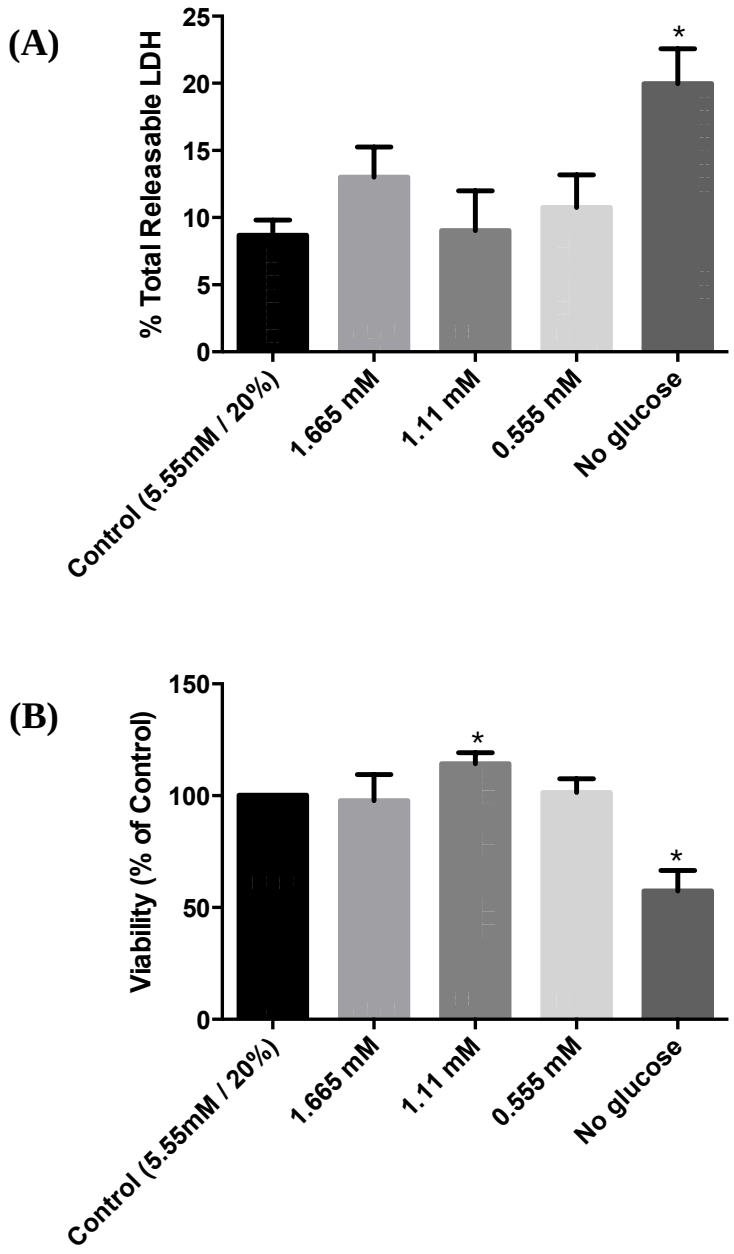


Figure 3.3: (A) Cytotoxicity (total releasable LDH) and (B) viability (MTS assay) of human Müller cells (MIO-M1) after 24hrs exposure to full glucose deprivation or reduced levels of glucose 0.56-1.67mM (mean ± s.e.m, * $p \leq 0.05$; n=4).

3.2.3 Effect of Glucose Deprivation on the Survival and Cytotoxicity of Human Müller Cells (MIO-M1)

Full deprivation of the MIO-M1 cells from glucose significantly for 24hrs increased (*p≤0.05; n=4) LDH levels by approximately 2-fold compared to control. No detectable alterations in the LDH levels were found when glucose was reduced to 10-30% (0.56-1.67 mM) of the control level (5.55 mM) (Figure 3.3 A).

Full glucose deprivation also reduced the viability by approximately 50%. On the other hand, viability was increased significantly with 1.11mM glucose (Figure 3.3B). These results were similar to those seen with reduced levels of oxygen and glucose deprivation (1.11mMglucose/4%O_2).

3.3 Long –term Oxygen Glucose Deprivation

Human MIO-M1 cells showed resistance when exposed to all levels of hypoxia and OGD conditions below full deprivation for 24hrs. It was interesting to find that hypoxia, glucose deprivation, and OGD at 20% of control (1.11mMglucose/4%O_2) caused MIO-M1 cell proliferation. This level was therefore chosen to study MIO-M1 viability and cytotoxicity in response to long-term deprivation (48 and 72hrs).

3.3.1 Effect of Long-term Oxygen-Glucose Deprivation on the Survival and Cytotoxicity of Human Müller Cells (MIO-M1)

At 72hrs, human MIO-M1 cells incubated in 1.11mM glucose/4%O_2 lost their integrity as evidence by 1.6-fold increase in total releasable LDH compared to control (*p≤0.05; n=4), while no detectable LDH release was found in 24 and 48hr experiments. Viability results confirmed the LDH data with a significant reduction in viability by 50% after 72hrs (Figure 3.4 A). MIO-M1 cells maintained at 1.11mM glucose/4%O_2 for 24hrs showed a significant 30% increase (*p≤0.05; n=4) in viability compared to control suggesting that MIO-M1 cells proliferated as indicated

earlier. This proliferation was maintained, but at lower rate of 14% after 48hrs (Figure 3.4B).

3.3.2 Effect of Long-term Oxygen Deprivation on the Survival and Cytotoxicity of Human Müller Cells (MIO-M1)

Cytotoxicity data showed no significant change of released LDH at any time point with oxygen deprivation to 4%. This was supported by viability data, which did not decrease with oxygen deprivation (4%) but slightly increased by approximately 10% at 24-48 hrs (Figure 3.5A, B).

3.3.3 Effect of Long-term Glucose Deprivation on the Survival and Cytotoxicity of Human Müller Cells (MIO-M1)

Long-term glucose deprivation to the MIO-M1 cells showed a similar pattern to OGD in which a significant 1.6-fold increase in LDH levels was seen at 72hrs (*$p \leq 0.05$; n=4). No significant increase in LDH levels was found at earlier time points (Figure 3.6A). A significant 24% increase in viability was seen at 24hrs (*$p \leq 0.05$; n=4). The cells maintained similar viability to control at 48hrs with no proliferation, while a significant reduction (*$p \leq 0.05$; n=4) in viability by 40% was seen at 72hrs (Figure 3.6B). These results were very similar to those seen with both oxygen and glucose deprivation (Figure 3.4B).

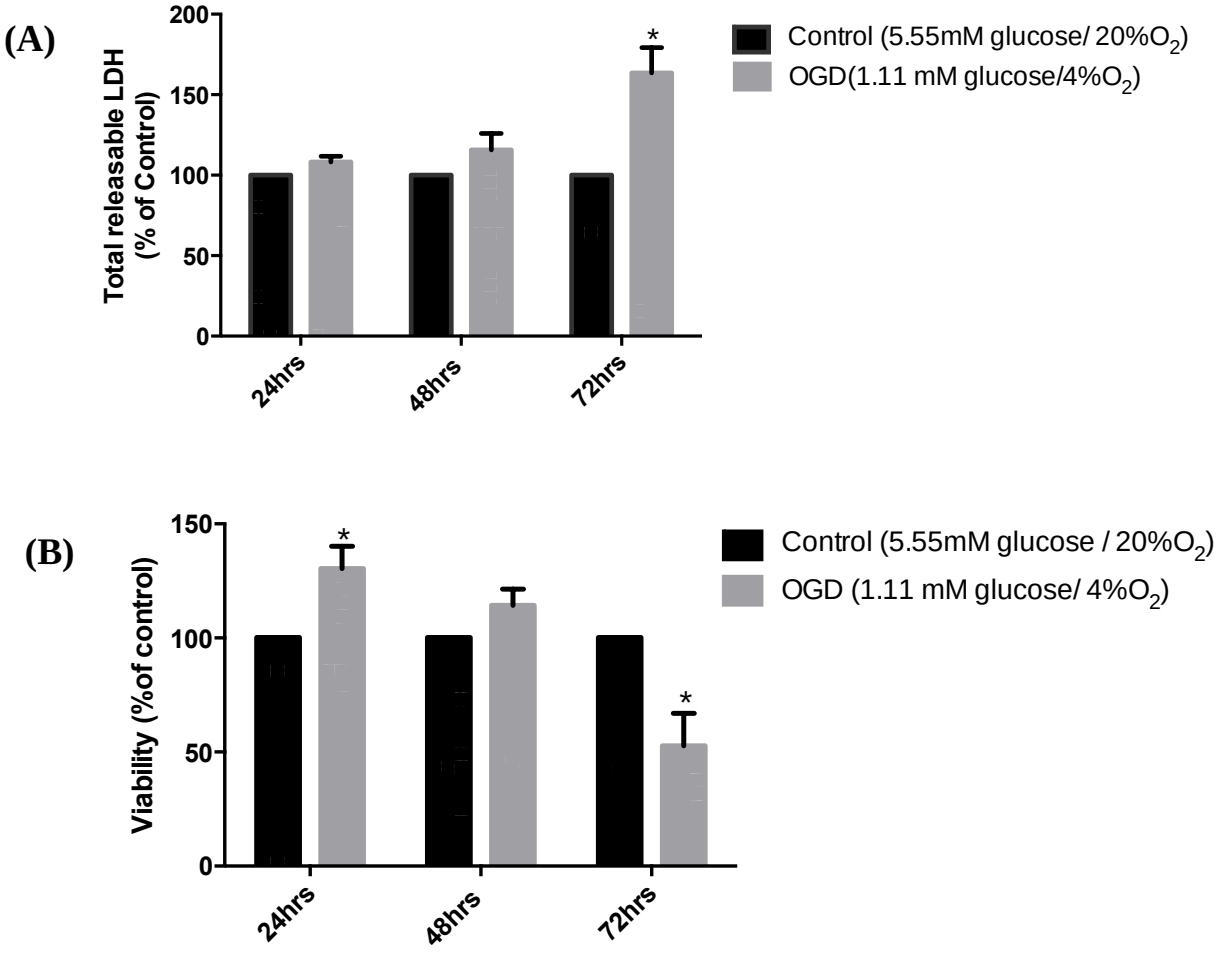


Figure 3.4: (A) Cytotoxicity (total releasable LDH) and (B) viability (MTS assay) of human Müller cells (MIO-M1) after 24hrs exposure to 20% OG level (1.11 mM glucose/4%O_2) for 24, 48 and 72hrs (mean ± s.e.m, * p≤0.05; n=4, t.test).

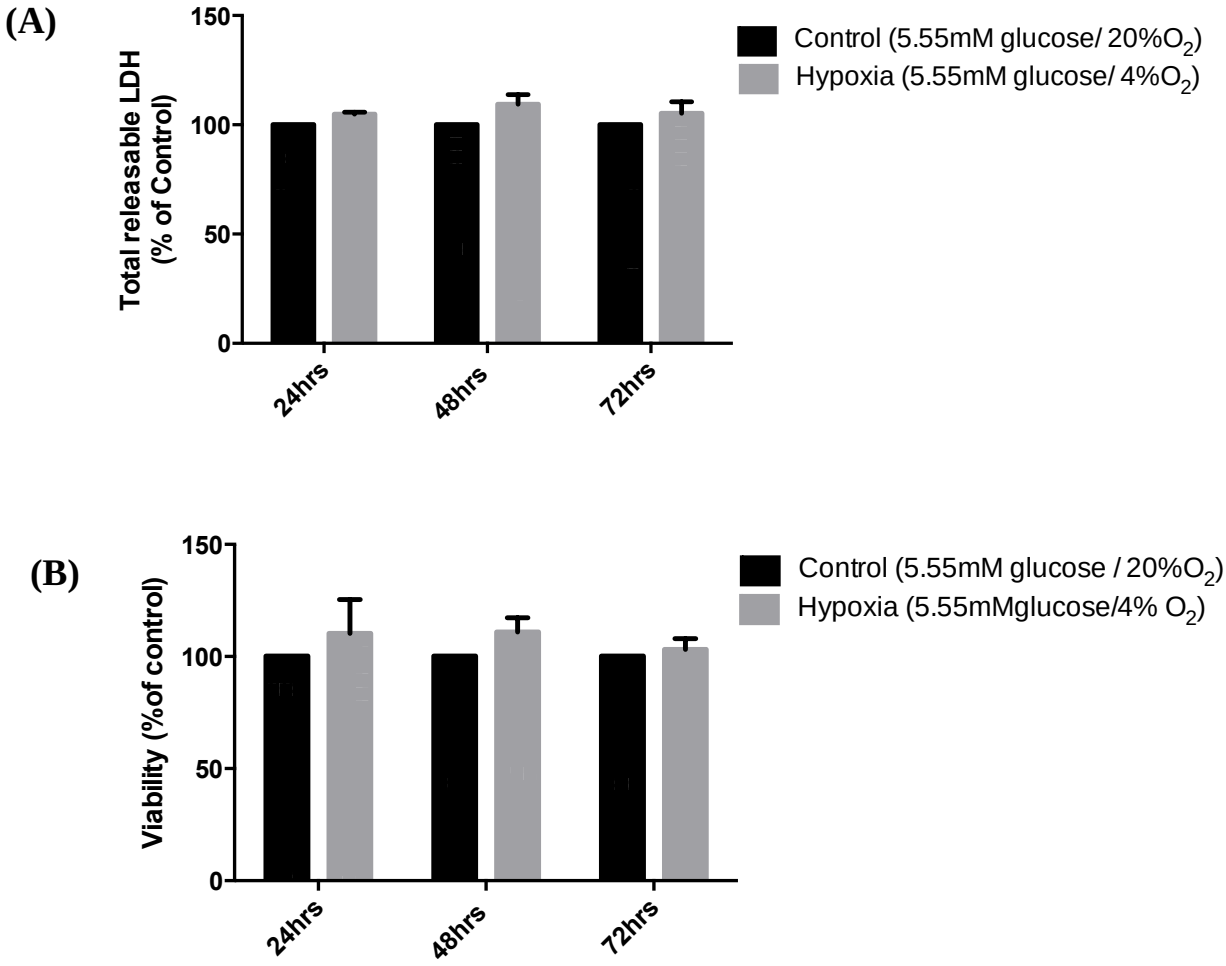


Figure 3.5: (A) Cytotoxicity (total releasable LDH) and (B) viability (MTS assay) of human Müller cells (MIO-M1) after 24hrs exposure to 4% oxygen for 24, 48 and 72hrs (mean ± s.e.m, * $p \leq 0.05$; n=4, t.test).

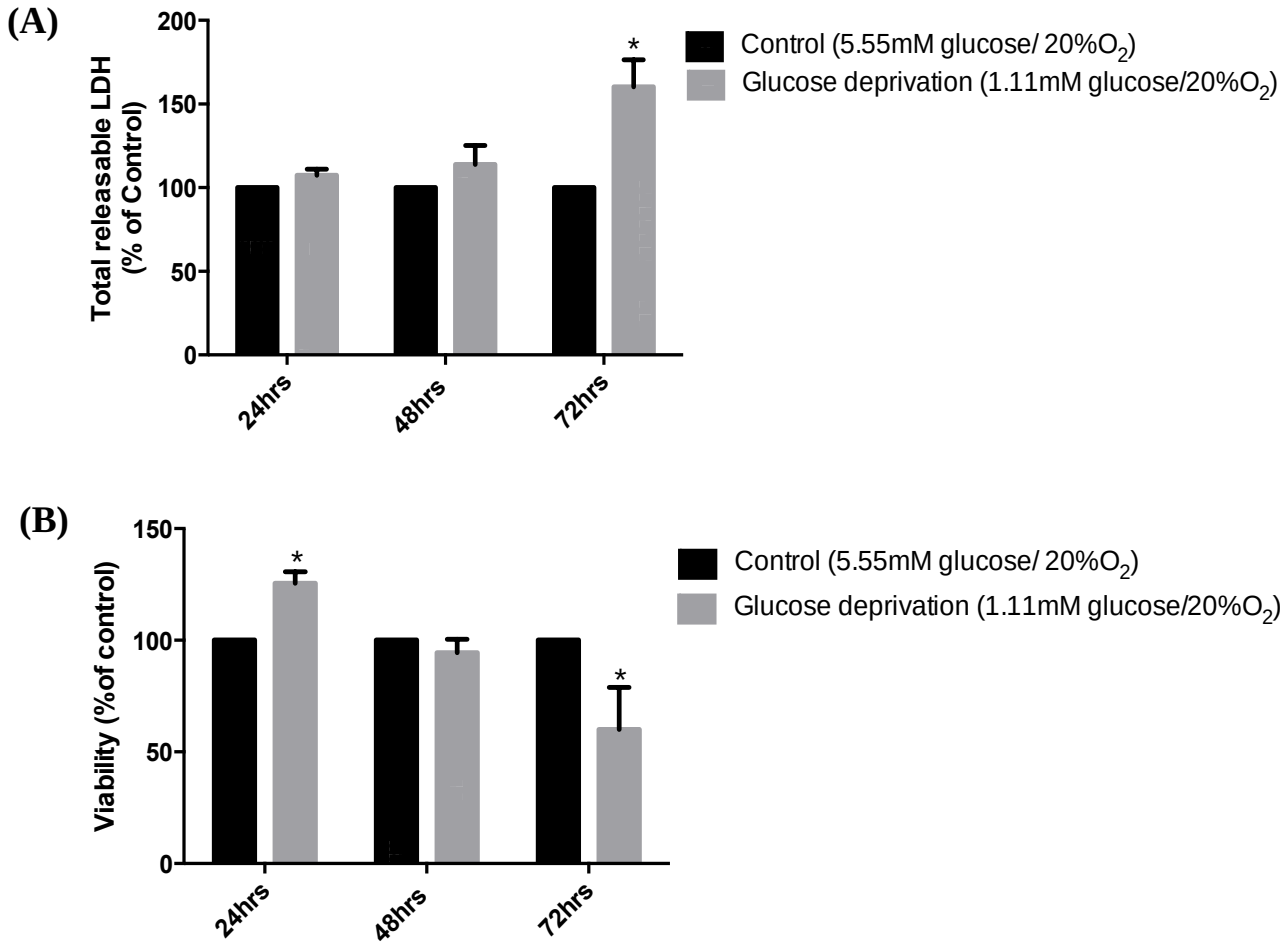
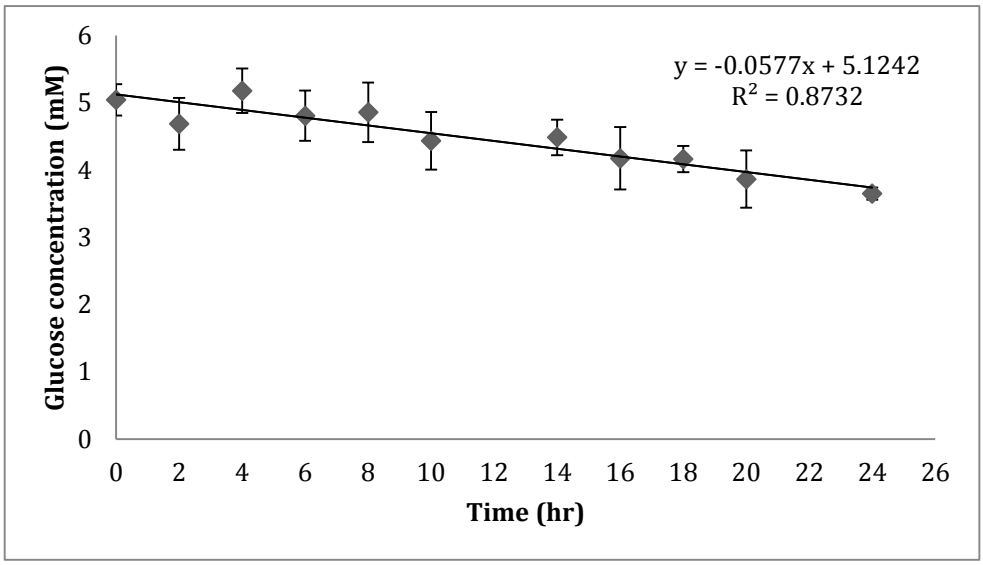


Figure 3.6: (A) Cytotoxicity (total releasable LDH) and (B) viability (MTS assay) of human Müller cells (MIO-M1) after 24hrs exposure to 1.11 mM glucose for 24, 48 and 72hrs (mean ± s.e.m, * p≤0.05; n=4, t.test).

The data, therefore, indicated that MIO-M1 cells were able to withstand oxygen deprivation for up to 72hrs with no effect on viability. In relation to glucose, 1.11mM caused a proliferation at 24hrs, but this proliferation was not maintained, and death occurred after 72hrs of glucose deprivation to this level. This loss of viability at 72hrs death may have been due to glucose utilization lowering the concentration in the bathing medium. The glucose utilization rate was therefore measured.

MIO-M1 cells were cultured at physiological glucose level (5.55mM) and also at 1.11mM glucose. Starting concentrations for control and treatment was measured at 5.0±0.23mM and 1.17±0.08 mM respectively. The concentration after 24hrs was 3.64±0.09mM and 0.14 ±0.05 mM for the control and reduced glucose respectively (Figure 3.7 A,B).

(A)



(B)

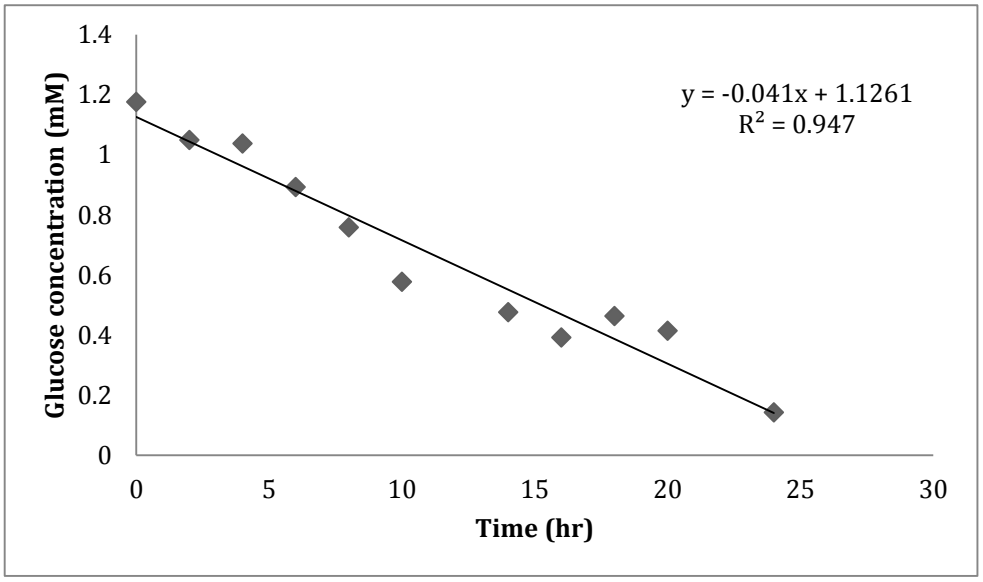


Figure 3.7 Glucose utilization by MIO-M1 under (A) control (5.55 mM) and (B) 1.11mM glucose for 24hrs.

3.4 Discussion

The human Müller cell line (MIO-M1) has been utilized to study glial cell function in vitro in relation to normal and diseased states. They express Müller cell markers and the authors also reported that the cells occasionally expressed GFAP after detachment from the monolayer indicating that they may be activated in vitro (Limb et al., 2002). Furthermore, Lawrence et al., (2007) reported that MIO-M1 cells also express stem cell markers, and they have been shown to differentiate into RGC precursors that were able to restore RGC function after *in vivo* transplantation (Singhal et al., 2012). This further demonstrates their stem cell characteristics.

As oxygen and glucose deprivation is part of the pathophysiology of glaucoma, the survival of Müller cells in a model of simulated ischemia was investigated using the MIO-M1 cell line. In an animal model of glaucoma, elevated IOP was associated with early astrocytes and Müller cell responses such as increased expression of GFAP, vimentin, nestin and glutamine synthetase (Lam et al., 2003; Xue et al., 2006). Activation of Müller cells in the human glaucomatous retina has been reported with early responses including increased immunostaining for GFAP (Tezel et al., 2003; Wang et al., 2002), and also glial proliferation (Thanos et al., 1991). In retinal stress, Müller cells initially respond by releasing survival factors in an attempt to rescue RGCs (Bringmann et al., 2009). As damage occurs to RGCs soma and the nerve fiber layer is affected, Müller cell processes replace the lost RGCs and form a glial scar (Nickells, 2007).

In the experiments reported here, the effect of OGD on cell survival was investigated in the MIO-M1 cells. They were subjected to four levels of oxygen and/or glucose deprivation compared to atmospheric oxygen (20%) and normal plasma glucose level

(5.55 mmol/L), which served as the experimental control. The deprivation levels were 10-30% of control levels.

3.4.1 Effect of Oxygen–Glucose Deprivation on the Survival and Cytotoxicity Human Müller Cells (MIO-M1)

Initial experiments assessing viability and cell death revealed that necrotic damage was detected when oxygen and glucose was withdrawn completely for 24hrs leading to a significant 5-fold increase in LDH levels. Viability results matched the LDH release data, with approximately 50% survival rate compared to control with full OGD. Viability studies of Müller cells in general and specifically for MIO-M1 cells under similar levels of OGD have not been presented previously in the literature, although studies have reported data from other glial cells derived from non-human species in relation to different diseases/conditions. For example, findings by Schmid-Brunclik et al., (2008) indicated that exposure of astrocytes to OGD caused death as early as 6hrs. Longer OGD treatment for 24hrs resulted in 90% of all astrocyte nuclei becoming TUNEL positive with obvious nuclear condensation and cell shape disruption (Schmid-Brunclik et al., 2008). In addition, exposure of astrocytes in culture to OGD caused cellular injury as measured by LDH efflux (Haun et al., 1992). Blocking of Ca^{2+} channels with nimodipine reduced astrocytes death indicating that influx of extracellular Ca^{2+} contributed to astrocyte death in ischemia (Haun et al., 1992).

With deprivation levels below full OGD, no change in the released LDH was observed which reflects that MIO-M1 cells withstand OGD at these levels with no loss of viability. Interestingly, exposure of MIO-M1 cells to 1.11mM glucose/4%O_2 for 24hrs caused an increase in viability indicating possible proliferation under this level of ischemia. The proliferation of Müller cells has previously been seen in other

conditions including retinal detachment (Lewis et al., 1992; Lewis et al., 1999), diabetic retinopathy (Guidry et al., 2009), proliferative vitreoretinopathy (Guidry, 2005) and glaucoma (Flammer and Mozaffarieh, 2007). Activation of Müller cells is described as a non-specific response to either mechanical or ischemic stress (Flammer and Mozaffarieh, 2007). Ischemia-induced proliferation of Müller cells has been reported *in vivo* when ischemia was induced in rats by central retinal artery occlusion (Stefansson et al., 1988). Furthermore, Müller cells isolated from rat retina after transient ischemia followed by reperfusion in vivo showed gliosis characteristics including cellular hypertrophy, alteration in osmotic swelling and expression of GFAP and CRALBP (Kuhrt et al., 2008). This response by Müller cells has also been found in adult zebrafish after an excision of the dorsal retina in which glial cells proliferated and filled the lesion in the retina (Yurco and Cameron, 2005).

With decreased oxygen-glucose levels (1.11mM glucose/4%O_2) for longer periods, there was no loss in viability at 48hrs, but the damage was apparent by the 72hrs time point. However, this most likely reflected that glucose levels had decreased due to glucose utilization by the cells (Figure 3.7). To assess if the changes seen were oxygen or glucose-dependent, each level of deprivation was repeated but the cells were deprived of either oxygen or glucose.

3.4.2 Effect of Oxygen Deprivation on the Survival and Cytotoxicity Human Müller Cells (MIO-M1)

Current results found that MIO-M1 cells were resistant to the absence of oxygen with all levels of deprivation. Even full oxygen deprivation for 72hrs did not alter viability or increased cell death. Interestingly, MIO-M1 cells slightly increased in number when they were maintained at 2 and 4% oxygen for 24hrs.

Wang et al., (2012) found that exposure of the of rat Müller cell line, rMC-1 to hypoxic conditions (2%O_2) for 24hrs, followed by three days of re-oxygenation, significantly induced cell proliferation (2-fold increase). The experiments investigated the mechanism of proliferation implicating the involvement of Notch signaling in the induction of Müller cell proliferation under hypoxic conditions by activating positive cell-cycle regulators such as cyclins A and D1, as well as the neural and retinal progenitor markers (Wang et al., 2012). In addition, anoxia has also been found to induce proliferation of primary astrocytes isolated from newborn rats (Schmid-Brunclik et al., 2008). They also reported that ATP levels dropped by 50–70% within the first 6–24 hrs of anoxia, but were subsequently maintained at 48hrs. However, in other studies exposure of a primary culture of astrocytes from newborn rat cerebral cortex to full oxygen deprivation for 24hrs resulted in astrocyte death with a 10-fold increase in LDH level (Yu et al., 1989). This was also found in primary glial culture from the cerebral hemispheres of newborn rats, with 24hrs hypoxia resulting in an 80% increase in LDH efflux (Callahan et al., 1990). The mechanism of tolerance of MIO-M1 cells to hypoxia was not investigated in the current experiments, but may be explained by Yu et al., (2008) findings which showed that increase expression of two major forms of glucose transporters (GLUT1 and GLUT3) occurred in cultured rat hippocampal neurons and astrocytes exposed to hypoxia (1% O_2) followed by anoxia. This suggests that glucose uptake is increased which helps to maintain ATP levels by glycolysis during hypoxic insult (Yu et al., 2008).

3.4.3 Effect of Glucose Deprivation on the Survival and Cytotoxicity Human Müller Cells (MIO-M1)

Glucose deprivation showed a similar response to OGD, highlighting the importance of glucose for the survival of glial MIO-M1 cells. Full glucose deprivation for 24hrs reduced MIO-M1 cell viability and increased cell death. In other studies using MIO-M1 cells, full glucose deprivation for a very short period of one hour did not change the viability or intracellular ATP level (Toft-Kehler et al., 2014). In another study by Schmid-Brunclik et al., (2008) exposure of astrocytes to full glucose deprivation under the normoxic condition for 6hrs induced proliferation by approximately 40% compared to control. Wang et al., (2012) showed that rat Müller cells withstand full glucose deprivation for 12hrs but experiments conducted for 24hrs with full glucose deprivation have not been reported.

Lower levels of deprivation of glucose maintained similar viability to control and increased proliferation was seen at 1.11mM glucose. In order to investigate this proliferation further, the cells were exposed to this level of glucose for a longer period of time. However, the proliferation was not increased but no loss in viability was found at 48hrs and the damage was apparent by the 72hr time point. Similarly, exposure of MIO-M1 cells to 2.2mM glucose for 24hrs did not alter the ATP levels (Emery et al., 2011). It was recognized that this is likely due to the fact that glucose would be being utilized over the incubation period. Therefore, glucose utilization by MIO-M1 cells was measured under control and 1.11mM glucose conditions over a 24hrs period (Figure 3.7 A,B). The results showed that after 24hr there was little glucose left in the cells, which were incubated in 1.11mM glucose at the start of the experiments. The results therefore indicated that MIO-M1 cells were able to cope with reduced glucose for 48hrs despite the consumption of glucose in the first 24hrs,

which was unexpected. If we consider that the cells were proliferating as a response to switching them from 5.55mM glucose to the reduced level 1.11mM glucose, they would actually consume more glucose than usual as they were increasing in number. After accommodating to the new condition, no more proliferation was seen, and they may switch to lower rate of glucose utilization, which enabled them to survive for 48hrs but failed to maintain them for 72hrs. Findings by Schmid-Brunclik et al., (2008) indicated that in cultured astrocytes fully deprived of glucose under normoxia for 6hrs, a 50% decrease in ATP levels occurred, but ATP levels recovered to 70% at 48 hrs. A study by Jelluma et al., (2006) showed that in human astrocytes, withdrawal of glucose enhanced mitochondrial respiratory chain activity of fatty acid oxidation and thus sustained ATP levels. Active production of lactate in the presence of glucose is a characteristic of astrocytes in culture (Pauwels et al., 1985; Swanson and Benington, 1996). Müller cells share similar metabolic activity as they are reported to depend 80–90% on glycolysis as their main pathway for energy production whether under aerobic and anaerobic conditions (Winkler et al., 2000), metabolizing glucose primarily to lactate (Poitry-Yamate et al., 1995). Blocking glycolysis by iodoacetate has been found to cause a decline of ATP to low levels and loss of viability in Müller cells indicating the importance of this pathway to survival (Poitry-Yamate and Tsacopoulos; 1995; Winkler et al., 2000). It has been proposed that lactate produced by glycolysis is then used as a source of oxidative energy metabolism for retinal neurons (Poitry-Yamate et al., 1995; Tsacopoulos et al., 1998; Xu et al., 2007). Because of this metabolic activity, Müller cells consume very low rates of oxygen thus preserving it for retinal neurons. This in turn, makes them more resistance to oxygen deprivation (Poitry-Yamate and Tsacopoulos; 1995; Winkler et al., 2000) as was seen in the experiments reported in this chapter. In the absence of glucose,

Müller cells use other substrates such as lactate, pyruvate, glutamate or glutamine and generate energy by metabolizing these substrates via tricarboxylic acid cycle, a non-dominant pathway which requires oxygen (Tsacopoulos et al., 1998; Winkler et al., 2000). Mechanisms requiring lactate or pyruvate would not be available to the cells in the current experiements and glutamate or glutamine from the culture medium were unable to support the cells as loss of viability occurred with full glucose deprivation in the presence of oxygen.

Glucose deficiency can be also compensated by the glycogen deposits in Müller cells, which allow them to withstand short periods of ischemia (Bringmann et al., 2006; Johnson, 1977), with Müller cells having abundant glycogen phosphorylase a key enzyme in glycogen degradation (Pfeiffer-Guglielmi et al., 2005). These mechanisms may have aided MIO-M1 cell survival in the early stress of glucose deprivation but *in vivo* experiments of ischemia for one hour caused depletion of the glycogen store in Müller cells (Gohdo et al., 2001).

Generally, the resistance of Müller cells to stress can be explained by several properties and functions that these cells can perform. These include the cells unique energy metabolism, glycogen deposits, ability to proliferate and release neurotrophic and growth factors, buffering of elevated potassium levels (Bringmann et al., 2006). Also, their glutathione content and ability to defend against oxidative stress (Garcia and Vecino, 2003) and their defense mechanism against glutamate excitotoxicity (Bringmann et al., 2009). The present research has supported the notion that Müller cells are relatively resistant to ischemic damage.

The fact that viability increased when oxygen and glucose level dropped to 20% of control level (1.11mM glucose/4%O_2) highlights that MIO-M1 cells may proliferate, perhaps protecting themselves by releasing growth/survival factors that could also be important to RGCs during ischemic insult. In the next chapter, gene expression of key growth factors, specific Müller cell markers, glutamate and ATP signaling related genes will be investigated.

Chapter 4

Effect of Oxygen and Glucose Deprivation on Gene Expression in Human Müller cells (MIO-M1)

4.1 Introduction

As previously discussed, human Müller (MIO-M1) cells were resistant to oxygen and glucose deprivation. Only complete withdrawal of glucose, rather than oxygen, was the trigger for cell loss suggesting the importance of glucose for survival of MIO-M1 cells. Results from previous experiments have also shown that MIO-M1 cells proliferate in response to reduction in glucose and oxygen to 20% (1.11mM glucose/4%O_2) of the control levels suggesting the possibility that Müller cells may respond to this level of ischemic stress by secreting growth/survival factors. Proliferation and gliosis is a cellular attempt to restore normal function and prevent damage (Liberto et al., 2004). In the central nervous system (CNS), astrocytes respond to insults by proliferation, increased production of GFAP, vimentin, nestin and cytokines (Liberto et al., 2004). Activated astrocytes become larger in size with nuclear hypertrophy and produce trophic and growth factors in an attempt to protect adjacent neurons and glia (Albrecht et al., 2002; Hudgins and Levison, 1998). These responses may result in the formation of astrogliotic scars (Norenberg, 1994; Norton et al. 1992). Similarly, microglial cells in the brain become activated during injury and release cytokines such as IL-1β, IL-3, IL-6, TNF-α, and VEGF (Stoll et al., 2002). In the retina, Müller cells are the main glial cells. They express numerous receptors and release neurotrophic and growth factors. Among these are VEGF, EGF, PDGF, IGF-1, bFGF, NGF, ciliary neurotrophic factor (CNTF) (Bringmann et al., 2001; Cao et al., 1997; Cao et al., 2001; Harada et al., 2000;Wen et al., 1995). The mitogenic activity of these growth factors has been examined in Müller cell cultures and *in vivo* (Ikeda and Puro, 1994; Lewis et al., 1992; Mascarelli et al., 1991; Uchihori and Puro,

1991). Müller cells also respond to anti-angiogenic factors such as PEDF and TGF-β (Eichler et al. 2004; Ikeda et al., 1998). In the following section, key growth factors will be introduced in relation to retinal and Müller cell function and their role during injury.

One of the Müller cell survival factors, which is released in response to hypoxia, is VEGF-A (Aiello et al., 1995; Eichler et al., 2000; Yafai et al., 2004). The VEGF family belongs to the platelet-derived growth factor (PDGF)/VEGF supergene family (Dvorak, 2002). It exists in six isoforms termed VEGF-A, B, C, D, E and placental growth factor (PIGF) (Dvorak, 2002; Shibuya et al., 2006; Takahashi et al., 2005). Exon splicing of the human VEGF-A gene results in the generation of four different isoforms: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$. $VEGF_{165}$, is the predominant isoform, with pro-angiogenic, as well as neuroprotective, properties and when the term "VEGF" is used, it refers to the VEGF-A/$VEGF_{165}$ isoform (Houck et al., 1991). VEGF binds and activates VEGFR1 and VEGFR2 tyrosine kinase receptors and both are involved in angiogenesis (Shibuya et al., 2006). These receptors are expressed predominantly on vascular endothelial cells and stimulate proliferation and chemotaxis (Neufeld et al., 1999). Disruption of genes encoding VEGFR1 and VEGFR2 results in severe abnormalities of blood vessel formation and fatal consequences during embryogenesis (Shalaby et al., 1995) Increased oxygen in the retina results in decreased expression of VEGF and endothelial cell death (Yamada et al., 1999).

In pathological conditions of the eye such as ischemia, VEGF is over-expressed and contributes to pathophysiological changes such as retinal vascular leakage and neovascularization. VEGF has been shown to exert neuroprotective effects, increasing neuronal cell survival through inhibition of apoptosis (Jin et al., 2000; Jin et al., 2001),

stimulation of neurogenesis and angiogenesis (Sun et al., 2003; Jin et al., 2000), increasing neuronal glucose uptake (Sone et al., 2000) and activation of antioxidants including induction of expression of heme oxygenases 1 (HO-1) (Chao et al., 2013; Zhu et al., 2007).

One factor that can potentiate VEGF action is PDGF as they share a low but significant sequence homology of 100 amino acids (Finkenzeller et al., 1997). The PDGF family consists of five isoforms: PDGF-A, B, C, and D, existing as homodimers (PDGF-AA, -BB, -CC, -DD) or as a heterodimer AB (Fredriksson and Eriksson, 2004). They act on tyrosine kinase receptors PDGFR-α and -β to induce angiogenesis and promote survival and proliferation of different cell types (Fredriksson and Eriksson, 2004). In the eye, PDGF-A is expressed by both neurons and astrocytes (Pringle et al., 1989). Glial cells possess PDGFR-α and a high expression of PDGF-A results in extensive proliferation of glial cells (Pringle et al., 1989). High expression of PDGF-A in the retina was associated with glial cells proliferation and traction, causing retinal detachment without vascular cell involvement (Mori et al., 2002).

Basic fibroblast growth factor (bFGF) is a potent mitogenic factor, which increases endothelial cell proliferation and migration, through activation of FGF receptors (Walsh et al., 2001). Basic FGF has been shown to regulate photoreceptor survival and differentiation and stimulate Müller cells proliferation (Mack and Fernald, 1993; Hicks and Courtois, 1990). Basic FGF stimulates angiogenesis *in vivo* and has been shown to promote neuronal survival in ischemic insult and glutamate toxicity (Anderson et al., 1988; Freese et al., 1992).

Another factor important for retinal homeostasis is pigment epithelium-derived factor (PEDF), a secreted 50-kDa glycoprotein and a member of the serpin superfamily

(Bouck, 2002). It was originally identified in conditioned medium from RPE and was found to be an endogenous inhibitor of angiogenesis in the eye (Bouck, 2002; Tombran-Tink and Johnson, 1989). PEDF expression has been detected in the retinal ganglion cell layer (Behling et al., 2002) and in Müller cells (Eichler et al., 2004). In addition, PEDF receptor was identified and isolated initially from retinoblastoma tumour cells (Singh et al., 1998) and neural retina (Aymerich et al., 2001). PEDF expression is regulated by hypoxia in a reciprocal direction to that of VEGF; as hypoxia is a stimulatory factor for VEGF expression, it decreases PEDF release (Eichler et al., 2004). Besides its function as an inhibitor of angiogenesis, PEDF has been shown to support the development and survival of photoreceptors (Cayouette et al., 1999; Jablonski et al., 2000), and also protect retinal ganglion cells from ischemia-induced death (Pang et al., 2007; Takita et al., 2003; Unterlauft et al., 2012).

Another important inhibitor of angiogenesis is leukaemia inhibitory factor (LIF), which is a member of the interleukin-6 cytokine family. The LIF receptor signals via glycoprotein 130, to activate the JAK (Janus kinase) tyrosine kinase family (Heinrich et al., 2003). Ash et al., (2005) studied the effect of LIF, in early retinal development and found that LIF inhibited retinal vascular development. This inhibition was independent of VEGF expression *in vivo*. In contrast, Kubota et al., (2008) showed that LIF-deficient mice exhibited increased microvessel density and upregulated VEGF *in vivo*. In a rat IOP-induced glaucoma model, increased expression of LIF mRNA was found (Johnson et al., 2011) and endogenous LIF produced by Müller cells, has been found to support survival of photoreceptors during light-induced injury (Bürgi et al., 2009; Joly et al., 2008).

As previously discussed, glutamate excitotoxicity plays a prominent role in hypoxic-ischemic insult in the retina. Major pathways of glutamate metabolism consist of

glutamate uptake by the glial glutamate transporter (GLAST) followed by enzymatic conversion to glutamine by glutamine synthetase (GS) (Ishikawa et al., 2013). In addition, the purinergic signaling system has been shown to be involved in pathogenesis of hypoxic-ischemic injury (Montero and Orellana, 2015; Niyadurupola et al., 2013). Pannexins are newly discovered channels expressed in a variety of tissues with multiple functions and have been implicated as ATP conduits in many cells (Bruzzone et al., 2003; Panchin et al., 2000; Phelan et al., 1998). These channels can be activated by membrane potential changes or by independent voltage stimuli such as low oxygen environment, mechanical stress or increased cytoplasmic calcium ions (Bao et al., 2004; Bruzzone et al., 2003). Reigada et al., (2009) has shown that ATP is released as a result of elevated pressure and pannexin hemichannels contribute to at least some of the ATP release. Sridharan et al., (2010) demonstrated that reduced oxygen tension causes ATP release from erythrocytes, which can be prevented by the pannexin-1 inhibitor. A recent study by Voigt et al., (2015) demonstrated that rat Müller cells expressed mRNAs for pannexin-1 and -2 and that glutamate induce ATP release from Müller cells occurs via these channels.

After ATP release and signaling, nucleotide needs to be inactivated to adenosine. This is achieved by the action of various enzymes including ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2; Autotaxin), which possess ATPase activity to produce ADP and Pi (Goding et al., 2003; Stefan et al., 2006). In addition, it has an ATP pyrophosphatase activity and thus produces AMP and PPi (Clair et al., 1997). NTPDase2 is the dominant ectonucleotidase expressed by rat astrocytes in the CNS and retinal Müller cells (Iandiev et al., 2007; Wink et al., 2006). In addition, ENPP2 expression has been found in ciliary, iris pigment, and retinal pigment epithelial cells (Fuss et al., 1997; Narita et al., 1994) and it is secreted by

oligodendrocytes (Stefan et al., 2006). ENPP2 protein with was found in human aqueous humour from normal eyes (Iyer et al., 2012; Tokumura et al., 2002), but it was found to be significantly elevated in aqueous humour from POAG patients (Iyer et al., 2012). Increased levels of ENPP2 were also found in the aqueous humour in an ocular hypertensive mice model (Wang et al., 2015). Animal studies have also shown that lysophospholipase D present in aqueous humour of porcine, rabbit and mouse (Iyer et al., 2012) and also released from healthy and injured corneal tissues and/or ciliary body into the aqueous humour in rabbits (Tokumura et al., 2012). Moreover, ENPP2 also catalyses the hydrolysis of lysophosphatidylcholine (LPC) to generate lysophosphatidic acid (LPA), which is referred to as lipophospholipase D activity. LPA acts on G protein-coupled receptors named LPA_{1-6} (Houben and Moolenaar, 2011; Moolenaar, 2002). LPA receptors have been found to be expressed in astrocytes, microglia, and oligodendrocytes (Rao et al., 2003; Tabuchi et al., 2000; Yu et al., 2003). LPA has been implicated in mediating cell proliferation, migration, apoptosis, inflammation, tumor metastasis, angiogenesis, fibrosis and secretion of matrix metalloproteinases (MMPs), cytokines and chemokines (Houben and Moolenaar, 2011; Nakanaga et al., 2010; Okudaira et al., 2010). Furthermore, LPA receptors have been found in the trabecular meshwork and Schlemm's canal and influence the aqueous humour outflow and potentially IOP in enucleated porcine eyes (Mettu et al., 2004). Therefore, expression of glutamate markers and purinergic related genes by Müller cells under conditions of OGD will be also investigated.

Finally, the expression of heat shock proteins (HSPs) under hypoxic-ischemic injury in Müller cells will be also investigated. HSPs are ubiquitous and highly conserved proteins whose expression is induced by different stressors (Garrido et al., 2001). They are divided into four major families according to their molecular size: HSP90,

HSP70, HSP60 and the small HSPs (Garrido et al., 2001). The HSP70 family constitutes the most conserved and best-studied class of HSPs functioning as ATP-dependant molecular chaperones (Lee et al., 2001). They assist in the folding of newly synthetized polypeptides, assembly and the transport of protein across cell membranes (Beckmann et al., 1990; Shi and Thomas; 1992). In pathological conditions, HSP70 enhances the ability of injured cells to overcome the increase in unfolded or denatured proteins (Nollen et al., 1999). Furthermore, HSP70 prevents stress-induced apoptosis by inhibition of stress-activated protein kinase SAPK/JNK (c-Jun N-terminal kinase) (Mosser et al., 1997) and inhibition of Bax activation, thereby preventing the release of proapoptotic factors from mitochondria (Stankiewicz et al., 2005).

As hypoxic-ischemic insult underlies retinal neuron degeneration in glaucoma, Müller cells are first responders to retinal insult, therefore, it is of great interest to identify the survival factors expressed by the human glial cell line, MIO-M1 cells in our *in vitro* model of oxygen and glucose deprivation.

4.2 Results

To investigate the effect of oxygen and/or glucose deprivation on gene expression *in vitro*, cultured MIO-M1 cells were exposed to control (5.55mMglucose/20%O_2), hypoxia (5.55mMglucose/0-4%O_2) and/or glucose deprivation (0-1.11mM/20% O_2), for 24hrs.

4.2.1 Effect of Oxygen and Glucose Deprivation on the Expression of Angiogenic Growth Factors

4.2.1.1 Effect of Oxygen and Glucose Deprivation on the Expression of VEGF mRNA

Deprivation of oxygen and glucose to the 20% oxygen-glucose level (1.11mM glucose/4%O_2) caused no significant changes in VEGF expression at 24hrs, although there was an indication of a small increase as a result of low glucose (Figure 4.1). In full OGD, VEGF mRNA was significantly (*$p \leq 0.05$; n=4) increased at each time-point (3,6,12 and 24hrs) with a 20-fold increase at the 24hrs time-point (Figure 42A). A significant increase in VEGF mRNA (*$p \leq 0.05$; n=4) was obtained when cells were totally deprived from oxygen at each time-point with 7-fold increase at the 24hrs (Figure 4.2B). Full glucose deprivation caused significant increase by 2 fold (*$p \leq 0.05$; n=4) in VEGF mRNA compared to control at 24hrs (Figure 4.2C). In full OGD, hypoxia and glucose deprivation, the increase in VEGF mRNA was observed as early as 3hrs and remained elevated over 24hrs experiment interval. In each case, VEGF mRNA level was highest at 12hrs. Comparing the levels of VEGF mRNA in oxygen deprivation versus glucose alone indicate that hypoxia was the key contributor to increasing levels seen in OGD; although it is notable that the absence of glucose did cause a significant increase in the VEGF expression.

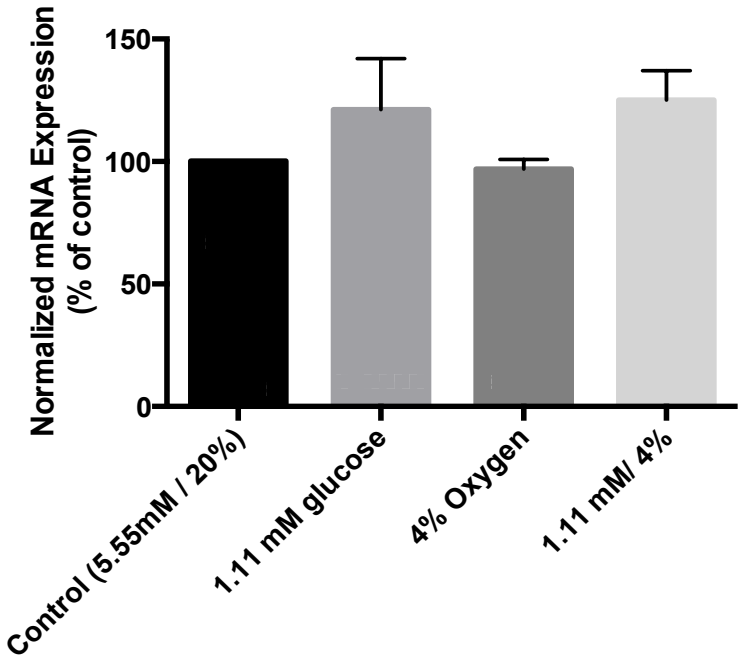


Figure 4.1: Expression of VEGF mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

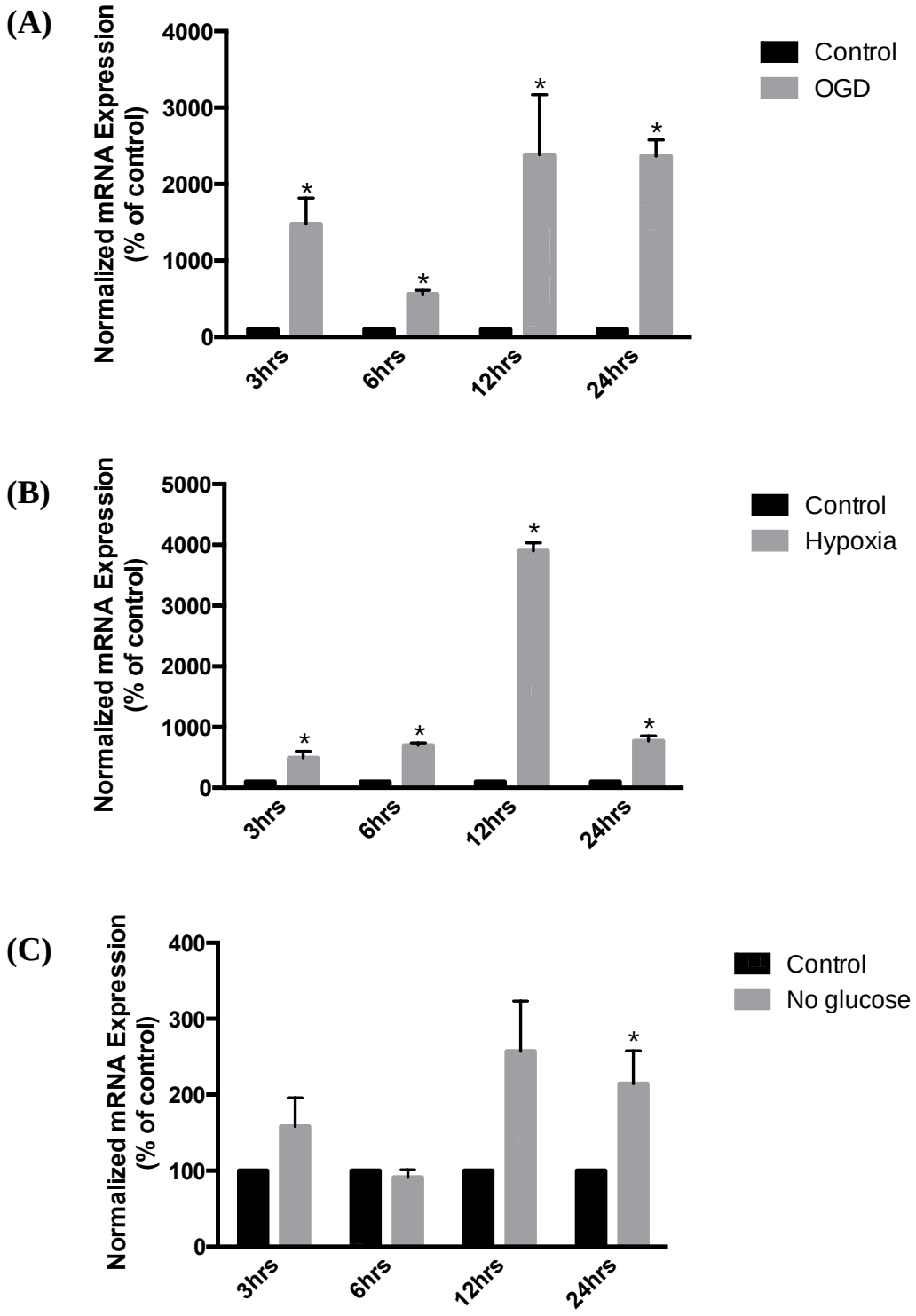


Figure 4.2: Expression of VEGF mRNA in human Müller cells (MIO-M1) after 3, 6, 12 and 24 hrs exposure to (A) full OGD (0mM glucose/0%O_2), (B) oxygen deprivation (5.55 mM glucose/0%O_2) and (C) glucose deprivation (0mM glucose/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; t-test).

4.2.1.2 Effect of Oxygen and Glucose Deprivation on the Expression of PDGF mRNA

As shown in Figure 4.3, PDGF mRNA did not change with different conditions of oxygen (4%O_2) glucose deprivation (1.11mM) or with a combination of both (1.11mM glucose/4%O_2). Complete withdrawal of oxygen and glucose deprivation did not result in an increase in PDGF expression in cultured MIO-M1 cells at any of the time-points measured (Figure 4.4 A, B & C) although, a significant decrease was seen with full OGD at the 24hrs time-point.

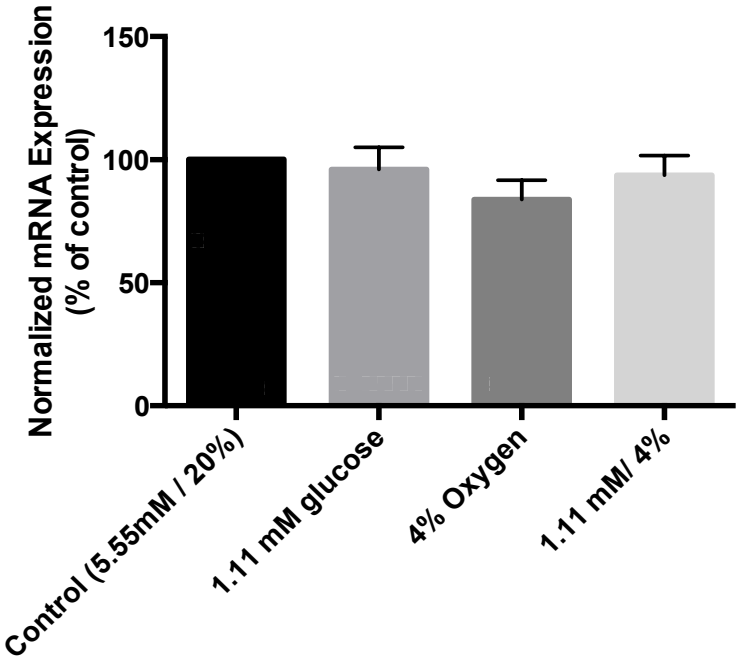


Figure 4.3: Expression of PDGF mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

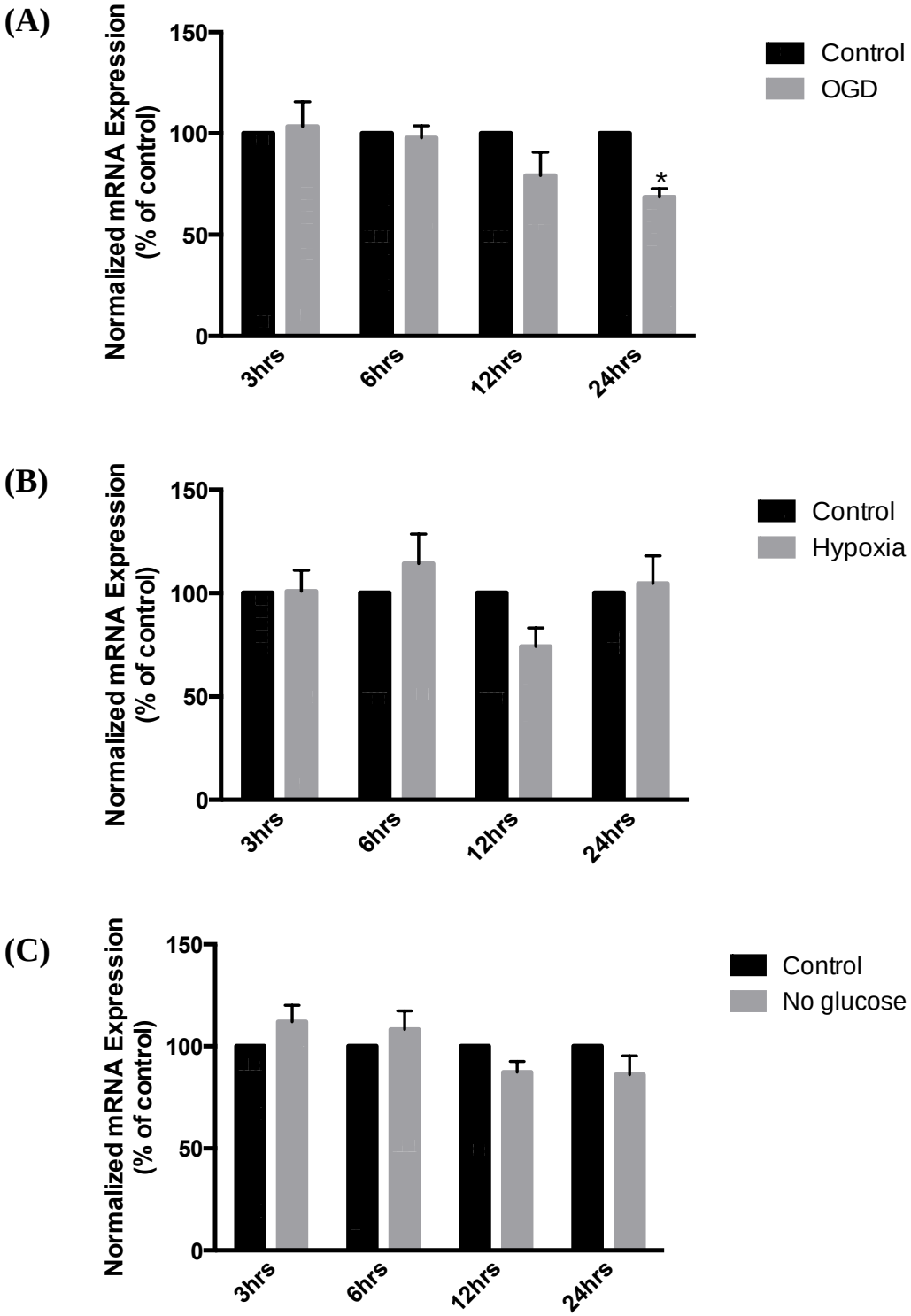


Figure 4.4: Expression of PDGF mRNA in human Müller cells (MIO-M1) after 3, 6, 12 and 24 hrs exposure to (A) full OGD (0mM glucose/0%O_2), (B) oxygen deprivation (5.55 mM glucose/0%O_2) and (C) glucose deprivation (0mM glucose/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; t-test).

4.2.1.3 Effect of Oxygen and Glucose Deprivation on the Expression of basic FGF mRNA

Similar to PDGF, bFGF mRNA proved to be unchanged after 24hrs exposure to (1.11mM glucose/ 4%O_2), oxygen deprivation (4%O_2) and glucose (1.11 mM) as compared control (Figure 4.5). Full OGD showed little overall change in bFGF mRNA expression, although there was a slight but significant reduction (*p≤ 0.05; n=4) in the level observed at 12hr time-point. Oxygen deprivation caused no significant changes. Glucose deprivation alone caused a significant 42% increase (*p≤ 0.05; n=4) in bFGF mRNA after 24hrs (Figure 4.6C).

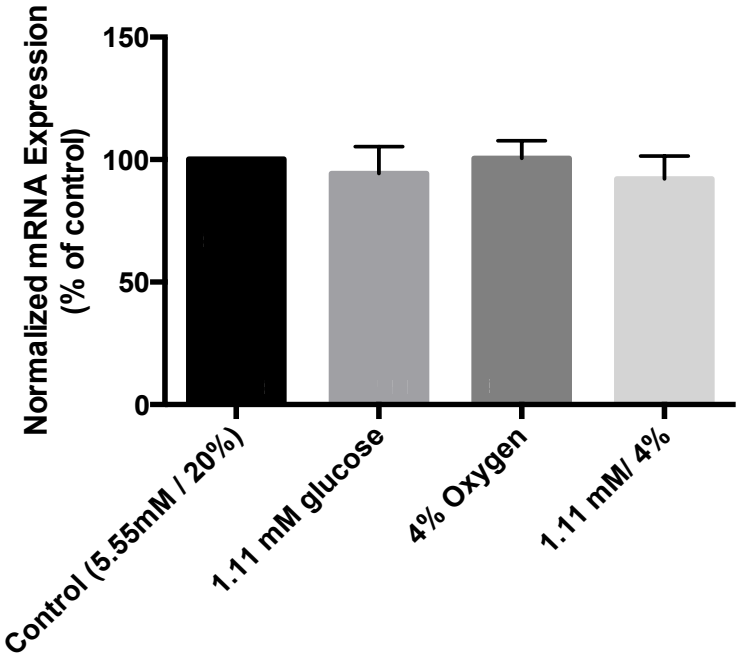


Figure 4.5: Expression of bFGF (FGF2) mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

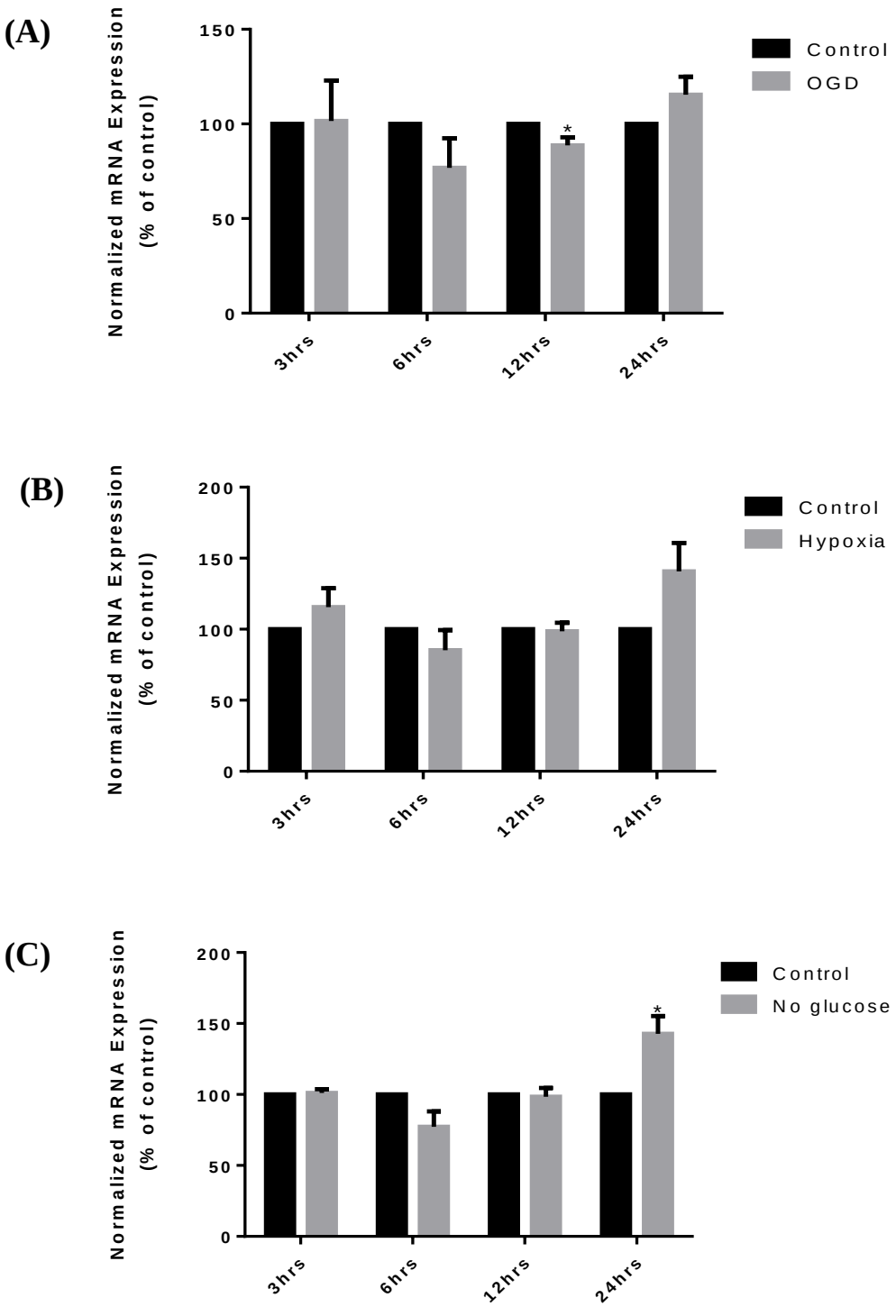


Figure 4.6: Expression of bFGF mRNA in human Müller cells (MIO-M1) after 3, 6, 12 and 24 hrs exposure to (A) full OGD (0mM glucose/0%O_2), (B) oxygen deprivation (5.55 mM glucose/0%O_2) and (C) glucose deprivation (0mM glucose/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; t-test).

4.2.2 Effect of Oxygen and Glucose Deprivation on the Expression of Anti-angiogenic Growth Factors

4.2.2.1 Effect of Oxygen and Glucose Deprivation on the Expression of PEDF

Exposure of MIO-M1 cells to reduced level of glucose and oxygen (1.11mM/4%O_2), hypoxia (4%O_2) and 1.11 mM glucose resulted in significant down-regulation (*p≤ 0.05; n=4) of PEDF mRNA by 36, 15 and 26% respectively as shown in Figure 4.7. Full OGD caused a significant reduction (*p≤ 0.05; n=4) in PEDF mRNA by 25% compared to control at 24hrs with no change in the expression at earlier time points (Figure 4.8A). Similarly, hypoxia caused a significant 20% reduction (*p≤ 0.05; n=4) in PEDF mRNA at 24hrs as compared to control that was proceeded by slight but significant (*p≤0.05; n=4) increase at 12hrs (Figure 4.8B). Complete deprivation of glucose resulted in no change in PEDF mRNA at any time point (Figure 4.8 C).

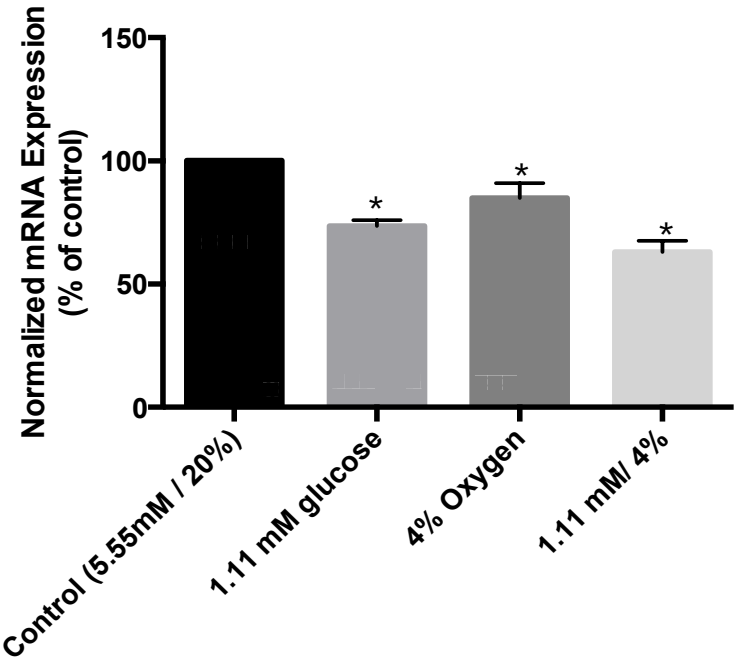


Figure 4.7: Expression of PEDF mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

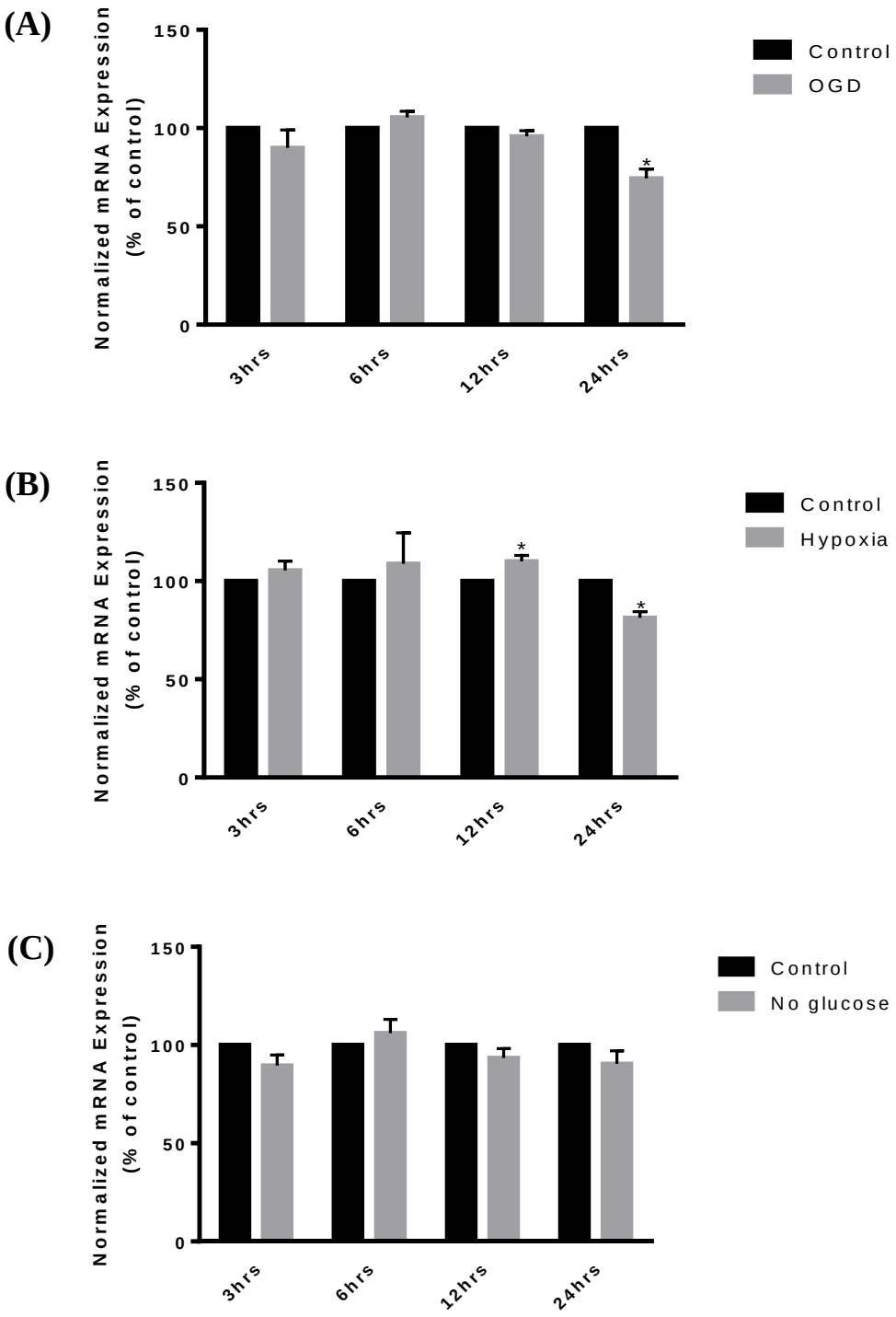


Figure 4.8: Expression of PEDF mRNA in human Müller cells (MIO-M1) after 3, 6, 12 and 24 hrs exposure to (A) full OGD (0mM glucose/0%O_2), (B) oxygen deprivation (5.55 mM glucose/0%O_2) and (C) glucose deprivation (0mM glucose/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; t-test).

4.2.2.2 Effect of Oxygen and Glucose Deprivation on the Expression of LIF

As shown in Figure 4.9, the expression of LIF mRNA with reduced glucose and oxygen level (1.11mM glucose/4%O_2), hypoxia (4%O_2) and 1.11mM glucose alone did not change significantly. Full OGD significantly (*p≤ 0.05; n=4) upregulated LIF mRNA peaking at 3hrs (7-fold) decreasing to 2.5- and 1.48- fold (*p≤ 0.05; n=4) at 6 and 12hrs to reach the control level after 24hrs (Figure 4.10A). Hypoxia (0%O_2) increased LIF mRNA with an approximate 1.7-fold increase at 12hrs only (Figure 4.10B). A similar pattern to full OGD was seen in glucose deprivation alone (Figure 4.10C) with a large significant increase (6.4-fold) seen at the earliest time-point (3hrs).

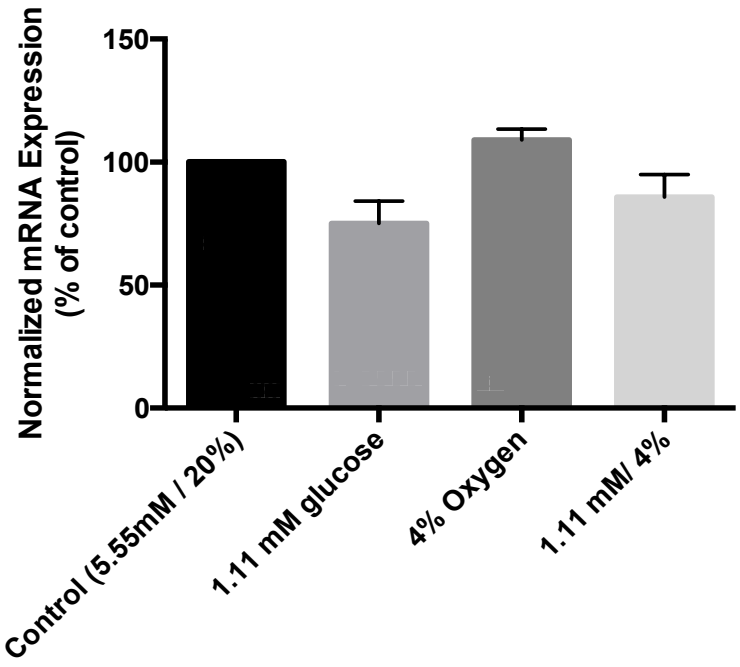


Figure 4.9: Expression of LIF mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

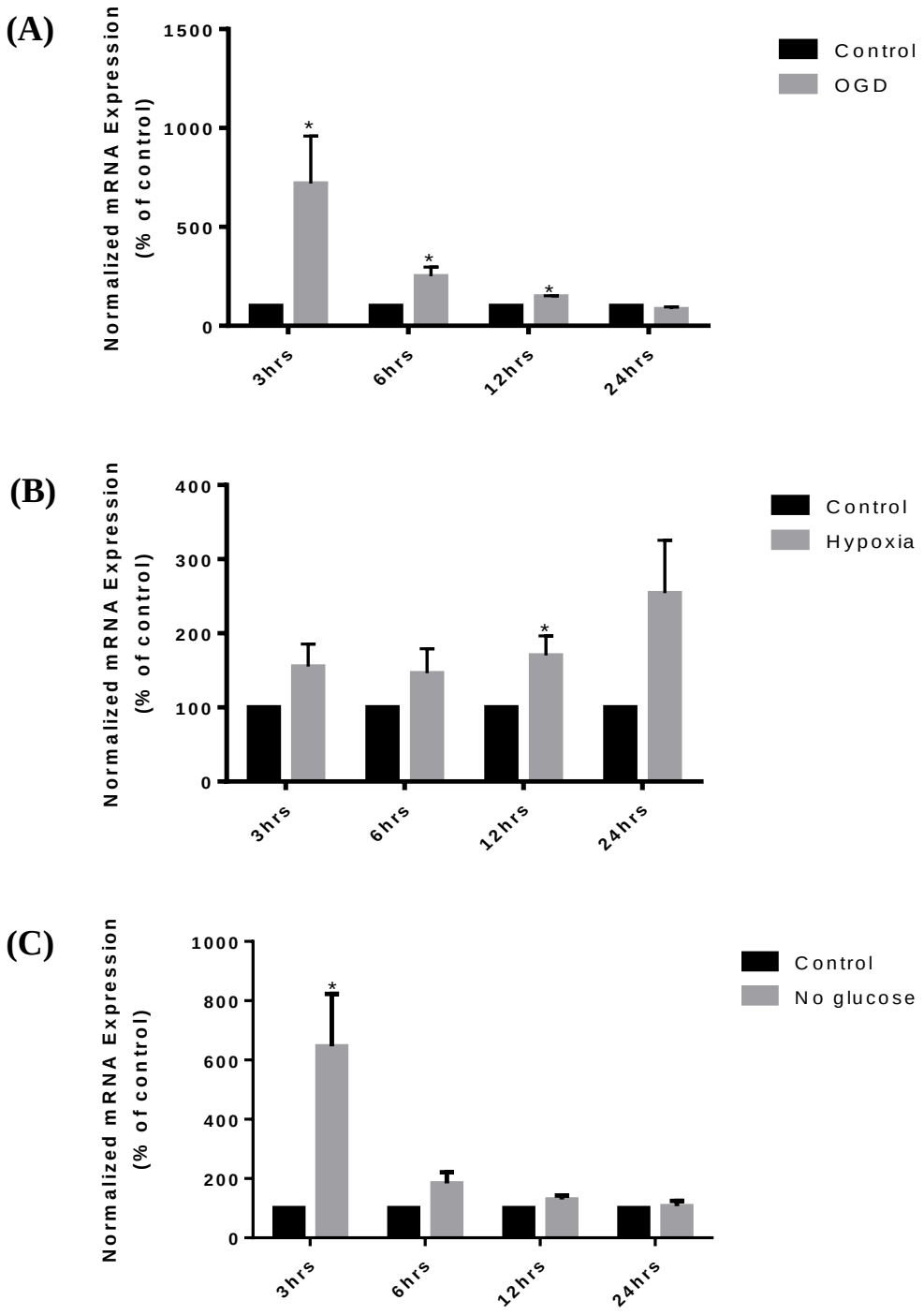


Figure 4.10: Expression of LIF mRNA in human Müller cells (MIO-M1) after 3, 6, 12 and 24 hrs exposure to (A) full OGD (0mM glucose/0%O_2), (B) oxygen deprivation (5.55 mM glucose/0%O_2) and (C) glucose deprivation (0mM glucose/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; t-test).

4.2.3 Effect of Oxygen and Glucose Deprivation on the Expression of Glutamate Markers

4.2.3.1 Effect of Oxygen and Glucose Deprivation on the Expression of Glutamine Synthetase (*GLUL*)

Reduced levels of glucose (1.11mM) and/or oxygen (4%) did not result in any significant change in *GLUL* mRNA levels (Figure 4.11). Full OGD significantly (*p≤ 0.05; n=4) down-regulated the levels by 20% at 3hrs followed by up-regulation by 49% at 24hrs as compared to control (Figure 4.12A). Complete deprivation of oxygen resulted in significant upregulation (*p≤ 0.05; n=4) of *GLUL* mRNA levels at 12 and 24hrs by 2- and 1.30- fold respectively as compared to control (Figure 4.12 B). No change in the expression of *GLUL* mRNA was found with full glucose deprivation alone (Figure 4.12 C).

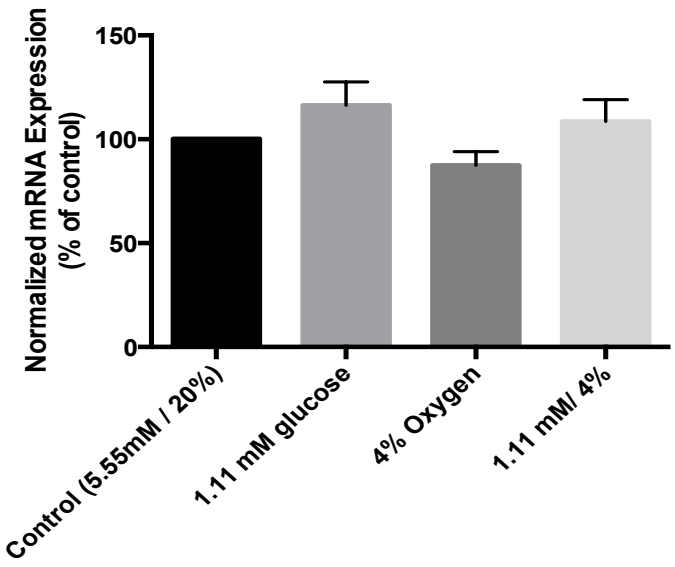


Figure 4.11: Expression of *GLUL* mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

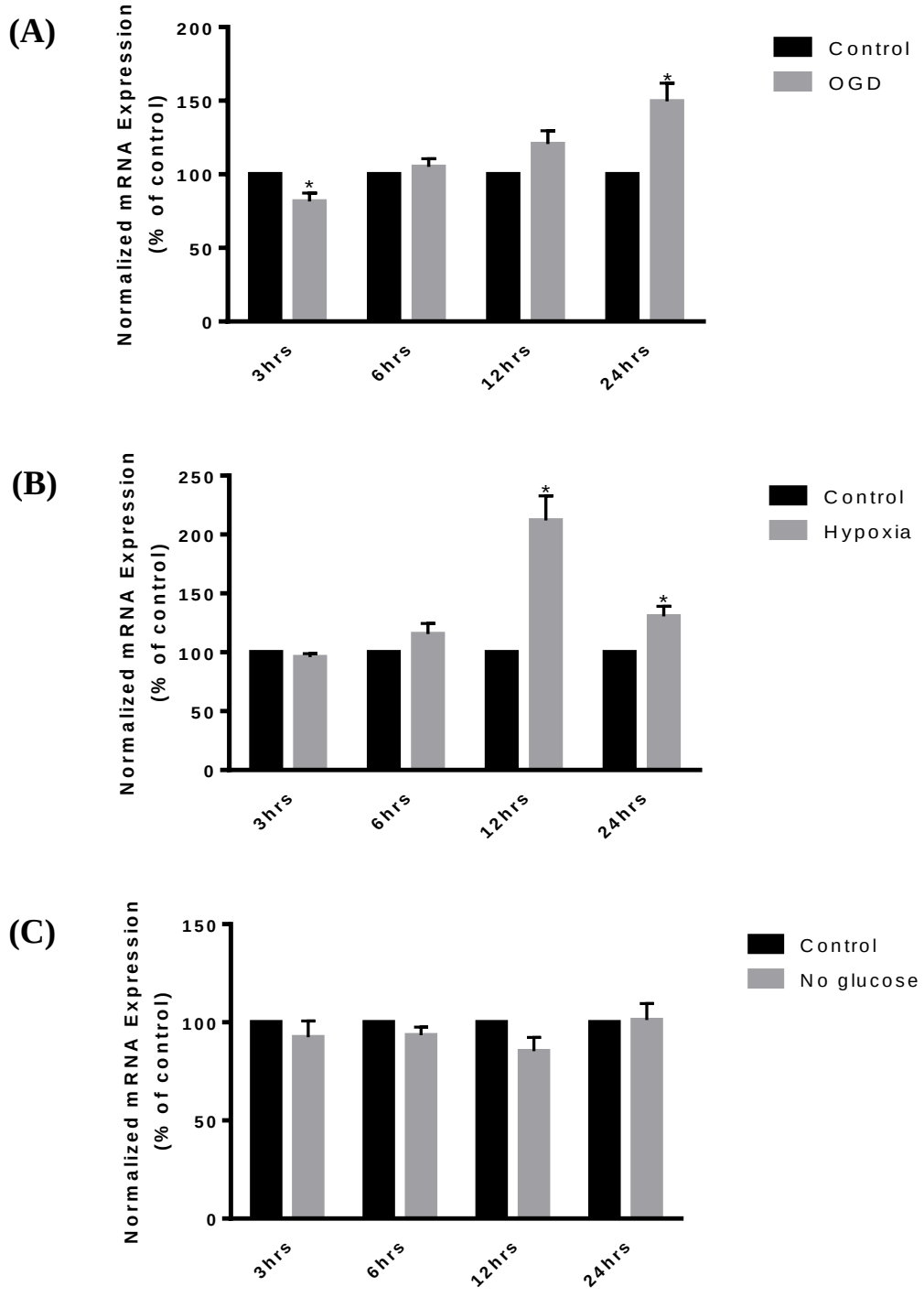


Figure 4.12: Expression of *GLUL* mRNA in human Müller cells (MIO-M1) after 3, 6, 12 and 24 hrs exposure to (A) full OGD (0mM glucose/0%O_2), (B) oxygen deprivation (5.55 mM glucose/0%O_2) and (C) glucose deprivation (0mM glucose/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; t-test).

4.2.3.2 Effect of Oxygen and Glucose Deprivation on the Expression of the Glutamate-Aspartate Transporter, GLAST (*SLC1A3*)

Expression of *SLC1A3* mRNA in MIO-M1 cells under reduced levels of glucose and oxygen (1.11mM glucose/4% O_2), hypoxia alone (4%O_2) and 1.11mM glucose did not exert a significant change on *SLC1A3* expression at 24hrs (Figure 4.13).

Full OGD resulted in a significant increase in *SLC1A3* expression at 12hrs by approximately 2-fold and continued to be strongly expressed (5-fold) at 24hrs (*$p \leq 0.05$; n=4) as compared to control (Figure 4.14A).

Full hypoxia reduced the levels at 3hrs by 40% as compared to control. This was followed by significant upregulation (*$p \leq 0.05$; n=4) at 24hrs by 1.5-fold (Figure 4.14B). Full glucose withdrawal significantly (*$p \leq 0.05$; n=4) reduced *SLC1A3* mRNA expression at 3hrs and although there was a trend of increasing levels with time, no significant changes were seen (Figure 4.14C).

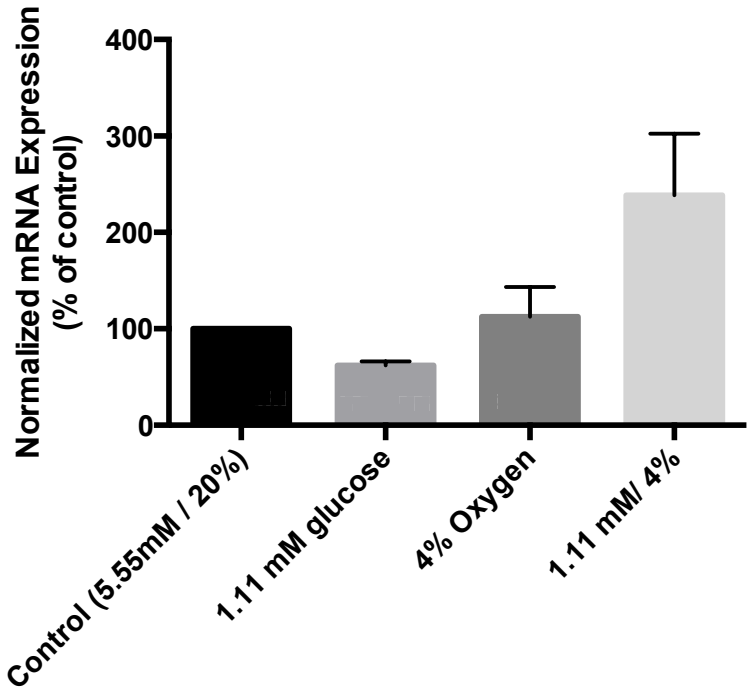


Figure 4.13: Expression of *SLC1A3* mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

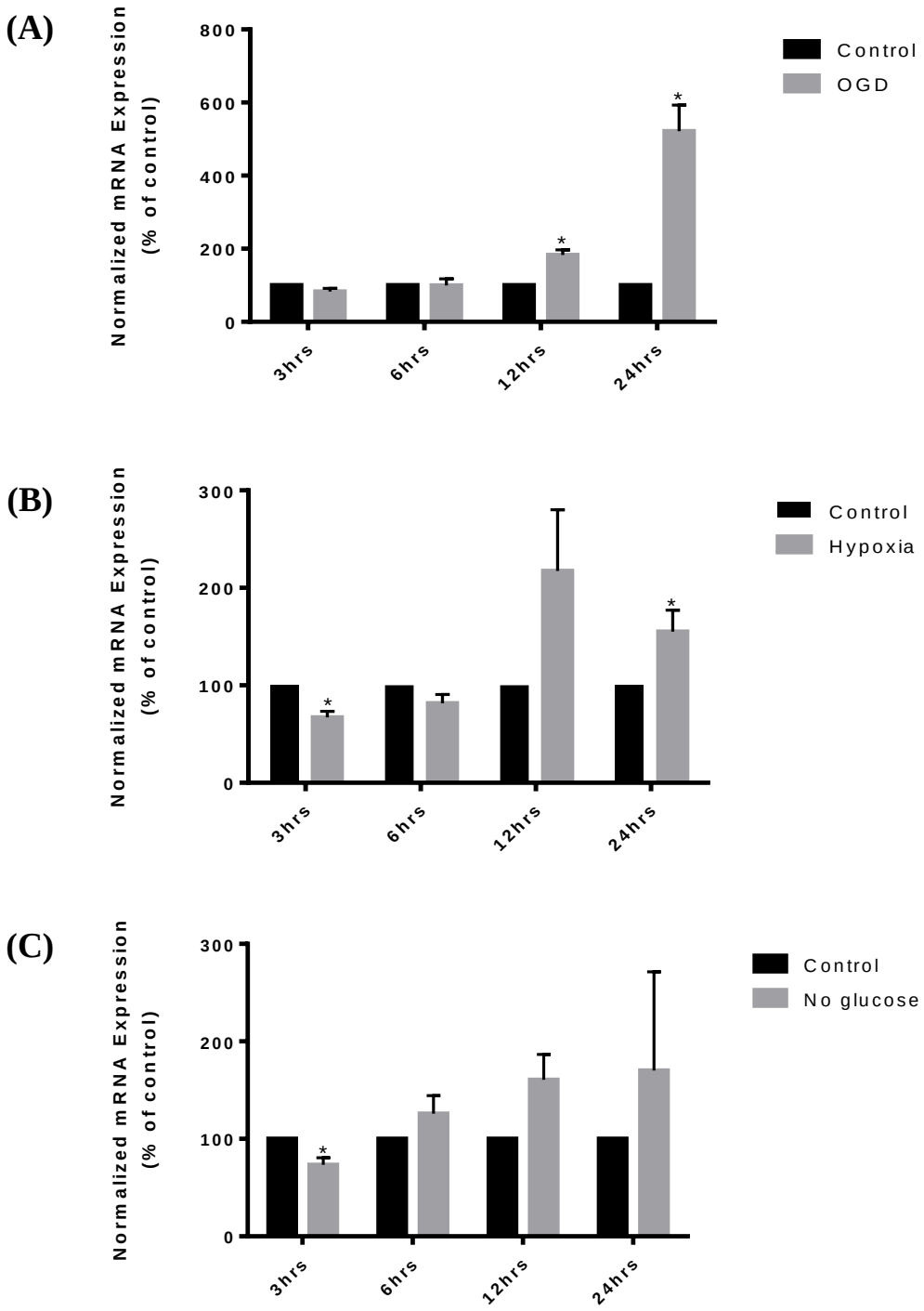


Figure 4.14: Expression of *SLC1A3* mRNA in human Müller cells (MIO-M1) after 3, 6, 12 and 24 hrs exposure to (A) full OGD (0mM glucose/0%O_2), (B) oxygen deprivation (5.55 mM glucose/0%O_2) and (C) glucose deprivation (0mM glucose/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; t-test).

4.2.3.3 Effect of Oxygen and Glucose Deprivation on the Expression of Glutamate Decarboxylase (*GAD1*)

As shown in Figure 4.14A, reduced levels of glucose and oxygen (1.11mMglucose/4%O_2), hypoxia (4%O_2) and 1.11mM glucose did not result in significant changes in *GAD1* mRNA expression. Full OGD significantly (*p≤0.05; n=4) upregulated the levels by 1.45-fold after 24hrs as compared to control (Figure 4.14B).

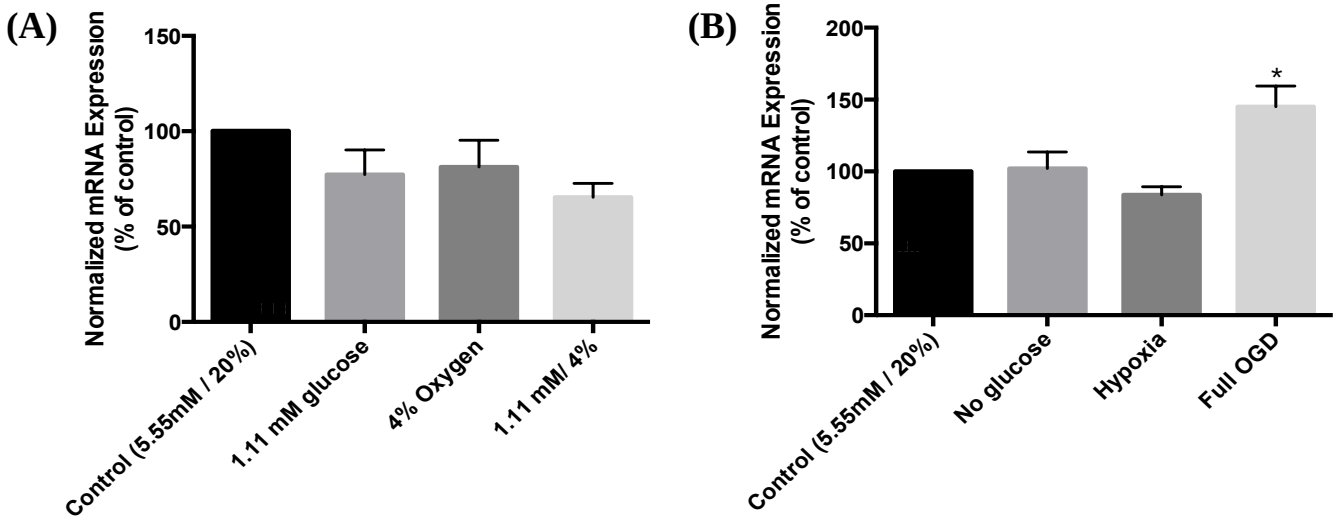


Figure 4.15: Expression of GAD mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2) and (B) control, no glucose (0mM/20% O_2), hypoxia (5.55mM/0% O_2) and full OGD. Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

4.2.4 Effect of Oxygen and Glucose Deprivation on the Expression of Purinergic Signaling Related Genes

4.2.4.1 Effect of Oxygen and Glucose Deprivation on the Expression of Pannexin1 (*PANX1*)

Reduced levels of glucose and oxygen (1.11mM glucose/4%O_2) resulted in significant (*p≤0.05; n=4) upregulation of *PANX1* by 1.5-fold as compared to control (Figure 4.16A). Similarly, Full OGD significantly (*p≤ 0.05; n=4) upregulated the expression by approximately 2-fold (Figure 4.16B).

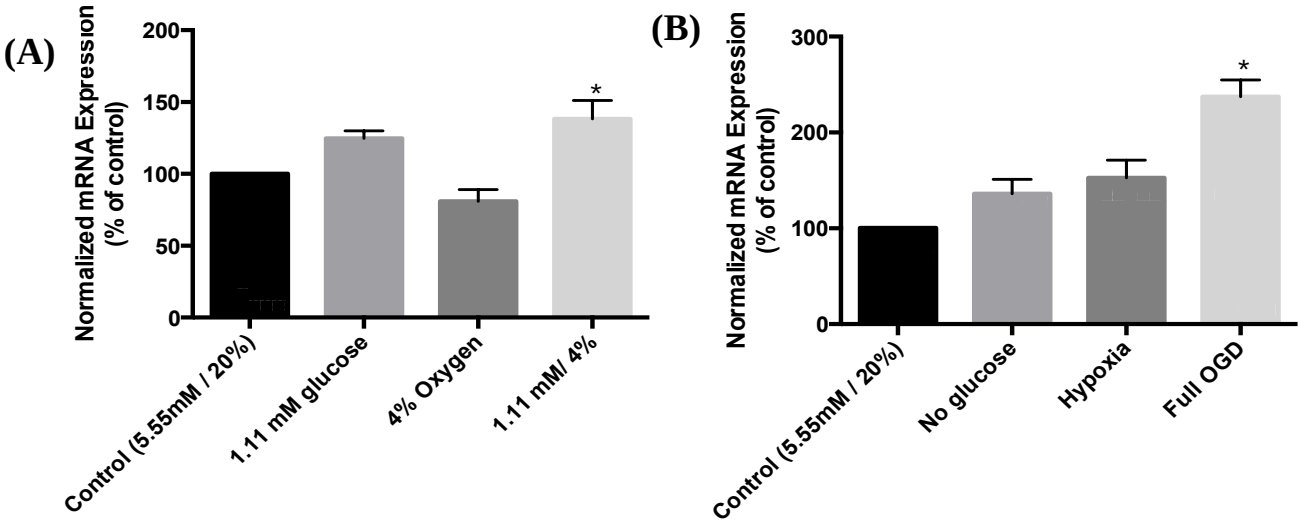


Figure 4.16: Expression of *PANX1* mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2) and (B) control, no glucose (0mM/20% O_2), hypoxia (5.55mM/0% O_2) and full OGD. Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

4.2.4.2 Effect of Oxygen and Glucose Deprivation on the Expression of Ectonucleotide Pyrophosphatase/Phosphodiesterase 2 (*ENNP2*)

Expression of *ENNP2* mRNA did not change after 24hrs exposure to 1.11mM glucose/4%O_2, hypoxia (4%O_2), and 1.11 mM glucose (Figure 4.17). Exposure of MIO-M1 cells to full OGD significantly upregulated (*p≤0.05; n=4) *ENNP2* mRNA by 1.5-fold as compared to control at 24hrs (Figure 4.18A). Significant upregulation (*p≤0.05; n=4) was obtained with full hypoxia at 12 and 24hrs by 57 and 46% respectively (Figure 4.18B) while complete glucose deprivation significantly (*p≤0.05; n=4) upregulated *ENNP2* mRNA by 57% at 24hrs compared to control (Figure 4.18C).

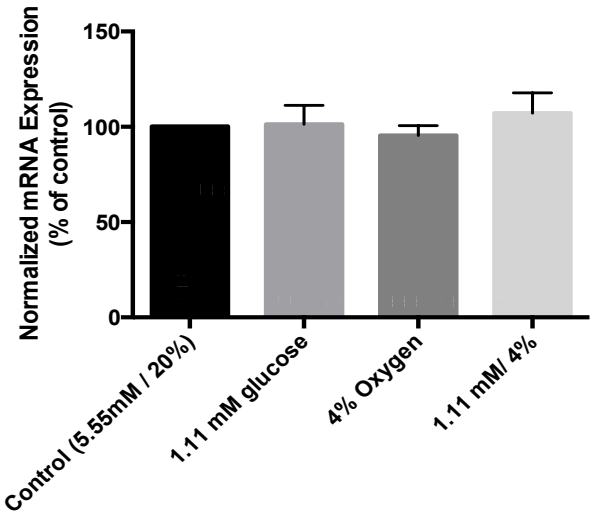


Figure 4.17: Expression of *ENNP2* mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

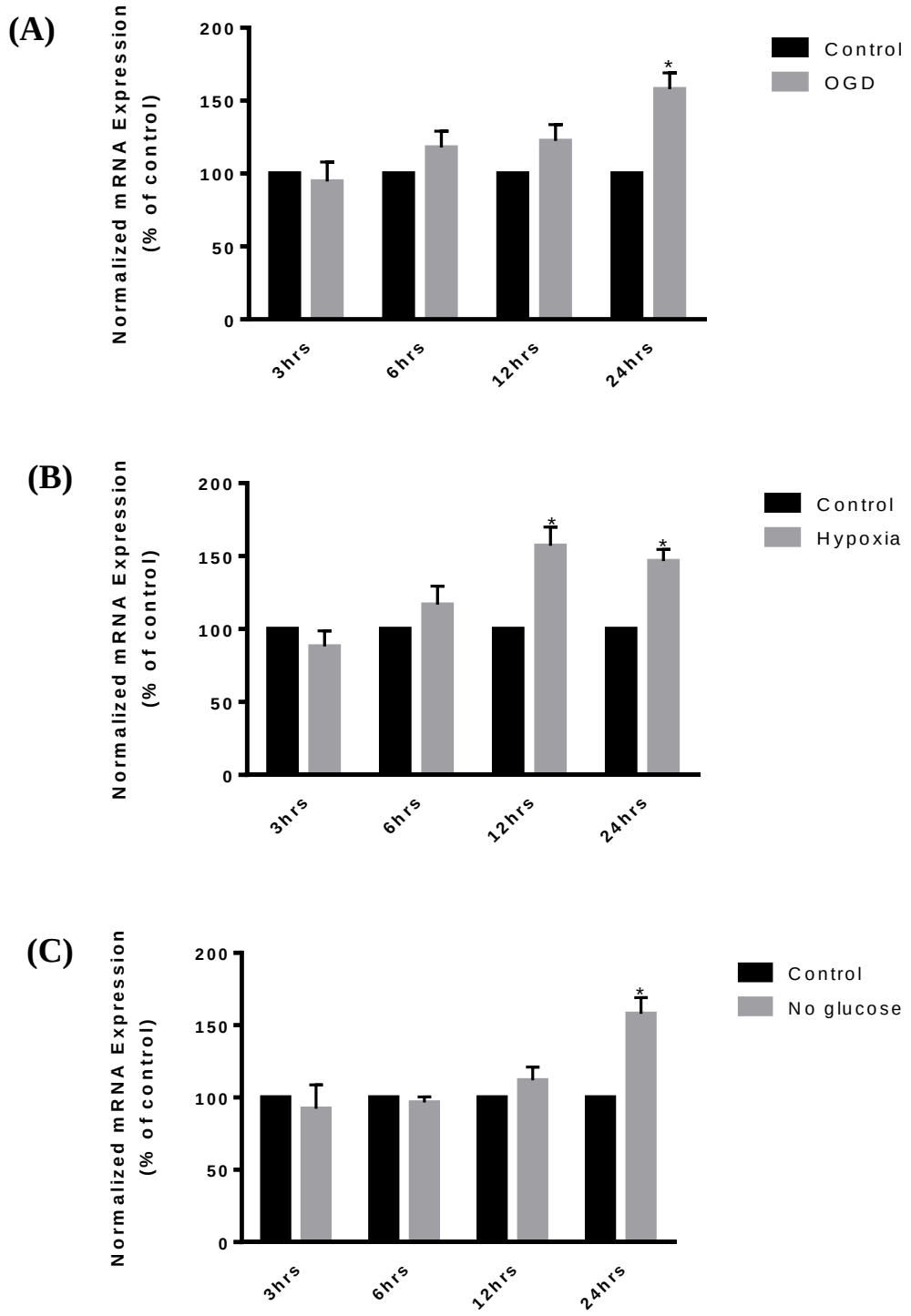


Figure 4.18: Expression of *ENNP2* mRNA in human Müller cells (MIO-M1) after 3, 6, 12 and 24 hrs exposure to (A) full OGD (0mM glucose/0%O_2), (B) oxygen deprivation (5.55 mM glucose/0%O_2) and (C) glucose deprivation (0mM glucose/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; t-test).

4.2.5 Effect of Oxygen and Glucose Deprivation on the Expression of Glial Müller Cells Markers

4.2.5.1 Effect of Oxygen and Glucose Deprivation on the Expression of GFAP

Reduction of oxygen level to 4% significantly upregulated GFAP mRNA by 1.5-fold (*p≤ 0.05; n=4) (Figure 4.19A,B&C). No change was detected with other levels of oxygen and/or glucose deprivation at all time points (Figure 4.19B&C).

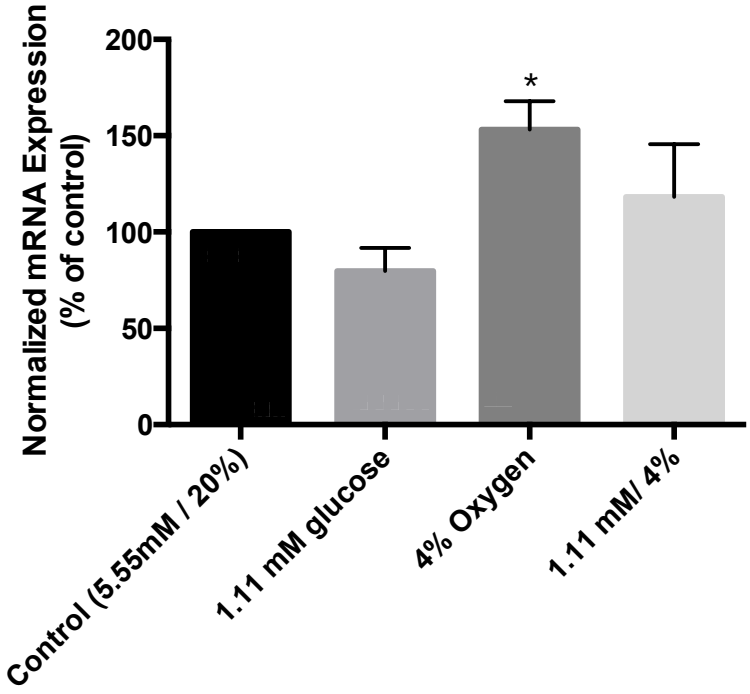


Figure 4.19: Expression of GFAP mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

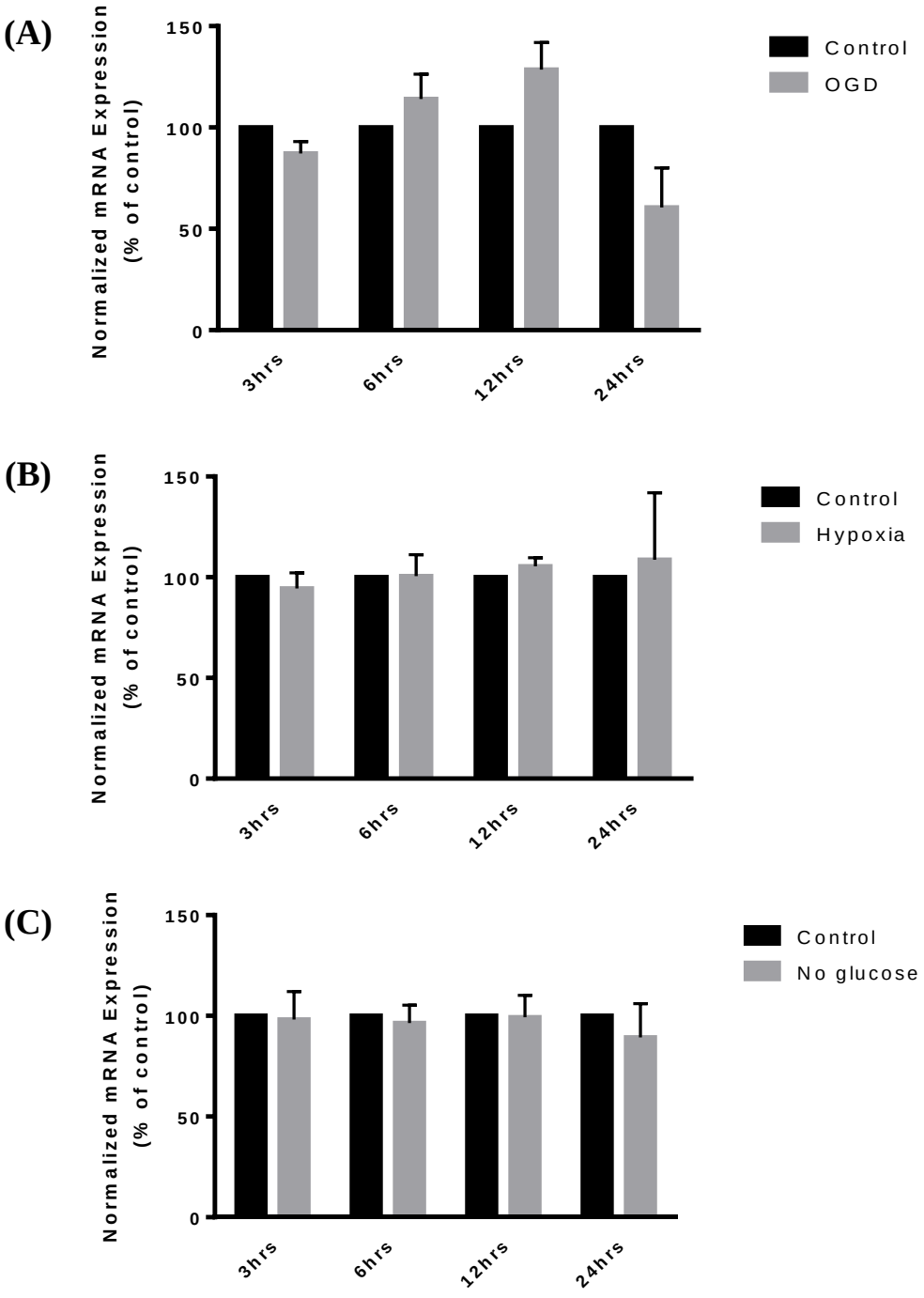


Figure 4.20: Expression of GFAP mRNA in human Müller cells (MIO-M1) after 3, 6, 12 and 24 hrs exposure to (A) full OGD (0mM glucose/0%O_2), (B) oxygen deprivation (5.55 mM glucose/0%O_2) and (C) glucose deprivation (0mM glucose/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; t-test).

4.2.6 Effect of Oxygen and Glucose Deprivation on the Expression of Heat-Shock Protein (*HSPA1B*)

Exposure of MIO-M1 cells to 1.11mM glucose/4%O_2, 4%O_2 and 1.11mM glucose did not alter the expression of *HSPA1B* mRNA (Figure 4.21). The upregulation of *HSPA1B* mRNA with full OGD did not follow an increasing pattern over time. Instead, it increased at 3hrs by 7-fold (*p≤0.05; n=4) and by 11-fold at 12 and 6–fold 24hrs respectively (*p≤ 0.05; n=4) (Figure 4.21A). Full hypoxia did not cause any change in *HSPA1B* mRNA levels at any time intervals (Figure 4.24B) while full glucose deprivation significantly upregulated the expression by 5.5–fold (*p≤ 0.05; n=4) at 3hrs as compared to control (Figure 4.21C).

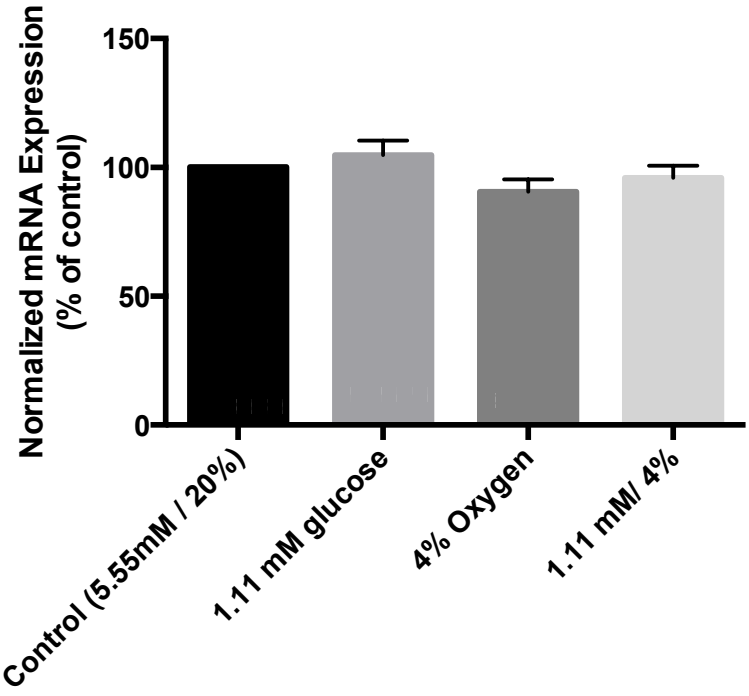


Figure 4.21: Expression of *HSPA1B* mRNA in human Müller cells (MIO-M1) after 24hrs exposure to control conditions (5.55mM glucose/ 20%O_2), glucose deprivation (1.11 mM glucose/20%O_2), hypoxia (5.55mM glucose/4%O_2) and OGD (1.11mM glucose/4%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; ANOVA).

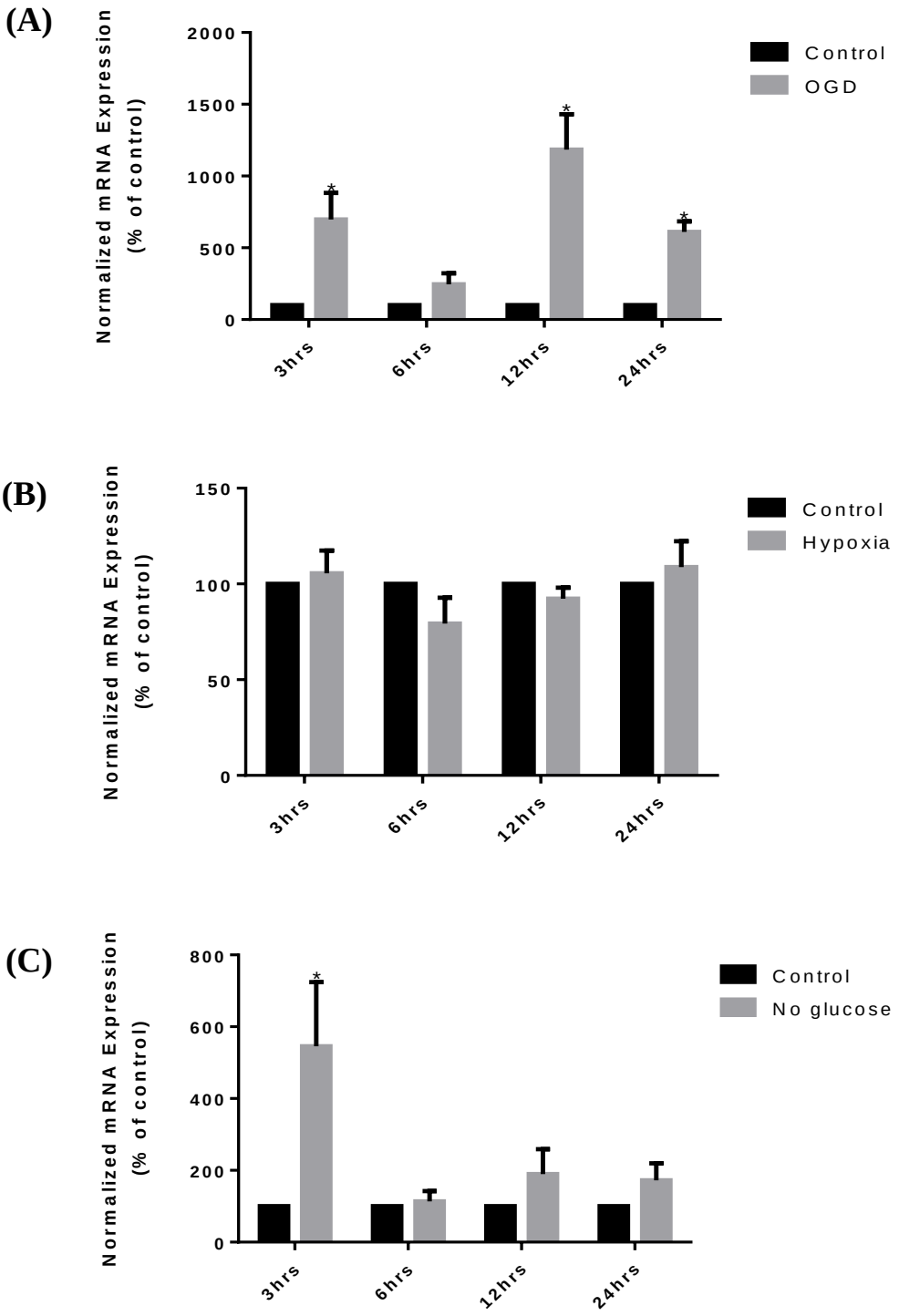


Figure 4.21: Expression of *HSPA1B* mRNA in human Müller cells (MIO-M1) after 3, 6, 12 and 24 hrs exposure to (A) full OGD (0mM glucose/0%O_2), (B) oxygen deprivation (5.55 mM glucose/0%O_2) and (C) glucose deprivation (0mM glucose/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4; t-test).

	MIO-M1 cell exposed to 24hrs to oxygen and glucose deprivation					
	Full OGD			1.11mM glucose/4%O_2		
Gene Name	**OGD**	**Hypoxia**	**Glucose**	**OGD**	**Hypoxia**	**Glucose**
VEGF	*↑ 3,6,12,24	*↑ 3,6,12,24	*↑ 24	↑ 24	↔	↑ 24
PDGF	*↓24	*↓24	↓24	↔	↔	↔
FGF	*↓12, ↑ 24	*↓12, ↑ 24	*↑ 24	↔		
PEDF	*↓24	*↓24	↔	*↓24	*↓24	*↓24
GFAP	↓24	↓24	↔	↑ 24	*↑ 24	↓24
GS	*↑ 24h	*↑ 24h	↔	↔	↓24	↑ 24
SLC1A3	*↑12, 24	*↑12, 24	↑ 6,12,24	↑ 24	↔	↔
ENNP2	*↑ 24h	*↑ 24h	*↑ 24h	↔	↔	↔
LIF	*↑ 3,6,12 ↓24	*↑ 3,6,12 ↓24	*↑ 3h	↓24	↔	↓24
HSPA1B	*↑3,12, 24	*↑3,12, 24	*↑3hr	↔	↔	↔
GAD	*↑ 24	*↑ 24	↔	*↓24	↓24	↓24
PANX-1	*↑ 24	*↑ 24	↑ 24	*↑ 24	↓24	↑ 24

Table 4.1: Summary of gene expression changes in MIO-M1 under oxygen and/or glucose deprivation for 24hrs (* indicate statistical significant change).

4.3 Discussion

Müller cells are the major macroglial cells in the retina, which respond to every pathological condition in the eye including light-induced damage, ischemia, retinal detachment, glaucoma and diabetic retinopathy (Bringmann and Reichenbach, 2001; Bringmann et al., 2006). Müller cell responses involve the production of neurotrophic factors, growth factors and cytokines, which may contribute to Müller cell proliferation and gliosis in an attempt to protect the retinal neurons (Bringmann et al., 2009). However, uncontrolled proliferation of Müller cells can lead to the formation of a gliotic scar, which is detrimental to retinal function (Bringmann et al., 2009). A balance between angiogenic and antiangiogenic factors in the eye is also essential to maintain a healthy status in the retina and pathological conditions can lead to an imbalance which may result in neovascularization in the retina. In the current study, the expression of important regulators of angiogenesis, excitotoxicity, and stress indicators were studied in the MIO-M1 cell line under conditions of simulated ischemia.

4.3.1 Effect of Oxygen and Glucose Deprivation on the Expression of Angiogenic Growth Factors

Vascular endothelial growth factor (VEGF) is a hypoxia-inducible angiogenic growth factor (Neufeld et al., 1999; Shibuya, 2008). Glial cells are the major source of VEGF in the retina (Pierce et al., 1995; Famiglietti et al., 2003; Kim et al., 1999). VEGF is also expressed by cultured MIO-M1 *in vitro* (Eichler et al., 2004; Holborn et al., 2004 a, b; Yafai et al., 2004) and studies have shown that MIO-M1 cells produce VEGF under normoxic conditions (Eichler et al., 2004; Yafai et al., 2004). In addition, hypoxia has been shown to increase VEGF production by rat Müller cells (Brook et al., 1998) and hypoxia (5%O_2) stimulates VEGF release from cultured rat and rabbit

retinal explants as well as guinea pig Müller cells and rMC-1 cell lines (Eichler et al., 2000). Furthermore, exposure of MIO-M1 cells to hypoxia (0.5%O_2) for 24hrs caused a 5-fold increase in VEGF level (Yafai et al., 2004). This is in agreement with Eichler et al., (2004) who demonstrated that exposure of MIO-M1 cells and guinea pig Müller cells to hypoxic condition (0.5% O_2) produced approximately 6- and a 25-fold increase in VEGF levels respectively. Results from the current research showed that MIO-M1 cells significantly increased the expression of VEGF mRNA when deprived fully of both oxygen and glucose (OGD) and hypoxia (0%O_2) for 24hrs by 20- and 7-fold respectively (Figure 4.1 & 4.2).

Further studies looking at interactions between Müller cells and other retinal cells in relation to VEGF have been carried out. For example, the released VEGF by MIO-M1 cells could be responsible for protecting the bovine retinal endothelial cells (BRECs) cells from apoptosis in co-culture (Yafai et al., 2004). Inhibition of VEGF expression in MIO-M1 cells under serum-free normoxic conditions led to an increase in the number of TUNEL-positive BRECs cells (Saint-Geniez et al., 2008). In a study by Kurihara et al., (2012) it has been found that knocking out VEGF in adult mouse RPE cells, caused rapid dysfunction of cone photoreceptors and led to vision loss. Indeed, low doses of VEGF (10ng/ml) were found to be a survival factor for isolated photoreceptor cells, decreasing apoptotic cell death (Saint-Geniez et al., 2008). VEGF may also be responsible for glial cell proliferation as reported by Schmid-Brunclik et al. (2008) who have shown that astrocytes proliferate in cultures deprived of both oxygen and glucose (OGD), and this was associated with an increase in the expression of HIF-1α and VEGF. In addition, exposure of guinea pig Müller cells to hypoxia increased the release of VEGF, which was thought to increase proliferation of endothelial cells when cultured in Müller cells derived hypoxic-medium (Eichler

et al., 2001). Others have reported that in normoxic glucose deficiency conditions, VEGF expression was upregulated in glioma cells in vitro (Shweiki et al., 1995). In a clonal glial cell line (C6 cells), total deprivation of glucose or oxygen for 12 hours resulted in 13-fold induction of VEGF mRNA as well as upregulation of the glucose transporter, GLUT1 (Stein et al., 1995). It has been also reported that glucose deprivation increased expression and stabilization of VEGF mRNA and prolong its half-life (Satake et al., 1998; Stein et al., 1995; Yun et al., 2005). In contrast, Brooks et al., (1998) reported that hypoxia increased VEGF production in rat retinal Müller cells after 48hrs, and it increased further by high glucose concentration. Findings by Eichler et al., (2004) disagree with Brooks et al., (1998) and reported that high glucose concentration (10-25mM) blocked the stimulatory effect of hypoxia on VEGF mRNA expression and protein release, both in retinal culture and Müller cell culture. The results presented here are in agreement with previous research showing that oxygen deprivation caused large increase in VEGF expression but also that full glucose deprivation alone significantly increased VEGF mRNA. These data suggest that oxygen is the main regulator of VEGF expression in MIO-M1 cells, but glucose also contributes to its regulation.

Platelet-derived growth factor (PDGF) is a known mitogenic growth factor for Müller cells (Milenkovic et al., 2003; Yamada et al., 2000). Several studies have reported that Müller cells express PDGF-α and β subunits (Cox et al., 2003; Cui et al., 2009; Milenkovic et al., 2003; Mudhar et al., 1993; Robbins et al., 1994). An early study by Uchihori and Puro, (1991) indicated that PDGF has both mitogenic and chemotactic effects on cultured human retinal glial cells from *post-mortem* eyes and that the calcium-channel blocker, nifedipine, inhibited these effects. In addition, transactivation of PDGF tyrosine kinase receptor has been found to cause P2Y-

receptor-induced mitogenic signaling in Müller cells (Milenkovic et al., 2003). Velez et al., (2012) has reported that MIO-M1 cells express the PDGF-α receptor, and that interaction between Müller cells and RPE can lead to upregulation of PDGF-α receptor and increased Müller cell pathogenicity in proliferative vitreoretinopathy (PVR). In a study by Moon et al., (2009), PDGF (10ng/ml) induced proliferation of rat Müller cells and increased phosphorylation of the PDGF receptor that could be blocked by PDGF receptor-selective tyrosine kinase inhibitor. In addition, impairing the expression of PDGF-β in retinal glial cells was found effective in the reversing retinal gliosis during experimental ischemic retinopathy (DeNiro et al., 2011). The evidence that PDGF is a mitogenic factor for Müller cells is therefore strong. Results from this study, however, did not find any regulation of PDGF mRNA under two levels of oxygen and glucose deprivation in MIO-M1 cells, suggesting that the autocrine release of PDGF is not responsible for the proliferation seen at 1.11mM glucose/4%O_2. PDGF was, however, down regulated by OGD, which may have an influence survival.

In the injured retina, Müller cells are the major source of bFGF (Morimoto et al., 1993; Walsh et al., 2001). It has been shown to stimulate the proliferation of cultured Müller cells (Cao et al., 1997; Geller et al., 2001; Walsh et al., 2001). In a study by Holborn et al., (2004), bFGF was found to increase the proliferation of cultured human MIO-M1 cells, as well as increase the secretion of VEGF. In cultured glial cells derived from adult *post-mortem* retina, bFGF increases calcium currents through L-type voltage-gated channels and nifedipine inhibits both the calcium current and the bFGF-induced proliferation (Puro and Mano, 1991). Immunoreactivity of bFGF was remarkably high in human retinal tissue from patients with ischemic PDR and also in transient retinal ischemia in rats (Yafai et al., 2013). bFGF has also been found to

rescue photoreceptors in retinal degeneration (Cao et al., 1997; Harada et al., 2000) and excitotoxic insults can also stimulate of bFGF expression and release (Nakamichi et al., 2003). Its release was strongly stimulated by the pro-inflammatory cytokines, IL-1β and TNF-α (Yafai et al., 2013; Yoshida et al., 2004) indicating its role in retinal neovascularization during ischemia. Current results show that (1.11mM glucose/4%O_2) and full OGD did not cause up-regulation of bFGF mRNA while complete glucose deprivation alone significantly upregulated bFGF expression. Hypoxic conditions (0.2%O_2) have previously been found to increase the release and expression of bFGF mRNA from Müller cells (Yafai et al., 2013) which is in accordance with the present data. It has been shown that bFGF could enhance release of VEGF from Müller cells and vascular endothelial cells during hypoxia (Stavri et al., 1995). Although bFGF is a well documented growth factor for Müller cells, the current data do not suggest that bFGF is playing a role in MIO-M1 cell proliferation under reduced levels of oxygen and glucose (1.11mM glucose/4%O_2).

4.3.2 Effect of Oxygen and Glucose Deprivation on the Expression of Anti-angiogenic Growth Factors

PEDF is an antiangiogenic factor, regulated in a reciprocal relation to VEGF. It has also been reported to exert a neuroprotective effect in cultured cerebellar granule cells and cultured RPE cells derived from rat (Malchiodi-Albedi et al., 1998; Taniwaki et al., 1995). Unterlauft et al., (2012) has shown PEDF is a glia-secreted factor that protects cultured adult mouse RGC during hypoxic insult (0.2%O_2) and found to be neuroprotective against glutamate-induced toxicity in the CNS, mediating its protective effect through restoring calcium homeostasis and blocking the activation of NMDA receptors (Taniwaki et al., 1997). In addition, Cao et al., (1999) found that PEDF protects retinal neurons against H_2O_2-induced cell death in a dose-dependent

manner. A balance in VEGF/PEDF ratio was found to be important in decreasing retinal oedema following ischemia/reperfusion injury (Tong et al., 2013) and to prevent retinal neovascularization (Eichler et al., 2004 a, b). It has also been reported that hypoxia and ischemia are associated with upregulation of VEGF mRNA and down regulation of PEDF mRNA in MIO-M1 cells (Eichler et al., 2004 a, b). This is in agreement with current data, which indicate that exposure of MIO-M1 cells to two levels of hypoxia (0 and 4%O_2) as well as low glucose levels (1.11mM) for 24hrs significantly down-regulated PEDF mRNA expression (Figure 4.7 and 4.8A,B). In agreement with current results, findings by Eichler et al., (2004) also demonstrated that MIO-M1 cells produce and secrete PEDF, and hypoxia was associated with down-regulation of PEDF mRNA. Increasing the severity of hypoxia (0.5-5% O_2) resulted in more down-regulation of PEDF release. These data suggest that MIO-M1 cell proliferation could be increased under the examined conditions, by the reduction in the PEDF, which then allows increased activity of the secreted VEGF.

Another inhibitor of angiogenesis is the Leukemia inhibitory factor (LIF), a member of the IL-6 family of cytokines, which has neuroprotective properties in the CNS promoting neuronal survival, neurogenesis and regeneration (Azari et al. 2006; Covey and Levison 2007; Heinrich et al., 2003; Ishibashi et al. 2009). Current result shows that LIF expression increased as early as 3hrs in MIO-M1 cells treated with full OGD and full glucose deprivation and decreased over time to reach control level by 24hrs, which indicates a reverse pattern to that showed for VEGF expression. Interestingly, hypoxia led to an increase in expression with time, which was similar to VEGF expression. In primary cultures of astrocyte from rats, exposed to OGD (glucose-free culture medium subjected to 6hrs of hypoxia, 1%O_2) treated with low dose of LIF inhibited astrocyte proliferation induced by OGD. The mechanism of the inhibitory

effect of LIF was attributed to down-regulation of HIF-1α and VEGF mRNA (Fan et al., 2013). Kubota et al., (2008) has also shown that LIF inhibited hypoxia-induced VEGF expression and proliferation in cultured astrocytes.

4.3.3 Effect of Oxygen and Glucose Deprivation on the Expression of Glutamate Markers

Glutamate clearance by Müller cells is mediated by the uptake and degradation by GLAST and GS respectively (Derouiche and Rauen, 1995). An increase in extracellular glutamate upregulates the expression of GLAST in Müller cells and has been found to protect retinal neurons from glutamate toxicity (Imasawa et al., 2005; Taylor et al., 2003). It was, therefore, interesting to look at the expression of GLAST in MIO-M1 cells under conditions of oxygen and/or glucose deprivation. The human Müller cell line (MIO-M1) used in this study has previously been shown to express GLAST (*SLC1A3*) (Hollborn et al., 2011). In the current experiments, MIO-M1 cells exposed to 24hrs-simulated ischemia caused a significant 5-fold increase of GLAST in full OGD. In agreement with the current results, *in vivo* retinal ischemia for 48hrs in rats caused a marked increase in GLAST mRNA in the INL with more than 90% of cells expressing GFAP, suggesting that Müller cells play a major role in the regulation of glutamate under ischemic conditions (Otori et al., 1994). This may contribute to the protection of ganglion cells when co-cultured with Müller cells, which were protected from glutamate toxicity and hypoxia damage (Kitano et al., 1996). Current results also showed that hypoxia ($0\%O_2$) caused significant up-regulation of GLAST in cultured MIO-M1 cells and 4% oxygen did not significantly alter GLAST expression. This is in agreement with Imasawa et al., (2005) study who reported that exposure of cultured rat retinal glial cells to hypoxia ($5\%O_2$) did not alter GLAST expression. To the contrary, more severe hypoxic conditions 2.5 and 1%

O_2 exposure for 24hrs, caused a significant reduction in GLAST protein and mRNA expression in astrocytes (Dallas et al., 2007), full hypoxia, however was not studied. Both hypoxia and glutamate treatment have been found to reduce the survival rate of cultured rat RGCs *in vitro* (Kitano et al., 1996). Damage from these stressors was reduced after treatment with a glutamate blocker and co-culture with cortical astrocytes and retina-derived Müller cells. These results suggest that glutamate is increased in hypoxic conditions, and that glial cells are playing a major role in retinal detoxification of glutamate (Kitano et al., 1996). In addition, Toft-Kehler et al., (2014) reported increased GLAST protein and mRNA expression following glucose deprivation for one hour in MIO-M1 cells. The results presented here did not show significant up-regulation of GLAST but increasing trend was seen with time in MIO-M1 cells.

Glutamine synthetase (GS) is the key glutamate-metabolizing enzyme expressed by Müller cells that convert glutamate to the non-excitotoxic amino acid glutamine (Riepe and Norenburg, 1977). The released glutamine is taken up by neurons where it is hydrolyzed by glutaminase to form glutamate again to be used in neurotransmission (Thoreson and Witkovsky, 1999). MIO-M1 cells express glial glutamine synthase (*GLUL*) as indicated by RT-PCR, protein and immunohistochemistry analysis (Holborn et al., 2011). Results from this study showed that MIO-M1 cells cultured in full OGD conditions significantly upregulated *GLUL* mRNA. Similarly, full oxygen deprivation alone significantly increased *GLUL* expression at 12 and 24hrs. Glucose deprivation (0mM) failed to regulate *GLUL* levels indicating that the increased levels seen in OGD are originating from the effect of oxygen rather than glucose. No expression change was found at lower levels of deprivation. It has been proposed that the function of GS is impaired in ischemia due to lack of intracellular ATP (Oliver et

al., 1990). In astrocytes cultured in glucose-deprived condition, the specific activity of GS decreased, and enzyme turnover increased (Rosier et al., 1996). In addition, in primary cultured cortical astrocytes exposed to OGD for 6 hours, decreased GS expression and increased the extracellular glutamate level were detected (Wang et al., 2013). Interestingly, there was a reduction in expression in full OGD at 3hrs, but after this time there was an increase in GS expression, which would contribute towards detoxification of glutamate.

Glutamate can be also converted to GABA by glutamic acid decarboxylase (GAD), and then GABA is converted to succinate semialdehyde by GABA transaminase (GABA-T) activity (Kobayashi et al., 1999). Although GAD has been reported to exist in cultured Müller cells (Kubrusly et al., 2005), other studies indicated that GAD is expressed in the neuronal retina, but not in Müller cells (Agardh et al., 1987; Nishimura et al., 1983). Expression of GAD mRNA under full OGD was significantly upregulated indicating that these cells express the enzyme and that it can be regulated by ischemia. No similar data has been found in other studies about the regulation of GAD expression under these conditions in the retina and this is the first study examining the effect of oxygen and/or glucose on the expression of GAD in MIO-M1 cells.

4.3.4 Effect of Oxygen and Glucose Deprivation on the Expression of Purinergic Signaling Molecules

Pannexin1, encoded by the *PANX1* gene, is a mammalian hemichannel expressed in the brain and the ocular tissues (Kurtenbach et al., 2014). It is activated by changes in membrane potential, ATP, an increase in intracellular calcium, glutamate, reduced oxygen, ischemia and following purinergic receptor activation (Kurtenbach et al., 2014) which mediates release of ATP from the cell, increasing extracellular ATP

concentration and stimulating purinergic signaling and possibly excitotoxicity (Kurtenbach et al., 2014).

Several studies have suggested that activation of purinergic signaling is involved in the proliferation of Müller cells. Human Müller cells isolated from donor retinas and activated by external ATP evoked the release of intracellular Ca^{2+} and increased density of $P2X_7$ receptor channels. This resulted in subsequent activation of big conductance K^+ channels (I_{BK}), which may contribute to the induction of proliferative activity in gliotic Müller cells during PVR (Bringmann et al., 2001). Similar findings were reported by Francke et al., (2002) who suggested that ATP evoked Ca^{2+} responses might support the proliferation of Müller cells during PVR in rabbit retina. Furthermore, extracellular ATP has been found to evoke the release of mitogenic growth factor, PDGF from Müller cells (Milenkovic et al., 2003). The current results show that exposure of MIO-M1 cells to full OGD and reduced levels 1.11mM glucose/4%O_2 significantly upregulated *PANX1* mRNA after 24hrs. Dvoriantchikova et al., (2012) found that *PANX1* deficiency protects RGCs from death induced by OGD in Panx1−/− mice thought to be mediated by inhibiting caspase-1 and the production of interleukin-1β as well as suppressing permeation of RGC plasma membranes (Dvoriantchikova et al., 2012). In addition, purinergic receptor-mediated Ca^{2+} responses in activated retinal glial cells have also been found in retinal detachment and retinopathy model in rabbits (Francke et al., 2003; Francke et al., 2005; Uckermann et al., 2003; Uhlmann et al., 2003). On the other hand, Newman (2003) demonstrated that activated glial cells might protect neurons in the retina by releasing ATP that is converted to adenosine and subsequently activating neuronal adenosine receptors. Similar findings were reported *in vivo* experiments by Hu et al., (2010) implicating that a balance between extracellular ATP and its protective

metabolite adenosine is essential to the survival of RGCs. In the current study, the expression of *PANX1* mRNA increased in ischemia suggesting that ATP may be released from MIO-M1 cells during through pannexin hemichannels but more experiments are needed to prove the ATP release and its role in MIO-M1 cell proliferation.

Autotaxin encoded by the gene *ENNP2*, possess lysophospholipase D activity as well as ATPase-like activity, converting lysophospholipids such as lysophosphatidylcholine to lysophosphatidic acid (LPA) (Clair et al., 1997; Tokumura et al., 2002). Results from the current study show that full OGD, hypoxia and glucose deprivation significantly increased *ENNP2* mRNA expression indicating that activation of this enzyme is taking place in MIO-M1 cells in response to stressors. This would facilitate degradation of ATP to ADP, which may indicate that ATP release is a significant event in ischemia. Under normal culture condition, human ARPE-19 were shown to express $ENNP_{1\text{-}3}$ mRNA and were able to degrade externally added ATP while ecto-ATPase inhibitors blocked ATP degradation (Reigada et al., 2005). More experiments are needed to investigate the ATP release from MIO-M1 cells and the involvement of LPA-mediated effect in survival and proliferation.

4.3.5 Effect of Oxygen and Glucose Deprivation on the Expression of Glial Cell Markers

GFAP is widely used as a stress marker expressed by reactive retinal glia in several pathological conditions in the retina. These include retinal ischemia (Kim et al., 1998), age-related macular degeneration (Diloreto et al. 1995), oxygen-induced retinopathy (Prentice et al., 2011; Smith et al., 1994), and diabetic retinopathy (Lieth et al. 1998). Hypoxia has been reported to cause upregulation of GFAP mRNA and protein as early as 3hrs (Kaur et al., 2007). MIO-M1 cells transfected with a GFAP promoter

and exposed to 40hrs hypoxia (1%O_2) resulted in a 16-fold induction of luciferase activity (Prentice et al., 2011) and cultured rat Müller cells showed 3-fold increases in GFAP mRNA after 72hrs of hypoxia (Tan et al., 2015). The results reported here indicate that human MIO-M1 cells showed no evidence of GFAP regulation in full OGD, hypoxia (0%O_2) or full glucose deprivation. However, oxygen deprivation to 4%O_2 showed a significant increase in GFAP mRNA at 24hrs as compared to control. Studies suggest that neuronal loss does not appear to be a prerequisite for GFAP induction (Fitzgerald et al., 1990; Osborne et al., 1991). Interestingly, it has been found that GFAP mRNA is transcribed for a limited time, and the gene is subsequently turned off (Sarthy and Egal, 1995). This could explain why GFAP expression did not change with full OGD.

4.3.6 Effect of Oxygen and Glucose Deprivation on the Expression of Heat-shock Protein HSPA1B (HSP70)

Heat-shock proteins are highly conserved proteins expressed in response to various insults to increase cell survival (Parcellier et al., 2003). Findings by Papadopoulos et al., (1996) showed that expression of HSP-70 enhances the survival of astrocytes challenged with heat or oxygen-glucose deprivation. Similarly, in primary culture of astrocytes pre-treated with heat followed by exposure to full OGD for 6 hours and reperfused with glucose at a concentration of 5.5 mmol/L at normoxia for 24hrs resulted in over-expression of HSP70. This expression protected against OGD by interrupting phosphorylation of the inhibitor of IκB, c-Jun N-terminal kinases (JNK) and p38 (Kim et al. 2015). Culture of rat retinal ganglion cells and Müller cells exposed to hyperthermia (42ºC) followed by hypoxia (9% oxygen) for 6 hrs or a sub-lethal dose of glutamate showed detectable level of HSP72 in control retinal cultures and significant increase was found in culture pre-treated with heat shock as evidence

by immunoreactivity (Caprioli et al., 1996). The survival rate of cells pre-treated with heat was significantly greater compared to cells that had no pre-treatment. This protection was abolished with inhibition of heat shock protein synthesis (Caprioli et al., 1996). The expression of the human inducible HSP70 in astrocytes protected them from glucose deprivation and oxidative stress with a well-preserved level of glutathione (Xu and Giffard, 1997). In the current experiments, exposure of cultured MIO-M1 cells to full OGD increased the expression of *HSPA1B* mRNA as early as 3hrs and continued to be raised over the 24hrs of the experiment. Oxygen deprivation, however, did not alter in *HSPA1B* expression, whilst glucose deprivation caused a large increase in expression, but only at the early time point (3hrs). Overall. it does appear that ischemia insult increases *HSPA1B* expression in MIO-M1 cells and this may be part of the mechanism that protect the cells from ischemic damage.

In conclusion, current experiments were conducted in an attempt to understand mechanisms that may be involved in the survival and proliferative state of MIO-M1 cells under conditions of simulated ischemia. More prominent changes in gene expression were found with complete deprivation of both oxygen and glucose rather than at the reduced levels (1.11mM glucose/4%O_2). In the next chapter it will be investigated whether such changes occur in the intact human retina, using human organotypic retinal culture (HORCs). Survival of RGCs under these conditions will also be investigated.

Chapter 5

Effects of Oxygen and Glucose Deprivation on the Survival and Gene Expression in Human Organotypic Retinal Cultures (HORCs)

5.1 Introduction

As previously discussed vascular dysregulation induced-reduction in blood flow is one proposed mechanism of retinal ganglion cell (RGC) loss, which occurs in glaucoma (Arnold, 1995; Harju and Vesti, 2001; Michelson et al., 1996; Michelson et al., 1998; Schwartz et al., 1977; Schwartz, 1994; Sugiyama et al., 2000; Tanaka, 1995; Yamazaki et al., 1996). Ischemic insult deprives the cells of oxygen and vital nutrients such as glucose and decreases waste removal. This deprivation will disturb tissue homeostasis and eventually leads to injury and death (Osborne et al., 2004). Retinal cells depend on glucose as a source of energy, and retinal Müller cells are the primary users, converting glucose into monocarboxylic acids, lactate, and pyruvate that are supplied to neurons and photoreceptors to meet their energy requirements (Poitry-Yamate, and Tsacopoulos; 1992; Poitry-Yamate et al., 1995). Deprivation of both oxygen and glucose deprives the retinal neurons of energy and induces deleterious effects on the tissue including ion and neurotransmitter disturbances, excitotoxicity, and angiogenesis (Osborn et al., 2004). For example, during retinal ischemia there is an accumulation of extracellular glutamate, which plays a role in excitotoxic neuronal death (Barnett et al., 2001; Boris-Moller and Wieloch, 1998).

The mechanism of glutamate release has been attributed to ischemia-induced depolarization and activation of voltage-dependent Ca^{2+} channels resulting in the exocytotic release of glutamate (Wahl et al., 1994). Also, Nishizawa, (2001) has suggested that in ischemia-induced energy failure, the glutamate release can also be independent of Ca^{2+} ions mediated by the glutamate transporter working in a reverse direction due to the imbalance of sodium ions across the neuronal membrane. As

discussed previously, glutamate is taken up by glial by glutamate transporters, and detoxified by glutamine synthetase and the activity of each these may increase to enhance the clearance of extracellular glutamate (Shaked et al., 2002). In addition, ATP-mediated excitotoxicity and overstimulation of purinergic $P2X_7$ receptors has been shown to increase RGC death during retinal ischemia in mechanisms similar to these occurring with glutamate toxicity (Niyadurupola et al., 2013). Ischemia-induced angiogenesis is a complex process characterized by vasodilation, increased vascular permeability and proliferation of endothelial cells (Witmer et al., 2003). Angiogenesis is controlled by growth factors such as VEGF and bFGF.

Furthermore, PDGF is responsible for recruitment of pericytes and smooth muscle cells for the developing vessels (Mignatti and Rifkin, 1996). With decreased blood supply, angiogenesis is a specific homeostasis response. However, upregulation of growth factors may also be part of the cytoprotective response (Bouck, 2002; Witmer et al., 2003). In the present experiments, human organotypic retinal cultures (HORCs) were used to assess the loss of RGCs as a result of simulated ischemic insult *in vitro*. This model was developed by Niyadurupola et al., (2010) and is a tool to investigate RGC loss in the human retina. The explants were taken from macular, para-macular and peripheral regions and assessed for the distribution of retinal RGCs using *THY-1* mRNA and counting RGCs using immunohistochemistry with another RGC marker, NeuN. The distribution of these markers was consistent with the proposed allocation of RGCs in the retina indicating that explants can be used to compare the loss of RGCs as a result of various insults. Furthermore, there were equivalent numbers of RGCs in the paramacular explants. Exposure of HORCs to glutamate or simulated ischemia (OGD) caused a reduction in both *THY-1* mRNA and the numbers of NeuN-labelled neurons (Niyadurupola et al., 2010). In this chapter, the effects of two levels

of OGD were investigated *in vitro* using HORCs to determine the survival of RGCs. Expression of angiogenic and antiangiogenic growth factors, excitotoxicity related genes and glial cell markers were also assessed.

5.2 Results

5.2.1 Effect of Graded Levels of Glucose Deprivation on the LDH Release and RGCs Death in HORCs

HORCs were incubated in glucose-deprived medium, ranging from 0-4.99mM under normoxic condition for 24hrs and LDH was measured from medium to assess overall of cell viability. The control condition was the physiological glucose level of 5.55mM and levels ranged from 10% deprivation to complete absence of glucose. Figure 5.1 shows glucose-dependent survival in HORCs, in which there was gradual increase in LDH release as the deprivation level increased. Significant levels of LDH release (2.5-fold) were found when the deprivation level reached 80% (1.11 mM). Maximum LDH release was seen with full glucose deprivation (3.67-fold).

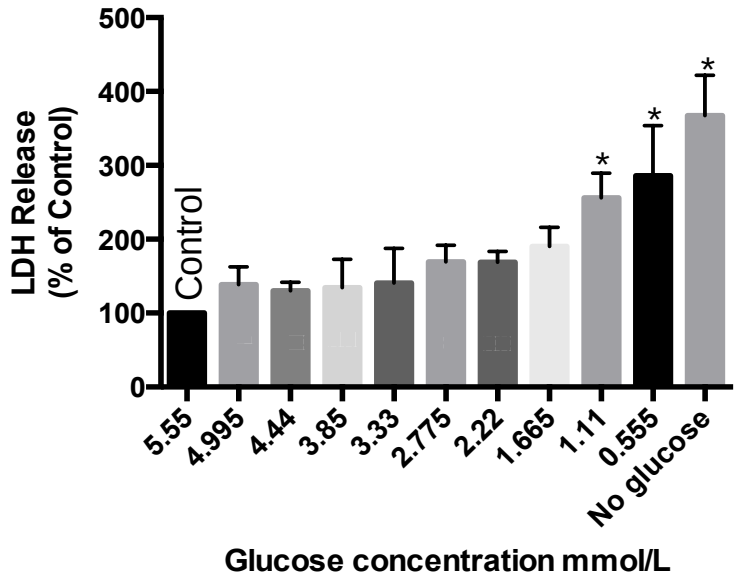


Figure 5.1: Effect of graded levels of glucose deprivation on LDH release from HORCs in medium after 24hrs (*p≤0.05; n=4. ANOVA)

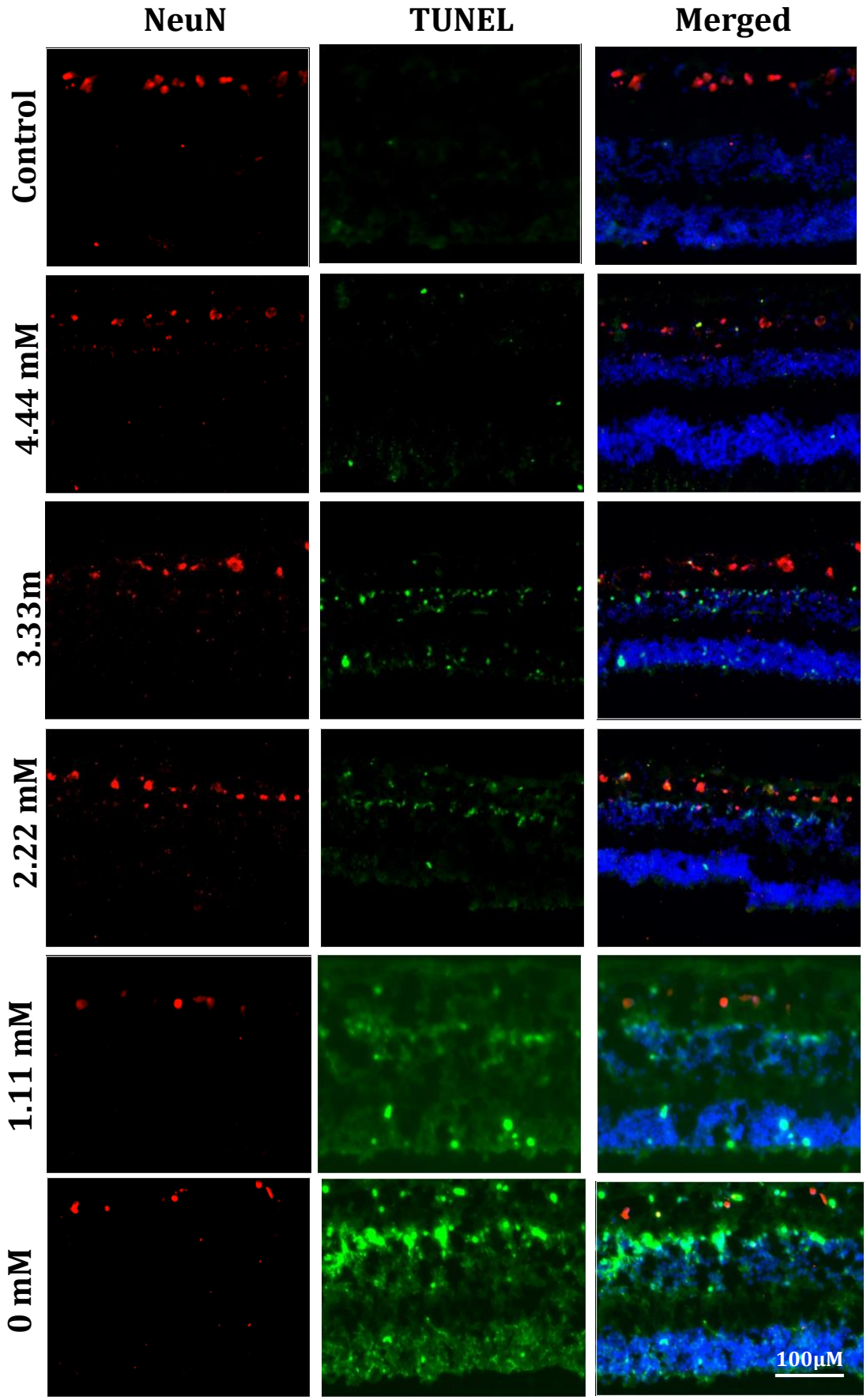


Figure 5.2: Representative immunofluorescence photomicrographs showing NeuN and TUNEL-labelling in HORCs with control and 20-60% glucose deprivation (4.44-2.22 mM). DAPI = blue, NeuN = red, TUNEL = green.

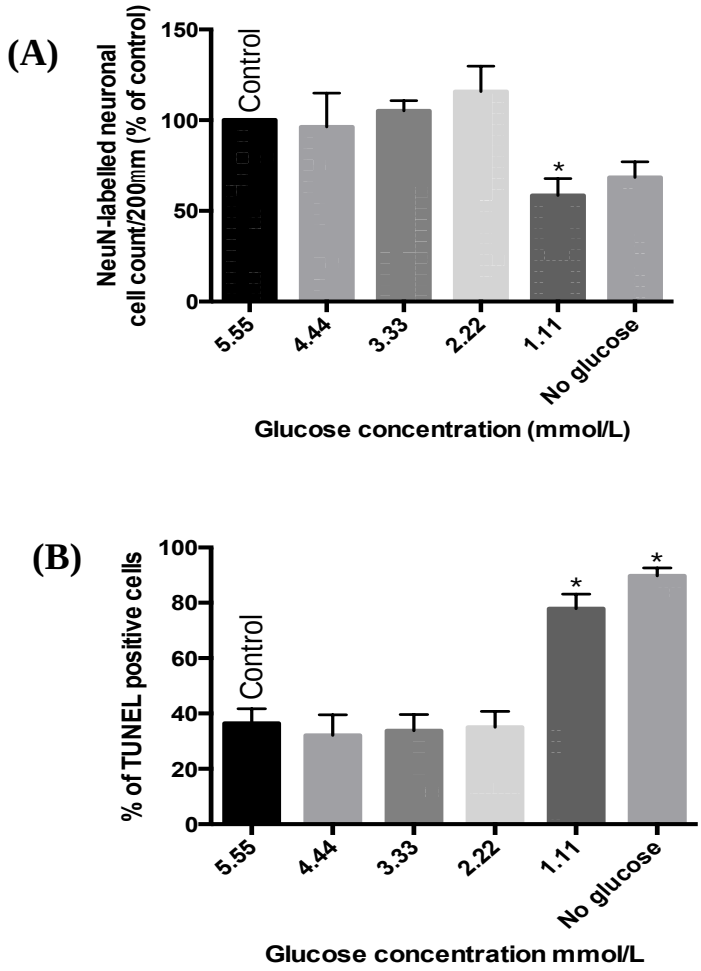


Figure 5.3: Effect of glucose deprivation on RGC death (A) Average number of NeuN-labelled cells from three intact 200μm from 8 sections per sample expressed as percentage of control and (B) Percentage of TUNEL-positive ganglion cells exposed for 24hr to glucose deprivation (0-555 mM) (*p≤0.05; n=4, ANOVA).

Intense TUNEL staining was observed in the INL, ONL and RGCs at glucose levels of 1.11 mM and no glucose-treated HORCs compared to controls (Figure 5.2). Although the staining increased in the INL and ONL as the deprivation increased, less staining was seen in the ganglion cell layer at lower glucose levels (2.22-4.44 mM). Evidence of RGCs loss was shown by immunohistochemistry analysis where the numbers of NeuN-labelled neurons in the retinal ganglion cell layer were counted in combination with TUNEL staining. A reduction of 48% (*p≤0.05; n=4) and 30% in

the mean numbers of NeuN-labelled was detected when the glucose deprivation was 80% of the control (1.11 mM) and full glucose deprivation (0mM) respectively. The number of NeuN-labelled neurons did not change in lower glucose levels (2.22-4.44mM) (Figure 5.3A). The percentage of TUNEL-positive RGCs was approximately 80% to 90% in HORCs exposed to 1.11mM and full glucose deprivation respectively (Figure 5.3B).

5.2.2 Effect of Oxygen Glucose Deprivation (OGD) on the LDH Release and RGCs Death in HORCs

Full deprivation of oxygen and glucose (OGD) and glucose deprivation alone resulted in significant 3- and 4-fold increases (*p≤0.05; n=4) in LDH release respectively compared to control (Figure 5.4). Although, oxygen deprivation alone for 24hrs did not cause LDH release there was an additive effect when both oxygen and glucose were deprived together.

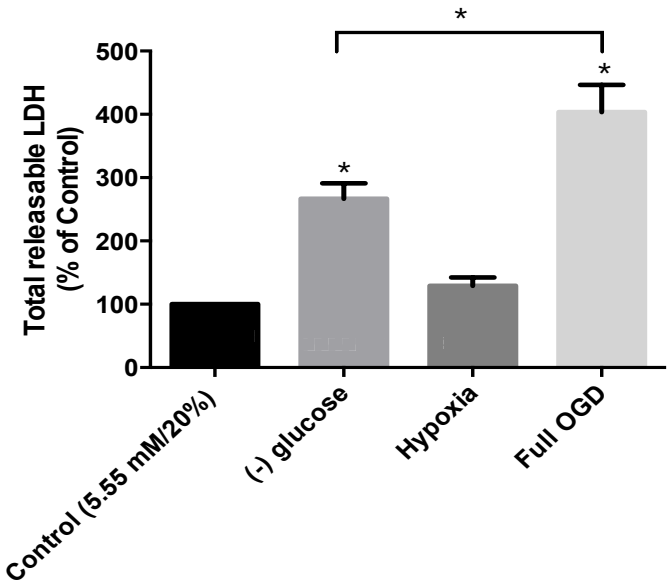


Figure 5.4: Effect of full glucose, oxygen deprivation and both (OGD) on LDH release from HORCs after 24hrs (*p≤0.05; n=4, ANOVA).

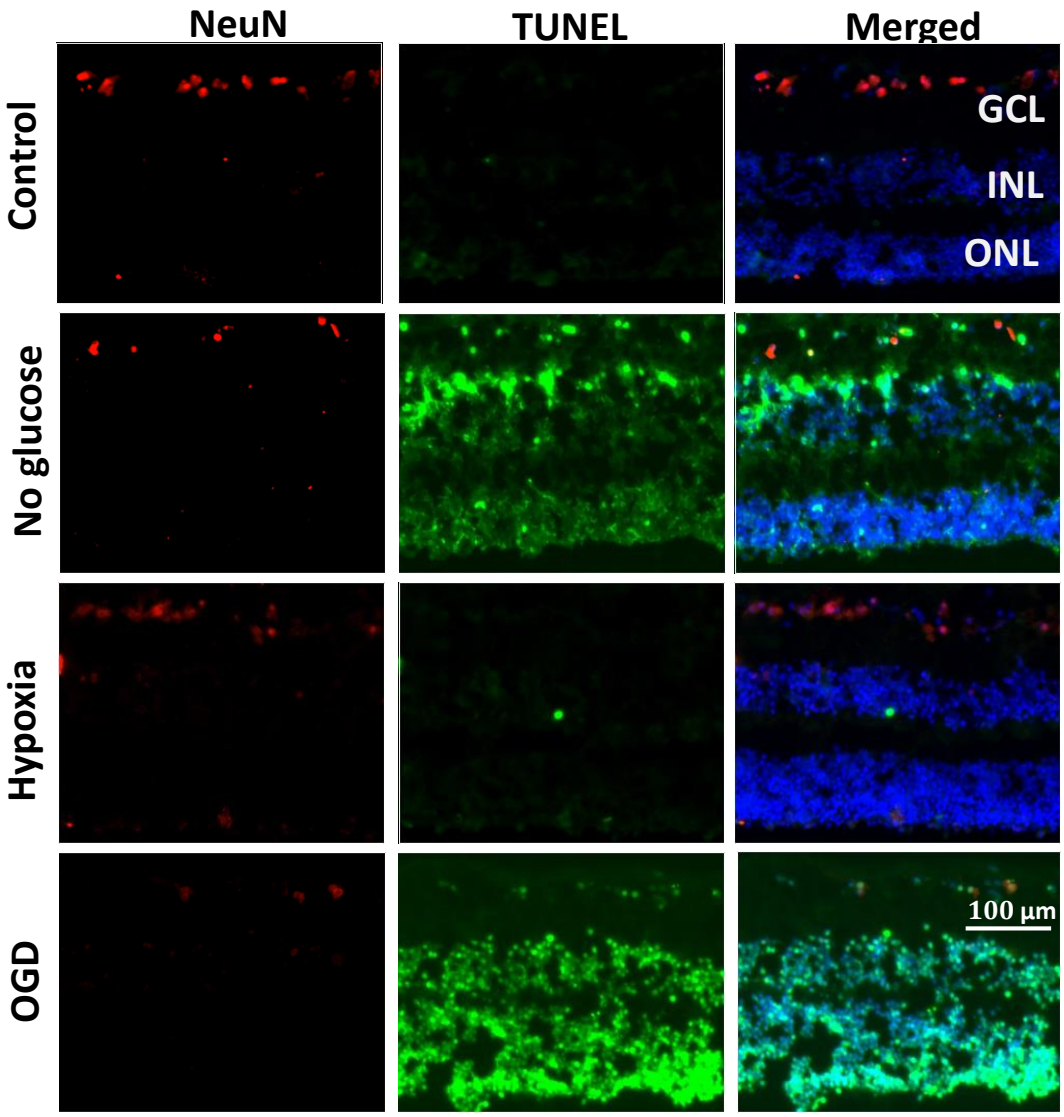


Figure 5.5: Representative immunofluorescence photomicrographs showing TUNEL-labelling in HORCs with control (5.55 mM glucose), glucose deprivation (0mM), oxygen deprivation (0%O_2), and full OGD. DAPI= blue, NeuN= red, TUNEL=green. GCL= ganglion cell layer, INL= inner nuclear layer, ONL= outer nuclear layer

TUNEL-labelling of HORCs exposed to oxygen indicated no apoptotic nuclei in the retina or TUNEL-positive retinal ganglion cells. Apoptotic-labelling was observed throughout all nuclei following 24hrs culturing in glucose deprived medium and exposure to full OGD (Figure 5.5). Similarly, the number of NeuN-labelled RGCs

decreased after exposure to glucose deprivation (0 mM) and full OGD (0mM/0%O_2) (*p≤0.05; n=4) (Figure 5.5B). Oxygen deprivation showed similar number of NeuN-labelled RGCs to control with no apoptotic nuclei.

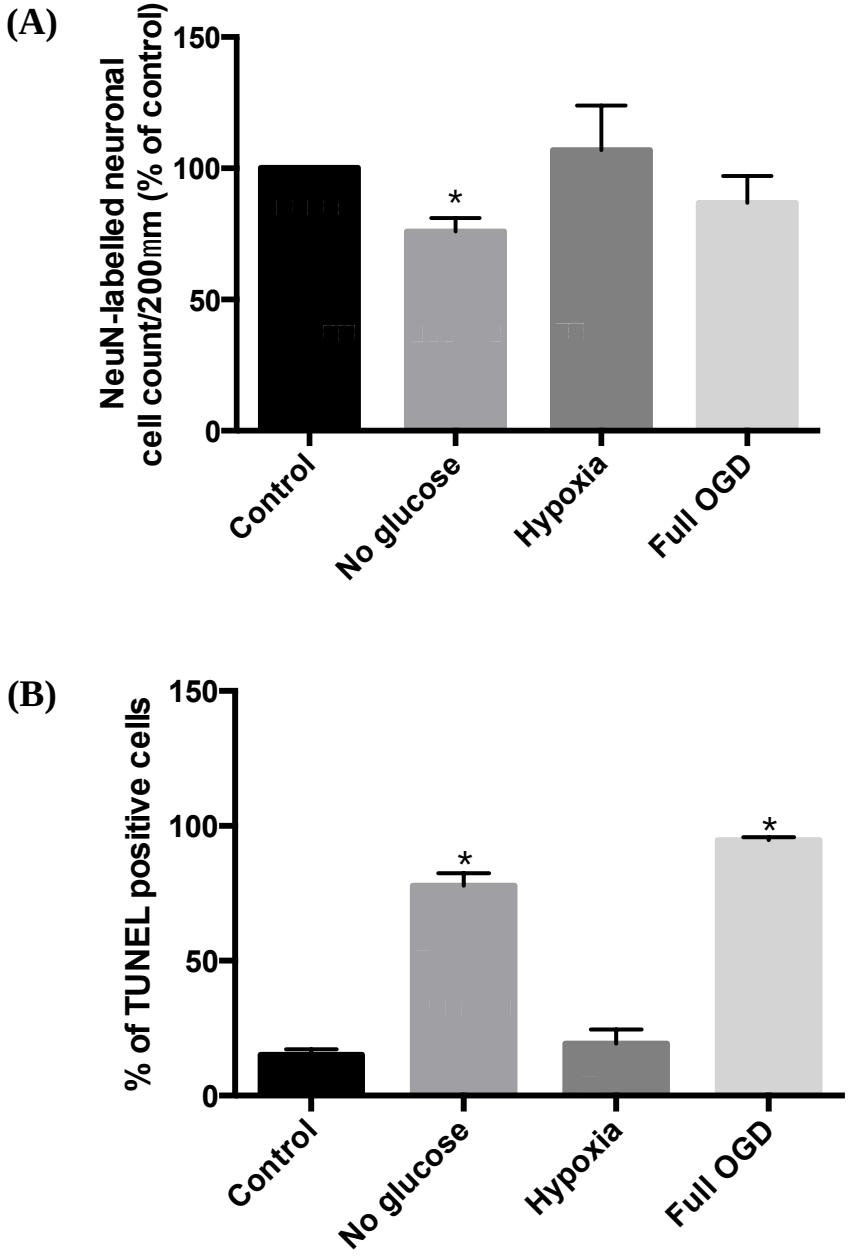


Figure 5.6: Effect of OGD on RGC death (A) Average number of NeuN-labelled cells from three intact 200μm from 8 sections per sample expressed as percentage of control and (B) Percentage of TUNEL-positive ganglion cells exposed for 24hr to control, full hypoxia, full glucose deprivation and full OGD (*p≤0.05; n=4, ANOVA).

5.2.3 Effect of Reduced Glucose and Oxygen Levels (1.11mM/4%O_2) on the LDH Release and RGCs Death in HORCs

In another set of experiments, HORCs were incubated in reduced levels of oxygen and/or glucose to 80% of the control level. In other words, oxygen was reduced to 4%, while glucose was reduced to 1.11mM. Results show that exposure of HORCs to reduced levels of glucose and oxygen (1.11mM glucose/4%O_2) and 1.11mM glucose resulted in a significant increase in the released LDH by 1.6- and 1.7-fold respectively (*p≤0.05; n=4) as compared to control. Oxygen deprivation alone (4%O_2) did not result in detectable LDH release (Figure 5.7).

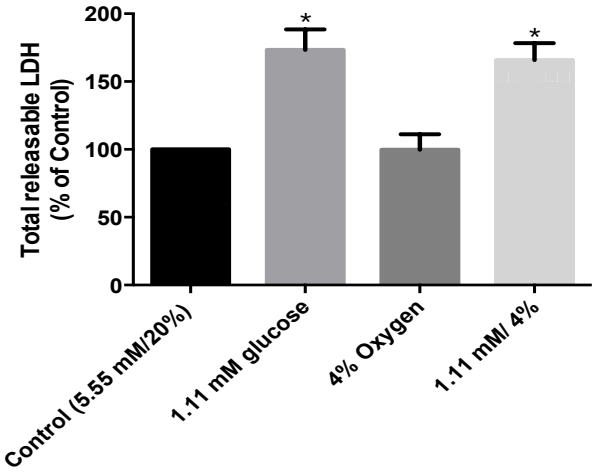


Figure 5.7: Effect of (A) control (5.55 mM glucose/20%O_2), 1.11 mM glucose, 4% oxygen and both (1.11mM glucose/4%O_2) on LDH release from HORCs after 24hrs (*p≤0.05; n=4, ANOVA).

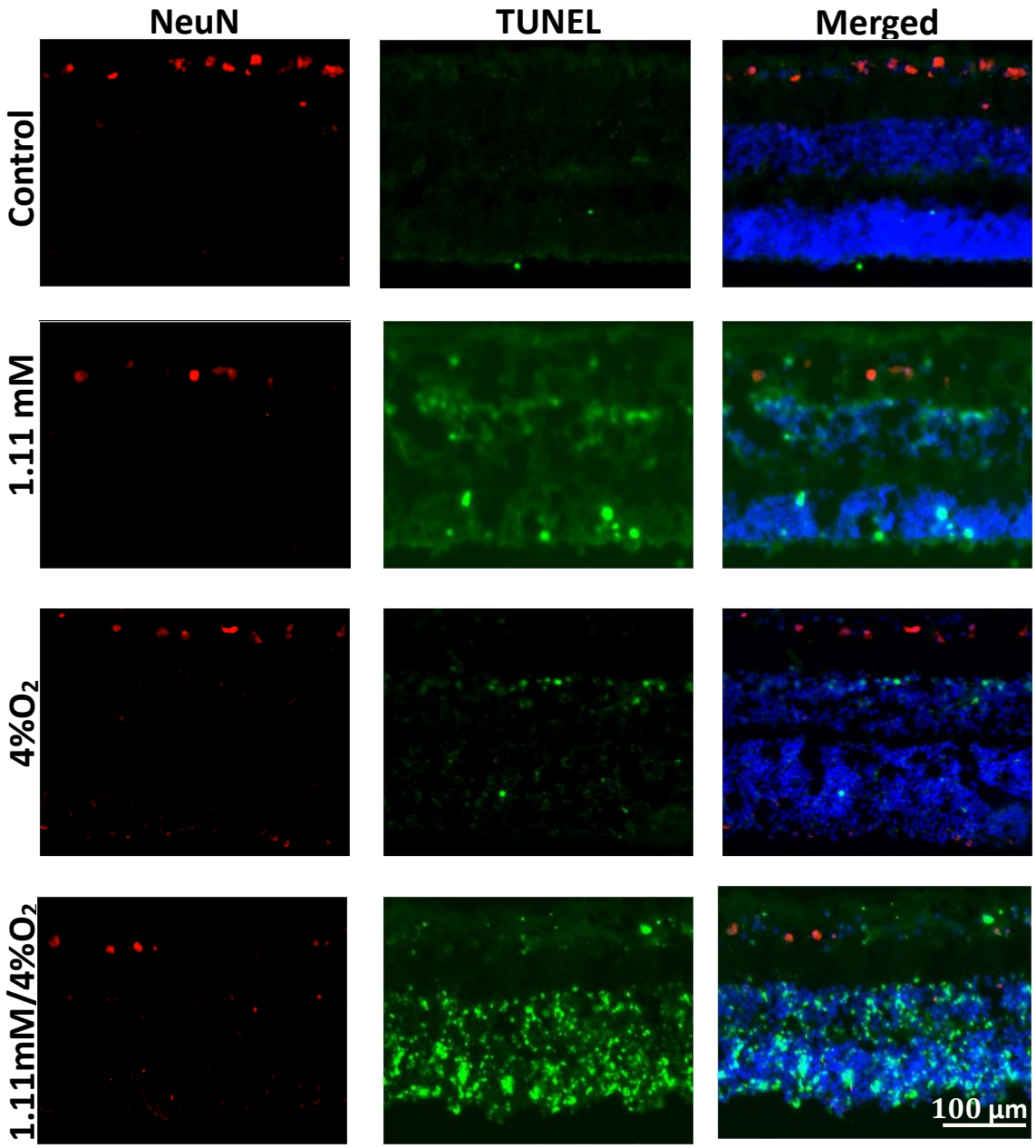


Figure 5.8: Representative immunofluorescence photomicrographs showing TUNEL-labelling in HORCs with control (5.55mMglucose/20%O_2), hypoxia (4%), glucose deprivation (1.11 mM) and 20% OG (1.11 mM glucose/4% O_2) for 24hrs. DAPI= blue, NeuN= red, TUNEL=green. GCL= ganglion cell layer, INL= inner nuclear layer, ONL= outer nuclear layer

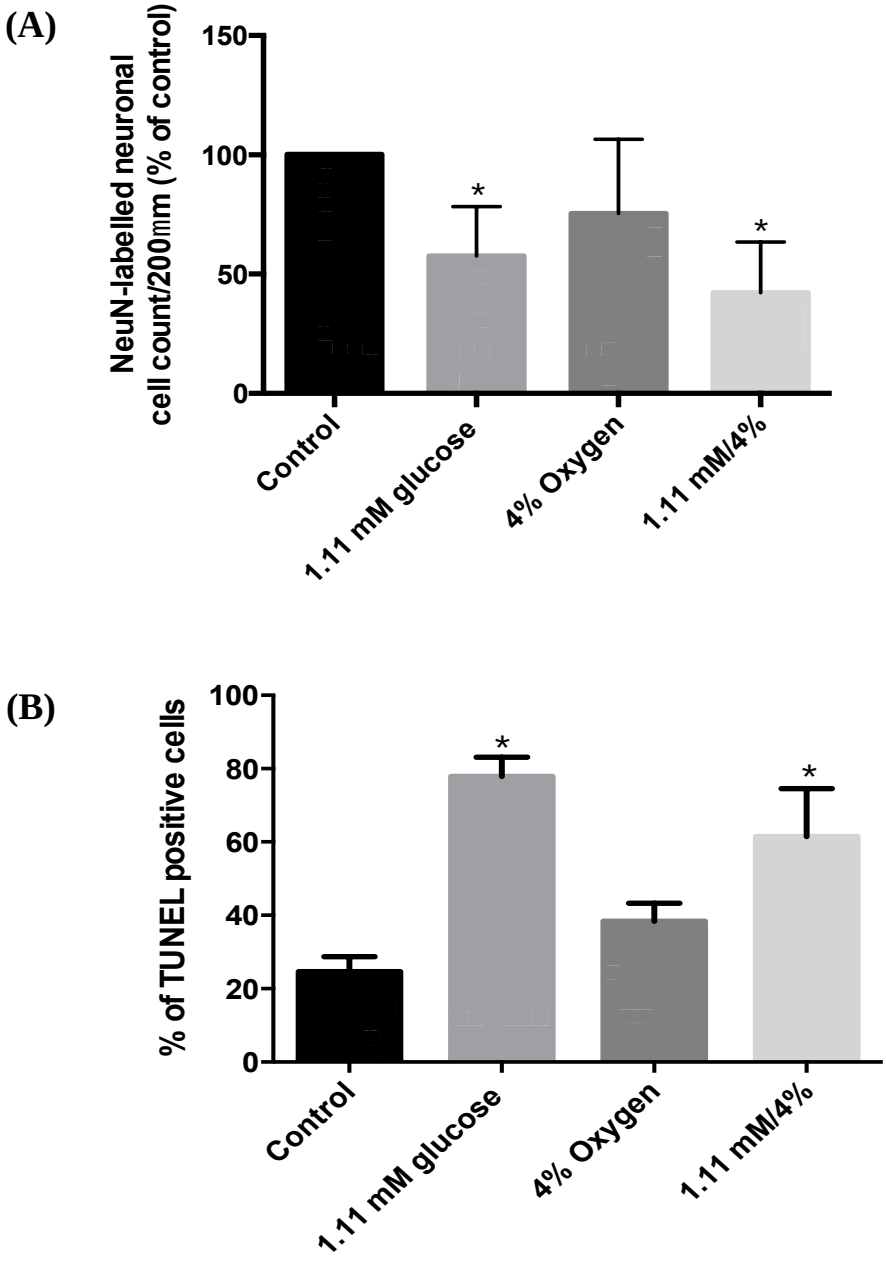


Figure 5.9: Effect of 1.11mM glucose/4%O_2 on RGC death (A) Average number of NeuN-labelled cells from three intact 200µm from 8 sections per sample expressed as percentage of control and (B) Percentage of TUNEL-positive ganglion cells exposed for 24hr to control (5.55mMglucose/20%O_2), glucose deprivation (1.11mM), hypoxia (4%O_2) and both (*$p \leq 0.05$; n=4, ANOVA).

The survival of RGCs under 1.11 mM glucose, 4% oxygen and a combination of both was investigated (Figure 5.8). Validation of RGC loss by immunohistochemistry showed that the mean numbers of NeuN-labelled neurones in the retinal ganglion cell layer were significantly decreased after 24hrs of glucose deprivation (1.11 mM) and 20% OG (1.11 mM glucose/4%O_2) compared with control (*$p \leq 0.05$; n=4) (Figure 5.9A). A significant 3.5- and 3- fold increase (*$p \leq 0.05$; n=4) in the TUNEL-positive neurons in glucose-deprived and oxygen and glucose deprived HORCs respectively were detected (Figure 5.9B). This was associated with TUNEL-positive nuclei found in the INL and ONL in addition to the RGC layer (Figure 5.8).

5.2.4 Effect of Oxygen and Glucose Deprivation on the Expression of Angiogenic Factors in HORCs

5.2.4.1 Effect of Oxygen and Glucose Deprivation on the Expression of VEGF mRNA

Expression of VEGF mRNA was significantly increased under full OGD by 2.5-fold (*$p \leq 0.05$; n=4) compared to control (Figure 5.10A). To determine whether this expression is oxygen or glucose dependent, VEGF mRNA was also determine under each nutrient deprivation alone. Exposure of HORCs to hypoxia (0%O_2) caused a significant (*$p \leq 0.05$; n=4) 2.59-fold increase the expression as compared to control, while full glucose deprivation (0mM) did not result in significant alteration in the expression. Changes seen under reduced levels of oxygen and/or glucose were not significant (Figure 5.10B)

5.2.4.2 Effect of Oxygen and Glucose Deprivation on the Expression of PDGF mRNA

Expression levels in HORCs were found to be upregulated significantly in full deprivation. Full OGD resulted in 2.6 fold up-regulation of PDGF mRNA expression compared to control (Figure 5.11A). Full oxygen deprivation alone caused 2-fold up-regulation in the PDGF mRNA expression (*$p \leq 0.05$; n=4). Glucose deprivation alone has less effect on the expression of PDGF compared to oxygen. Under reduced levels of oxygen and/or glucose, no significant changes were found (Figure 5.11B)

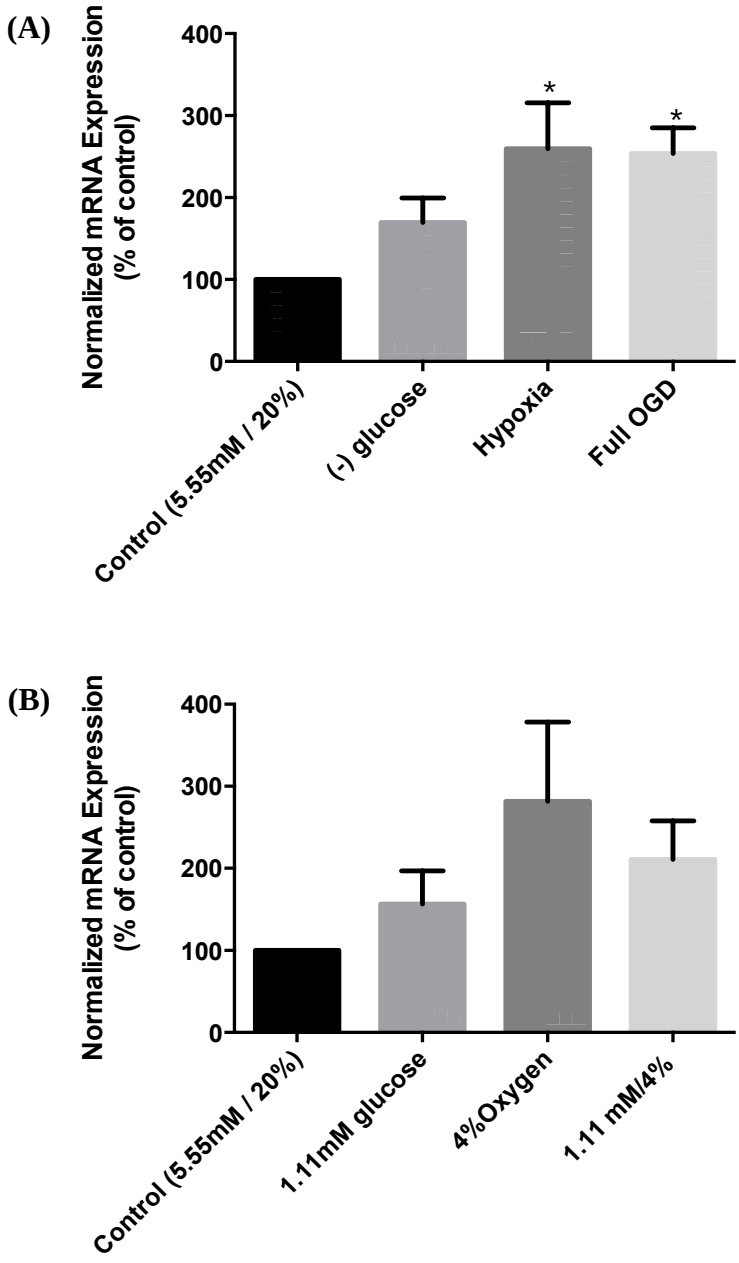


Figure 5.10: Expression of VEGF mRNA in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA)

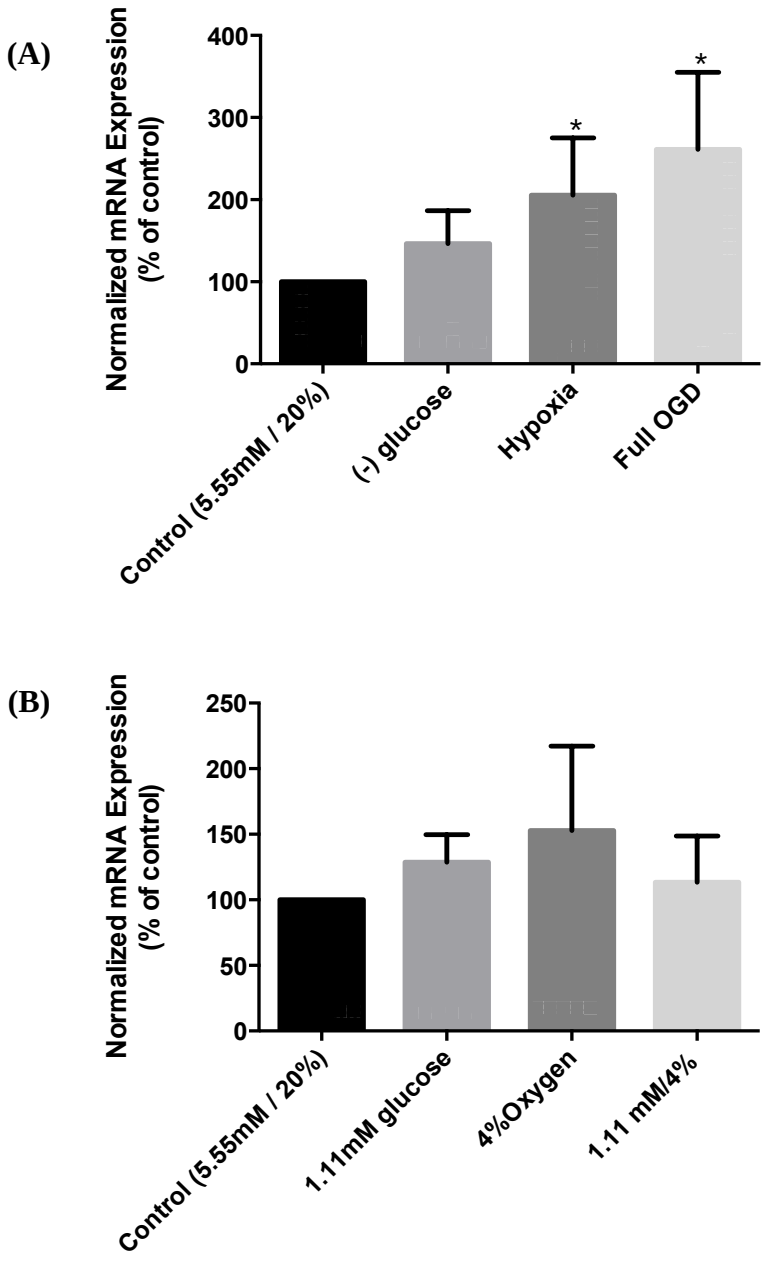


Figure 5.11: Expression of PDGF mRNA in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA)

5.2.4.3 Effect of Oxygen and Glucose Deprivation on the Expression of bFGF mRNA

Exposure of HORCs to 1.11mM glucose caused a significant down-regulated bFGF mRNA expression by approximately 50% compared to control (Figure 5.12B). The changes seen under other deprivation conditions were not statistically significant.

5.2.5 Effect of Oxygen and Glucose Deprivation on the Expression of Anti-angiogenic Factors

5.2.5.1 Effect of Oxygen and Glucose Deprivation on the Expression of PEDF

The expression of PEDF mRNA levels did not change at any level of oxygen and glucose deprivation (Figure 5.13A, B).

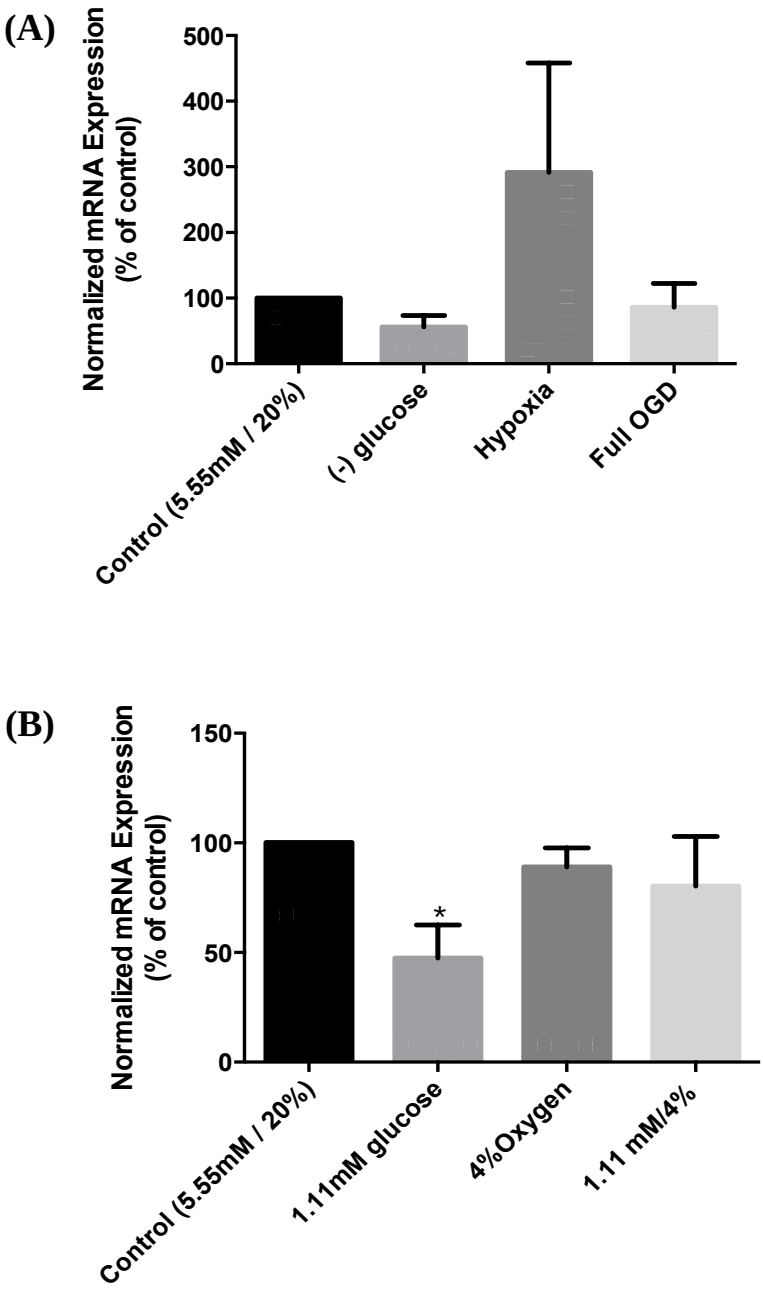


Figure 5.12: Expression of bFGF mRNA in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA).

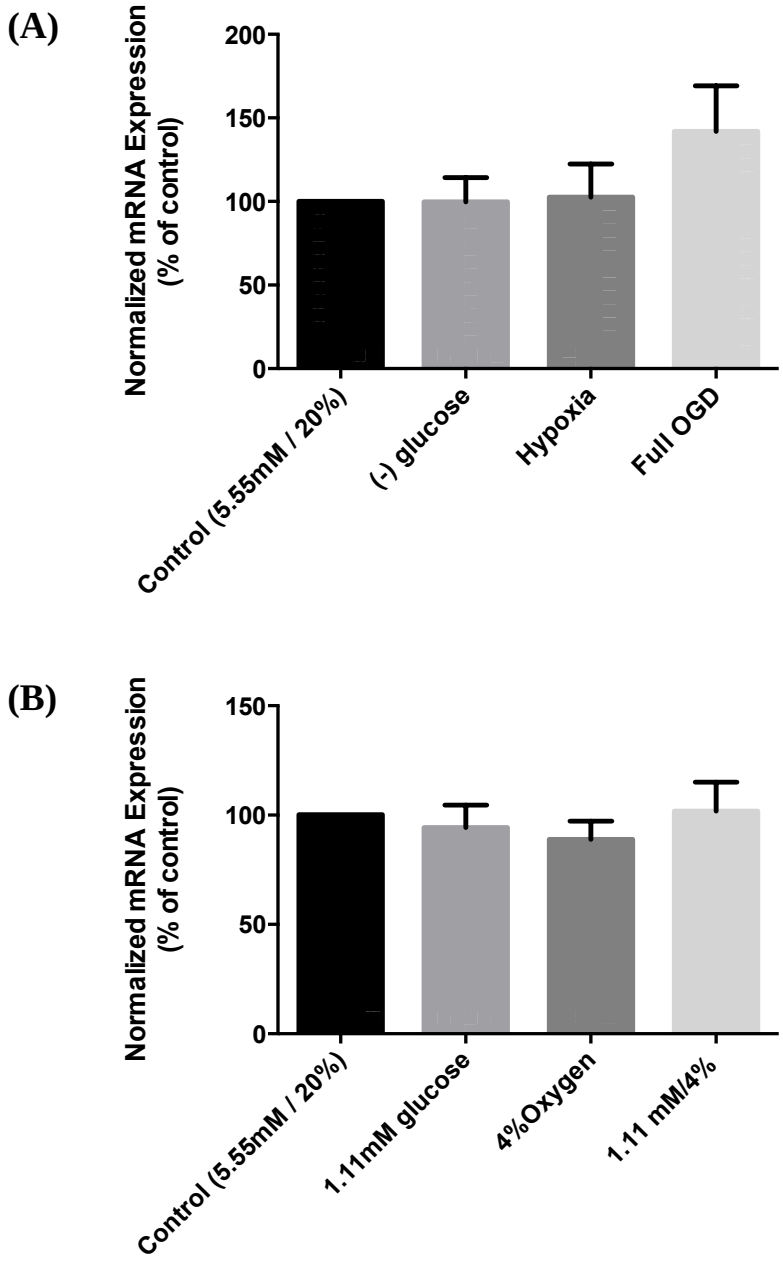


Figure 5.13: Expression of PEDF mRNA in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA).

5.2.5.2 Effect of Oxygen and Glucose Deprivation on the Expression of LIF

Full OGD and glucose deprivation resulted in a significant reduction in LIF mRNA to approximately 16 and 36% of control (Figure 5.14A). Although reduced levels of oxygen and/or glucose to 20% (1.11mM glucose/4%O_2) showed a similar reduction pattern in expression of LIF, it was statistically non-significant (Figure 5.14B).

5.2.6 Effect of Oxygen and Glucose Deprivation on the Expression of Glutamate Markers

5.2.6.1 Effect of Oxygen and Glucose Deprivation on the Expression of Glutamine Synthetase (*GLUL*)

Exposure of HORCs to full glucose deprivation significantly (*$p \leq 0.05$; n=4) increased *GLUL* mRNA by approximately 3-fold compared to control. Full hypoxia and OGD did not result in any significant regulation (Figure 5.15A). A similar pattern of expression was found with reduced levels of 1.11mM glucose, 4% oxygen and both (1.11mM glucose/4%O_2) but was statistically non-significant (Figure 5.15B).

5.2.6.2 Effect of Oxygen and Glucose Deprivation on the Expression of the Glutamate-Aspartate Transporter, GLAST (*SLC1A3*)

The expression of glutamate transporter, GLAST in HORCs was not significantly altered under any of the treatments (Figure 5.16A,B). Although a trend towards an increased expression in glucose deprivation and OGD was evident.

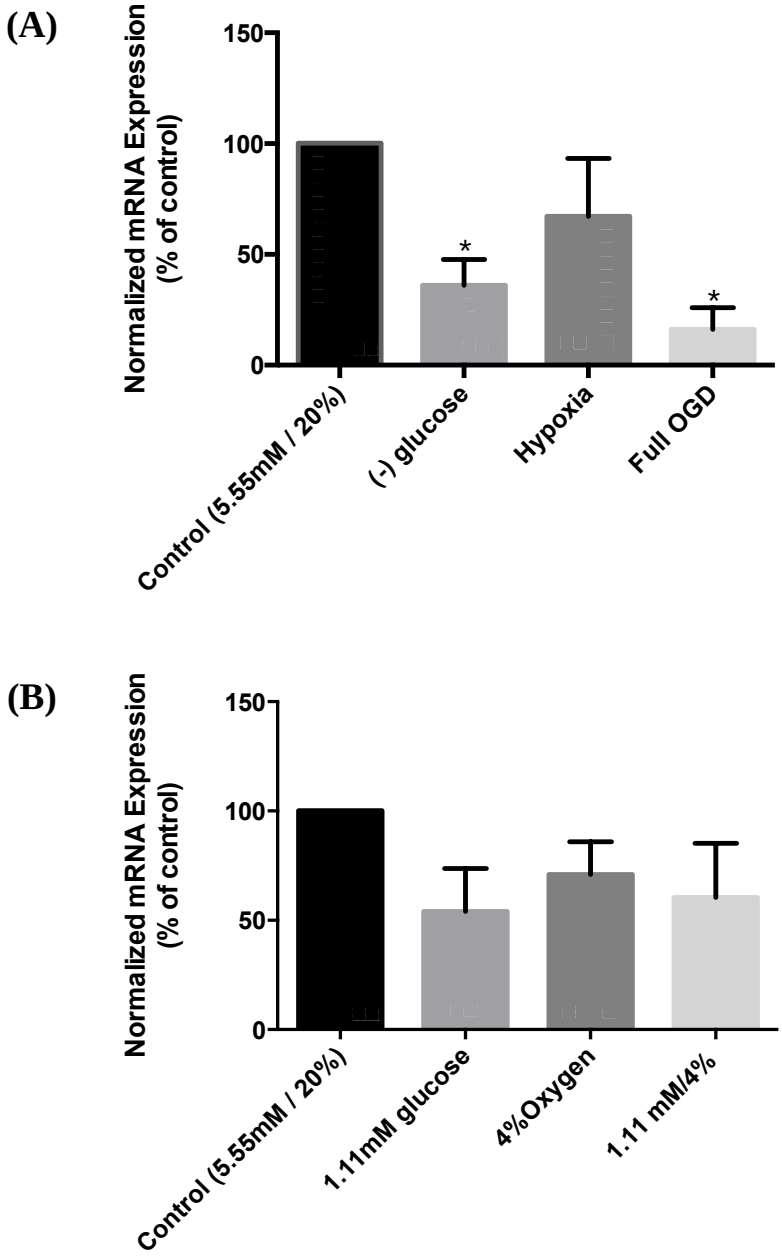


Figure 5.14: Expression of LIF mRNA in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA)

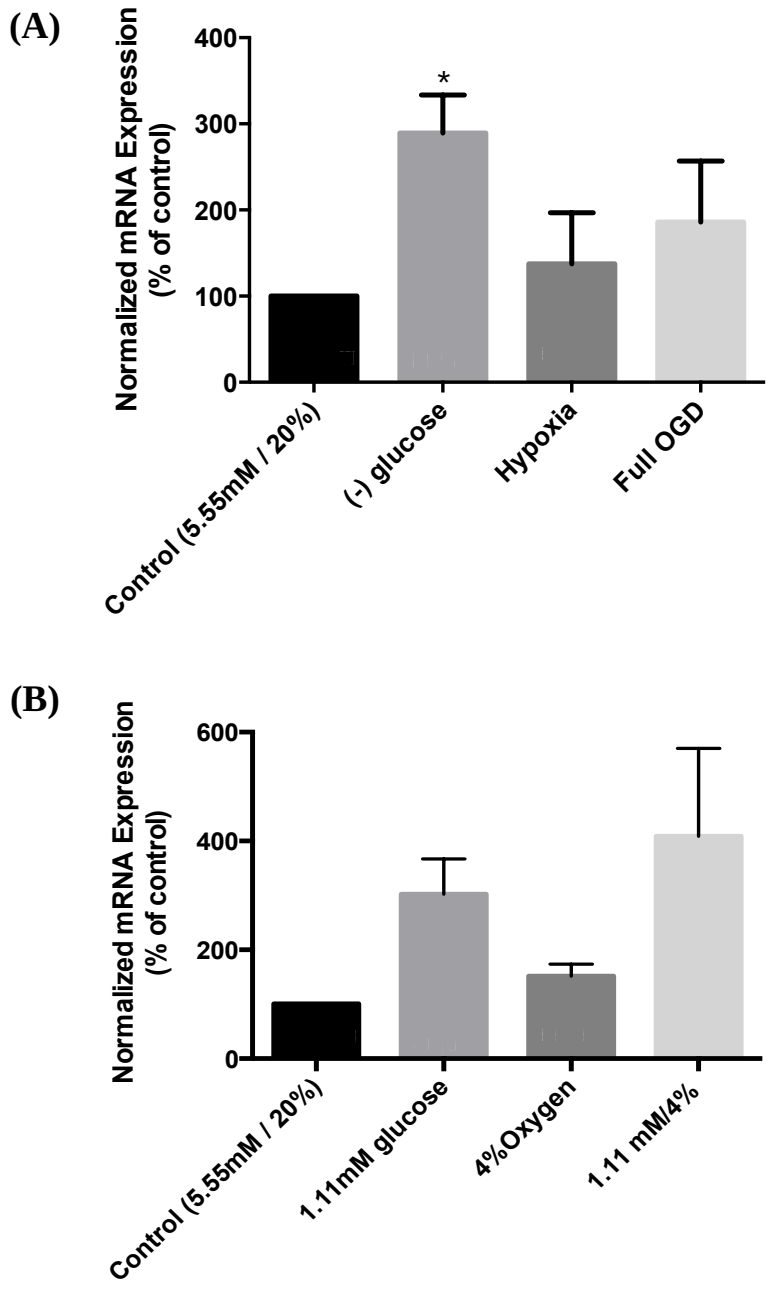


Figure 5.15: Expression of GS (*GLUL*) mRNA in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA)

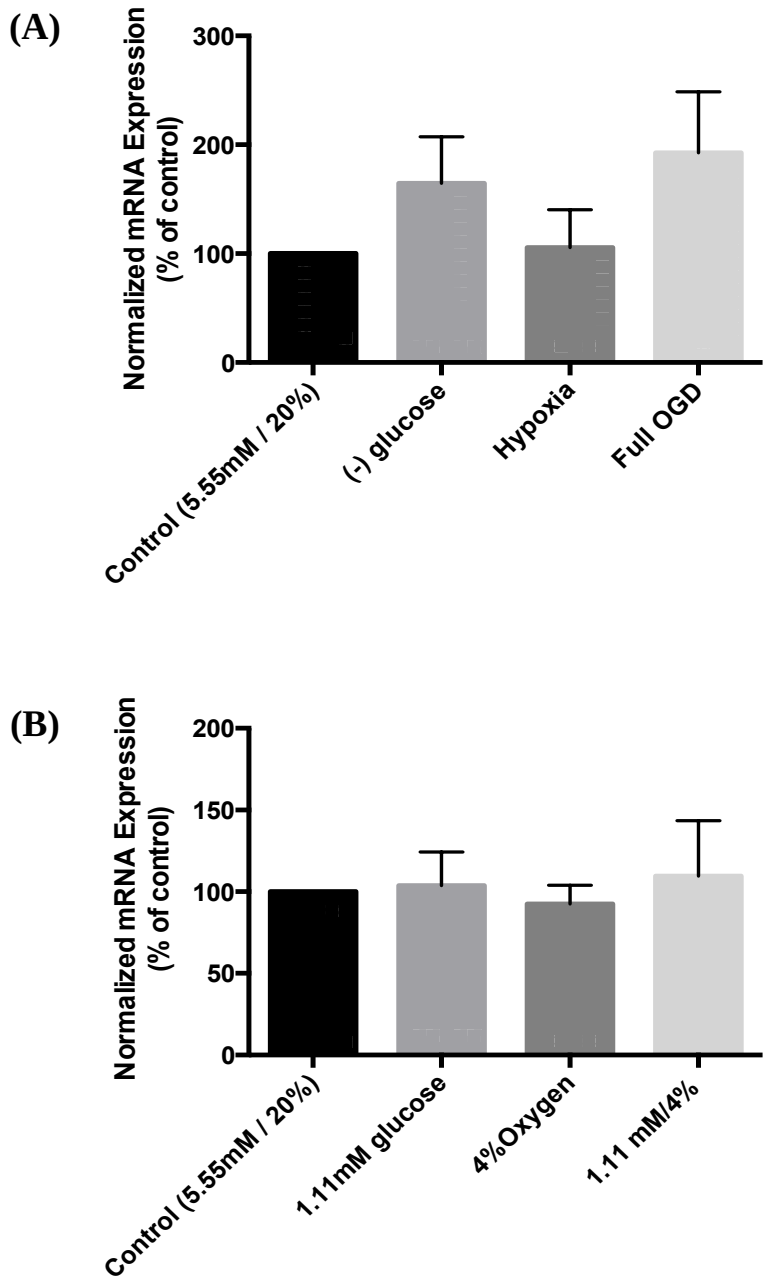


Figure 5.16: Expression of *SLC1A3* mRNA in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA).

5.2.6.3 Effect of Oxygen and Glucose Deprivation on the Expression of Glutamate Decarboxylase (*GAD1*)

Exposure of HORCs to full OGD caused a significant 2-fold upregulation (*$p \leq 0.05$; n=4) in *GAD1* mRNA. Full glucose deprivation and hypoxia (0%O_2) did not alter the levels significantly (Figure 5.17A). Lower deprivation conditions showed a similar pattern to full deprivation, but the levels were not significant (Figure 5.17B).

5.2.7 Effect of Oxygen and Glucose Deprivation on the Expression of Purinergic Signaling Related Genes

5.2.7.1 Effect of Oxygen and Glucose Deprivation on the Expression of Pannexin1 (*PANX1*)

No significant changes were seen in expression of *PANX1* mRNA under any of the conditions investigated. Although a trend towards an increase in full OGD and glucose deprivation was apparent (Figure 5.18).

5.2.7.2 Effect of Oxygen and Glucose Deprivation on the Expression of Autotaxin (*ENNP2*)

Expressions of *ENNP2* mRNA did not significantly change under any of the conditions investigated (Figure 5.19).

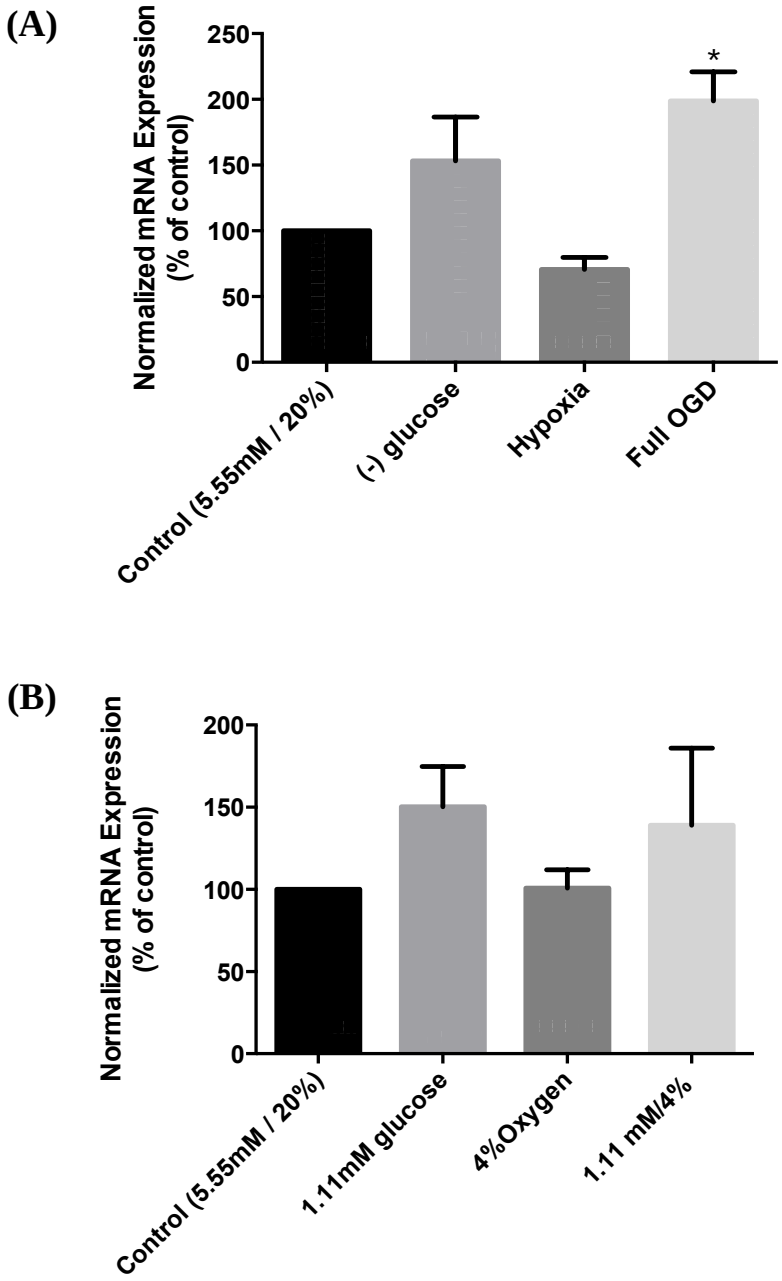


Figure 5.17: Expression of *GAD* mRNA in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA).

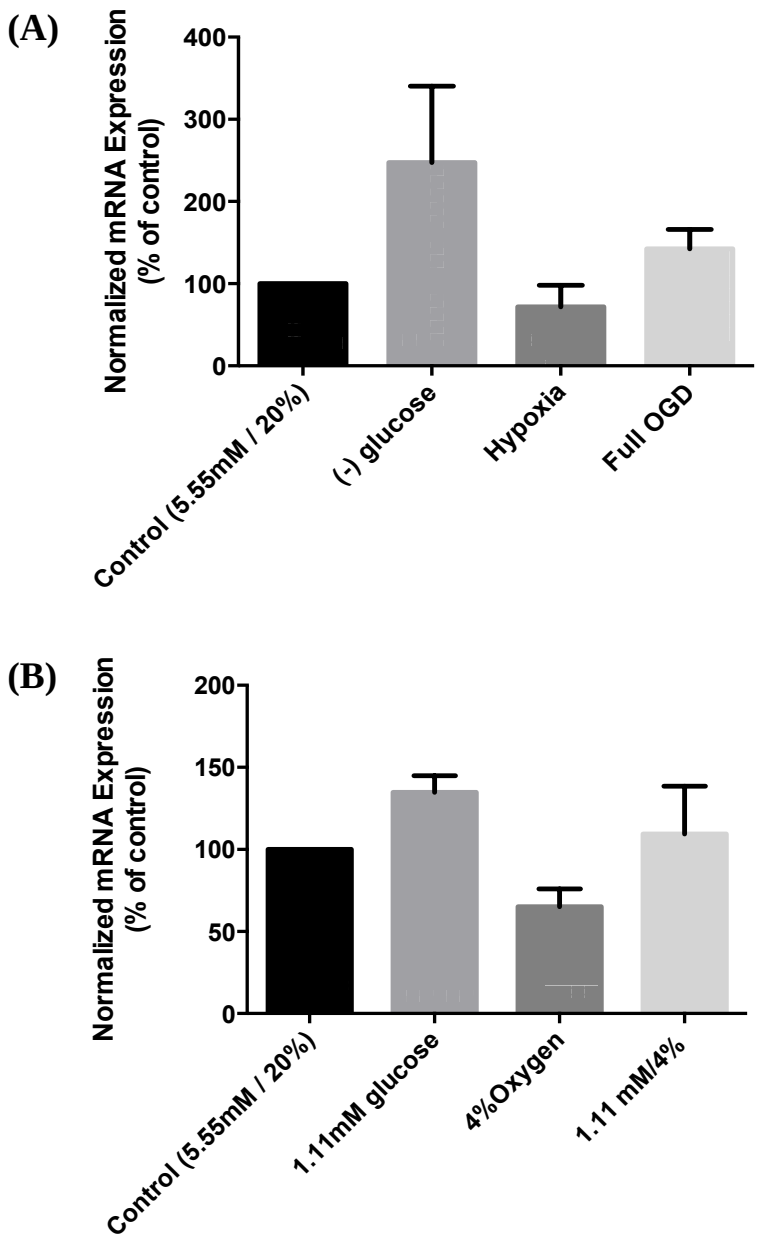


Figure 5.18: Expression of *PANX1* mRNA in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA).

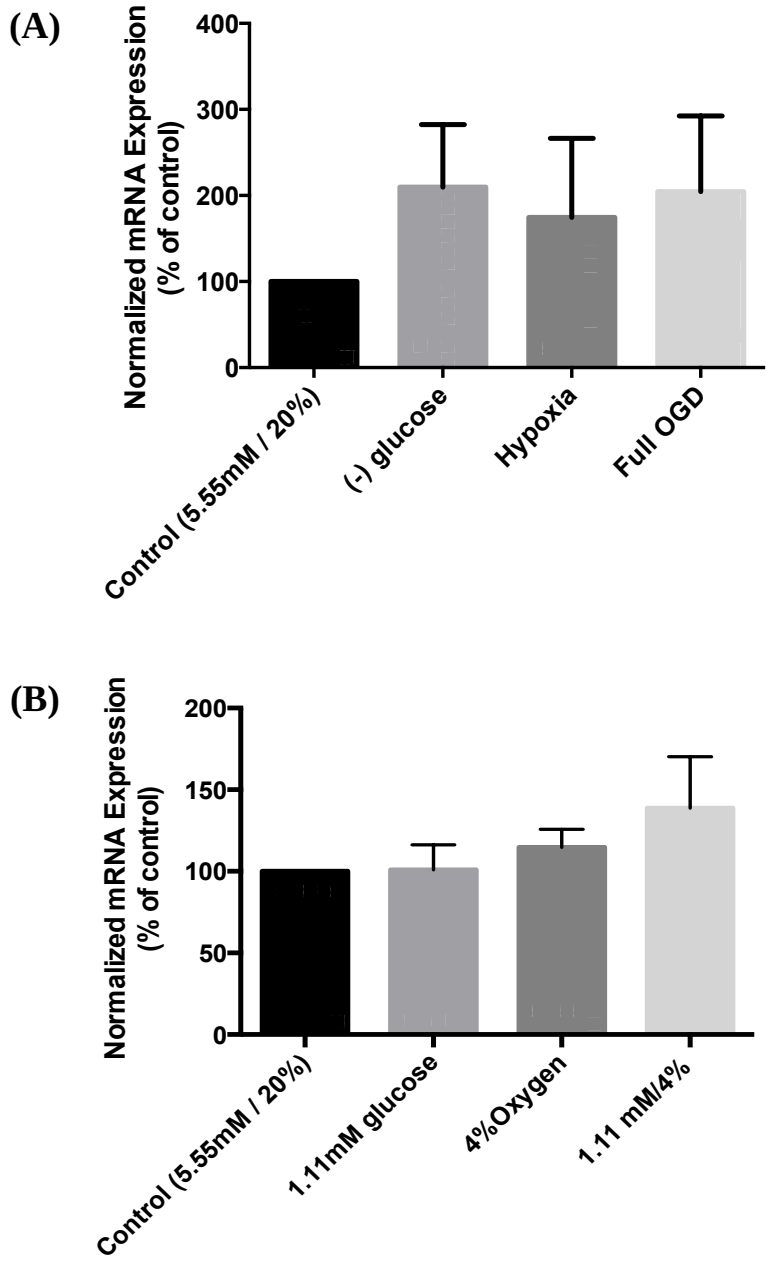


Figure 5.19: Expression of *ENNP2* mRNA in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA).

5.2.8 Effect of Oxygen and Glucose Deprivation on the Expression of Glial Müller Cells Markers

5.2.8.1 Effect of Oxygen and Glucose Deprivation on the Expression of GFAP

No significant changes to the levels of GFAP mRNA levels were seen under the conditions of these experiments (Figure 5.20 A, B).

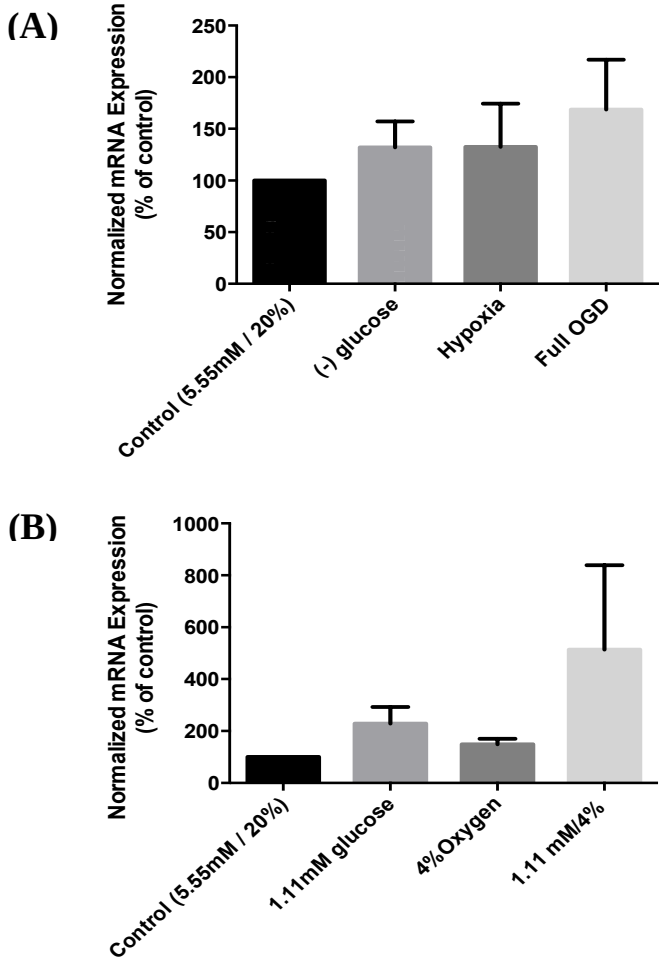


Figure 5.20: Expression of GFAP mRNA in HORCs after 24hrs exposure to: in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA).

5.2.9 Effect of Oxygen and Glucose Deprivation on the Expression of Heat-Shock Protein (*HSPA1B*)

No significant changes to the levels of heat-Shock Protein (*HSPA1B*) were found at any level of oxygen and/or glucose deprivation (Figure 5.24A,B). Although a trend towards an increase in low glucose and OGD was apparent.

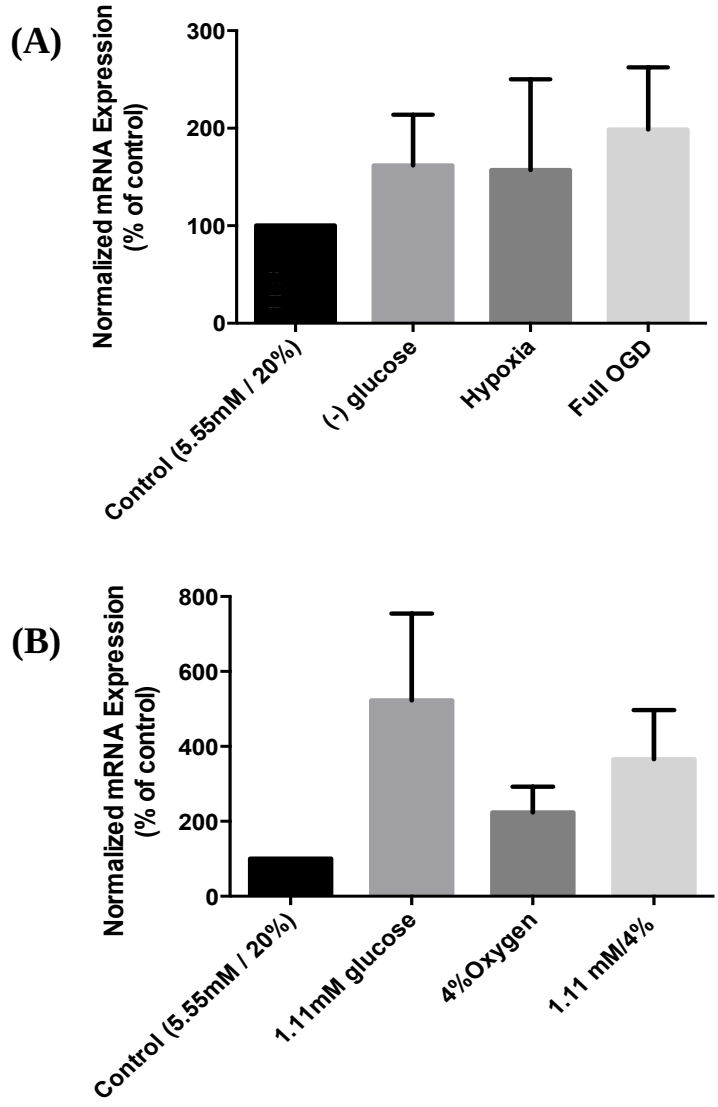


Figure 5.21: Expression of *HSPA1B* mRNA in HORCs after 24hrs exposure to: **(A)** full glucose deprivation (0mM glucose/20%O_2), full hypoxia (5.55mM glucose/0%O_2) and full OGD (0mM glucose/0%O_2) and **(B)** glucose deprivation (1.11mM glucose/20%O_2), hypoxia (5.55mMglucose/4%O_2) and deprivation of both oxygen and glucose (1.11mMglucose/4%O_2), All conditions were compared to control (5.55mM/20%O_2). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (n=4; *p≤0.05; ANOVA).

	HORCs exposed to 24hrs to oxygen and glucose deprivation					
	Full OGD			1.11mM glucose/4%O_2		
Gene	**OGD**	**Hypoxia**	**Glucose**	**OGD**	**Hypoxia**	**Glucose**
VEGF	*↑ 24	*↑ 24	↑ 24	↑ 24	↑ 24	↑ 24
PDGF	*↑ 24	*↑ 24	↑ 24	↑ 24	↑ 24	↑ 24
FGF	↔	↑ 24	↔	*↓24	↔	↔
PEDF	↑ 24	↔	↔	↔	↔	↔
GFAP	↑ 24	↑ 24	↑ 24	↑ 24	↔	↑ 24
GS	↑ 24	↑ 24	*↑ 24	↑ 24	↔	↑ 24
SLC1A3	↑ 24	↔	↑ 24	↔	↔	↔
Autotaxin	↑ 24	↑ 24	↑ 24	↑ 24	↔	↔
LIF	*↓24	↓24	*↓24	↓24	↓24	↓24
Heat shock	↑ 24	↑ 24	↑ 24	↑ 24	↑ 24	↑ 24
GAD	*↑ 24	↓24	↑ 24	↑ 24	↔	↑ 24
PANX-1	↑ 24	↓24	↑ 24	↑ 24	↓24	↑ 24

Table 5.1: Summary of gene expression changes in HORCs under oxygen and/or glucose deprivation for 24hrs (* indicate statistical significant change).

5.3 Discussion

5.3.1 Effect of Oxygen and Glucose Deprivation on the Survival of Retinal Ganglion Cells

The current experiments assessed LDH release, as an indicator of necrotic retinal damage in HORCs and apoptosis was assessed by using TUNEL assay. Deprivation of glucose only under the normoxic condition for 24hrs led to increasing in the release of LDH. The greater the deprivation, the greater the release of LDH highlighting the importance of glucose for retinal cells survival. A significant release of LDH was obtained with 1.11mM and 0.555mM glucose and full glucose deprivation indicating necrotic damage. Although there was no significant increase in the released LDH at lower levels of glucose deprivation, the LDH release started at a 10% level of deprivation highlighting that small decreases in glucose are detrimental to retinal cell survival. Retinal explants treated with graded levels of glucose showed TUNEL-positive cells in the ganglion cell layer at higher levels of deprivation. The number of TUNEL-positive cells was increased in the INL and ONL as the glucose deprivation increased correlating with LDH data. This data in human retina is supported by numerous experiments in animal models. Chronic hypoglycaemia, induced by a mutation in the glycogen receptor gene in mice resulted in losses of visual activity by the age of 10 months and increased TUNEL-positive cells in the INL of the retina (Umino et al., 2006). In addition, isolated retina from mice after exposure to hyperinsulinemic/hypoglycemic clamp to induce five-hour hypoglycemia (2.2 mM glucose) showed significant TUNEL-positive cells in the retina compared to fewer cells in control (6mM glucose) (Emery et al., 2011). Cleaved caspase-3 positive cells, together with TUNEL-positive cells, were found in the ONL and GCL (Emery et al., 2011).

Based on LDH and TUNEL data from glucose deprivation experiments, two levels of oxygen and glucose deprivation were chosen to examine apoptotic RGCs death. Several animal studies have reported a loss of inner retinal cells as a result of simulated ischemia. A loss of RGC was evident after IOP-induced ischemia (Osborne et al., 1999b; Wang et al., 2002) and ligation of ophthalmic vessels (Lafuente et al., 2002). Loss of photoreceptors was also reported in a carotid artery occlusion model (Osborne et al., 1999b). In the present experiments, complete withdrawal of oxygen and glucose (OGD) for 24hrs caused a significant 4-fold increase in released LDH. Reduced levels of oxygen and glucose (1.11mM glucose/4%O_2) resulted in a 1.6-fold increase in LDH release. In IOP-induced ischemia in rats, TUNEL-positive cells appeared in the INL and GCL within one day of initiation of the ischemia, marked by cell body shrinkage, aggregation, condensation of nuclear chromatin and expression of p53 mRNA, the tumor suppressor gene known to induce apoptosis (Loo et al., 1999). Current results show that full OGD and reduced levels (1.11mM glucose/4%O_2) for 24hrs caused 90% and 60% TUNEL-positive RGCs respectively. This was associated with a reduction in the number of NeuN-labelled neurons in both treatments. Apoptotic death in a rat model of retinal ischemia-reperfusion injury was detected in RGC and INL layers (Lam et al., 1999). TUNEL-positive cells also appeared in the INL and ONL. Moreover, in *post-mortem* eyes of patients with primary open-angle glaucoma and secondary glaucoma, positive TUNEL cells were observed in the GCL (Kerrigan et al., 1997; Okisaka et al., 1997). Similar results were obtained with experimental glaucoma in monkeys (Quigley et al., 1995). In the present study, oxygen deprivation (full and 4%O_2) did not alter the LDH release as compared to control and did not cause RGCs death as evidenced by RGC counting and TUNEL staining. This is in contrast to other reports, where, *in vitro* hypoxia-

induced RGCs death has been shown to occur. Short period (2 hours) of hypoxia ($5\%O_2$) in rats followed by re-oxygenation caused RGCs death by caspase-3–mediated apoptosis (Rathnasamy et al., 2014). In addition, Sivakumar et al., (2013) found that following exposure of neonatal rats to 2hrs hypoxia ($5\%O_2$) an increased amount of glutamate accumulates in the retina. This caused RGC damage through activation of AMPA receptors, increased Ca^{2+} accumulation, and ROS generation. It should however, be noted that experiments. As shown previously, human Müller cells (MIO-M1) resisted oxygen deprivation ($4\%O_2$) and/or 1.11mM glucose. They also survived full hypoxia whereas loss of viability only occurs when the cells were deprived fully of glucose or oxygen and glucose.

5.3.2 Effect of Oxygen and Glucose Deprivation on the Expression of Angiogenic Growth Factors

In the retina, VEGF is expressed by endothelial cells (Aiello et al., 1995), Müller cells (Pierce et al., 1995), astrocytes (Stone et al., 1995), ganglion cells (Stone et al., 1996) and the retinal pigment epithelium (Miller et al., 1997). Moreover, VEGF is upregulated in several ocular pathologies including age-related macular degeneration (AMD) (Ferrara et al., 2007), and proliferative diabetic retinopathy (PDR) (Duh et al., 2004). Findings by Bai et al., (2009) suggest that retinal Müller cell-derived VEGF is a major contributor to ischemia-induced retinal vascular leakage and pre-retinal and intraretinal neovascularization. Following induction of ischemia by vein occlusion, VEGF mRNA was upregulated in the retina and increased in the aqueous fluid (Miller et al., 1994) and oxygen-induced retinopathy in rats increased VEGF immunoreactivity was seen in the ganglion cell layer (Vinores et al., 1997). Current results show that retinal explants significantly up-regulate VEGF mRNA under conditions of hypoxia ($0\%O_2$) and full OGD. Such upregulation might be expected to

lead to pathogenic vascular changes although they could be considered as an attempt by the retinal cells to survive during the severe insult. In the previous chapter, MIO-M1 cells showed an increase expression of VEGF mRNA when exposed to complete oxygen, glucose deprivation and oxygen-glucose deprivation. The retinal explants increased expression under total deprivation conditions; therefore it is possible that Müller cells are playing a role in this regulation.

Another important angiogenic factor in the eye is PDGF. Under physiological states, PDGF is expressed at a low level in perivascular cells, ganglion cells, and in the RPE (Campochiaro et al., 1994; Mudhar et al., 1993) in addition to retinal glia (Uchihori and Puro 1991). PDGF has been implicated in the pathogenesis of different retinal conditions including retinal detachment (Seo et al., 2000), proliferative vitreoretinopathy (Campochiaro, 1997) and ischemic retinopathy (DeNiro et al., 2011). In the current *in vitro* model of ischemia, expression of PDGF mRNA in HORCs increased with hypoxia (0%O_2) and full OGD. As with VEGF, it is possible that the retinal cells increased expression of PDGF in an attempt to survive the insult. Previous experiments in this work showed that MIO-M1 cells did not alter the expression of PDGF under similar conditions, which may indicate that the source of PDGF in retinal explants is derived from other retinal cells. Although the response to ischemia may be an attempt at survival, there was no evidence of cell survival in HORCs exposed to full OGD. Therefore, the expression of PDGF and VEGF could exacerbate death rather than survival and blocking of these growth factors could be a strategy to reduce RGC loss and this require further investigation. It may be, however, that blocking these factors leads to increased degeneration. These would be interesting experiments to perform in the HORC model.

Expression of bFGF was also measured in the HORC simulated ischemia model. In addition to Müller cells as an important source of bFGF in the retina, it is also present in astrocytes, ganglion cells and pigment epithelium cells (Walsh et al., 2001). The release of bFGF from Müller cells increases in the retina after detachment as indicated by activation of extracellular signal-regulated kinase (ERK) (Geller et al., 2001) and it has been found that bFGF mRNA is upregulated and released after retinal stress. In the retinas of cats and rabbits, intravitreal injection of bFGF resulted in a significant elevation in the number of Müller cells four days after injection and continued to increase after four weeks (Lewis et al., 1992). Despite the number of findings of the neuroprotective effect of bFGF, there have also been reports to the contrary, for example that exacerbation of glutamate-mediated neurotoxicity was mediated by the endogenous release of bFGF through a down-regulation of glutamine synthetase (Kruchkova et al., 2001). Results show that both levels of simulated ischemia and glucose deprivation reduced bFGF mRNA levels compared to control. This may indicate that reduction of bFGF expression and release is detrimental to retinal cell survival.

5.3.3 Effect of Oxygen and Glucose Deprivation on the Expression of Anti-angiogenic Growth Factors

Pigment epithelium derived growth factor (PEDF) is a known potent angiogenesis inhibitor (Eichler et al., 2004) secreted from retinal pigment epithelial cells, and Müller cells in the retina (Gettins et al., 2002; Ogata et al., 2002). Studies have shown that PEDF protects RGCs from ischemia-induced neuronal apoptotic death (Takita et al., 2003), reduces RGC loss and vision decline in an animal model of human inherited glaucoma (Zhou et al., 2009). However, current results showed that

expression of PEDF mRNA in HORCs did not change as a response to experimental conditions.

Leukemia inhibitory factor (LIF) is another inhibitor of angiogenesis and a member of the interleukin-6 family of cytokines (Heinrich et al., 2003). It can be induced in the brain by ischemia-reperfusion injury in neurons and astrocytes (Suzuki et al., 2000) and has been reported as a survival factor in cultured neurons and astrocytes (Gadient et al., 1998). In the present experiments, LIF mRNA was significantly reduced with full OGD and glucose deprivation indicating that LIF down-regulation may exacerbate RGCs loss under these conditions. In cultured MIO-M1 cells, initial upregulation was found as early as 3hrs, but the levels decreased by 24hrs, which may reflect the importance of LIF in mediating cell survival. It would have been interesting to look at earlier time points in HORCs to see if there was an increase in the expression prior to the decrease seen at 24hrs and in situ hybridization techniques could have been used to determine which retinal cells were expressing LIF.

5.3.4 Effect of Oxygen and Glucose Deprivation on the Expression of Glutamate Markers

Glutamate is the main retinal excitatory amino acid released by Müller cells, astrocytes, photoreceptors, bipolar cells and ganglion cells (Heidinger et al., 1998; Hernandez et al., 2000). Increased glutamate levels are associated with excitotoxicity and neurodegenerations in human and animal models of glaucoma (Brooks et al., 1997; Honkanen et al., 2003; Levkovitch-Verbin et al., 2002; Källberg et al., 2007; Kim et al., 2000). In order to try to protect against elevated glutamate, its detoxification systems GLAST, and GS may be upregulated in an attempt to maintain glutamate at low concentration at the extracellular space. In HORCs exposed to simulated ischemia (full OGD) and complete glucose deprivation, but not oxygen

deprivation alone there was a trend of increasing expression of GLAST mRNA. Whether this indicates that glutamate is elevated, it remains a question that need to be confirmed, because measuring glutamate level was not attempted in this study. Data presented here showed that reduced levels of glucose and oxygen (1.11mM glucose/4%O_2) did not alter the expression of GLAST regardless the fact that at this level, RGCs death was noticed.

It is possible that reduced levels of glutamate and/or glutamate transporter could also occur due to loss of the retinal cells in that glutamate and glutamate transporters reside (Lehre et al., 1997; Martin et al., 2002). The authors further explained that glutamate levels could increase by released from dying neurons or decrease as the synaptic transmission declines as ganglion cells disappeared. A reduction in the level could also reflect Müller cell injury (Martin et al., 2002). These explanations show the complexities of the system, but could be an explanation for the unchanged expression seen under reduced levels of glucose and oxygen.

Another method of glutamate detoxification in the retina after the up take by glia is the conversion of glutamate by glutamine synthetase (GS) into glutamine (Thoreson and Witkovsky, 1999). It has been reported that ischemia-induced ATP depletion inhibits GS activity, because ATP is required to convert glutamate to glutamine (Oliver et al., 1990). Decreased GS activity has been seen in IOP-induced glaucoma in rats (Moreno et al., 2005). Current results indicate that GS mRNA is upregulated with both levels of ischemia, hypoxia and glucose deprivation. In agreement with the findings presented here, Shaked et al., (2002) reported an increase in GS activity in retinal explants isolated from chicken embryos exposed to glutamate, and this enhanced the clearance of extracellular glutamate (Shaked et al., 2002).

A final pathway of glutamate metabolism is the conversion to GABA by GAD. In normal retina, GAD immunoreactivity was found in the inner plexiform layer, inner nuclear layer and ganglion cell layer (Lin et al., 1983; Pusateri et al., 1984; Schnitzer and Rusoff, 1984; Tyler et al., 1995). Similar studies indicated the presence of GAD immunoreactivity in horizontal, amacrine and bipolar cells (Agardh et al., 1987; Mosinger et al., 1987; Yang, 1997; Mariani and Caserta, 1986), but not in the ganglion cell layer (Tyler et al., 1995). It was shown here that GAD mRNA followed the pattern of GLAST and GS expression, specifically an increase in the expression was found in simulated ischemia and glucose deprivation while hypoxia did not alter the expression levels.

5.3.5 Effect of Oxygen and Glucose Deprivation on the Expression of Purinergic Related Signaling Markers

Stimulation of the $P2X_7$ receptor kills retinal ganglion cells in several *in vitro* and *in vivo* models (Niyadurupola et al., 2013; Resta et al., 2005, 2007; Zhang et al., 2005, 2007). The expression of the $P2X_7$ receptor is found in Müller cells and ganglion cells in the retina (Brändle et al., 1998; Ishii et al., 2003). In addition, stimulation of the $P2X_7$ receptors as well as simulated ischemia, mediated RGC death in human organotypic retinal cultures (HORCs) (Niyadurupola et al., 2013). It has also been found that stimulation of the $P2X_7$ receptor opens the pannexin-1 (*PANX1*) hemichannel and activate the caspase-1 cascade (Pelegrin and Surprenant, 2006). In a study by Thompson et al., (2006), oxygen-glucose deprivation resulted in hemichannel-like activity in hippocampal neurons and in experimental retinal ischemia followed by reperfusion in *PANX1* knockout mice, RGCs were protected from death with reduced Ca^{2+} accumulation, inflammation and apoptosis in the absence of pannexin 1 activity (Dvoriantchikova et al., 2012). Current results showed

that simulated ischemia and glucose deprivation rather than hypoxia upregulated *PANX1* mRNA expression in HORCs indicating that glucose rather than oxygen is responsible for this regulation. Upregulation of *PANX1* could lead to enhanced ATP release and activation of $P2X_7$-mediated cell death.

Another gene involved in purinergic signaling is *ENNP2*, codes for the enzyme autotaxin, which has ecto-ATPDase activity. No literature was found related to the expression of this gene in the retina under normal and ischemic conditions. Current results showed full OGD, hypoxia and glucose deprivation all upregulated *ENNP2* mRNA. It is possible that the function of autotaxin in production of LPA may be more important in relation to survival mechanisms than its activity to remove extracellular ATP.

5.3.6 Effect of Oxygen and Glucose Deprivation on the Expression of Müller Cells Marker Expression

In retinal disease, astrocytes, and Müller cells react to injury by increasing GFAP protein expression (Bringmann et al., 2006; Fernandez et al., 2009; Lewis and Fisher, 2003). In IOP-induced ischemic insult in rats, reperfusion caused increased GFAP immunoreactivity in Müller cells as early as one hour and lasted for two weeks (Kim et al., 1998). Similarly, retinal ischemia induced by elevated IOP in mice increased GFAP expression 24hrs after the injury (Ji et al., 2014). GFAP upregulation in ischemia-reperfusion has been linked to glial cells stiffening, the formation of glial scar and exacerbation of injury (Lu et al., 2011). In experimental glaucomatous eyes in rats, GFAP increased in the first two months post-operation and decreased at three and four months (Wang et al., 2000). Similar findings were reported in experimental glaucoma in rats (Xue et al., 2006), after laser-induced elevated IOP (Woldemussie et al., 2004) and in an optic nerve crush rat model (Chen et al., 2002). GFAP has also

been observed to increase in retinal tissue from glaucomatous human eyes (Wang et al., 2002). It might therefore be expected that GFAP would be upregulated in the HORC model with simulated ischemia. However, results from the current study showed that exposure of HORCs to full OGD, hypoxia, and glucose deprivation, as well as reduced levels, did not significantly alter the expression.

5.3.7 Effect of Oxygen and Glucose Deprivation on the Expression of Heat-Shock Protein 1B (*HSPA1B*)

Heat-shock proteins are complex proteins of different molecular weights, which are expressed under stress including ischemia (Dillmann, 1999). Ischemic injury results in rapid HSP-70 mRNA expression of the retina (Lewden et al., 1998). Induction of heat shock proteins in RGCs was found to be protective under conditions of chronic ocular hypertension (Ishii et al., 2003; Ostling et al., 2007) and NMDA-induced excitotoxicity (Ahn et al., 2008; Marcuccilli et al., 1996; Rordorf et al., 1991). In the present study, *HSPA1B* mRNA expression was upregulated under all conditions of oxygen and/or glucose deprivation but the induction seen was not sufficient to fully protect RGCs since OGD was found to cause RGC death.

Overall, the data presented here has indicated that HORCs can be used to investigate RGC death and gene expression following 24hrs insults of oxygen glucose deprivation. It was shown that glucose rather oxygen is a critical factor for retinal survival as evidence by TUNEL staining primarily in RGCs in HORCs. Changes in gene expression have been found such as VEGF, PDGF, LIF, and PEDF. It is possible that some growth factors might be neuroprotective to retinal RGCs and secreted by Müller cells. Therefore, it is interesting to investigate the potential protective effect of these cells when co-cultured with HORCs under stress condition of glucose deprivation.

Chapter 6

Effect of VEGF-A Treatment or Müller cells Co-culture on the Survival of Retinal Ganglion Cells Under Glucose Deprived Conditions

6.1 Introduction

Müller cells span all the retinal layers composing columnar units, a position that enables them to communicate with all retinal neurons and influence their activities (Bringmann et al., 2006; Newman, and Reichenbach, 1996). As previously discussed, neuroprotective effects of Müller cells can be mediated by protection against excitotoxicity as well as the release of neurotrophic factors, growth factors and cytokines (Bringmann et al., 2001, 2009).

Several studies have indicated that VEGF is expressed and released under conditions of glucose deprivation in different cell types (Iida et al., 2002; Satake et al., 1998; Schmid-Brunclik et al., 2008; Shweiki et al., 1995; Stein et al., 1995; Yun et al., 2005). In addition, VEGF-derived from activated Müller cell is one of the growth factors that possess both protective and damaging effects on the survival of retinal neurons (Eichler et al., 2000, 2004; Yafai et al., 2004; Hollborn et al., 2002; Gora-Kupilas and Josko, 2005). It is also important for the retinal and choroidal circulation (Stalmans et al., 2002; Marneros et al., 2005). VEGF has been also found to be a survival factor for MIO-M1 cells and inhibition of VEGF increased apoptosis in these cells (Saint-Geniez et al., 2008). On the other hand, retinal Müller cell-derived VEGF is a major contributor to ischemia-induced neovascularization (Bai et al., 2009). It is involved in the pathogenesis of eye disorders such as diabetic retinopathy (Miller et al., 1997), age-related macular degeneration (Ferrara et al., 2007), retinal ischemia (Bai et al., 2009) and neovascular glaucoma (Horsley and Kahook, 2010).

In chapter 3, it was reported that MIO-M1 cells proliferate in response to oxygen deprivation (4%O_2), glucose deprivation (1.11mM) and reduced levels of both (1.11mM glucose/4%O_2). This proliferation may be as a result of increased production of mediators acting as growth factors. They have also been shown to have effects on neuronal survival (Bringmann et al., 2009). A significant upregulation of VEGF mRNA was seen with complete deprivation of oxygen and/or glucose, and increases were seen with reduced oxygen and glucose, although these were not found to be statistically significant. Investigating VEGF protein secretion from MIO-M1 cells and its involvement in survival and proliferation under conditions of glucose deprivation (1.11mM) was therefore of interest.

The interaction between Müller cells and other cells can be simulated *in vitro* by the co-culture technique. Several studies have shown that glial cells in co-culture release stimulatory or inhibitory factors, which are important for the proliferation or survival of other cells as well as protecting against different stresses. For example, co-culture of astrocytes with cerebellar neurons enabled uptake of glutamate by astrocytes (Brown, 1999; Amin and Pearce, 1997) and protected neurons against oxidative stress-induced damage (Drukarch et al., 1998). Similarly, co-culture of rat RGCs with Müller glia has also been found to safeguard the RGCs against glutamate and NO toxicity (Kitano et al., 1996; Kawasaki et al., 2000). In addition, co-culture of neonatal rat RGCs with rat Müller glia or in Müller cells derived conditioned medium enhanced the survival of RGCs (Raju and Bennett, 1986). It was also found to enhance survival of RGCs in co-culture in different species (García et al., 2002; García et al., 2003; Unterlauft et al., 2014).

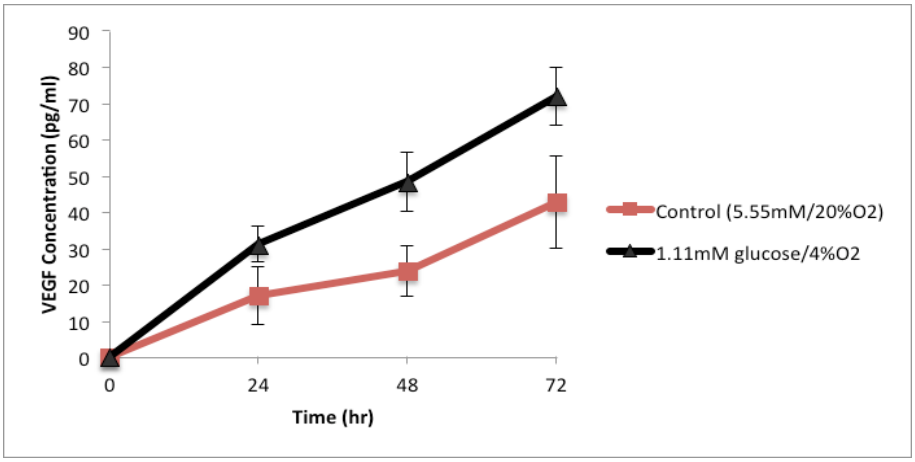
The specific aims of the present experiments were to investigate the VEGF release from MIO-M1 under conditions of oxygen and glucose deprivation. In addition, to investigate the possible neuroprotective effects of exogenous VEGF (50ng/ml) treatment on the survival of RGC in in HORCs under conditions of glucose deprivation for 24hrs. Finally, to investigate the effects of co-culture of MIO-M1 cells with HORCs under reduced glucose (1.11mM), a level of glucose deprivation that had been found to cause MIO-M1 cell proliferation; to see if this interaction between MIO-M1 cells and the retinal explants in culture may protect RGCs from death under glucose-deprived condition.

6.2 Results

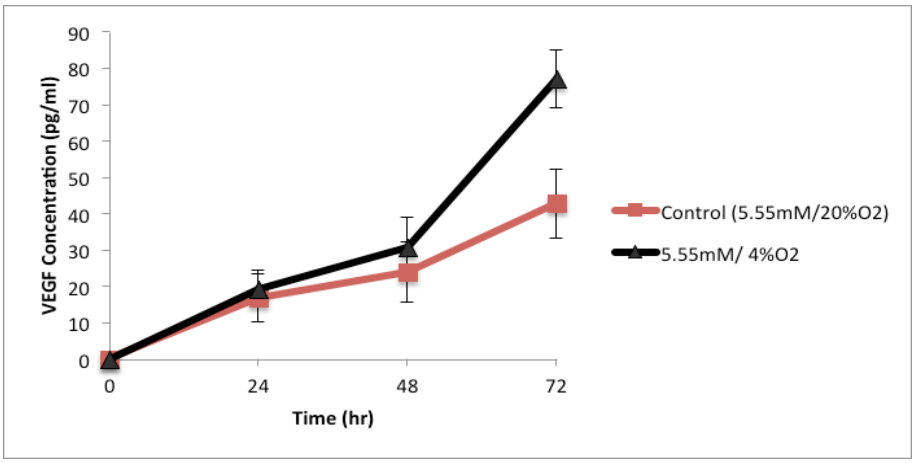
6.2.1 Effect of Reduced Glucose and Oxygen Levels on VEGFA Release From Human Müller Cells (MIO-M1)

Under control conditions (5.55mM glucose/20%O_2), MIO-M1 cells secreted VEGF and it seems that VEGF accumulated with time (Figure 6.1A). With reduced glucose and oxygen (1.11mM glucose/4%O_2), the rate of increase was greater (Figure 6.1A). Similar trends were seen with oxygen deprivation and glucose deprivation alone (Figure 6.1 B&C).

(A)



(B)



(C)

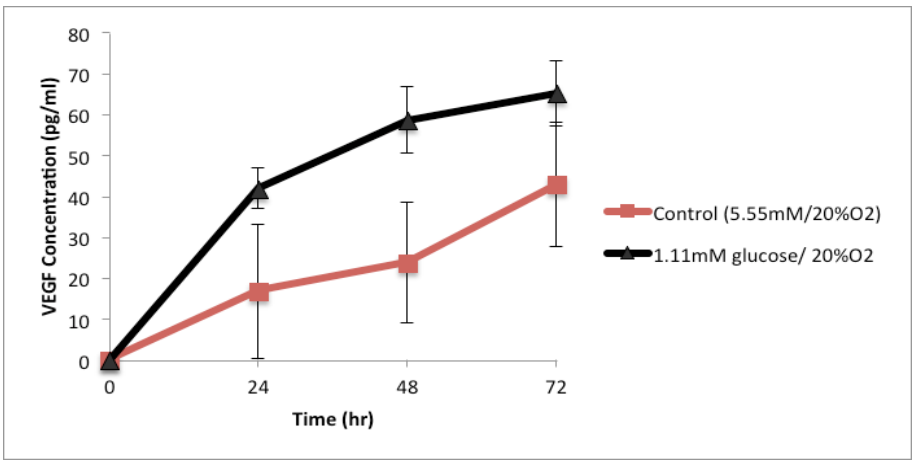


Figure 6.1: Release of VEGF-A (pg/ml) by human Müller cells (MIO-M1) after 24, 48, and 72hrs exposure to (A) reduced levels of glucose and oxygen (1.11mMglucose/4%O_2), (B) oxygen deprivation (5.55mMglucose/4%O_2) and glucose deprivation (1.11mMglucose/20%O_2) compared to control (5.55mM glucose/20% O_2) (mean ± s.e.m, n=4, t-test).

6.2.2 Effect of Exogenous VEGF-A Treatment (50ng/ml) on the LDH Release and RGCs Survival in HORCs

Human Müller cells (MIO-M1) released VEGF under the examined conditions and this could potentially play a role in neuronal survival. The effect of exogenous VEGF (50ng/ml) on the survival of RGCs in HORCs was therefore examined under 1.11mM glucose.

Retinal injury was initially evaluated by LDH release in VEGF-treated HORCs exposed to glucose deprivation (1.11mM) for 24hrs. HORCs treated with normal glucose level (5.55mM) and VEGF caused no alteration in LDH release compared to control. Glucose deprivation of HORCs resulted in a significant (*$p \leq 0.05$; n=4) increase (3-fold) in LDH release. Treatment with VEGF (50ng/ml) did not reduce the increase in LDH release caused by glucose deprivation with no significant difference between 1.11mM glucose-VEGF and 1.11mM glucose alone (Figure 6.2).

Immunohistochemistry of NeuN and TUNEL staining from HORCs treated under control and glucose deprivation (1.11mM) condition with and without VEGF (50ng/ml) are shown in Figure 6.4.

The number of NeuN-positive RGCs was significantly reduced by approximately 25% in glucose-deprived HORCs treated with VEGF (50ng/ml) (*$p \leq 0.05$; n=4) compared to control. Although the NeuN count was not significantly reduced with 1.11mM glucose (Figure 6.4A), a significant 3-fold increase (*$p \leq 0.05$; n=4) in the number of TUNEL-positive cells was observed (Figure 6.4B). In glucose- deprived HORCs treated with VEGF, there appeared to be a decrease in TUNEL-positive RGCs, but this difference was not significant. Likewise, under control conditions VEGF appeared to reduce apoptotic RGCs but significant again was not gained.

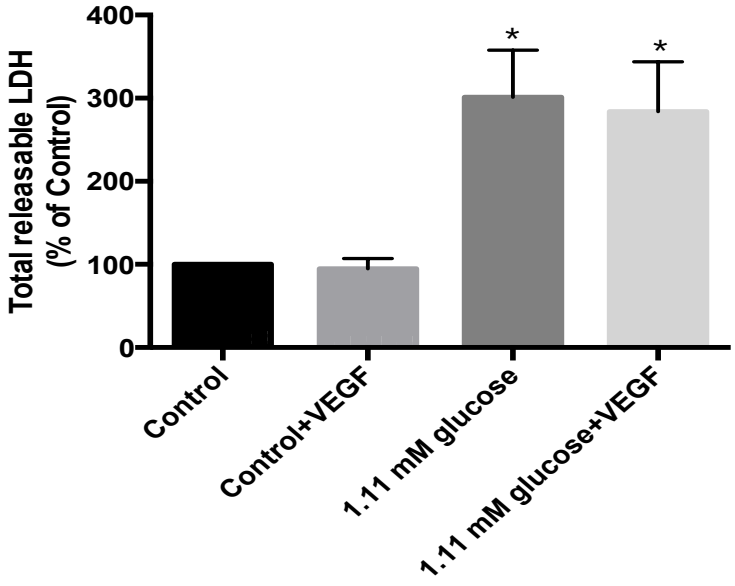


Figure 6.2: Effect of VEGF-A treatment (50ng/ml) on the release of LDH by HORCs under control (5.55mM glucose/20%O_2) and glucose deprivation (1.11mMglucose/20%O_2) for 24hrs (mean ± s.e.m, * $p \leq 0.05$; n=4, ANOVA).

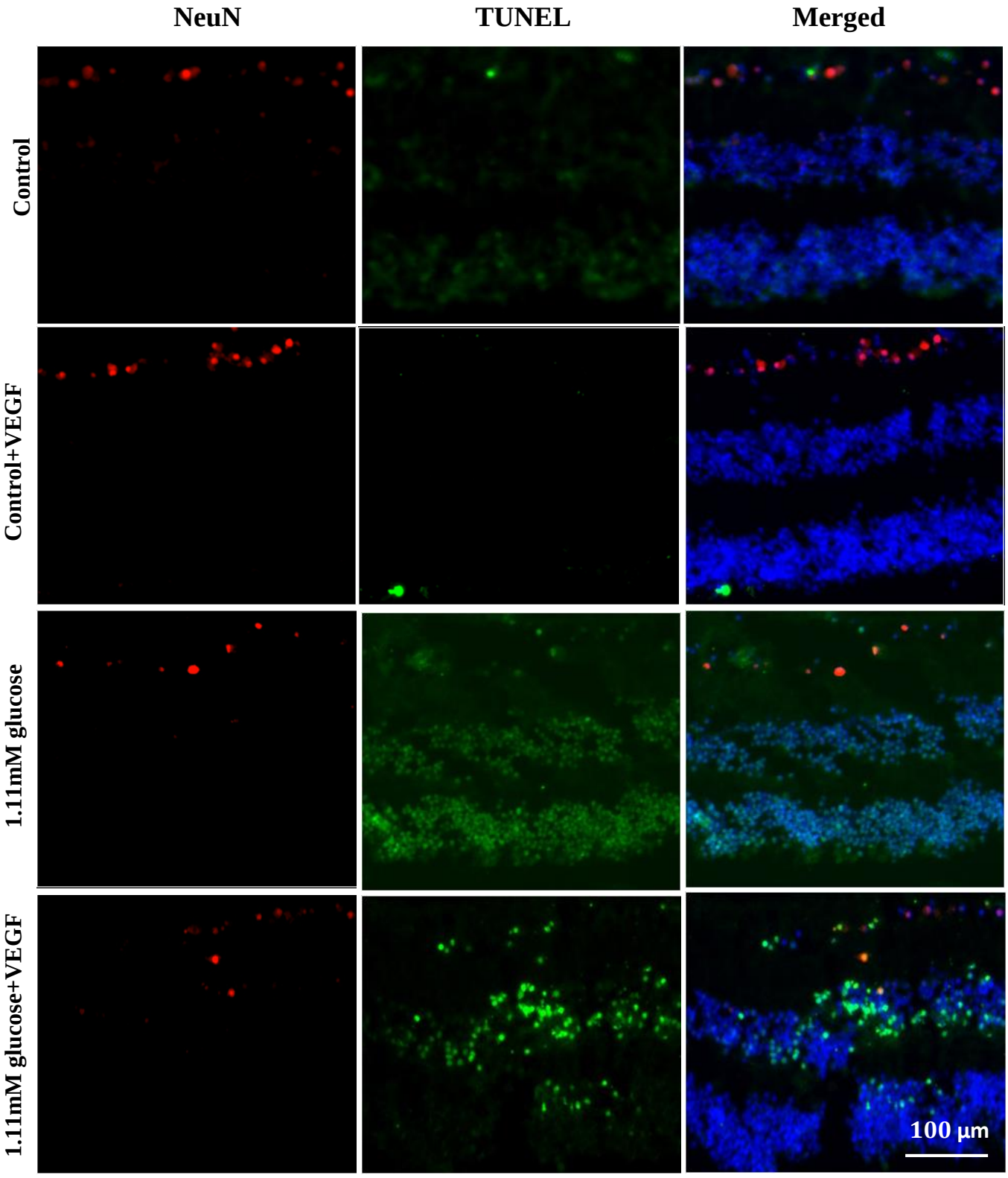


Figure 6.3: Representative immunofluorescence photomicrographs of NeuN and TUNEL-labelling in HORCs labelling after 24hrs exposure to control, control +VEGFA, 1.11mM glucose, 1.11mM glucose +VEGF-A. DAPI = blue, GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer.

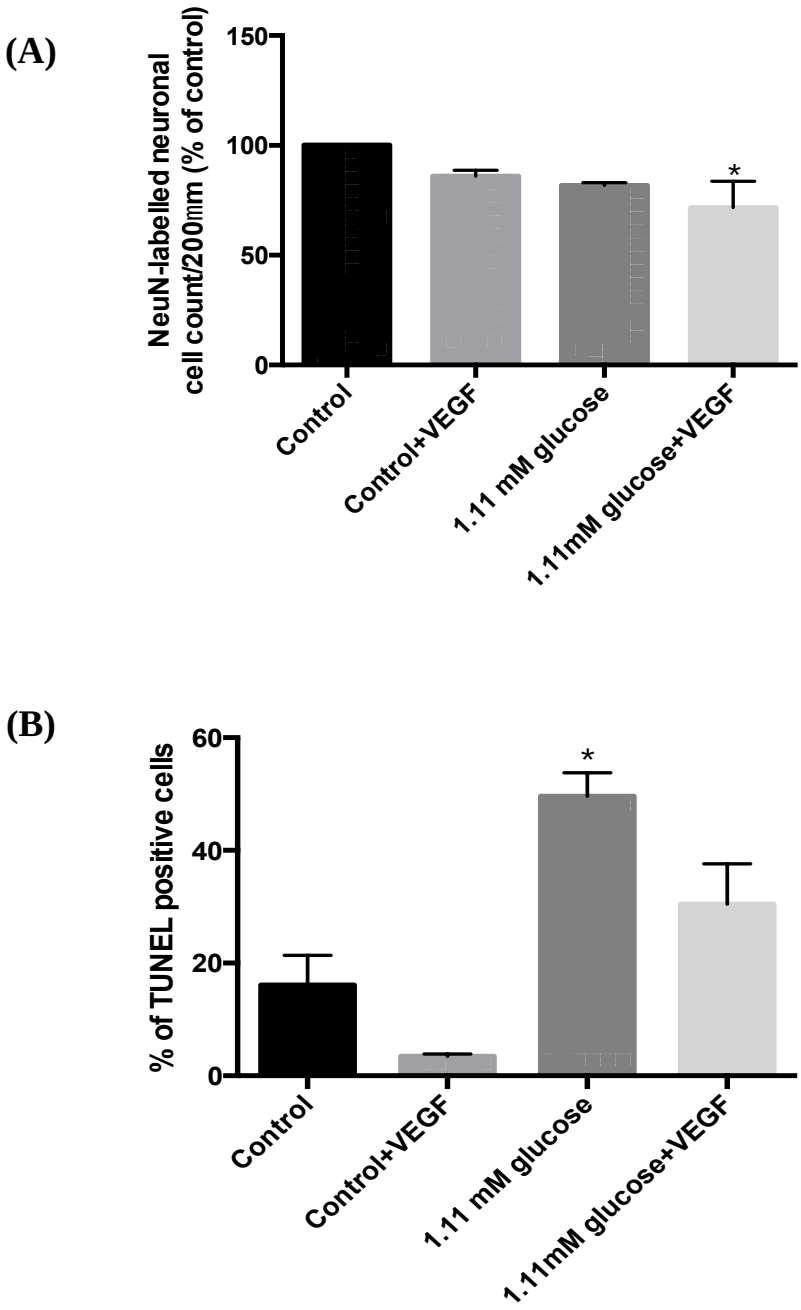


Figure 6.4: Effect of VEGF-A (50 ng/ml) RGCs death under glucose deprivation on (A) Average number of NeuN-labelled cells from three intact 200μm from 8 sections per sample expressed as percentage of control and (B) Percentage of TUNEL-positive ganglion cells, exposed for 24hr to glucose deprivation (1.11mM) (*$p \leq 0.05$; n=4, ANOVA).

6.2.3 Effect of Glucose Deprivation and MIO-M1 Co-culture on LDH Release and Survival of Retinal Ganglion Cell in HORCs

Initially, LDH measurement was used to assess the overall effect of co-culture with MIO-M1 cells on HORC integrity with 1.11mM glucose. From previous experiments, it would be expected that 1.11mM glucose have no effect on the cytotoxicity of MIO-M1 cells. Data presented here show similar results in which the LDH release from MIO-M1 cells was similar under control and 1.11mM glucose conditions. Culturing HORCs with MIO-M1 cells at normal glucose levels (5.55mM) for 24hrs did not increase the releasable LDH significantly compared to control (HORCs only). As shown previously, exposure of HORCs to 1.11mM glucose for the same time period resulted in a significant increase (*$p \leq 0.05$; n=4) in LDH levels compared to control HORCs. When HORCs were cultured with MIO-M1 cells, a significant increase (*$p \leq 0.05$; n=4) in LDH was found compared to control alone (Figure 6.5). With no significant difference between HORCs cultured in the presence or absence of MIO-M1 cells

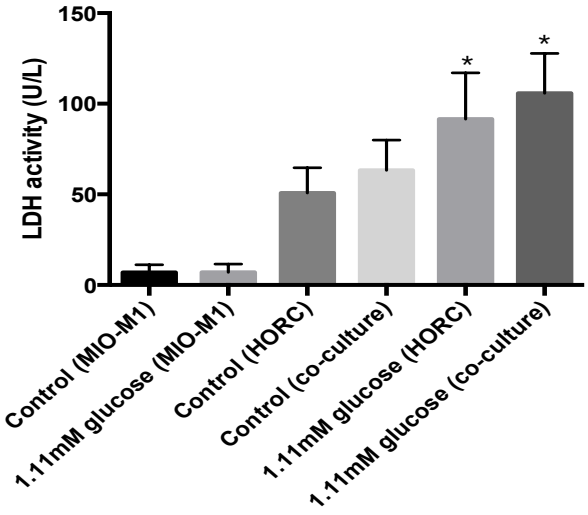


Figure 6.5: Effects of co-culture of MIO-M1 cells with HORCs on LDH release (U/L) when exposed to control (5.55mM glucose/20%O_2) or glucose deprivation (1.11mMglucose/20%O_2) conditions for 24hrs (mean ± s.e.m, *$p \leq 0.05$; n=4, ANOVA).

RGCs loss was assessed by immunohistochemistry where the numbers of NeuN-labelled neurons in the retinal ganglion cell layer were counted in combination with TUNEL staining. Representative images are shown in Figure 6.6 and quantification in Figure 6.8.

Control HORCs cultured alone under normal glucose level (5.55mM) for 24hrs did not affect the retinal layer integrity or increased TUNEL staining. Increased TUNEL staining was however noted in control HORCs in co-culture. In HORCs-treated with 1.11mM glucose, TUNEL staining was also found in all layers which appeared more intense in the RGCs layer, inner nuclear layer, outer nuclear layer in HORCs with MIO-M1 co-culture under glucose deprivation compared to controls indicating that MIO-M1 cells increased retinal damage (Figure 6.5). A significant reduction in the mean number of NeuN-labelled by approximately 25% (*$p \leq 0.05$; n=4) was detected when HORCs were co-cultured with 1.11mM glucose-treated in co-culture with MIO-M1 cells (Figure 6.7A). The number of NeuN-labelled neurons was not reduced significantly different to control in HORCs treated with 1.11mM glucose or 5.55mM in combination with MIO-M1 cells (Figure 6.7A). A similar percentage of TUNEL-positive cells in the RGCs layer was found in HORCs co-cultured under control conditions and 1.11mM glucose. Control HORCs co-cultured with MIO-M1 cells in 1.11mM glucose has however more TUNEL detected in other layers (Figure 6.7B).

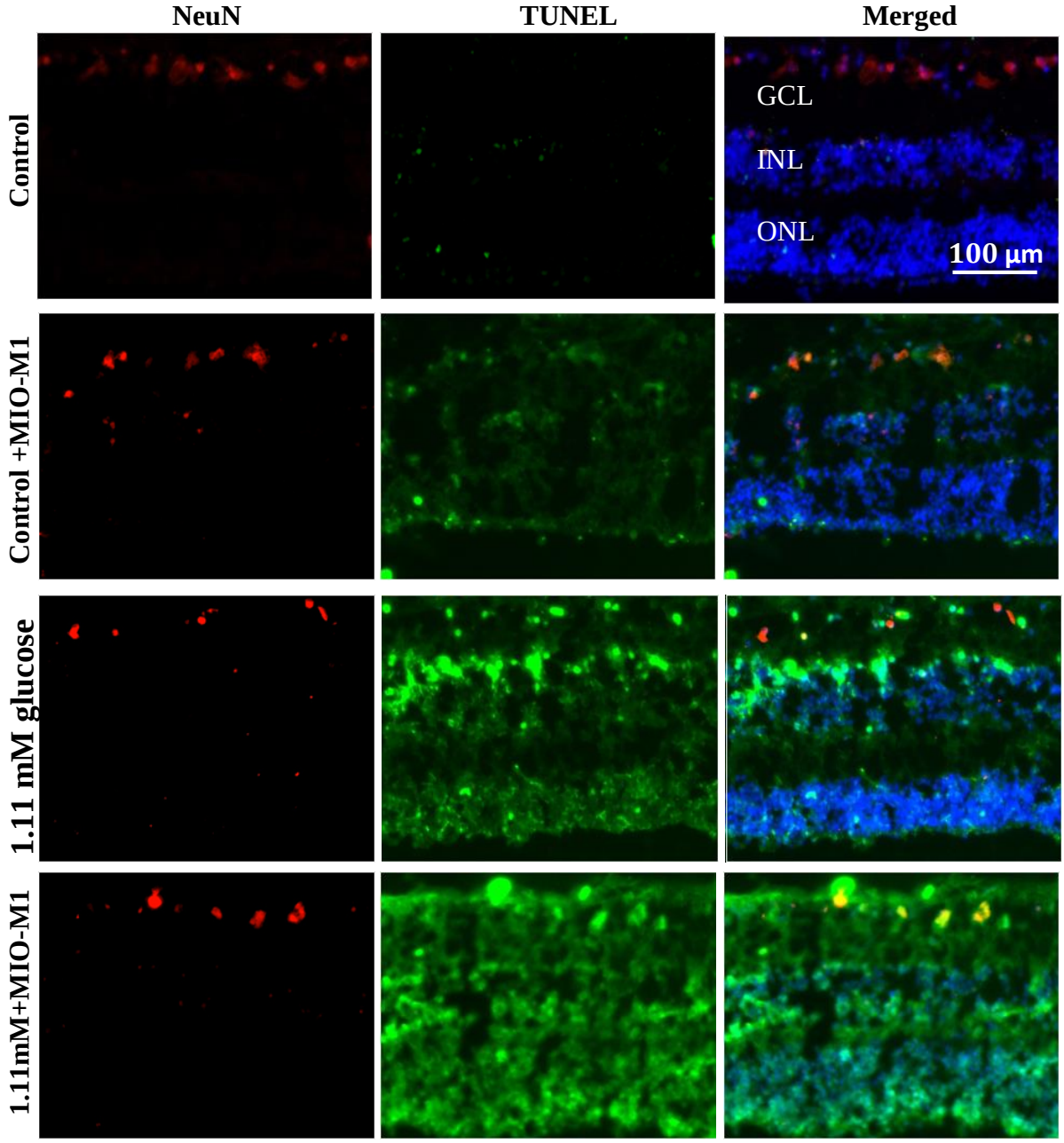


Figure 6.6: Representative immunofluorescence photomicrographs of NeuN and TUNEL-labelling in HORCs labelling after 24hrs exposure to control, control co-cultured with MIO-M1, 1.11mM glucose, 1.11mM glucose co-cultured with MIO-M1. DAPI = blue, GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer.

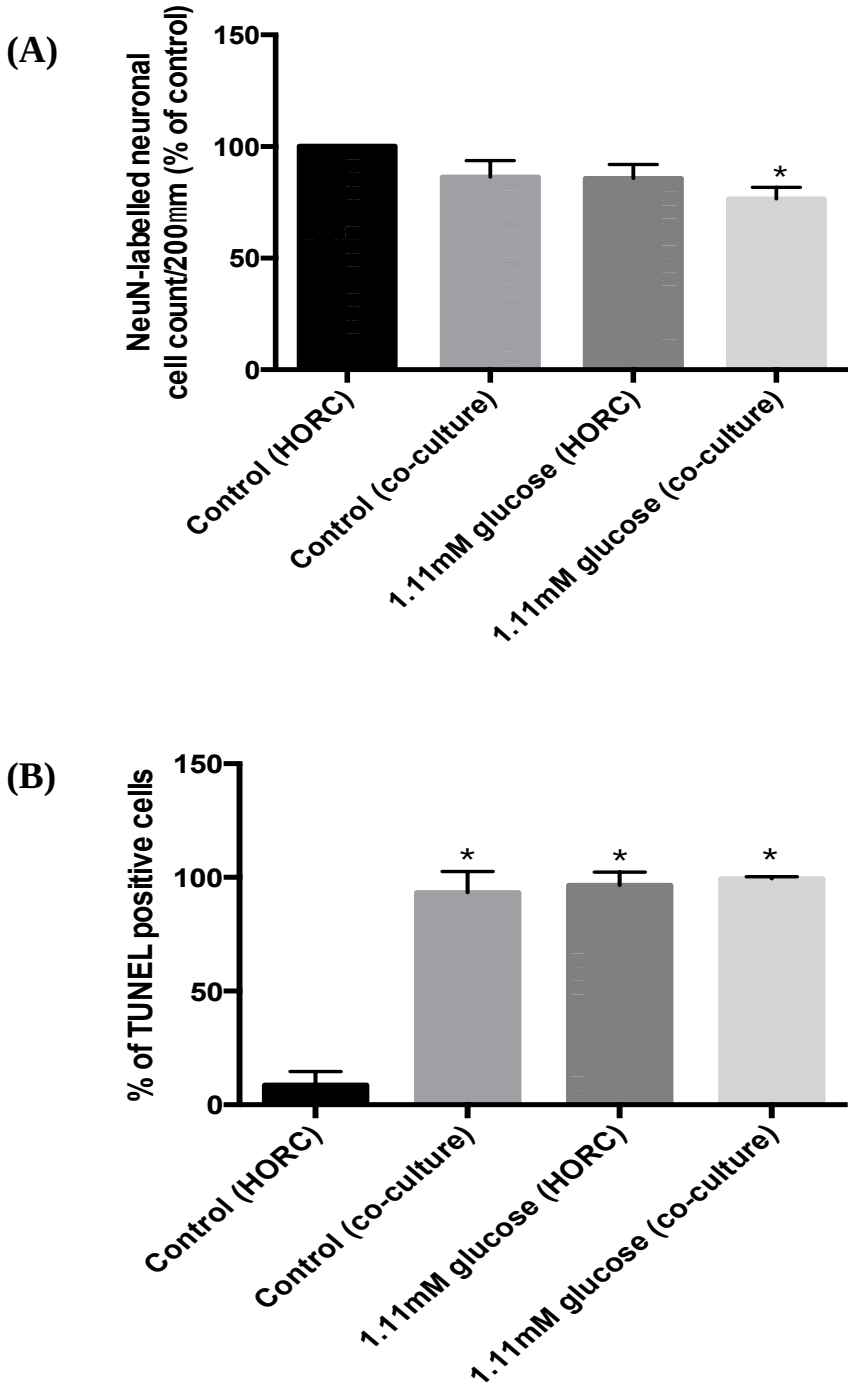


Figure 6.7: Effect of co-cultured MIO-M1 and HORCs under control (5.55mM) and glucose deprivation (1.11mM) for 24hrs on the survival of RGCs (A) Average number of NeuN-labelled cells from three intact 200μm from 8 sections per sample expressed as percentage of control and (B) Percentage of TUNEL-positive ganglion cells, exposed for 24hr to glucose deprivation (1.11 mM) (*$p \leq 0.05$; n=4, ANOVA).

6.2.4 Effect of Glucose Deprivation and MIO-M1 Co-culture on the VEGF-A Release from HORCs

In earlier experiments, the regulation of VEGF under different glucose deprivation was investigated and it was found that VEGF mRNA in cultured primary Müller cells and HORCs is upregulated when glucose deprivation conditions were applied for 24hrs. Secretion of VEGF protein therefore was also investigated in the co-culture experiments.

Under 1.11mM glucose, VEGF released by MIO-M1 cells increased by approximately 2-fold (41.85±16.24 pg/ml) after 24hrs compared to control (16.85±4.87) while retinal explants cultured alone under control condition (5.55mM glucose/20%O_2) resulted in VEGF level of 23.17±7.98 pg/ml. Control HORCs in co-culture caused 3-fold increase in VEGF concentration of approximately 60.1± 15.37 pg/ml. Further increase was detected in HORCs treated with 1.11mM glucose alone of approximately 4-fold (80.67±18.14 pg/ml) compared to control. When HORCs were cocultured with MIO-M1 cells under similar level of glucose deprivation this caused approximately 10-fold increase in VEGF (251.76±196 pg/ml) compared to HORC control (Figure 6.8). This suggests that both cultured MIO-M1 cells and HORCs release VEGF under condition of glucose deprivation. It is worth mentioning that there was variation in the released protein between retinal explants under same condition indicating donor variability.

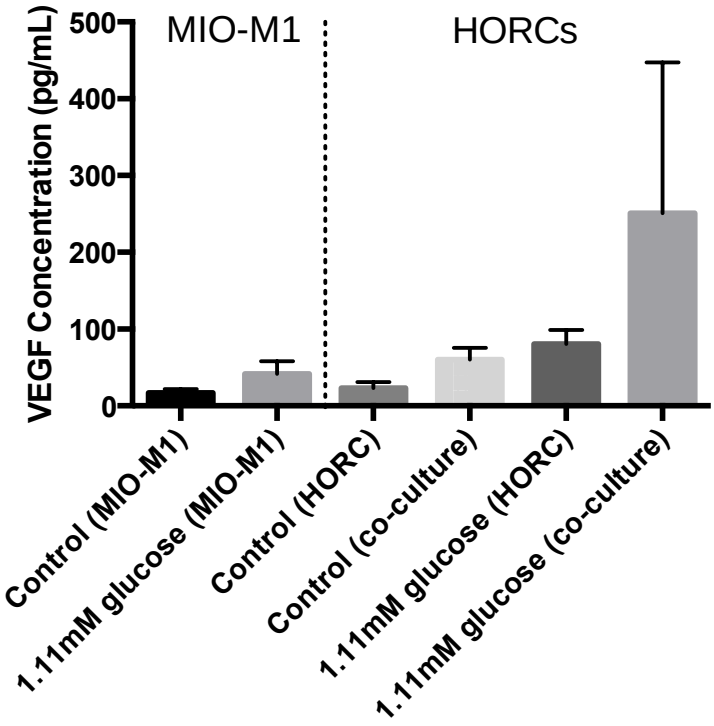


Figure 6.8: VEGF-A release from MIO-M1 and HORCs under control condition and glucose deprivation (1.11mM) when cultured alone and together (*$p \leq 0.05$; n=4, ANOVA).

6.3 Discussion

Retinal glial cells can exert both neuroprotective and neurotoxic effects through releasing different factors (Bringmann et al., 2006; Heidinger et al., 1999; Kawasaki et al., 2000; Tezel and Wax, 2000). Müller glia activation has been reported to occur in different retinal injuries and conditions such as ischemia-reperfusion (Pannicke et al., 2005), axotomy of the optic nerve (Chun et al., 2000), glutamate excitotoxicity (Honjo et al., 2000; Kawasaki et al., 2000) and experimental glaucoma (Carter-Dawson et al., 1998). In Chapter 3, it was reported that MIO-M1 cells proliferate in response to glucose deprivation (1.11mM), hypoxia (4%O_2) and glucose/oxygen deprivation (1.11mM glucose/4%O_2). This proliferation may be associated with increased production of protective mediators such as growth factors. In an attempt to protect RGCs against glucose deprivation-induced loss, retinal explants were treated with VEGF and in other experiments, co-cultured with MIO-M1 cells under the condition of glucose deprivation (1.11mM).

It has been shown in different cell lines that glucose deprivation increased VEGF expression and release (Brooks et al., 1998; Eichler et al., 2000; Iida et al.2002; Satake et al., 1998; Stein et al., 1995; Shweiki et al., 1995; Yun et al., 2005). The VEGF concentration in astrocytes after exposure to hypoxia for 24hrs was approximately 10pg/ml in cytoplasmic fraction, and 350pg/ml in the medium and higher levels of VEGF were found after exposure to anoxia (Schmid-Brunclik et al., 2008). Current data show that MIO-M1 cells release VEGF after exposure to oxygen deprivation (4%O_2), glucose deprivation (1.11mM) and reduced levels of both (1.11mMglucose/4%O_2) at different time points. Interestingly, MIO-M1 cells also showed increasing levels of VEGF over time at normal glucose and oxygen conditions (control conditions). Although, it is well known that hypoxia is the most

powerful stimulant to VEGF release (Witmer et al., 2003), the level of protein production by MIO-M1 cells was found in the present study to increase as a result of glucose deprivation alone as well as by reduced levels of both glucose and oxygen (1.11mM glucose/4%O_2). The increase in VGEF proteins by MIO-M1 cells under 1.11mM glucose is thought to be one factor responsible for cell proliferation and survival and thus it is also possible that the released growth factor may protect RGCs in HORCs.

In vitro studies have shown that VEGF could protect neurons against hypoxia, glutamate excitotoxicity and deprivation of oxygen, or glucose (Jin et al., 2000; Tolosa et al., 2008). In addition, VEGF was found effective in protecting primary cultures of rat cerebral cortical neurons from hypoxia and glucose deprivation (Jin et al., 2000) while a higher dose of VEGF (100ng/ml) reduced apoptosis in neuronal cultures exposed to hypoxia (Ding et al., 2005; Jin et al., 2001). Another isoform of VEGF (VEGF120; 100ng/ml) inhibited apoptosis of retinal explant cultures in an ischemia-reperfusion model in rats (Nishijima et al., 2007). Therefore, we sought to explore the neuroprotective effect of VEGF (50ng/ml) on HORCs exposed to 1.11mM glucose. Similar elevated levels of LDH were released from HORCs exposed to 1.11mM glucose with and without VEGF indicating that addition of the growth factor did not protect against glucose deprivation-induced damage. Although RGC numbers significantly reduced with VEGF treatment under glucose deprivation, a slight reduction in apoptosis was seen in both control and glucose deprivation in VEGF-treated HORCs. It is therefore not possible to say that protection was found, but it is worth considering that the current concentration of VEGF used would not be effective in overcoming the damage induced by glucose deprivation, and further experiments would be needed to determine the appropriate and neuroprotective dose.

On the other hand, VEGF cannot be excluded as a cause of damage rather than a protective agent as the low glucose and VEGF together had less RGC.

Another approach used here to investigate the effects of secreted factors from the MIO-M1 cells on RGCs survival was the co-culture of MIO-M1 cells with retinal explants. Co-culture of HORCs with MIO-M1 did not result in RGCs protection, but rather an increase in cell death even in control HORC was observed. It therefore appears that there are factors secreted into the medium by MIO-M1 cells that diffuse into the retinal explant to activate apoptotic death pathways as evidence by TUNEL data presented here. VEGF released by activated Müller cells has been shown to have both neuroprotective and detrimental effects (Bringmann et al., 2006). Glial activation has been reported to exacerbate disease progression, increasing vascular permeability, and causing neovascularization by releasing VEGF (Penn et al., 2008). Schmid-Brunclik et al., (2008) have also shown that glucose-free astrocyte-conditioned media contained an elevated concentration of VEGF, which increased retinal injury. VEGF causes endothelial cell hyperplasia resulting in capillary non-perfusion, and more damage follows by inducing vessel dilation and leakage, focal hemorrhages, microaneurysms, and retinal neovascularization (Tolentino et al., 2002).

In the present study, VEGF protein was detected in HORCs exposed to glucose deprivation alone and when co-cultured with MIO-M1 cells. Previous results (Chapter 5) also showed that VEGF mRNA is upregulated in HORCs exposed to 1.11mM glucose. The source of VEGF in current experiments originates from both retinal explants and MIO-M1 cells with some evidence of synergistic effects. Also there was a large variation in the VEGF level between retinal explants suggesting donor variability in response to the experimental condition. It is possible that the released VEGF by MIO-M1 cells and HORCs contributed to more damage to retinal explants

and caused RGCs loss. In co-culture experiments under different conditions (rat Müller cells with RGC under hypoxic condition 0.2%O_2) RGC protection was not seen but viability was increased in normoxic condition. The author explained that reduced neuroprotection of Müller glial cells might be linked to changes in the level of released survival factors and/or to the deleterious effect of other factors secreted by reactive glial cell (Unterlauft et al., 2012). This may explain in part the increase in apoptotic cell death in control HORCs co-cultured with MIO-M1 cells. The exposure of MIO-M1 cells to HORC in co-culture may release different factors from both which led to activation of the cells and enhanced RGCs death even in control conditions. Therefore, anti-VEGF treatment may be considered as a potential protective agents as favorable visual outcome was obtained in different conditions including age-related macular degeneration (Campochiaro, 2007), angiomatous proliferation (Cho et al., 2015), proliferative diabetic retinopathy (Avery et al., 2006), ischemic central retinal vein occlusion (Brown et al., 2014), neovascular glaucoma (Horsley and Kahook, 2010; Park et al., 2012) and for the treatment of glioblastoma (Gerstner et al., 2009). More experiments are needed to investigate the role of anti-VEGF for protection against glucose deprivation induced damage as well as retinal ischemia. Experiments using the inhibitor would help determine the role of VEGF in either protecting the cells mediating neuronal cell death.

Other mechanisms should also be considered for mediation of neurotoxic effects by glial cells in culture. Chronic activation of Müller cells leads to inflammatory processes by the production of cytokines such as TNF-α and interleukin (IL)-1β and these mediate RGC death (Tezel and Wax, 2000; Kitaoka et al., 2007; Tezel et al., 2012). Excess production of NO increases oxidative stress and contributes to toxic damage in neurons and photoreceptors (de Kozak et al., 1997; Kashiwagi et al., 2001).

Kashiwagi et al., (2001) suggested that when glial cells proliferate, they may express fewer receptors and possess limited enzyme activity, which may exert neurotoxic effects on retinal RGCs. This may explain the current results as MIO-M1 cells usually undergo proliferation when exposed to 1.11mM glucose and, therefore, were unable to eliminate toxins such as glutamate or ROS derived from HORCs and MIO-M1 cells if any in the medium.

Further studies are needed to clarify if pro-inflammatory cytokines are released in the current model under a condition of glucose deprivation (1.11mM) and thus contribute to RGCs death in HORCs when co-cultured with MIO-M1 cells.

In conclusion, alterations in the cellular functions of glial cells when exposed to glucose deprivation that led to proliferation as well as co-culture with HORCs can result in toxic rather than beneficial effects on the RGCs. VEGF is released from MIO-M1 cells and possibly by retinal explants when exposed to 1.11mM glucose and it may contribute to apoptotic RGCs death. Anti-VEGF may provide therapy for the management of RGC degeneration in retinal ischemia. Although the relationship between neurodegeneration and neuroprotection would need further investigation. Pro-inflammatory cytokines may play a role, and further investigation is needed to reveal the secreted factors by MIO-M1 cells, which led to more damage to retinal explants in the current model.

Chapter 7

Effect of High Glucose on the Gene Expression and Survival of Human Müller Cells (MIO-M1) and RGCs in HORCs

7.1 Introduction

As shown previously, VEGF is not only regulated by hypoxia. VEGF mRNA was also found to be upregulated under full glucose deprivation and reduced glucose (1.11mM) both in MIO-M1 cells and HORCs. Furthermore, the influence of VEGF in retinal diseases is not limited to conditions where the oxygen and glucose are deprived; it has been implicated in other retinal conditions such as diabetic retinopathy (DR) in which glucose levels are raised and responsible for vascular complications (Antonetti et al., 2012) as well as loss of neuronal cells in the retina (Martin et al., 2004; Park et al., 2003). In common with glaucoma, several studies have provided evidence suggesting diabetes-induced degeneration of RGCs in humans showing that RGCs undergo apoptosis and that there was increased expression of Bax, caspase-3 and caspase-9 in RGCs from diabetic patients (Abu-El-Asrar et al. 2004; Abu El-Asrar et al. 2007; Barber et al. 1998; Oshitari et al., 2008). Moreover, neovascularization is a serious complication of several retinal diseases including DR and neovascular glaucoma (Gao et al., 2001; Hamanaka et al., 2001; Lim et al., 2009; Mason et al., 2015; Miller et al., 1997; Tripathi et al., 1998). VEGF is part of the pathophysiological changes and anti-VEGF therapy is suggested as a potential therapy in treatment of these diseases (Dong et al., 2014; Horsley and Kahook, 2010; Jo et al., 2006; Park et al., 2012; Simo and Hernandez, 2008). The data presented so far in this thesis has related to deprivation of glucose. In this final chapter, the effects of high glucose are investigated in research relating predominantly to diabetic retinopathy.

DR is a microvascular complication of diabetes mellitus (Liew et al., 2014). In the UK, 3.3 million people were suffering from diabetes in 2014 (Quality and Outcomes Framework 2012/3), and this number is expected to reach 5 million by 2025 (AHPO diabetes prevalence model). DR affects almost three-quarters of patients who have had diabetes for more than 15 years (Klein et al., 1984) and it is the leading cause of vision loss in adults of working age (Liew et al., 2014). Elevated blood glucose is the main pathogenic factor contributing to the development of biochemical and cellular alterations in DR and leads to two major complications, macular oedema, and neovascularization (The Diabetes Control and Complications Trial Research Group, 1993). The initial alteration of DR leading to macular oedema is termed microangiopathy (Mohamed et al., 2007), in which vascular permeability increases causing a passive influx of plasma into the retina (Aiello et al., 1998). The term "proliferative diabetic retinopathy" or PDR describes the advanced stage of retinopathy in which signs of neovascularization or abnormal, newly present blood vessels are formed in the retina and can spread into the vitreous. These vessels may bleed into the vitreous, and as they are attached to the retina with fibrous tissue, they can pull and detach the retina (Antonetti et al., 2012; Crawford et al., 2009; Fong et al., 2004). High glucose has been shown to cause a direct deleterious effect to both pericytes and endothelial cells leading to damage of vessel walls, and occlusion of some vessels can occur (Nehls and Drenckhahn, 1991). Blockage of retinal capillaries can then cause hypoxia-ischemia, which stimulates signalling pathways and further increases vascular permeability that exacerbates damage (Hamanaka et al., 2001; Linsenmeier et al., 1998; Schroder et al., 1991).

Beyond the vascular abnormalities, neurodegenerative changes occur as a consequence of chronic high glucose in DR; increased apoptosis of ganglion cells,

glial cell reactivity, and altered glutamate metabolism have all been reported (Barber et al., 1998; Lieth et al., 1998; Mizutani et al., 1998). Studies have shown that glutamate concentration in the retina can increase as a result of blood-retinal barrier impairment in which an increase as little as 5μM glutamate can be lethal to neurons (Castillo et al., 1997; Do carmo et al., 1998; Gowda et al., 2011; Lieth et a., 1998). Furthermore, Müller cell dysfunction has been reported in diabetes in which the ability to convert glutamate into glutamine is reduced which also would increase retinal glutamate concentration (Lieth et al., 1998). A study by Yu et al., (2009) revealed that GS mRNA expression and activity progressively decreased over a period of 12 months after induction of diabetes in rats. It has also been found that GLAST activity is reduced in diabetes, compromising the ability of Müller cells to regulate glutamate (Li et al., 2002). Investigators have found that GLAST has redox-sensing sites and oxidation reduces GLAST function, which may contribute to the changes were seen in the diabetic retina (Lieth et al., 2000; Kowluru et al., 2001; Trotti et al., 1998).

Activation of Müller cells has been shown to occur in human and rat diabetic retina (Mizutani et al., 1998; Lieth et al., 1998; Rungger-Brandle et al., 2000; Barber et al., 2000; Zhao et al., 2015). Rungger-Brandle et al., (2000) suggested that glial activation is an early feature of retinopathy that follows leakage of the blood-retinal barrier. In an animal model of diabetes, overexpression of GFAP was observed three months after induction of diabetes in rats (Lieth et al., 1998, Rungger-Brandle, Barber et al., 2000, Mizutani et al., 1998). Müller cell activation in diabetes, as in other retinal injuries, results in release of growth and neurotrophic factors including VEGF (Eichler et al., 2000), PEDF (Eichler et al., 2004), TGFβ (Ikeda et al., 1998), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (Ali et al., 2011).

Much research has focused on VEGF as the main growth factor participating in the pathogenesis of DR because of its potent angiogenic activity, and its role in the control of vascular permeability and endothelial cell proliferation (Aiello et al., 1994; Simo et al., 2002). VEGF was detected in the vitreous fluids from patients with diabetic retinopathy (Aiello et al., 1994), proliferative diabetic retinopathy (Shinoda et al., 2000; Hernandez et al., 2001; Simo et al., 2002) and ischemic occlusion of the central retinal vein in diabetes (Aiello et al., 1994). In chronic diabetes in rats, VEGF expression increased in the GCL, INL, and ONL, with an increase in the number of capillaries and VEGF protein, detected in vascular and perivascular structures (Hammes et al., 1998). Also, expression of VEGF receptors was found to increase in retinas of diabetic rats (Gilbert et al., 1998). In *post-mortem* human eyes of individuals with diabetes, those without microvascular leakage, as well as controls, were negative for VEGF staining (Hofman et al., 2001) while VEGF receptors were expressed in the presence of leaky microvessels (Witmer et al., 2002). In addition, the number of VEGF-stained vessels in human diabetic retinas was higher than non-diabetics (Mathews et al., 1997). This may indicate that VEGF expression and release is linked to vascular changes. Furthermore, Amin et al., (1997) found that VEGF expression precedes retinal neovascularization in *post-mortem* human retinas and optic nerves of humans with diabetes without proliferative retinopathy. Multiple retinal cells express PEDF to counterbalance the angiogenic effect of VEGF (Barnstable and Tombran-Tink, 2004). In patients with DR, decreased levels of PEDF in ocular fluids and vitrectomy specimens suggest that the loss of PEDF contributes to diabetes-induced neovascularization and neuronal loss (Ogata et al., 2002; Barnstable and Tombran-Tink, 2004). Down-regulation of PEDF in DR also contributes to inflammation and vascular leakage that increases VEGF and TNF-α secretion by

retinal Müller cells (Zhang et al., 2006) and over-expression of PEDF inhibits retinal inflammation and neovascularization (Park et al., 2011). The imbalance between VEGF and PEDF is proposed to lead to active proliferative DR (Ogata et al., 2002).

In addition to VEGF and PEDF, other growth factors or cytokines are also involved in regulating angiogenesis including PDGF A/B and bFGF (Praidou et al., 2010; Simo et al., 2006). PDGF has been implicated in retinal neovascularization in different pathologies including age-related macular degeneration and proliferative diabetic retinopathy (Bo et al., 2014; Cox et al., 2003; Mori et al., 2002; Velez et al., 2012). The levels of PDGF isoforms, -AA, -AB, and -BB were elevated in serum and vitreous of patients with PDR (Praidou et al., 2009) and in a study by Gong et al., (2014), serum levels of PDGFA/B and bFGF were increased significantly in diabetic compared to non-diabetic rats. Studies have shown that a combination of anti-VEGF and anti-PDGF-BB is more effective than blocking VEGF-A alone for the treatment of ocular neovascularization in experimental mice (Dong et al., 2014; Jo et al., 2006) and similar results were reported in human trials in which co-administration of anti-VEGF and an anti-PDGF agent together was very effective for the management of AMD (Mones et al., 2011).

One of the pathways associated with hyperglycemia-induced vascular injury is the production of reactive oxygen species (ROS). Several studies have indicated that ROS formation increases retinal vascular damage, which can be reduced with antioxidants (Du et al., 2003; Kowluru et al., 2000). The increase in ROS occurs by multiple pathways including glucose auto-oxidation; the polyol pathway and increased advanced glycation end products (AGEs) (Brownlee, 2001 and Brownlee, 2005; Inoguchi and Sonta et al., 2003; Jain, 2006). Several studies have found that oxidative stress contributed to increased production of VEGF under *in vitro* conditions and

therefore it has been proposed to be involved in upregulation of VEGF expression in diabetes (Ellis et al., 1998; Ellis et al., 2000; Kuroki et al., 1996; Lu et al., 1998) and several studies also demonstrate a role for PKC activation in this process (Kowluru et al., 1997; Kowluru et al., 1999; Lee et al., 1989). Furthermore, Siflinger-Birnboim et al., (1992) found that non-toxic concentration of H_2O_2 increased permeability of cultured endothelial cells by activation of PKC and similar findings were reported by Konishi et al., (1997).

Protein kinase C is a group of enzyme members of the serine/threonine-related protein kinase family. Isoforms α, βI, βII and γ are the conventional PKCs and are widely distributed in various tissues. Their activity is dependant on Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (DAG) (Geraldes and King, 2010). Another subclass is the novel PKCs (δ, ε, η, and θ), which are regulated by PS and DAG, but are Ca^{2+}-independent. The last subclass is the atypical PKCs (ζ and λ) in which activity is regulated by PS, but is independent of Ca^{2+} and DAG (Parekh et al., 2000). Hyperglycemia has been found to activate several PKC isoforms in retinal tissues, including PKC-α, -β- δ and ε (Idris et al., 2001; Ishii et al., 1996) and PKC isoforms have been shown to play a key role in processes such as endothelial dysfunction, vascular permeability, angiogenesis, cell growth and apoptosis (Geraldes and King, 2010; Newton, 2003).

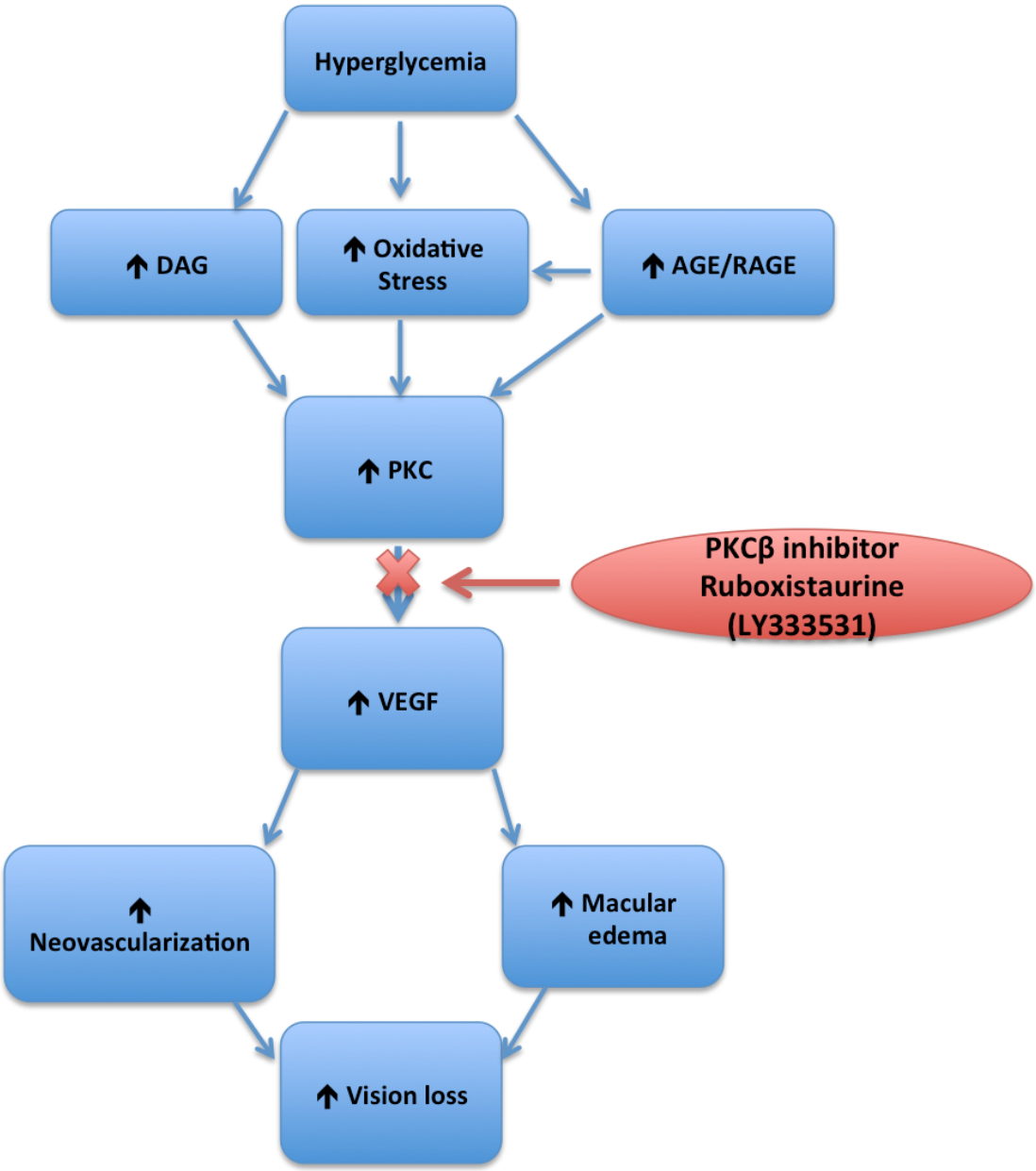


Figure 7.1: Hyperglycemia-induced activation pathways that can lead to production of vascular endothelial growth factor (VEGF) causing retinal complications (see text for details). PKCß activation can be inhibited using inhibitors for example LY33531 (Ruboxistaurine)

Pathways involved in hyperglycemia induced VEGF activation via PKC are summarized in Figure 7.1. Hyperglycemia-induced vascular damage and PKC activation is proposed to occur via increased flux of glucose through the polyol and hexosamine pathways and intracellular formation of AGEs, all of which cause an increase in the glycolytic intermediate dihydroxyacetone phosphate that is responsible for elevated DAG levels. In the polyol pathway, glucose is converted to sorbitol by the enzyme aldose reductase, using NADPH as a cofactor. NADPH is also required to regenerate reduced glutathione. Depletion of NADPH by the polyol pathway is proposed as a mechanism of increased intracellular oxidative stress (Lee and Chung, 1999). The AGEs modify protein function, interacting with AGE receptors (RAGE) on vascular endothelial cells, which leads to ROS production (Giacco and Brownlee, 2010; Kowluru and Chan, 2007).

Each of these (DAG, oxidative stress and AGE/RAGE) have been shown to activate PKC and activation of PKC, in turn, can activate expression of VEGF (Geraldes and King, 2010; Giacco and Brownlee, 2010). Expression of VEGF is responsible for diabetic retinopathy complications such as macular oedema and neovascularization. Blocking of PKC could be a useful strategy to reduce VEGF secretion and may improve retinal condition. The PKC-β isoform has been suggested as a specific target in DR (Aiello, 2002).

PKC-β inhibitors have been used in several experimental systems to investigate the role of PKC-β in DR. For example, treatment with the PKC-β inhibitor LY379196 reduced NADPH oxidase (Quagliaro et al., 2003). Another PKC-β inhibitor, LY333531 (Ruboxistaurin), reduced VEGF-induced vascular permeability and neovascularization in bovine aortic endothelial cells (Xia et al., 1996). In the retina, LY333531 also attenuated leukocyte entrapment in the retinal microcirculation

(Nonaka et al., 2000) and intravitreal administration of LY333531 decreased retinal PKC-β activation and improved retinal blood flow (Bursell et al., 1997). Oral administration of LY333531 has also been shown to reduce VEGF-induced retinal and optic nerve neovascularization in a pig model of branch retinal vein occlusion (Danis et al., 1998) and improved retinal blood flow in diabetic rats (Ishii et al., 1996). High glucose, oxidative stress and hypoxia all occur in diabetes and have been shown to induce expression and release of VEGF. Due to the detrimental vascular effects of VEGF in DR, the use of anti-VEGF therapy may have beneficial effects (Simo and Hernandez, 2008). Therefore, the aim of the present experiments was to explore the effect of long-term high glucose treatment on survival, growth factor expression and VEGF release in MIO-M1 cells. In addition, high glucose treatment was combined with hypoxia and/or H_2O_2 to induce oxidative stress to examine if they work synergistically to further induce VEGF release. In the later experiments, a PKC-β inhibitor LY333531 was used to examine its ability to reduce VEGF levels. Experiments were also performed in HORCs to study the effect of high glucose as on the survival of RGCs.

7.2 Results

High glucose experiments in this chapter were carried out using commercially available high glucose medium with the highest concentration of 25mM. This concentration is approximately five times higher than the physiological glucose levels of 5.55mM. It should be noted that some cells in cultures are routinely grown in high glucose concentration of 25mM (Russell et al., 1999; Vincent et al., 2005) but as MIO-M1 cells are routinely cultured in 5.55mM glucose then the higher level of 25mM was considered the best concentration to produce a hyperglycemic insult without causing death.

7.2.1 Effect of High Glucose (HG) on the Cytotoxicity and Viability of Human Müller Cells (MIO-M1) and RGC Survival in HORCs

As shown in Figure 7.2A, exposure of MIO-M1 cells to raised glucose levels (10-25mM) for 24hrs did not alter the release of LDH compared to physiological levels of glucose (5.55 mM). The viability of MIO-M1 cells (Figure 7.1B) was also comparable to control indicating that HG treatment at these concentrations and for this time period does not affect the survival of MIO-M1 cells.

Exposure of HORCs to HG levels (10-25mM) did not cause any elevation of LDH release (Figure 7.2A). Assessment of RGC numbers in retinal explants exposed to the highest level of glucose (25mM) showed a similar number of NeuN-positive cells to control (5.55mM). This indicates that exposure of HORCs to this level of glucose for 24hrs does not affect the survival of RGCs (Figure 7.2B). Immunohistochemistry revealed normal structure of the retina treated with high glucose (Figure 7.2C).

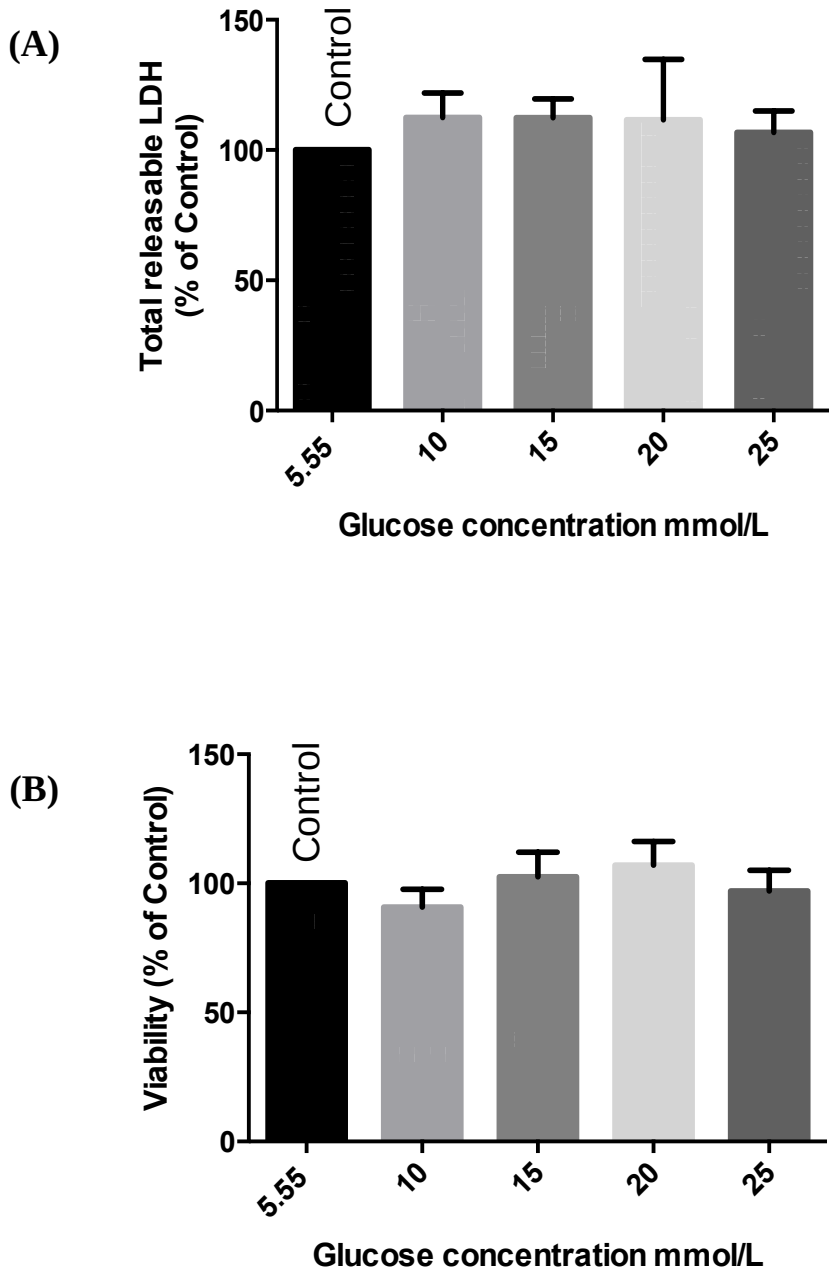


Figure 7.2: (A) Cytotoxicity (total releasable LDH) and (B) viability (MTS assay) of human Müller cells (MIO-M1) after 24hrs exposure to control (5.55mM glucose) or raised levels of glucose (10-25 mM) (mean ± s.e.m, * $p \leq 0.05$; n=4).

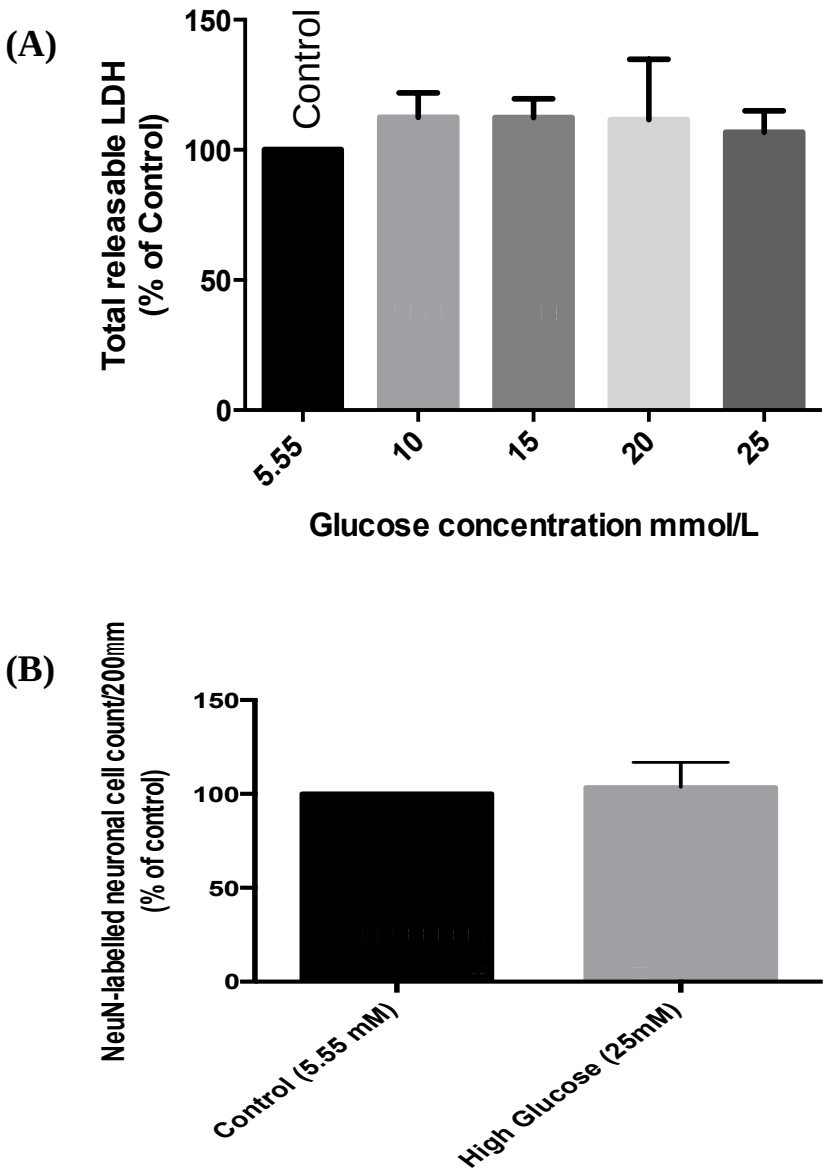


Figure 7.3: (A) Cytotoxicity (total releasable LDH) in HORCs after 24hrs exposure to control (5.55mM glucose) or raised levels of glucose (10-25 mM) and (B) NeuN counting in HORCs after 24hrs exposure to control and high glucose (25mM) under normoxia (mean ± s.e.m, * $p \leq 0.05$; n=4).

(C)

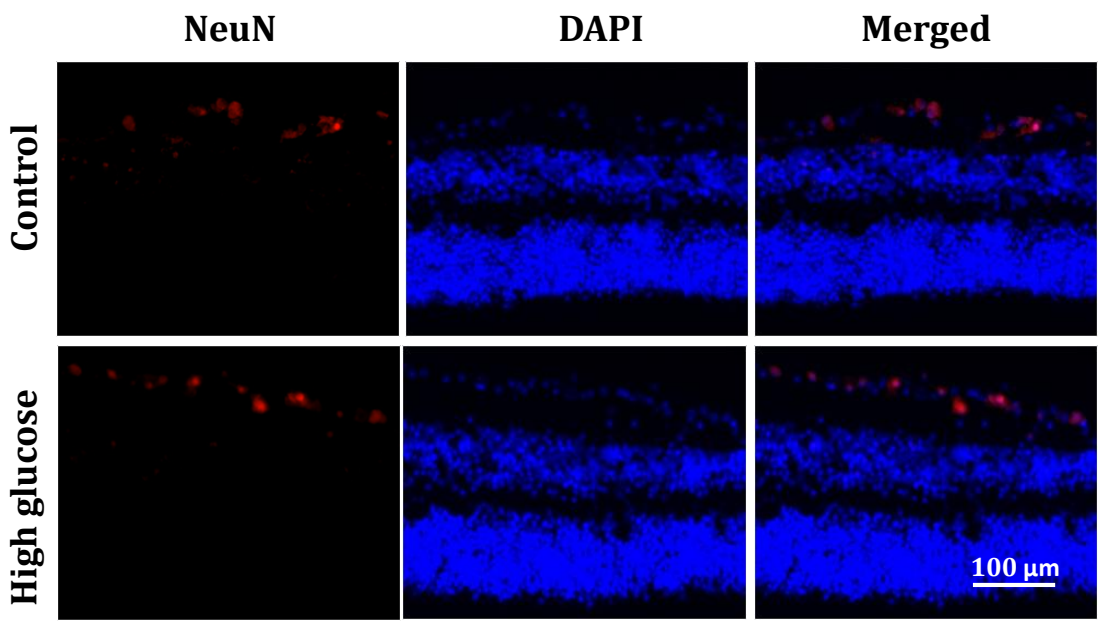


Figure 7.3C: Representative immunofluorescence photomicrographs of NeuN labelling in HORCs after 24hrs exposure to control or high glucose (25mM). DAPI = blue, GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer.

7.2.2 Effect of High Glucose (HG) on Genes Expression in Müller cells (MIO-M1) and HORCs

7.2.2.1 Effect of High Glucose (HG) on the Expression of VEGF mRNA in Human Müller cells (MIO-M1) and HORCs

Exposure of MIO-M1 cells to 25mM glucose for 24hrs significantly (*$p<0.05$, n=4) increased VEGF mRNA expression by 4-fold as compared to normal glucose (5.55mM). Glucose levels of 10-20mM did not alter VEGF expression (Figure 7.3A). In HORCs, no significant increases in VEGF mRNA expression were seen in 25mM glucose compared to control (5.55mM) (Figure 7.3B).

7.2.2.2 Effect of High Glucose (HG) on the Expression of PDGF mRNA in Human Müller cells (MIO-M1) and HORCs

In MIO-M1 cells, a similar pattern of change to VEGF was found with PDGF in which the highest glucose level of 25mM caused a significant 2.6-fold (*$p<0.05$, n=4) upregulation in PDGF mRNA expression compared to control (Figure 7.4A). No regulation of PDGF mRNA was seen under high glucose concentrations in HORCs (Figure 7.4B).

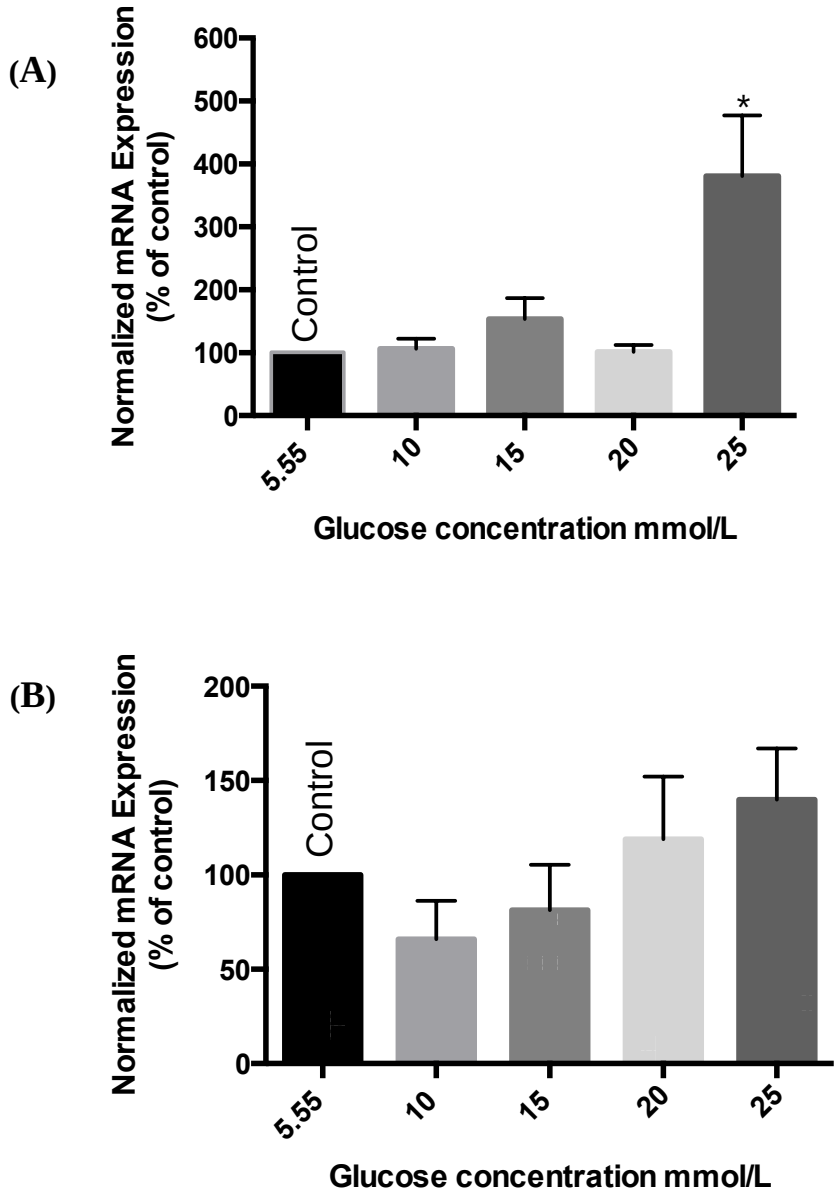


Figure 7.4: Expression of VEGF mRNA in (A) human Müller cells (MIO-M1) and (B) HORCs after 24hrs exposure to control (5.55mM glucose) or raised levels of glucose (10-25mM). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4).

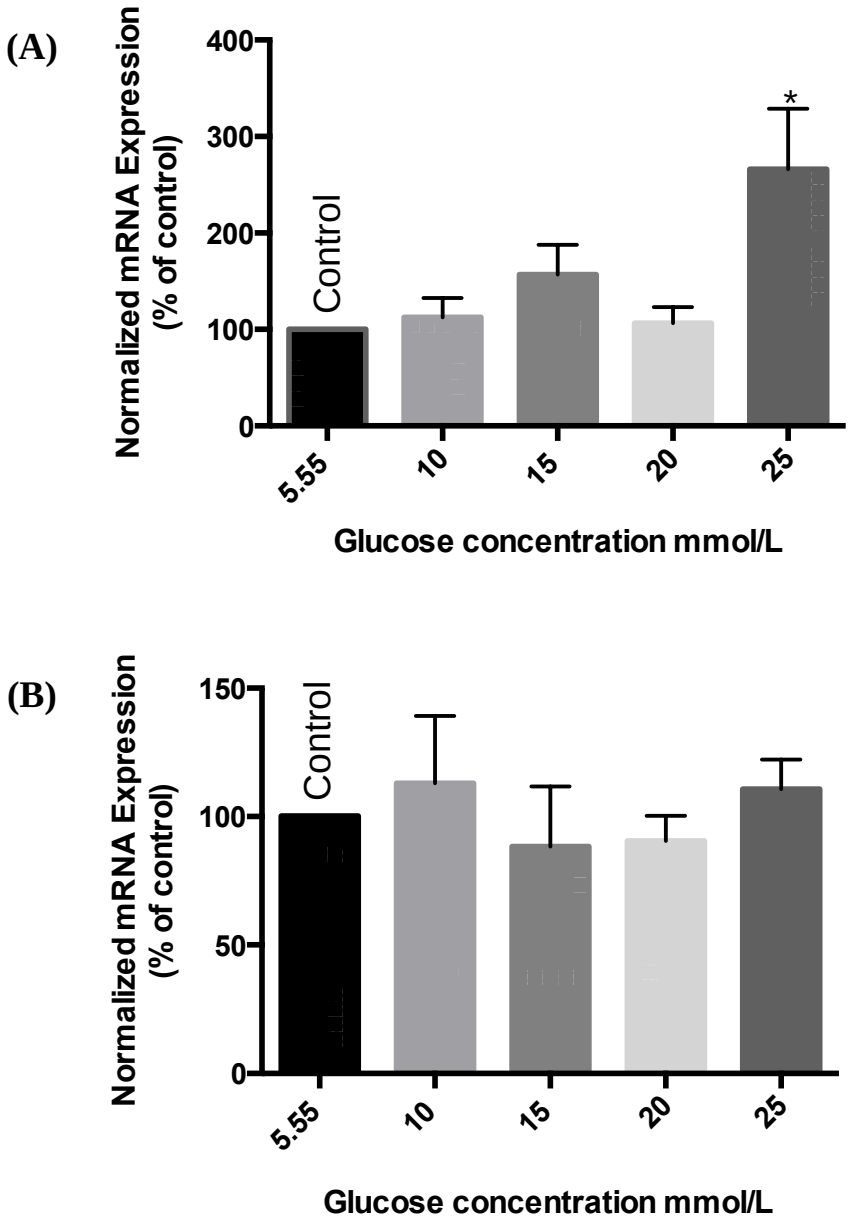


Figure 7.5: Expression of PDGF mRNA in (A) human Müller cells (MIO-M1) and (B) HORCs after 24hrs exposure to control (5.55mM glucose) or raised levels of glucose (10-25 mM). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4).

7.2.2.3 Effect of High Glucose (HG) on the Expression of bFGF and PEDF mRNA in Human Müller Cells (MIO-M1)

The expression of bFGF mRNA showed a trend towards an increase at 25mM glucose but a decrease at 10-20mM, however, no changes were found to be significant (Figure 7.5A). In addition, the expression of the anti-angiogenic factor, PEDF, did not change under raised glucose concentration (10-25mM) compared to control (Figure 7.5B).

7.2.2.4 Effect of High Glucose (HG) on the Expression of Glutamine Synthetase (*GLUL)* and Glutamate Transporter (*SCL1A3*) mRNA in Human Müller Cells (MIO-M1) and HORCs

Expression of glutamine synthetase (*GLUL*) mRNA in MIO-M1 cells showed no significant changes with increasing levels of glucose (Figure 7.6A). *GLUL* and GLAST (*SCL1A3)* expression in HORCs showed trend towards increasing, but no significant changes were found (Figure 7.6B&C).

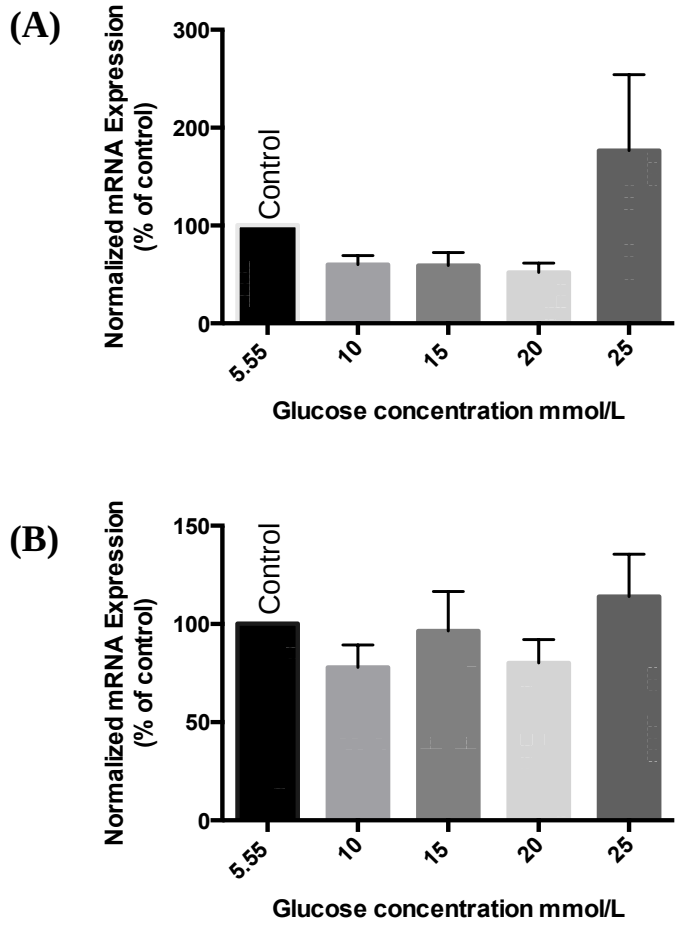


Figure 7.6: Expression of (A) bFGF mRNA and (B) PEDF in human Müller cells (MIO-M1) after 24hrs exposure to control (5.55mM glucose) or raised levels of glucose (10-25 mM). Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *$p \leq 0.05$; n=4).

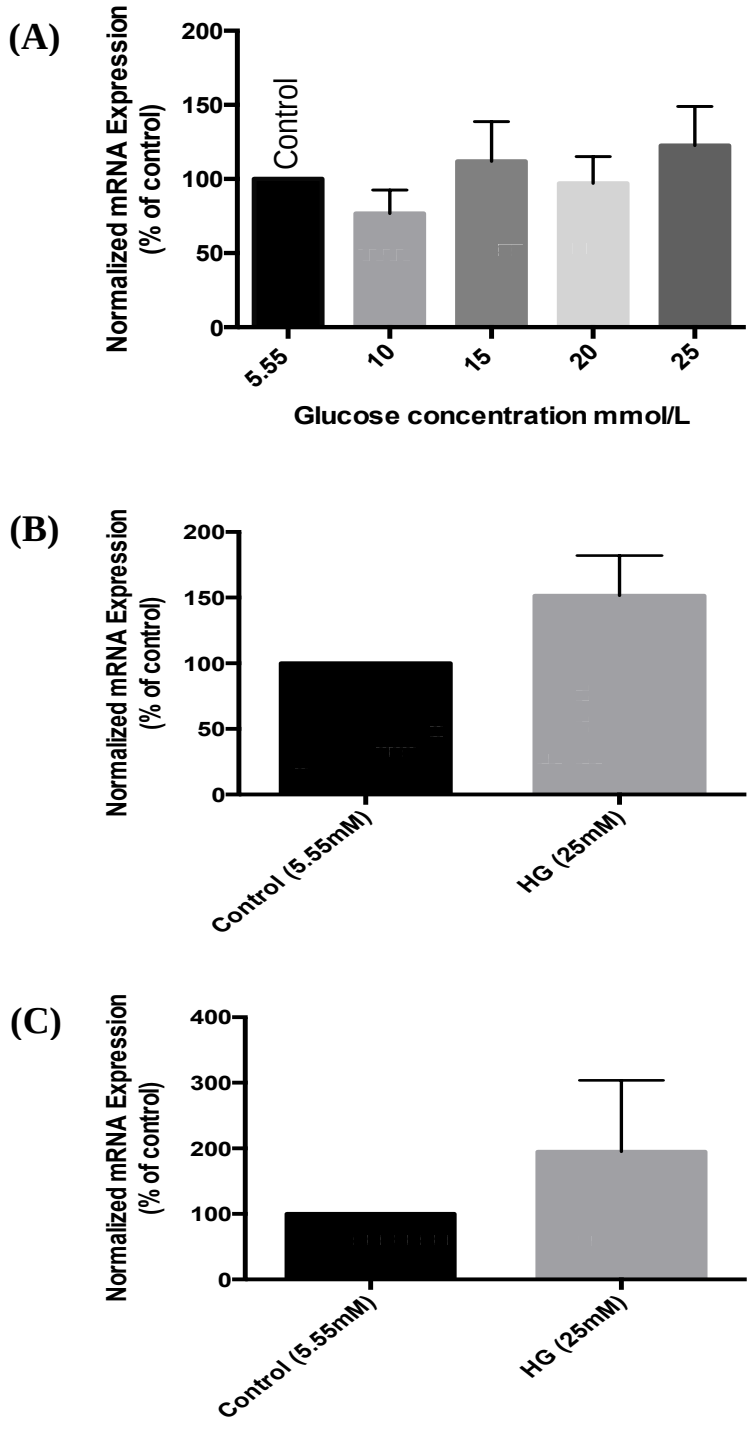


Figure 7.7: Expression of *GLUL* mRNA in (A) human Müller cells (MIO-M1) and (B) HORCs. (C) Expression of the glutamate transporter GLAST (*SCL1A3*) in HORCs after 24hrs exposure to control (5.55mM glucose) or raised levels of glucose (10-25 mM) in MIO-M1 or 25mM glucose in HORCs. Gene expression was normalized to the housekeeping genes *TOP1* and *CYC1* (mean ± s.e.m, *p≤0.05; n=4).

7.2.3 Effect of HG on the Release of VEGF from Human Müller Cells (MIO-M1) and HORCs

VEGF is the main growth factor involved in the pathology of diabetic retinopathy therefore levels of VEGF protein were measured using ELISA in both MIO-M1 cells and HORCs treated with high glucose.

In MIO-M1 cells, the release of VEGF was studied over a period of 72hrs both under normal glucose (5.55mM) as a control and high glucose (25mM) conditions. A significant increase in VEGF (*$p<0.05$, $n=4$) of approximately 2.6-fold was seen with HG after 72hrs compared to its control. In HORCs, the levels were measured only at 24hrs. They showed concentration dependant increase in VEGF release. A significant increase (*$p<0.05$, $n=4$) of approximately 4.5-fold was seen with 20mM glucose (Figure 7.7B).

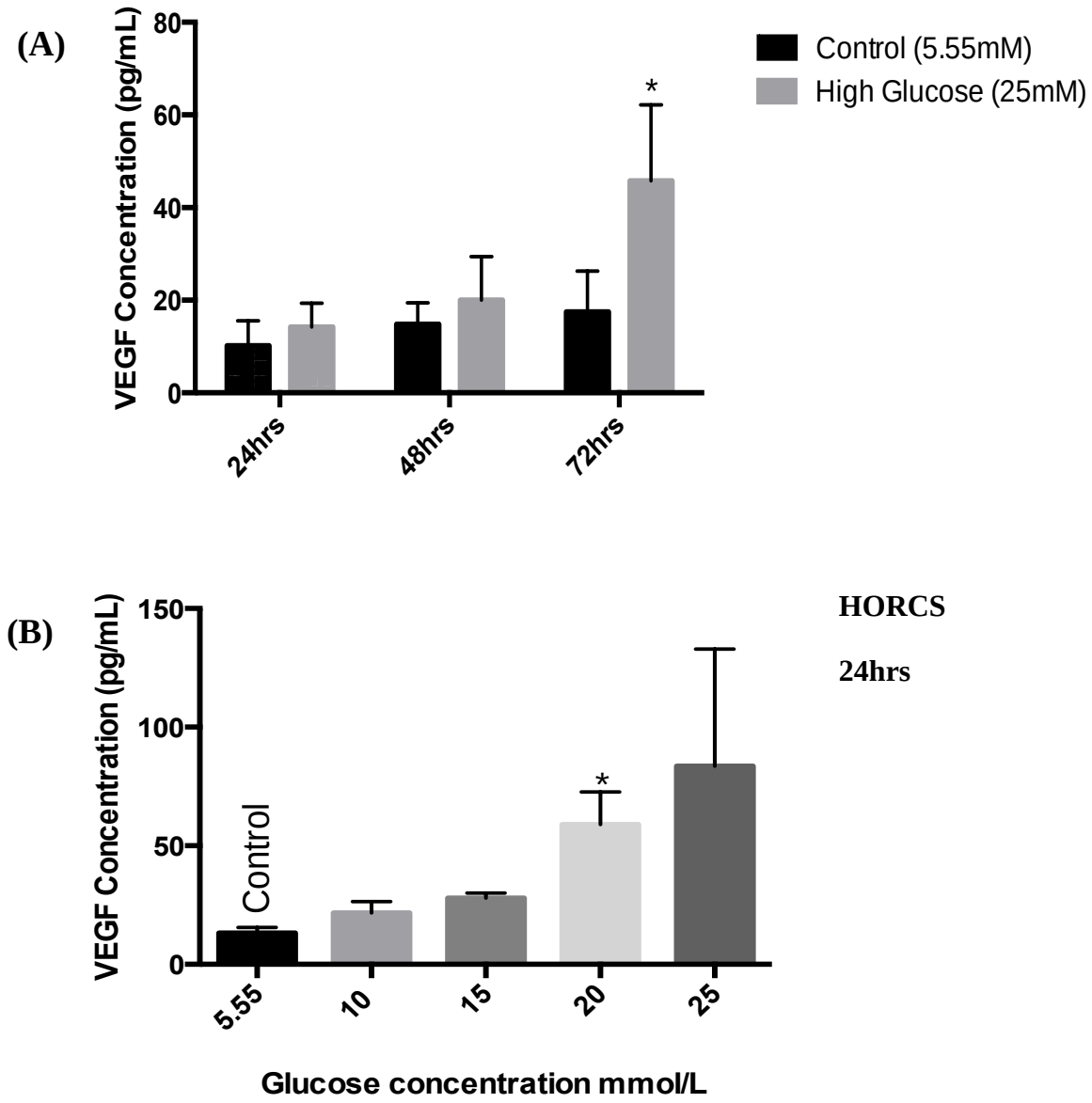


Figure 7.8: Release of VEGF from (A) human Müller cells (MIO-M1) after 24, 48, and 72hrs exposure to control low glucose (5.55mM) and high glucose (25mM) and (B) HORCs after 24hrs exposure to control low glucose (5.55mM) and raised levels of glucose (10-25 mM) (mean ± s.e.m, *$p \leq 0.05$; n=4).

7.2.4 Effect of Oxygen Deprivation on VEGF Release Human Müller Cells (MIO-M1) Under Normal and High Glucose Condition.

In DR, raised glucose is often combined with decreased oxygen. The effects of hypoxia and high glucose were therefore investigated on VEGF secretion from MIO-M1 cells.

Significant stimulation of VEGF release was found under full hypoxia in both low (5.55mM) and high glucose (25mM) (*$p<0.05$, n=4) at all time points (Figure 7.8). In the first 24hrs, VEGF level was 67.1±20.7pg/ml under hypoxia-low glucose compared to 148.5±41.9 pg/ml with the combination of hypoxia high glucose although this difference was not significant. The levels of VEGF release with hypoxia and hypoxia/HG continued to increase up to 72hrs, but no synergism was apparent.

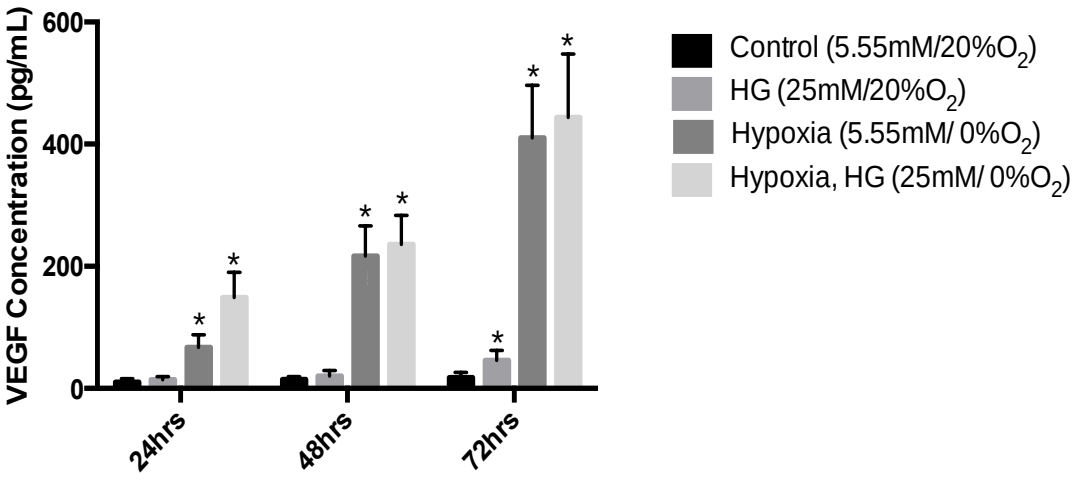


Figure 7.9: Release of VEGF by human Müller cells (MIO-M1) after 24, 48, and 72hrs exposure to (A) 5.55mMglucose and (B) high glucose (25mM) under normoxia ($20\%O_2$) and full hypoxia ($0\%O_2$) and (C) combination of both (mean ± s.e.m, * $p\leq0.05$; n=4). Comparison made to respective control.

7.2.5 Effect of Hydrogen Peroxide (H_2O_2) on the Cytotoxicity and Viability of Human Müller Cells (MIO-M1) Under Normal and High Glucose

The mechanism of VEGF upregulation in high glucose has been proposed to be via generation of ROS. The effects of oxidative stress on VEGF release was therefore of interest. Initial experiments assessed the survival of MIO-M1 cells in H_2O_2 at 24hrs in order to determine a concentration that caused oxidative stress without causing cell death.

Treatment of MIO-M1 cells with H_2O_2 (100μM- 1mM) at control (5.55mM) glucose levels caused no change in LDH release (Figure 7.9A) or viability (Figure 7.9B) at 24hrs. Increase in LDH release and reduction in viability was seen with 2mM H_2O_2 (Figure 7.10A). Interestingly, MIO-M1 cells under high glucose (25mM) were more sensitive to the oxidative stress induced by H_2O_2. Under those conditions, 500μM and 1mM H_2O_2 showed elevated levels of LDH (Figure 7.10A) and significant loss of viability (Figure 7.10B) at the 24hrs time point.

From these experiments, lower doses of H_2O_2 (50 and 100 μM) for 24hrs were chosen and to induce oxidative stress without loss of viability at either level of glucose.

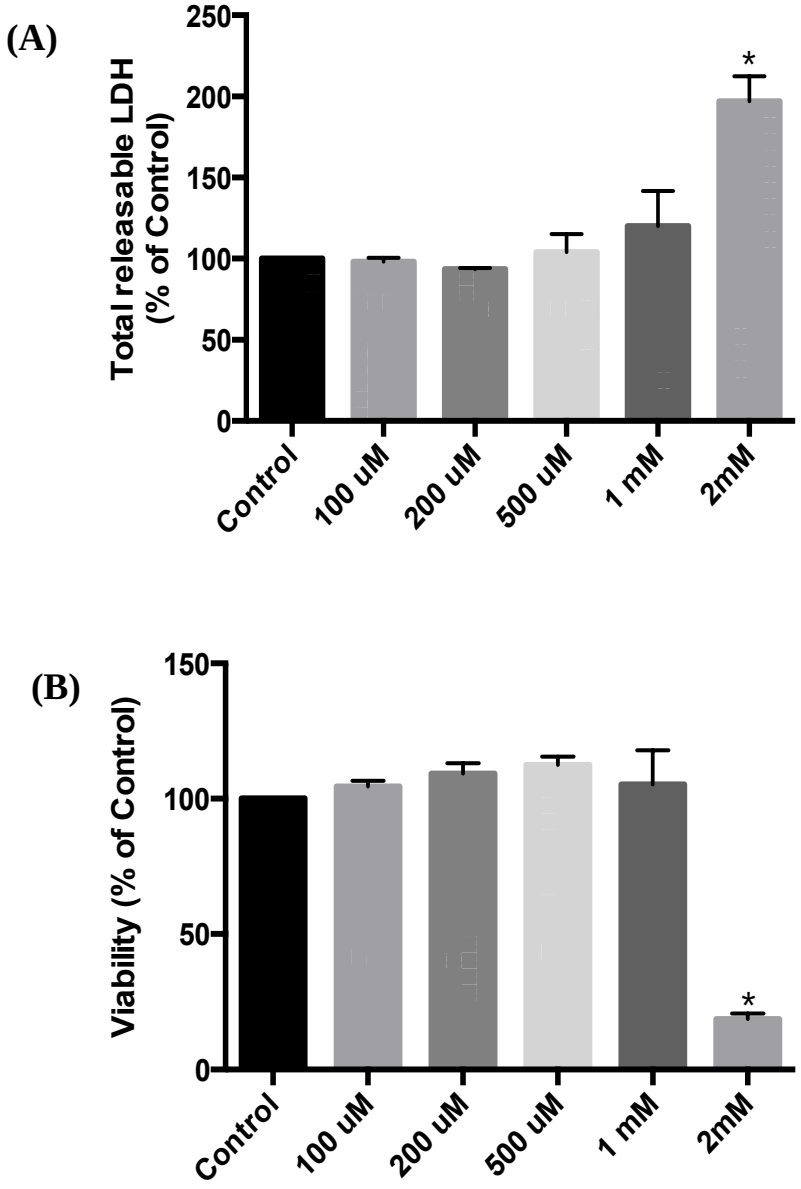


Figure 7.10: (A) Cytotoxicity (total releasable LDH) and (B) viability (MTS assay) of human Müller cells (MIO-M1) after 24hrs exposure to H_2O_2 (100-2000nM) under 5.55mM glucose and normoxia (mean ± s.e.m, * $p \leq 0.05$; n=4).

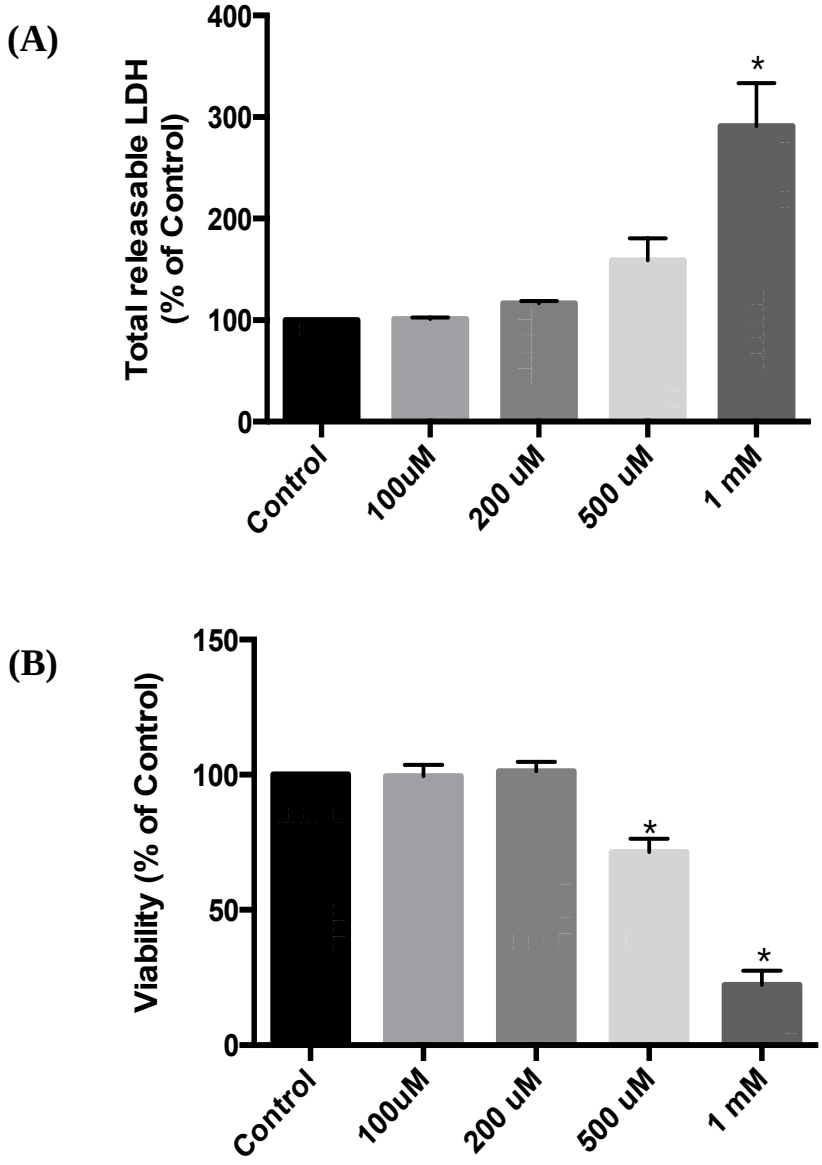


Figure 7.11: (A) Cytotoxicity (total releasable LDH) and (B) viability (MTS assay) of human Müller cells (MIO-M1) after 24hrs exposure to H_2O_2 (100-1000nM) under 25mM glucose and normoxia (mean ± s.e.m, * $p \leq 0.05$; n=4).

7.2.6 Effect of Hydrogen Peroxide (H_2O_2) and Glucose on VEGF Release Under Normoxic Condition in Human Müller cells (MIO-M1)

Exposure of MIO-M1 cells to H_2O_2 (50 and 100 μM) for 24hrs showed an increasing trend in VEGF secretion both under normal (5.55mM) and HG (25mM) conditions, but no significant increases were seen at this time point (Figure 7.11).

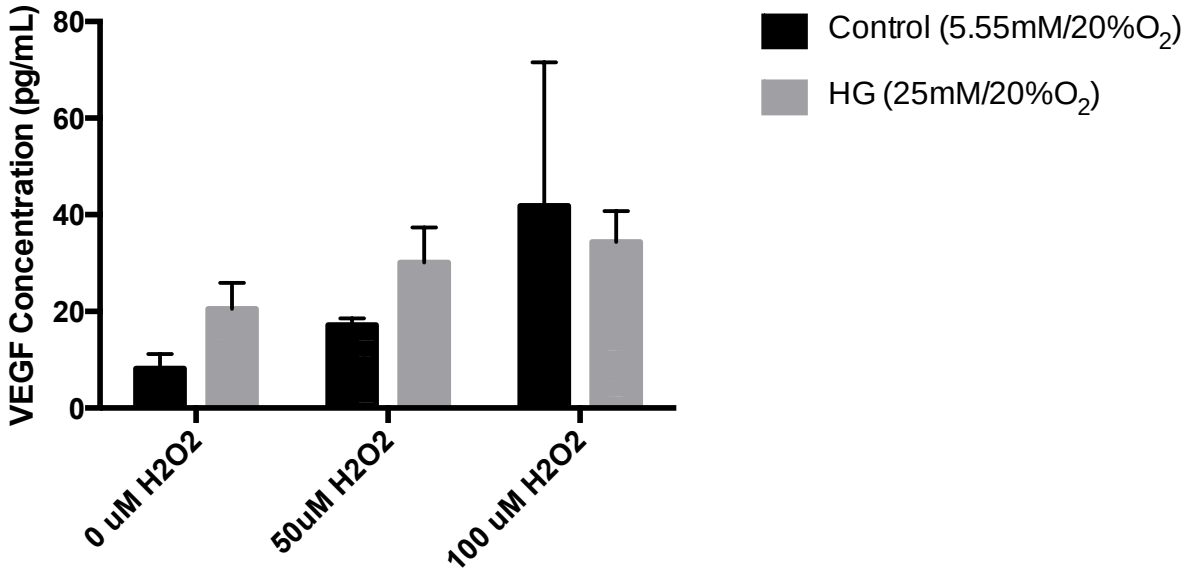


Figure 7.12: Release of VEGF by human Müller cells (MIO-M1) after 24hrs exposure to low glucose (5.55mM glucose/20%O_2) and high glucose (25mM/ 20%O_2) either alone or in combination with 50μM or 100μM H_2O_2, under normoxia (mean ± s.e.m, * $p \leq 0.05$; n=4, t.test).

7.2.7 Effect of Hydrogen Peroxide (H_2O_2) and Glucose on VEGF Release Under Hypoxic Condition in Human Müller cells (MIO-M1)

Exposure of MIO-M1 cells to hypoxia under normal glucose and HG increased VEGF secretion compared to normoxia (Figure 7.12). The highest level of H_2O_2 (100μM) significantly increased (*$p<0.05$, $n=4$) VEGF secretion from a control level of 8.17± 3.00 pg/ml to 99.3±25.43 pg/ml under hypoxic conditions (Figure 7.12A). HG conditions, hypoxia and oxidative stress significantly increased (*$p<0.05$, $n=4$) the VEGF secretion with low dose H_2O_2 (50μM) from a control level of 20.52±5.35 pg/ml to 84.9±24.5 pg/ml and to 74.5±8.34 pg/ml with the highest level of H_2O_2 (100μM). The higher concentration of H_2O_2 did not appear to increase VEGF secretion compared to the lower concentration (Figure 7.12B). Hypoxia and oxidative stress (100μM H_2O_2) significantly increased VEGF secretion from 34.35± 6.38 pg/ml compared to 74.5± 8.34 pg/ml with high glucose and oxidative stress, alone.

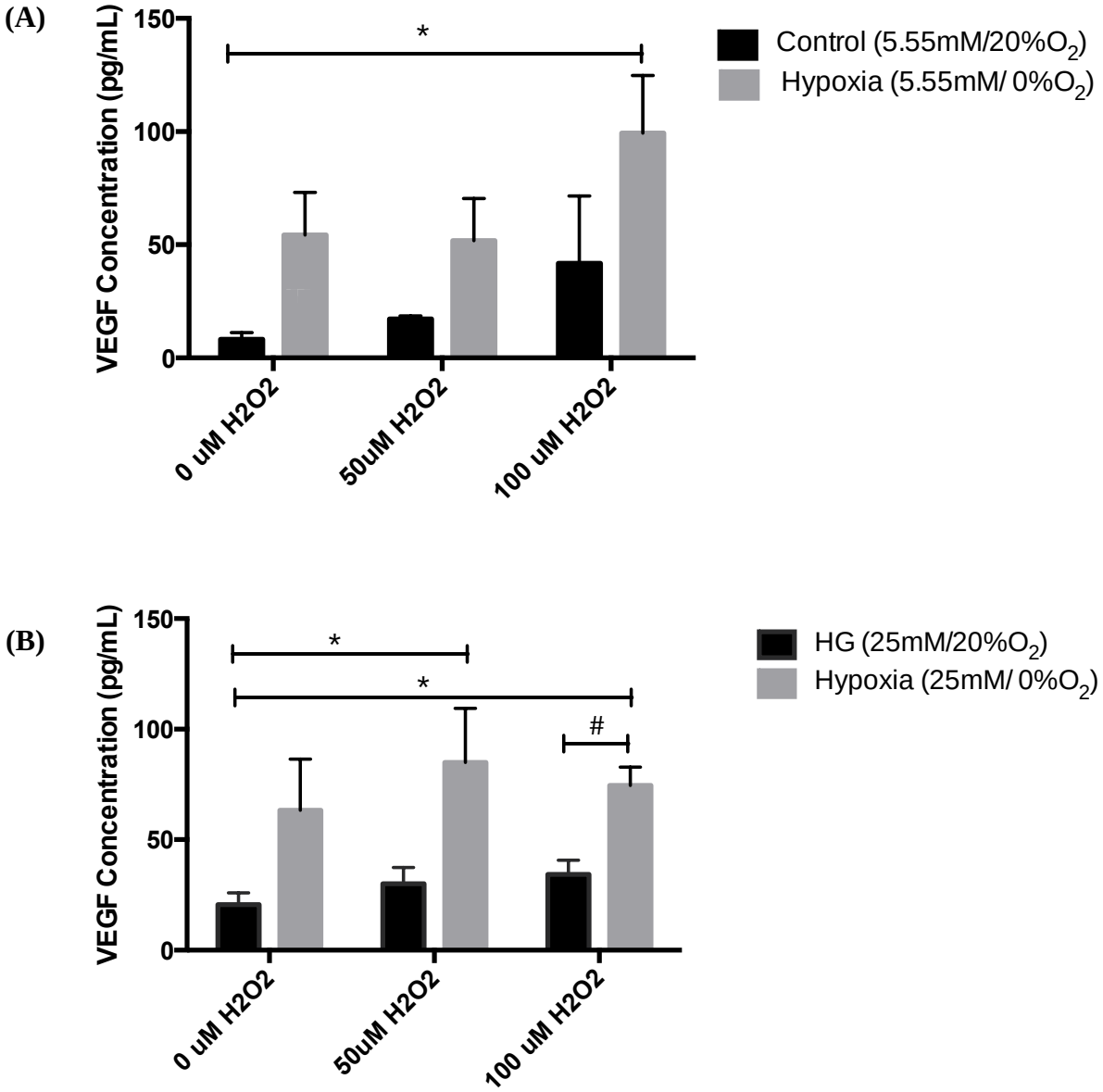


Figure 7.13: Release of VEGF by human Müller cells (MIO-M1) after 24hrs exposure to (A) low glucose (5.55mM) and (B) high glucose (25mM) under normoxia and full hypoxia either alone or in combination with 50µM or 100µM H_2O_2 (mean ± s.e.m, *, # $p \leq 0.05$; n=4, t.test). * Compared to respective control (no H_2O_2), # compared to HG+100µM H_2O_2.

7.2.8 Effect of PKC-β Inhibitor (LY333531) on VEGF Release Under High Glucose Condition in Human Müller Cells (MIO-M1)

Initially, MIO-M1 cells were treated with PKC-β inhibitor (LY333531) in a range of 100nM-1μM under control low glucose conditions. Viability and cytotoxicity tests were carried out for these experiments. As shown in Figure 7.13A, the LDH level was similar to control with all concentrations of LY333531. The viability of MIO-M1 cells was not affected with any of the concentrations used (Figure 7.13B). Therefore, the highest dose (1μM) and a lower concentration (500nM) were chosen for the next experiments.

Treatment of MIO-M1 cells under control conditions with LY333531 showed a dose-dependent trend of reduced VEGF secretion from the initial level of 10.2±5.3 pg/ml to 2.96±1.73 pg/ml with 500nM and 1.25±0.44 pg/ml with 1μM LY333531 at 24hrs (Figure 7.14A). However, no reduction in VEGF secretion was significant. Under high glucose conditions (25mM), no significant changes were seen with either concentration or at any time point (Figure 7.14B).

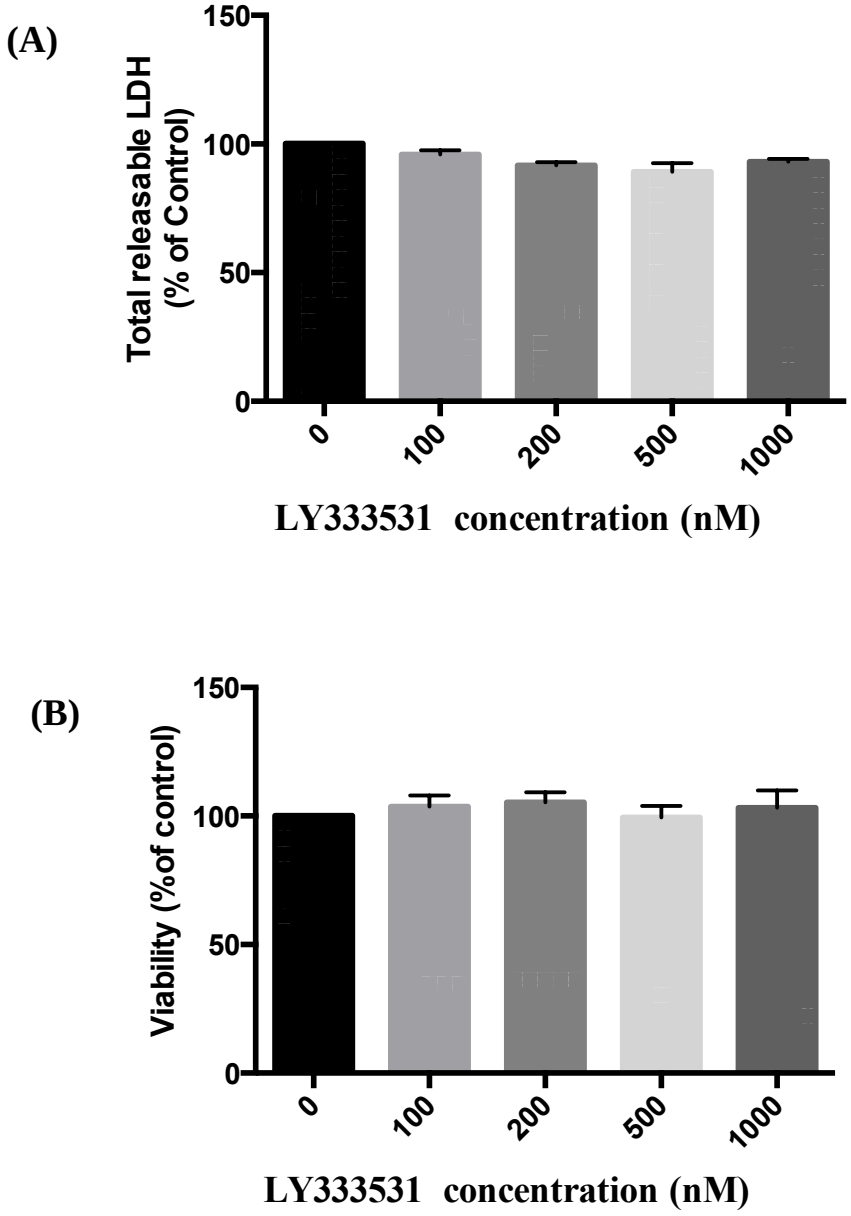


Figure 7.14: Effect of the PKCβ inhibitor (LY333531) on the survival of human Müller cells (MIO-M1) under control conditions (A) Cytotoxicity (total releasable LDH) and (B) Viability (MTS assay) after 24hrs (mean ± s.e.m, *p≤0.05; n=4).

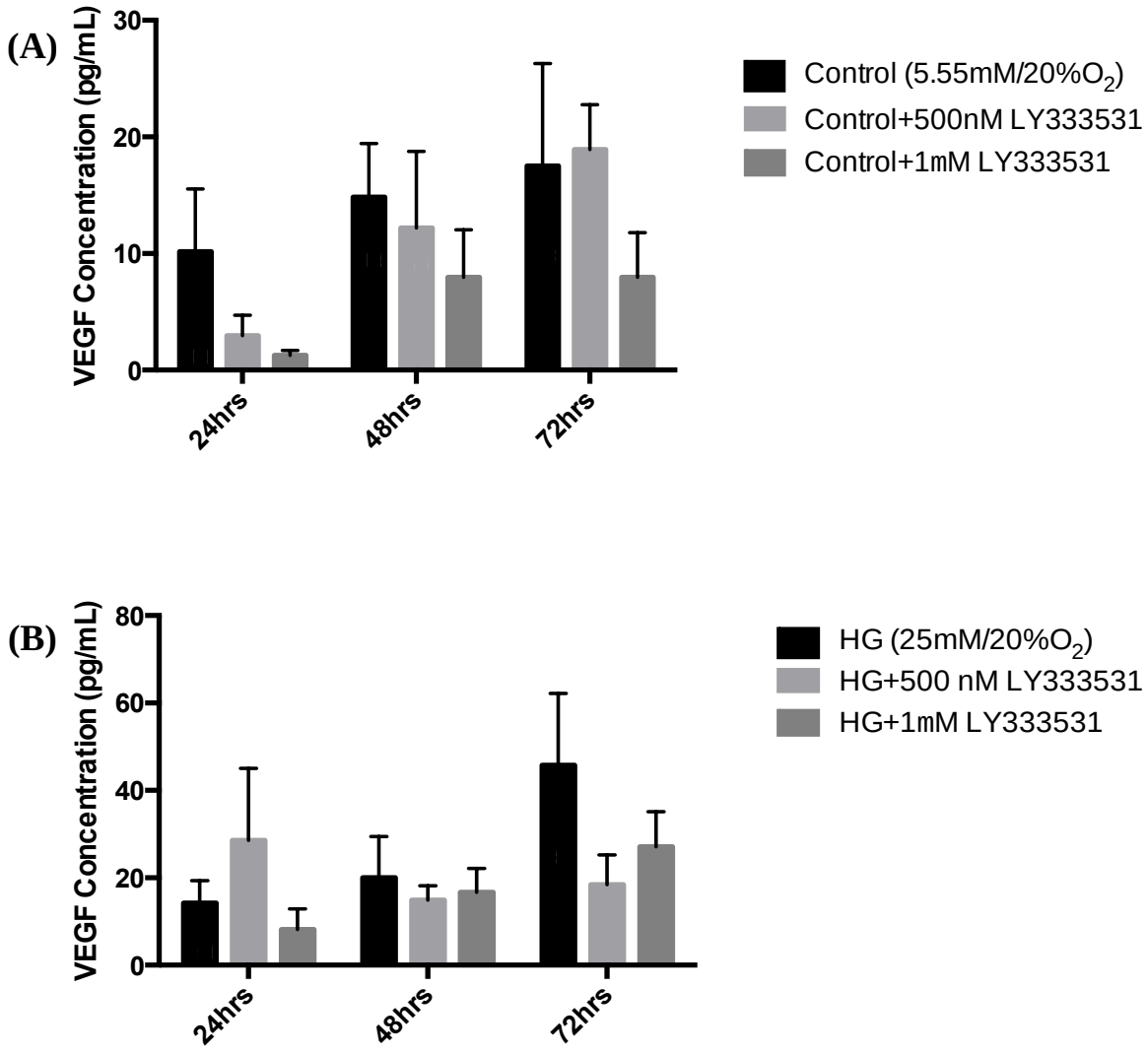


Figure 7.15: Release of VEGF by human Müller cells (MIO-M1) after 24, 48, and 72hrs exposure to (A) 5.55mMglucose/20%O_2 and (B) 25mMglucose/20%O_2 either alone or in combination with 500nM or 1μM PKCβ inhibitor (LY333531) (mean ± s.e.m, * $p \leq 0.05$; n=4).

7.2.9 Effect of PKC-β Inhibitor (LY333531) on VEGF Release Under Hypoxia in Human Müller Cells (MIO-M1)

Exposure of MIO-M1 cells to LY333531 (500nM and 1μM) did not reduce VEGF secretion as a result of full hypoxia (0%O_2) at 24hrs (Figure 7.15A). At 48 and 72hrs there were trends towards a dose-dependant decrease in VEGF secretion as a result of PKC-β inhibition, but no significant reduction were found. Under HG and hypoxia, a significant reduction in VEGF was obtained with 1μM LY333531 at 24hrs (Figure 7.15B). A dose dependent decrease could be seen at later time points, but these were not found to be significant.

7.2.10 Effect of Hydrogen Peroxide (H_2O_2) and Hypoxia with and without PKC-β Inhibitor (LY333531) on VEGF Release in Human Müller Cells (MIO-M1)

Under control conditions, treating MIO-M1 cells with H_2O_2 and 1μM LY333531 did not reduce the VEGF levels (Figure 7.16A). Actually, an increase in VEGF secretion was apparent with 50μM H_2O_2 although this was not found to be significant. Under conditions of high glucose, no effect of PKC inhibition was seen in the presence of H_2O_2 (Figure 7.16B). Likewise, with hypoxia and HG in the presence of H_2O_2, no significant inhibition was seen (Figure 7.17).

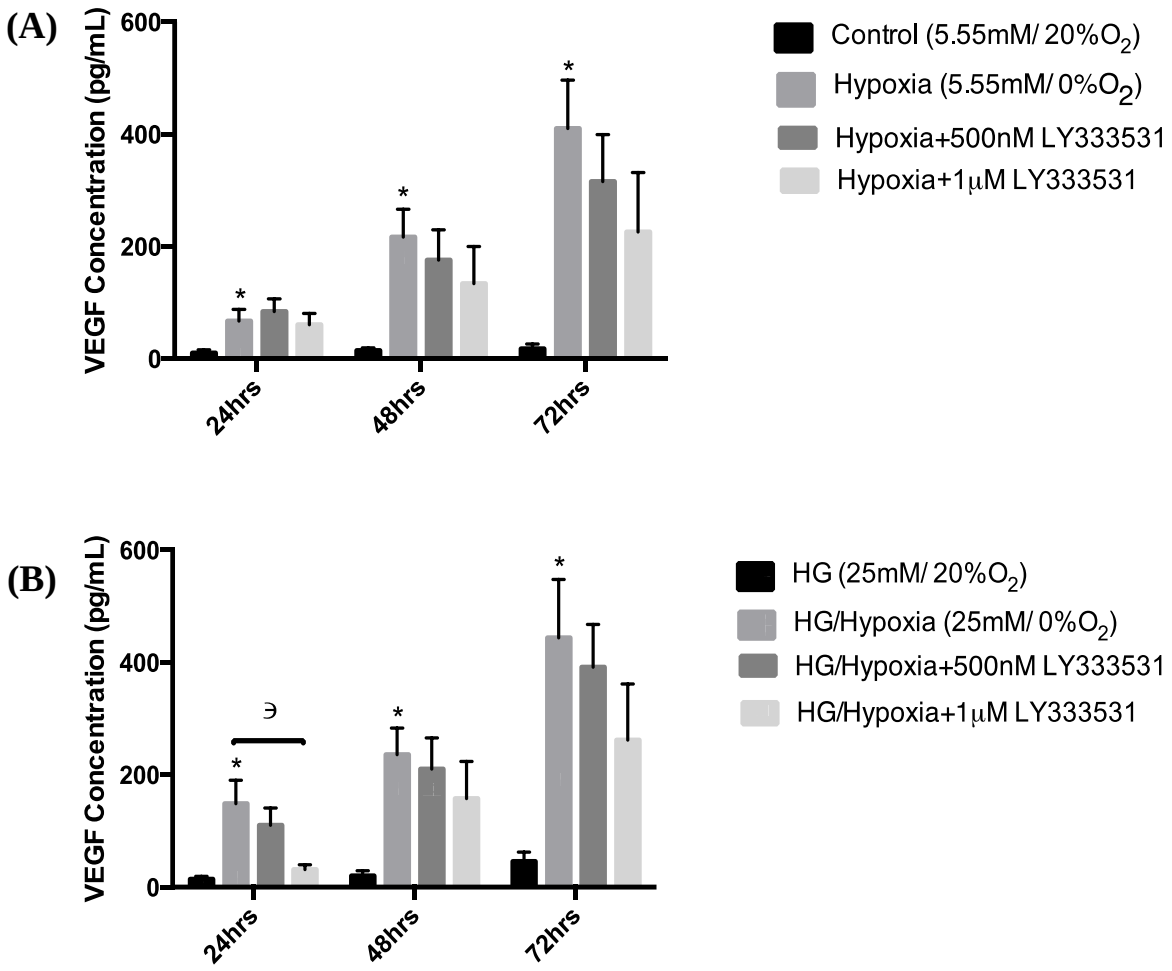


Figure 7.16: Release of VEGF by human Müller cells (MIO-M1) after 24, 48, and 72hrs exposure to (A) 5.55mMglucose/0%O_2 and (B) 25mM/0%O_2 either alone or in combination with 500nM or 1μM PKCβ inhibitor (LY333531) (mean ± s.e.m, * $p \leq 0.05$; n=4). *Comparison to respective control, ɜ comparison to hypoxia.

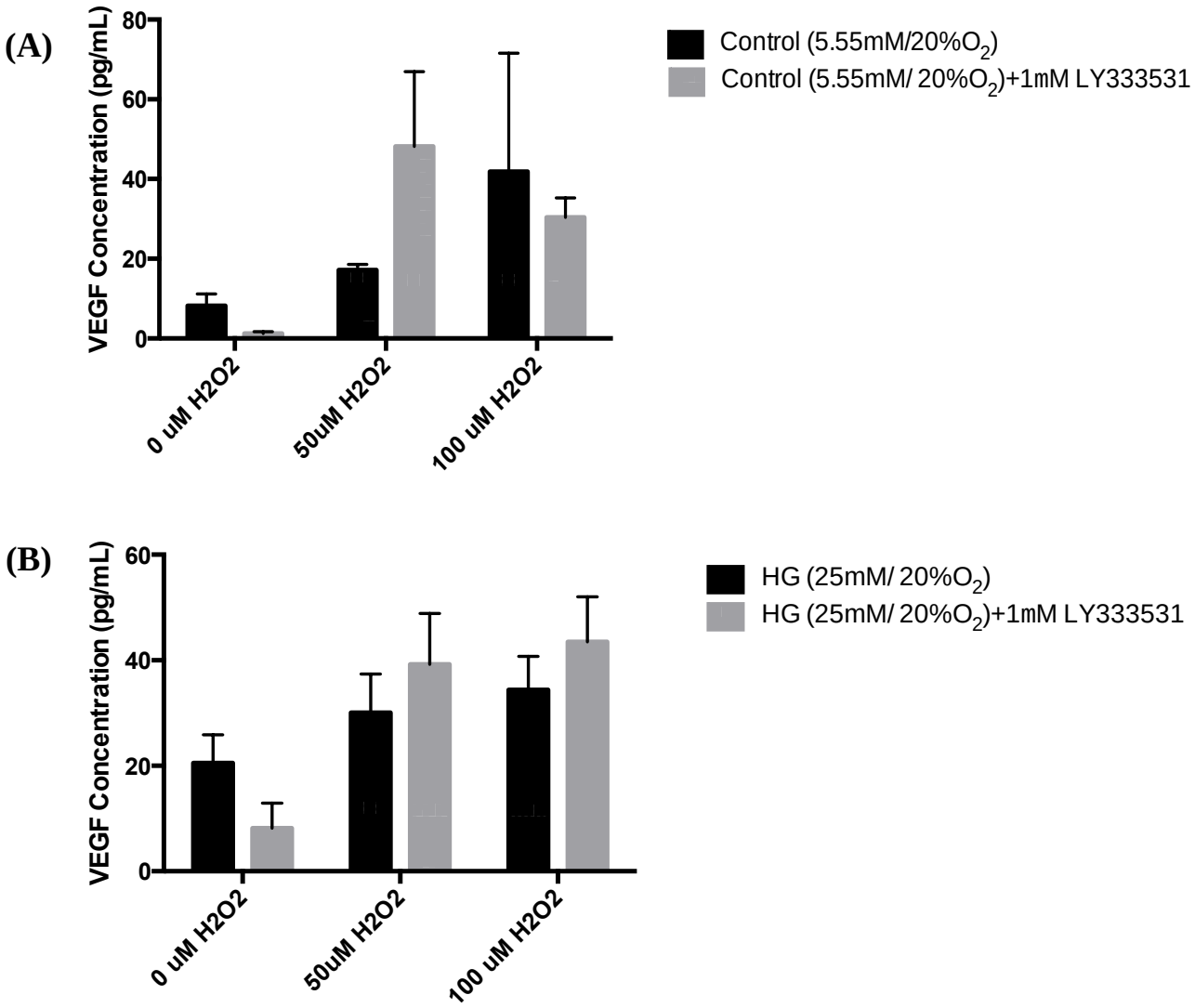


Figure 7.17: Release of VEGF by human Müller cells (MIO-M1) after 24hrs exposure to (A) low glucose (5.55mM) and (B) high glucose (25mM) under normoxia either alone or in combination with 50μM or 100μM H_2O_2 in addition to 1μM PKCβ inhibitor (LY333531) (mean ± s.e.m, * $p \leq 0.05$; n=4, t.test).

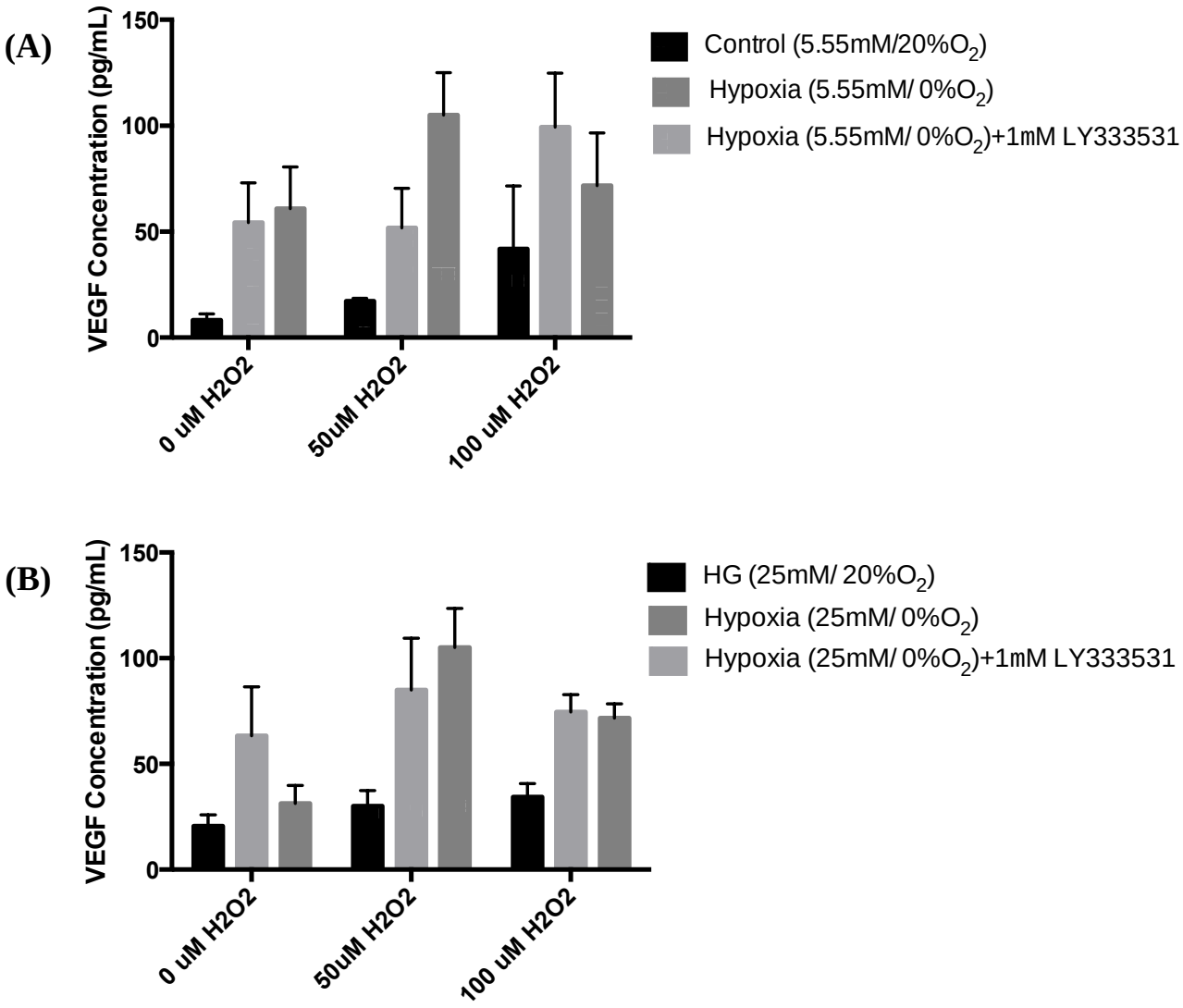


Figure 7.18: Release of VEGF by human Müller cells (MIO-M1) after 24hrs exposure to (A) low glucose (5.55mM) and (B) high glucose (25mM) under hypoxia either alone or in combination with 50μM or 100μM H_2O_2 in addition to 1μM PKCβ inhibitor (LY333531) (mean ± s.e.m, * $p \leq 0.05$; n=4, t.test).

7.3 Discussion

Diabetic retinopathy (DR) is a common complication of diabetes and a leading cause of blindness (Aiello et al., 1998). It is worth mentioning that diabetes is a metabolic disease, and its complications are developed over years. It is difficult to simulate and match all the clinical course of DR using experimental models but using cell culture models can be useful to study the cellular mechanisms underlying diabetic retinopathy (Matteucci et al., 2014). Proteomics studies on vitreous humour samples from patients with diabetic retinopathy identified many proteins responsible for biological processes and could be considered clinical biomarkers (Kim et al., 2007). These proteins are responsible for biological or pathological functions including but not limited to: cell growth and transport, metabolism and, immune response (Kim et al., 2007). It is also important to note that hyperglycemia accelerates different pathological pathways such as advanced glycation end products (AGEs), which formed by the non-enzymatic reaction of glucose and other glycating compounds derived both from glucose and from fatty acids (Giacco and Brownlee, 2010; Kowluru and Chan, 2007). Production of AGEs increases oxidative stress and disturbs protein function through modifying of the extracellular matrix component and causes the interaction between the modified proteins and AGE receptors (RAGE), which induced ROS production and mediate deleterious effects of hyperglycemia (Giacco and Brownlee, 2010; Kowluru and Chan, 2007). Most knowledge about the pathophysiology and therapeutic strategies were derived from *in vivo* experimental models of DR (Matteucci et al., 2014). However, retinal culture can have a significant contribution to the understanding of mechanisms of toxicity and treatment (Matteucci et al., 2014). Müller cells are the first responders in retinal diseases including diabetic retinopathy (Barber et al., 2000; Barber et al., 2003). In addition to vascular abnormalities, activation of Müller cells occurs in DR

and both lead to increased VEGF expression and exacerbation of the condition (Barber et al., 2003). Oxidative stress also plays an important role in exacerbating the condition (Kowluru and Chan, 2007). In the present study, the human Müller cell line MIO-M1 and HORCs were used to investigate cell survival under conditions of high glucose and also expression changes in key genes. In addition, MIO-M1 cells were used to determine the influence of glucose, hypoxia and oxidative stress on VEGF release and the role of PKCβ in the process was investigated.

7.3.1 Effect of High Glucose on the Cytotoxicity and Viability Human Müller Cells (MIO-M1) and RGCs Survival in HORCs

In the present study, elevated glucose levels (10-25mM) for 24hrs did not affect viability or increase LDH levels in MIO-M1 cells compared to the culture at normal glucose levels (5.55mM). Shin et al., (2014) reported similar results in mouse retinal astrocytes, where exposure to high glucose (40mM) had no effect on apoptosis of retinal astrocytes, although it did enhance their proliferation. On the other hand, several studies have reported reduced viability with equal or higher level of glucose in different Müller cell lines. For example, exposure of transformed rat retinal Müller cells (the rMC-1 cell line) to high glucose (25mM) resulted in decreased viability with a 1.5-fold increase apoptotic cells compared to control (Du et al., 2003), a finding confirmed by other investigators (Muto et al. 2014; Trueblood et al., 2011). Other researchers have used a higher level of glucose (Han et al., 2015) showing that the exposure of primary rat Müller cells to 55mM glucose resulted in a 50% reduction in viability with significant increases in LDH release compared to the control (25mM). Another study by Zhao et al., (2015), in which exposure of primary rat Müller cells to high glucose (15-55mM) for 24, 48, and 72hrs, revealed that exposure to 35mM for 48hrs caused an approximate 40% reduction in viability. It is possible that

transformed or primary rat Müller cells respond to high glucose stress differently than human Müller cells. Also if MIO-M1 cells were exposed to a higher glucose concentration of more than 25mM cell death may occur. However, whether these levels would be considered to be relevant to levels seen in diabetes would have to be considered. Exposure of HORCs to high glucose for 24hrs did not cause any death as evidence by a comparable level of LDH release and RGC number.

Other researchers have found that hyperglycemia-induced apoptosis in neuronal and Müller cells in rat and human retinas as well as Müller cell activation (Hammes et al., 1995; Kerrigan et al., 1997). Furthermore, GFAP staining presented along the entire length of the Müller cell processes in retinas obtained from *post-mortem* diabetic patients as well as streptozotocin-induced diabetic rats (Mizutani et al., 1998; Zeng et al., 2000). Reduction in the NeuN-positive retinal ganglion cells with increases in TUNEL staining was also observed in streptozotocin-diabetic rats (Barber et al., 1998; Zeng et al., 2000) and human diabetes (Barber et al., 1998). A significant reduction in Brn3a has been found after three months of inducing diabetes in rats (Zhao et al., 2015). In patients with DR, a thinning of the RGC layer was found with type 1 diabetes indicating an early neurodegenerative effect of high glucose on the retina (van Dijk et al., 2010). In the current study, high glucose stress in HORCs was induced over a very short time of 24hrs. This may not be enough to induce death compared to animal studies. More sensitive measures may be required to see initial changes using the human retinal culture.

7.3.2 Effect of High Glucose on the Expression of Growth Factors in Human Müller Cells (MIO-M1) and HORCs

Müller cells are considered one of the major sources of VEGF in the retina (Bandello et al., 2013; Reichenbach and Bringmann, 2013; Wang et al., 2010). As mentioned

earlier, several studies have shown increased expression of VEGF mRNA and protein under the condition of HG (Bai et al., 2009; Dong et al., 2014; Hofman et al., 2001; Ke et al., 2012; Mu et al., 2009). Expression of VEGF mRNA and release by rat Müller cells increased after exposure to HG (20mM) (Ke et al., 2012). Others reported that HG (25mM) causes VEGF secretion with a peak at 24hrs (Ye et al., 2012). In primary cultures of rat Müller cells exposed to HG (30mM), VEGF secretion increased at 48 and 72hrs compared to control (5.55mM) (Jiang et al., 2012). Similarly, expression of HIF-1α and VEGF mRNA in cultured rat retinal Müller cells increased after 24hrs exposure to 30mM HG (Li et al., 2012). In the present study, a significant 4-fold increase of VEGF mRNA in MIO-M1 cells was obtained under the highest glucose level of (25mM). Current results also showed that VEGF expression was increased in MIO-M1 cells under HG with no evidence of death or damage in contrast to other studies that reported high glucose-induced cell death. Findings by Sueishi et al., (1996) also showed that VEGF was expressed by RGCs under hypoxia and diabetic condition in rats and has been found that vitreous VEGF level is significantly higher in patients with PDR compared to non-diabetic patients (Ambati et al., 1997). In HORCs, expression of VEGF mRNA was upregulated by increasing the glucose levels and was found to be significantly elevated at 20mM. From literature searches this appears to be the first time that glucose has been shown to regulate VEGF secretion in the human retina.

In addition to changes in VEGF, MIO-M1 cells exposed to HG for 24hrs, showed an increase in expression of PDGF-A mRNA by 2.6-fold, highlighting the involvement of PDGF in Müller cells response in diabetic injury. No alteration in PDGF expression was found under HG treatment in HORCs. Other studies have reported that cultured human vascular endothelial cells increase production of PDGF in

response to high glucose (Mizutani et al., 1992; Mizutani et al., 1995; Okuda et al., 1996). Elevated PDGF levels have also been found retinas from diabetic patients (Gong et al., 2014; Praidou et al., 2009). This is the first time that changes in PDGF have been investigated in Müller cells. It may be therefore that the use of anti-PDGF in combination with anti-VEGF could exert a beneficial effect for the management of DR (Dong et al., 2014; Jo et al., 2006).

In addition, anti-angiogenic factors should be considered. The levels of VEGF mRNA and protein increased, and the levels of PEDF mRNA and protein decreased in rat Müller cells cultured under HG (10-30mM). This suggests that the balance of these factors is disturbed under hyperglycemia that may contribute to neovascularization (Mu et al., 2009; Xie et al., 2012). In addition, gliotic Müller cells from diabetic patients displayed decreased PEDF immunoreactivity (Lange et al., 2008). However, this reciprocal regulation between VEGF and PEDF in high glucose was not seen by Zhang et al., (2006) using rat retinal Müller cells. Current results show that no alteration to the expression of PEDF was found MIO-M1 cells under HG conditions.

The expression of bFGF under HG (25mM) in cultured MIO-M1 cells was found to follow a similar pattern to VEGF and PDGF in this study. Current results support the view that VEGF, PDGF, and bFGF produced by human Müller cells might be involved in the development and the progression of diabetic retinopathy.

7.3.3 Effect of High Glucose on Expression of Glutamate Markers in Human Müller Cells (MIO-M1) and HORCs

In vitro and *in vivo* studies indicated that glial dysfunction occurs in DR, and the activity of GS and glutamate transporters is reduced. Shen and Xu, (2009) suggested that an increase in IL-1β in rat Müller cells decreased the expression of GS under HG condition. In Müller cells isolated from diabetic rats, GLAST activity was reduced,

and this decrease was attributed to hyperglycemia–induced oxidative stress (Li and Puro, 2002). Furthermore, in rat Müller cells cultured under HG (25mM), GLAST expression decreased significantly suggesting a consequent reduction in glutamate uptake (Xie et al., 2012). Lieth et al., (1998, 2000) reported that GS activity was reduced in diabetic rat retinas with increased in GFAP expression and glutamate levels indicating that glutamate metabolism is impaired in activated Müller cells in diabetes. Moreover, in a study by Ambati et al., (1997), the vitreous concentration of glutamate was higher in patients with PDR than non-diabetic patients. Data presented here showed that the expression of glutamine synthetase (*GLUL*) did not change in MIO-M1 cells under HG treatment. In HORCs, the expression of *GLUL* and GLAST (*SLC1A3*) tended to increase under 25mM glucose compared to control HORC cultured under physiological glucose level (5.55mM). Although this was not found to be significant, it would be interesting to investigate the expression of these genes at different time periods in HORCs as maximal changes may not occure at 24hrs.

7.3.4 Effect of High Glucose, Oxidative Stress and Hypoxia on the Release of VEGF in Human Müller cells (MIO-M1)

Initial experiments suggested that MIO-M1 cells released VEGF under hyperglycemic conditions. As hypoxia also occurs in the advanced stages of diabetic retinopathy (DR) (Arden and Sivaprasad, 2011; Hamanaka et al., 2001), MIO-M1 cells were also exposed to hypoxic conditions, which is a known potent stimulant of VEGF expression and release. Data showed that full oxygen deprivation ($0\%O_2$) strongly influenced the release of VEGF both under physiological and high glucose concentrations in the MIO-M1 cells. Aiello et al., (1995) showed similar results with retinal pigment epithelial cells, pericytes, and microvascular endothelial cells in which hypoxia (0% to 5% O_2), caused an increase in VEGF expression.

In a study by Hirata et al., (1997), human retinal Müller cells cultured in HG condition (17.5mM) followed by exposure to advanced glycation end products (AGE) for 12hrs increased expression of VEGF mRNA and protein. Introducing another important factor in diabetes, oxidative stress, VEGF release was found higher under normal glucose at 24hrs than with HG, and it does not seem that oxidative stress and HG work synergistically to induce VEGF release. Several studies have shown that high glucose conditions alone can increase the production of ROS in glial cells. Furthermore, high glucose conditions had a significant impact on production of ROS and production of inflammatory mediators in cultured mouse astrocytes (Shin et al., 2014). High glucose treatment (30mM) depleted the cellular glutathione content of rat Müller cells by 60% after 48hrs (Jiang et al., 2012). In diabetic rat retinas and rMC-1 cells exposed to elevated glucose concentrations (25mM), superoxide production was significant increased (Du et al., 2003). In addition, MIO-M1 cells cultured with chronically HG concentrations (17.5mM) demonstrated an increase in H_2O_2 levels compared to those cultured in normal glucose (5.55mM) conditions (Giordano et al., 2015). The PKCβ inhibitor ruboxistaurin (LY333531) is a bisindolylmaleimide compound, which shows selectivity for PKCβI and βII over PKCα and other PKCs classes when administered in the nM range (Ishii et al., 1996; Jirousek et al., 1996). A study by Cha et al., (2000) found that a protein kinase C inhibitor -calphostin-C- inhibited glucose-induced increases in VEGF production in cultured in rat mesangial cells. In an attempt to block the stimulatory effect of glucose on VEGF release, PKC-β-specific inhibitor LY-333531 was used in two doses in MIO-M1 cells exposed to conditions associated with DR. In the present study, the inhibitory effect of LY333531 on VEGF release was tested under conditions of HG, oxidative stress, and hypoxia. Results suggest that LY-333531-induced a trend towards reduction in VEGF

obtained under normal glucose (5.55mM) in the first 48hrs, which was not seen under HG (25mM).

In addition, current data showed that high dose of LY-333531 (1μM) was effective in reducing hypoxia-induced VEGF release both under normal and high glucose levels. A significant reduction was found in the first 24hrs under high glucose condition but not at 48 and 72hrs. As the cells were treated once at the beginning of the experiments it is possible that the treated cells metabolize the drug, or it is broken down and would be less effective at later time points. These results can be compared to dose from primary cultured RPE, which showed that VEGF expression and release under HG and hypoxia is PKC-dependent (Young et al., 2005). In this case an inhibitor of PKC-delta was used to reduce VEGF secretions under the examined condition (Young et al., 2005).

Considering the role of oxidative stress, studies have found that both factors increase VEGF expression. Exposure of rat mesangial cells to HG (25mM) for 48hrs increased oxidative stress and VEGF mRNA and protein, an effect that was suppressed by LY-333531 (Xia et al., 2007). Treatment of human RPE and rat glioblastoma to H_2O_2 (400μM) and hypoxia-induced the expression of VEGF mRNA and increased protein production (Kuroki et al., 1996). *In vivo* hypoxia in rats increased oxidative stress and VEGF protein expression (Sasaki et al., 2000). In the current experiments, no significant inhibition was seen when investigating the role of PKCß with HG and oxidative stress. The attempt to block VEGF release from MIO-M1 cells under the condition of hypoxia and oxidative stress with LY-333531 remained inconclusive. It would be particularly interesting to investigate whether PKC inhibition could block high glucose-induced VEGF secretion in HORC model.

Chapter 8

General Discussion

The aim of the present study was to investigate the effect of potential stressors in glaucoma, specifically oxygen and glucose deprivation (OGD; simulated ischemia) on the survival, proliferation and response of Müller cells, the major glial cell in the retina. This aim was achieved using the human Müller cell line (MIO-M1). These cells express markers of mature Müller cells, including cellular retinaldehyde-binding protein (CRALBP), glutamine synthetase, vimentin and epidermal growth factor receptor (EGF-R) and, therefore, represent a good model to study Müller cell response to pathological stimuli (Limb et al., 2002). Furthermore, simulated ischemia and its effects on the survival of RGCs and gene expression were also investigated in the human retina, using the HORCs model. The model of organotypic culture enables to study the effect of different stressors on the retina such as OGD and could serve as a link between *in vitro* and *in vivo* models (Niyadurupola et al., 2011; Osborne et al., 2015). A summary of the major findings presented in this thesis is given in Figure 8.1. As discussed earlier, ischemia is part of the pathophysiology of glaucoma and may occur due to a reduction in blood flow as a consequence of the increase in IOP (Osborne et al., 1999) or vascular dysregulation (Ghanem et al., 2011). The model of simulated ischemia used in *in vitro* studies is based on the complete removal of oxygen from the glucose-free medium and growing the culture in a nitrogen atmosphere causing complete OGD. From a clinical standpoint, this is not a good correlation to *in vivo* ischemia, as the extent of blood flow reduction may be variable and may not reach the full deprivation.

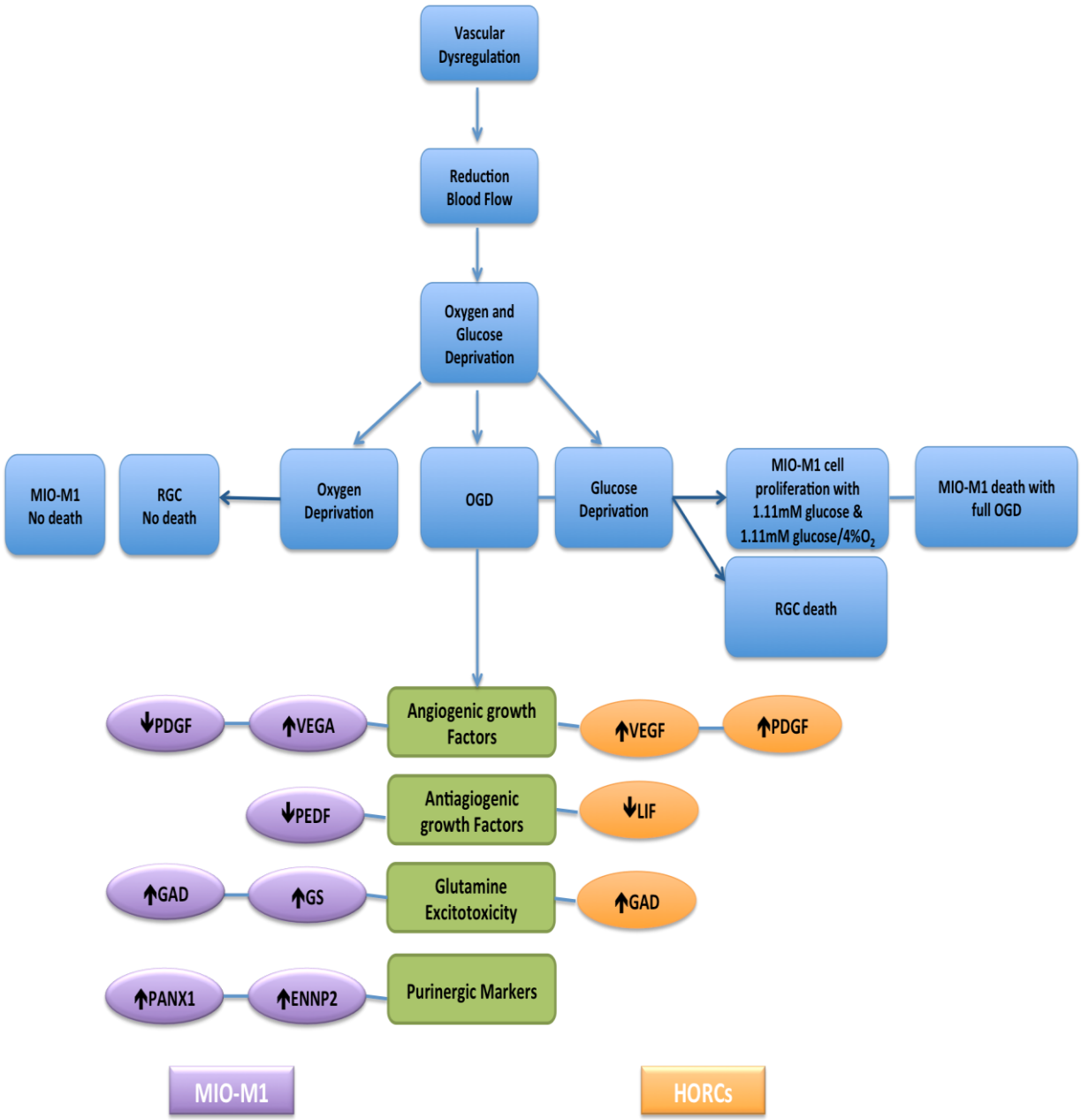


Figure 8.1: Summary of the effect of oxygen and glucose deprivation on RGC and Müller cell survival and gene expression. For details about changes in gene expression under each condition refer to Table 4.1 and 5.1.

Full deprivation causes very fast damage while graded levels of deprivation give more insight to how cells behave under stress without losing viability. Graded-levels of oxygen and glucose deprivation gave detailed information about the survival of MIO-M1 cells and showed how they are resistance to these insults. A key finding of this research was that Müller cells (MIO-M1) respond to a degree of oxygen and glucose deprivation (1.11mMglucose/4%O_2) and glucose deprivation (1.11mM) by proliferation. It is now well documented that glial cells proliferate in response to injury (Bianchi et al., 2015; Cho et al., 2015; Fan et al., 2013; Hollborn et al., 2004; Mascarelli et al., 1991; Moon et al., 2009; Romo et al., 2011). Their specialized energy metabolism, which depends up to 80-90% on anaerobic glycolysis, contributed to their survival in anoxia and ischemia (Poitry-Yamate et al., 1995; Tsacopoulos et al., 1998; Winkler et al., 2000). Studies have found that the utilization of Müller cells of lactate, pyruvate, glutamine or glutamate to generate energy by the tricarboxylic acid cycle increasing their survival in the absence of glucose (Winkler et al., 2000). However, the later amino acids did not support survival in the current experiments under full OGD.

The results in this thesis support that glucose is of key importance for MIO-M1 cells and they can withstand oxygen but not glucose deprivation. In the retina, low glucose supply as a result of an ischemic event or intense activity causes Müller cells to shift into anaerobic metabolism to provide the monocarboxylates such as lactate and pyruvate to photoreceptors and neurons (Winkler et al., 2000; Winkler et al., 2003). This process has also been proposed to occur in the brain and is called activity-linked neuron lactate shuttle hypothesis (ANLSH) and occurs in rat C6 glioma cells and astrocytes (Bouzier et al., 1998; Bouzier-Sore et al., 2003; Genc et al., 2011; Pellerin, 2003). Frenzel et al., (2005) showed that rabbit Müller cells incubated in 0.555mM

glucose maintained similar ATP levels to control (11mM) and survived oxidative stress induced by NO. It has been found that the presence of pyruvate or other monocarboxylates can protect Müller cells when cultured in deprived glucose conditions. This protection is abolished in the complete absence of glucose (Frenzel et al., 2005). This is supported by the findings presented in this thesis where MIO-M1 cells survived different levels of deprivation, but not full glucose deprivation. It is of particular interest to find that glucose is the main factor for MIO-M1 cells survival rather than oxygen. Others have reported that oxygen deprivation causes glial cell death (Bondarenko and Chesler, 2001; Callahan et al., 1990; Kelleher et al., 1993; Tan et al., 2015; Yan et al., 2005; Yu et al., 1989). In this research, it has been reported that MIO-M1 cells survive hypoxia (0 and 4%) for up to 72hrs with no evidence of death.

In the present research, exposure of MIO-M1 cells to 2% and 4%O_2 caused significant cell proliferation compared to the atmospheric control level although most gene expression including VEGF did not alter under reduced oxygen level (4%O_2), most changes were obtained with full hypoxia (0%O_2). Other researchers have also found that low O_2 concentrations (1–5%O_2) stimulate cell proliferation in different cell lines (Cipolleschi et al., 2000; Guzy and Schumacher, 2006; Hermitte et al., 2006; Ivanovic et al., 2000; Jiang et al., 1996). These low oxygen concentrations are thought to be enough to produce energy by mitochondrial respiration allowing cell proliferation while full oxygen deprivation or anoxia would permit the shift into anaerobic glycolysis to maintain the energy level without allowing cell proliferation (Guzy and Schumacher, 2006; Ivanovic, 2009; Jiang et al., 1996). However, this is not the case with Müller cells, as they are known to prefer anaerobic glycolysis to obtain energy even in the presence of glucose and oxygen, and they are characterized by a very low

rate of oxygen consumption (Winkler et al., 2000). It has been found that the oxygen concentration in the retina varies from 1 to 5% (Buerk et al., 1993; Yu and Cringle, 2005). This level of oxygen refers to "physiological hypoxia" and is thought to occur during development and is well tolerated by the tissue being sufficient for the normal function and does not induce any pathologic events (Chan-Ling et al., 1995; Guzy and Schumacher 2006). It has been argued that the atmospheric O_2 concentration is too elevated for the cells of most tissues, and experimental conditions should be compared to physiological hypoxia, and the atmospheric oxygen culture condition should be considered as hyperoxia (Ivanovic et al., 2009). Most research data is derived from experiments at 20–21%O_2 including the experiments presented in this thesis but certainly it is an important point to consider. There are many technical difficulties, which prevented experiments under physiological hypoxia including the availability of hoods and incubators set at that level of oxygenation. It is also important to consider that the cells were previously exposed to levels of 20–21%O_2 during isolation, growing, and splitting stages making this the control level they were exposed to, and, therefore, any level below that would be considered as hypoxia.

To further understand if cell proliferation under 1.11mM glucose and 4% oxygen could cause expression of survival factors, important neuroprotective angiogenic growth factors, and excitotoxicity (glutamate and ATP) related genes were investigated. Changes in gene expression were seen. Before discussing these it is worth considering that the increase in expression in mRNA may be due to either enhanced mRNA stability or de novo transcription. As the level of mRNA within a cell depends on both synthesis and decay rate, both can regulate the levels of cellular messenger RNA transcripts and, therefore, affect the expression of specific genes such as cytokines and growth factors. Interestingly, the regulation of mRNA stability

is found to occur in the pathways whereby tissues and organs respond to stresses including starvation, infection and inflammation (Ross, 1995). From the current experiments it is not possible to determine whether changes in synthesis or turn over were responsible for changes in expression that were seen.

Significant upregulation of VEGF mRNA was found with complete deprivation of oxygen and/or glucose. Although the VEGF mRNA was not significantly elevated under reduced levels (1.11mMglucose/4%O_2), evidence of VEGF protein secretion by MIO-M1 cells was found for up to 72hrs. Studies have indicated that VEGF expression and release play an important role in ischemic injury in neovascular glaucoma (Chalam et al., 2014; Lim et al., 2009; Pe'er et al., 1998; Tripathi et al., 1998). Therefore, the use of anti-VEGF treatments could be used in future experiments to test their effect on cell survival and preventing ischemia-induced damage. The intracellular signaling pathways of different growth factors were not investigated in the current study, but it is important to discuss possible pathways. Studies have shown that ERK1/2, the p38 kinase family, and the c-Jun N-terminal kinase (JNK) family are the major mitogen-activated protein kinases (MAPKs) involved in the proliferation and stimulatory effects of growth factors and cytokines (Hollborn et al., 2004; Milenkovic et al., 2003). Activation of MAP kinases occurs in the cytoplasm and causes translocation into the nucleus (Roskoski et a., 2012). Activation of ERK1/2 and c-Fos in Müller cells are important mechanisms, which rescue retinal neurons from cell death (Peng et al., 1998; Rohrer et al., 1999; Akiyama et al., 2002; Nakazawa et al., 2008). In the current research, the expression of PDGF did not change with reduced levels of oxygen and glucose but were significantly reduced with full OGD. It is less likely that PDGF is involved in MIO-M1 cells proliferation in contrast to the findings of Moon et al., (2009) that PDGF-stimulated

Müller cell proliferation through JNK and Akt signaling pathways. Also, it was found that bFGF is significantly increased in full glucose deprivation while reduced with 1.11mM glucose. Studies found the neuroprotective effects of bFGF to occur via activation of ERK1/2 and c-Fos in Müller cell (Walhin et al., 2000, Walhin et al., 2001). Current data showed that the expression of PEDF was significantly reduced with full OGD and hypoxia. The beneficial effect on neuronal survival by PEDF has been proposed through different pathways including p38 MAP kinase (Chen et al., 2006), ERK-1/-2 MAP kinase (Sanchez et al., 2012), stress-activated phospho-kinase (JNK) (Konson et al., 2011), and phosphatidylinositol 3-kinase/Akt signaling cascades (Haribalaganesh et al., 2010). From the discussion above, it is clear that there is significant overlap of the intracellular signaling cascades activated in response to different growth factors. These pathways may play a role in MIO-M1 cells response to hypoxia and/or glucose deprivation, which needs to be determined in future experiments. Importantly, the present research has found that human Müller cells may release cytotoxic factors under a stressful condition such as glucose deprivation, which may kill RGCs rather than protect them. As MIO-M1 cells survived for 24hrs under conditions of 1.11mM glucose and, indeed proliferate, it was assumed that the cells were releasing survival factors. Culturing retinal explants on a monolayer of MIO-M1 cells caused increased damage to HORCs even under control condition. The molecular mechanisms by which glial cells modulate neuronal death have not been investigated in the current study and remain to be determined in future experiments. Many investigators have reported that activation of glial cells causes production of neurotoxic molecules such as nitric oxide, ROS, TNF-α and IL-1 (Cotinet et al., 1997; Fuchs et al., 2005; Kawasaki et al., 2000; Yoshida et al., 2004). Therefore, It would be interesting to analyze the conditioned medium from MIO-M1

cells for released cytotoxic molecules or cytokines to try to determine the factors responsible for the observed damage. Interestingly, Xie et al., (2004) found that co-culture of glial cell (astrocytes and microglia) caused neuronal death possibly via increased upregulation of iNOS and IL-1 with activation of MAPK signaling pathways. Inhibition of p38 and JNK partially protected neurons from glia-induced death. In activated Müller cells (MIO-M1), IL-6, IL-8 production is mediated through the p38 MAPK pathway (Liu et al, 2014; Liu et al, 2015). Therefore, it is essential to investigate the contribution of these pathways to glial activation and subsequent neuronal death as modulation of these responses may help in the development of therapeutic interventions.

To simulate glaucomatous retinal ganglion cell (RGC) degeneration *in vitro*, oxygen and/or glucose deprivation was also investigated using the human organotypic retinal culture (HORC) model. In HORCs, graded levels of glucose provided a useful indicator of how glucose can cause death to retinal cells. The greater the deprivation, the more TUNEL-positive cells were absent in different retinal layers. Oxygen-glucose deprivation (OGD) was capable of reducing NeuN and significantly increases TUNEL-positive RGCs. It is interesting to find that oxygen deprivation alone was unable to increase LDH levels or cause significant loss of RGCs in the HORC model. Depletion of ATP stores due to the absence of glucose and oxygen causes energy failure and initiation of disruption of the ionic balance and changes in levels of neurotransmitter as well as metabolites (Lipton, 1999). Excitotoxic damage induced by excess glutamate is proposed to play a central role in ischemic retinal death (Lipton, 1999). Glucose deprivation under normoxic conditions increased the susceptibility of cerebral neuron neurons to low glutamate concentration (Kimura et al., 1999; Nishizawa, 2001). Other responses found by other researchers to contribute

to retinal ischemic injury include the increase in potentially toxic inflammatory mediators such as TNF-α, NO and IL-1 (Morgan et al., 1999; Rathnasamy et al., 2014; Roberge et al., 1988; Yoshida et al., 2004). Hypoxia is a potent stimulator of angiogenic factors, most importantly VEGF, a growth factor whose expression is controlled by hypoxia-inducible factor (HIF). Furthermore, glucose deprivation (Chen et al., 2015; Nishimoto et al., 2014) and sub-lethal OGD (Huang et al., 2014) has also been found to increase HIF1-α and leads to anti-apoptotic effects in cancer cells (Nishimoto et al., 2014). Findings by Stein et al., (1995) showed that exposure of C6 cells, a clonal glial cell line, to hypoxia or hypoglycemia resulted in increase expression of VEGF. Data from this research showed that expression of VEGF increases in MIO-M1 cells and HORCs under certain deprivation conditions. Future experiments are needed to investigate the expression of HIF1-α under conditions of glucose deprivation and reduced level (1.11mM glucose/4%O_2) in MIO-M1 cells and HORCs. This research also found that the MIO-M1 cell line could be utilized in a model of simulated hyperglycemia *in vitro* and to investigate the mechanisms of high glucose, oxidative stress, and hypoxia induced-VEGF production. One of the shortcomings of this model is the time required for simulating the pathology of diabetes. In the current model, MIO-M1 cells were exposed to high glucose for a relatively short period compared to that which they would be exposed to in *in vivo* models or in human diabetes. In addition, oxidative stress and hypoxia develop in advance stage of the disease accompanied with microvascular abnormalities so mimicking the entire pathophysiological progression of the disease is difficult in cell culture (Arden and Sivaprasad, 2011). Cultured Müller cells (MIO-M1) were sensitive to the combination of HG and H_2O_2 and lost viability after 48hrs exposure, and this limited the experimental condition to 24hrs period only.

The experimental hyperglycemia was induced in this thesis by high glucose medium of a maximum 25mM. It should be noted that some cells in cultures are routinely grown in this concentration (Russell et al., 1999; Vincent et al., 2005) but as MIO-M1 cells are routinely cultured in 5.55mM glucose then the higher level of 25mM was considered enough to produce a hyperglycemic insult without causing death. In contrast, other researchers have found that 25-50mM can induce apoptotic cell death in neuronal retinal culture (Santiago et al., 2006; Costa et al., 2012; Oshitari et al., 2010) as well as in Müller cell lines (Du et al., 2003; Han et al., 2015; Muto et al. 2014; Trueblood et al., 2011, Xi et al., 2005). The time of exposure to HG, which caused cell loss, was variable between different studies. In some studies, death was reported after 24hrs (Trueblood et al., 2011), 48hrs (Han et al., 2015; Matteucci et al., 2014), or 72hrs (Xi et al., 2005). Others reported longer survival time for five days exposure (Chavira-Suarez et al., 2011; Du et al., 2003).

The current research has reported 72hrs survival after exposure to HG but not if it is combined with oxidative stress. This evidence of different susceptibility highlights the need for more experiments to characterize the precise time course of HG-induced stress and cell death. In conclusion, the present research has provided insight to the survival and alteration in gene expression of different growth factors, glutamate and glial activation markers in human Müller cells (MIO-M1) when exposed to stressors associated with the development of glaucoma. More evidence was obtained from exposure of HORCs to oxygen and glucose deprivation, which caused a reduction in RGC survival and changes in genes expression. Moreover, both models can be also used to simulate hyperglycemia in diabetic retinopathy. These models may therefore be of benefit in investigation of potential therapeutic interventions to retinal neurodegenerative disease, including glaucoma

List of Abbreviation

AGEs	Advanced glycation end products
α-SMA	Alpha-smooth muscle actin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	A one-way analysis of variance
ADP	Adenosine diphosphate
A_{1-3}	Adenosine receptors 1-3
AQP4	Aquaporin-4 channels
ATP	Adenosine 5′-triphosphate
BDNF	Brain-derived neurotrophic factor
bFGF (FGF2)	Basic fibroblast growth factor
CAIs	Carbonic anhydrase inhibitors
cDNA	Complementary Deoxyribonucleic acid
COAG	Chronic open angle glaucoma
CNS	Central Nervous System
CNTF	Ciliary neurotrophic factor
CRBP	Cellular retinol binding protein
CRALBP	Cellular retinal binding protein
CYC-1	Cytochrome c-1
DAG	Diacylglycerol
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline solution
DR	Diabetic retinopathy
$EAAT_{1-5}$	Excitatory amino acid transporter
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELAM-1	Endothelial leukocyte adhesion molecule-1
ELISA	Enzyme Linked-Immuno-Sorbent Assay
EMEM	Eagle's Minimum Essential Medium
eNOS	Nitric oxide synthase
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2

ERK	Extracellular-signal-regulated kinases
ET	Endothelin
FBS	Foetal bovine serum
GABA	Gamma-aminobutyric acid
GAD1	Glutamate decarboxylase 1
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GLAST	Glial glutamate transporter
GLUL	Glutamate-ammonia ligase (Glutamine Synthetase)
GLUT 1-4	Glucose transporters 1-4
GS	Glutamine synthetase
H_2O_2	Hydrogen perodxide
HBSS	Hank's balanced saline solution
HG	High glucose
HGF	Hepatocyte growth factor
HO-1	Heme oxygenases 1
hONAs	Human optic nerve head astrocytes
HORC	Human organotypic retinal culture
hRPE	Human retinal pigment epithelium
HSPs	Heat shock proteins
HSPA1B	Heat shock 70kDa protein 1B
HIF-1α	Hypoxia-inducible factor-1α
IGF-1	Insulin-like growth factor-1
IL-1β	Interleukin-1 beta
INL	Inner nuclear layer
Kir4.1	Inwardly rectifying K^+ channel of the
IOP	Intraocular pressure
IPL	Inner plexiform layer
JNK	c-Jun N-terminal kinases
LDH	Lactate dehydrogenase
LIF	Leukemia inhibitory factor
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine

LY333531	The PKCβ inhibitor ruboxistaurin
MFC	Mass flow controllers
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MTS	CellTiter 96® AQueous One Solution Cell Proliferation
MIO-M1	Moorfields/Institute of Ophthalmology-Müller 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NeuN	Neuronal Nuclei
NGF	Nerve growth factor
NHS	National health service
NMDA	N-methyl-D-aspartate receptor
NO	Nitric oxide
NTG	Normal tension glaucoma
OCT	Optimal cutting temperature medium
OGD	Oxygen-glucose deprivation
ONH	Optic nerve head
ONL	Outer Nuclear Layer
OPL	Outer plexiform layer
PACG	Primary angle-closure glaucoma
PANX1	Pannexin 1
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PEDF	Pigment epithelium derived factor
PKC	Protein kinase C
POAG	Primary open angle glaucoma
PS	phosphatidylserine
PVR	Proliferative vitreoretinopathy
QRT-PCR	Quantitative reverse transcription polymerase chain reaction (RT-PCR)
RGCs	Retinal ganglion cells
RLT	RNeasy lysis buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium

TESPA	3-triethoxysilylpropylamine
TGF-β	Transforming growth factor beta
THY-1	Thy-1 Cell Surface Antigen
TM	Trabecular meshwork
TNF-α	Tumor necrosis factor alpha
TOP-1	Topoisomerase I
TSP-1	Thrombospondin-1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

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Appendix A: Average threshold cycle (C_T) values from QRT-PCR of different genes investigated in MIO-M1 cells under control, 1.11mM glucose, hypoxia (4%O_2) and 1.11mMglucose/4%O_2 conditions at 24hrs (n=4).

Genes	Control	1.11mM glucose	Hypoxia (4%O_2)	1.11mMglucose/4%O_2
VEGF	27.99	27.56	27.86	27.58
PDGF	29.77	29.67	29.87	29.79
bFGF	28.59	28.56	28.30	28.95
PEDF	30.29	30.59	30.38	30.98
LIF	32.18	32.44	31.88	32.34
SLC1A3	28.50	28.60	28.40	28.92
GLUL	26.16	25.78	26.20	25.98
GAD	31.12	31.36	31.31	31.67
PANX1	22.91	22.38	23.07	22.34
ENNP2	29.67	29.43	29.55	29.45
GFAP	29.54	29.69	28.91	29.36
HSPA1B	25.36	25.10	25.34	25.33
CYC1	25.53	25.15	25.20	25.28
TOP1	27.18	27.16	27.15	27.23

Appendix B: Average threshold cycle (C_T) values from QRT-PCR of different genes investigated in MIO-M1 cells under control, no glucose, hypoxia and full OGD conditions at 3,6,12 and 24hrs (n=4).

	Control				No glucose				Hypoxia				Full OGD			
Genes	3hrs	6hrs	12hrs	24hrs	3hrs	6hrs	12hrs	24hrs	3hrs	6hrs	12hrs	24hrs	3hrs	6hrs	12hrs	24hrs
VEGF	26.71	26.14	26.38	25.53	25.93	26.18	24.97	24.77	25.95	23.15	21.55	23.15	25.40	23.40	22.35	21.48
PDGF	27.24	27.41	27.28	27.63	26.87	27.20	27.34	28.13	27.07	27.11	28.26	28.17	27.02	27.27	28.03	28.74
bFGF	29.86	29.85	29.62	29.94	29.61	30.17	29.50	29.69	29.48	30.02	30.20	30.07	29.73	30.19	30.18	30.33
PEDF	30.26	30.75	29.41	29.03	30.19	30.52	29.36	29.54	29.96	30.48	29.89	30.09	30.23	30.44	29.92	30.23
LIF	31.99	32.15	30.58	30.38	29.20	30.25	30.07	30.61	31.24	30.98	30.10	29.32	29.07	29.69	30.38	31.27
SLC1A3	26.99	27.21	27.61	28.17	26.76	27.58	28.33	28.97	26.60	27.05	28.00	28.43	26.91	27.30	28.08	29.30
GLUL	24.32	24.93	24.26	24.70	24.22	24.92	24.36	24.96	24.20	24.59	23.75	24.90	24.42	24.68	24.37	24.70
GAD				31.24				31.51				32.10				31.30
PANX1				22.09				21.93				22.10				21.40
ENNP2	27.64	28.13	27.57	28.01	27.31	28.06	27.18	27.97	27.10	27.68	27.44	28.06	27.18	27.61	27.69	27.91
GFAP	26.49	26.80	27.19	26.55	26.36	26.72	27.10	26.97	26.42	26.69	27.58	27.07	26.49	26.50	27.21	27.76
HSPA1B	25.19	24.88	24.96	24.87	23.02	24.80	24.13	24.49	24.94	25.14	25.63	25.38	22.28	23.63	21.78	22.8
CYC1	24.42	24.66	24.42	24.48	24.30	24.50	24.16	24.78	24.24	24.50	24.87	25.17	24.35	24.53	24.74	24.95
TOP1	26.18	26.65	26.15	26.36	25.73	26.57	26.10	26.61	25.88	26.50	26.81	26.88	25.73	26.40	26.58	27.06

Appendix C: Average threshold cycle (C_T) values from QRT-PCR of different genes investigated in HORCs under control, 1.11mM glucose, hypoxia (4%O_2) and 1.11mMglucose/4%O_2 conditions at 24hrs (n=4).

Genes	Control	1.11mM glucose	Hypoxia (4%O_2)	1.11mMglucose/4%O
VEGF	23.42	23.42	22.84	23.44
PDGF	28.41	28.42	28.81	29.49
bFGF	27.32	28.86	27.62	28.94
PEDF	26.07	26.45	26.42	27.35
LIF	23.31	25.29	24.63	26.01
SLC1A3	24.37	24.62	24.61	25.47
GLUL	22.69	21.46	22.25	22.15
GAD	25.64	25.25	25.15	25.23
PANX1	27.51	27.26	27.43	27.79
ENNP2	30.38	30.70	30.29	31.32
GFAP	24.33	24.86	24.67	24.44
HSPA1B	20.30	18.79	19.66	19.09
CYC1	24.55	24.69	24.81	25.36
TOP1	25.47	25.81	25.98	26.38

Appendix D: Average threshold cycle (C_T) values from QRT-PCR of different genes investigated in HORCs under control, No glucose (0mM), hypoxia (0%O_2) and full OGD conditions at 24hrs (n=4).

Genes	Control	No glucose	Hypoxia (0%O_2)	Full OGD
VEGF	23.01	22.07	21.49	22.28
PDGF	28.54	29.09	28.58	28.48
bFGF	27.76	29.58	26.98	28.90
PEDF	30.35	31.02	29.70	29.97
LIF	25.54	28.52	27.00	29.84
SLC1A3	24.35	24.38	24.59	24.65
GLUL	22.31	21.27	22.07	21.88
GAD	25.49	25.14	25.43	25.11
PANX1	27.67	26.77	27.34	27.57
ENNP2	26.69	26.32	26.86	26.70
GFAP	24.03	24.38	23.85	24.27
HSPA1B	19.82	19.43	19.67	19.90
CYC1	25.15	25.36	25.02	25.49
TOP1	26.50	26.46	26.22	27.04