Development of Novel Techniques for Measuring Bulbar Conjunctival Red Blood Cell Velocity, Oximetry and Redness

by

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Authors Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis including any final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Introduction

The ocular surface provides a unique opportunity to study hemodynamics since the vessels can be visualized directly, without treatment and non-invasively. The availability of instruments to measure various hemodynamic parameters on the ocular surface in an objective manner are lacking. The quantification of red blood cell velocity, blood oxygen saturation and conjunctival redness on the ocular surface using novel, validated techniques has the potential of providing useful information about vascular physiology.

The specific aims of each chapter are as follows:

Chapter 3: The objective was to design, develop and validate a system that would non-invasively quantify the red blood cell velocity in the conjunctival vessels. A tool was developed to automatically analyze video sequences of conjunctival vessels, digitally imaged with high enough magnification to resolve movement of the blood within the vessel.

Chapter 4: The objective was to: a) design and develop a method in order to non-invasively quantify the changes in blood oxygen saturation (SO_2) in the conjunctival vessels and demonstrate reliability of the measures and, b) demonstrate the application of the method by showing a response to an isocapnic hyperoxic provocation and compare those values to the results from a valid instrument.

Chapter 5: The aim of this experiment was to examine variations in ocular redness levels, red blood cell velocities and oxygen saturation levels over time in clinically healthy participants and also to compare differences between two age groups.

Chapter 6: The aim of this experiment was to examine the ocular redness levels, red blood cell velocities and oxygen saturation levels in clinically healthy participants when a topical ophthalmic decongestant was instilled onto the eye and to demonstrate the validity of the use of two novel techniques.

Chapter 7: The aim of this experiment was to examine ocular redness, red blood cell velocity and oxygen saturation in participants who were habitual soft contact lens wearers (study) compared to those that did not (control) and also to compare differences in silicone (SH) and non-silicone hydrogel wearers.

Methods

Chapter 3: Simulations representing moving RBCs within a vessel and the random variation of each cell in terms of speed, shape and intensity were created in order to evaluate the performance of the algorithm. For each vessel, a signal that correlated to blood cell position was extracted from each frame, and the inter-frame displacement was estimated through a modified dynamic time warping (DTW) algorithm. This provided the red blood cell velocity over time in each point of the vessels. Thus, from these estimates, the mean red blood cell velocity for each vessel was easily evaluated. The true mean velocity from the simulation with the one estimated by the algorithm was compared and the system accuracy was determined.

Chapter 4: a) Conjunctival vessels were imaged with two narrow-band interference filters with O_2-sensitive and O_2-insensitive peak transmissions using a Zeiss slit lamp at 32x magnification. Optical densities were calculated from vascular segments using the average reflected intensities inside and outside the vessels. Optical density ratios were used to calculate relative oxygen saturation values. Video images of the bulbar conjunctiva were recorded at three times of the day. Measurement repeatability was assessed over location at each time and across consecutive frames. b) Subjects initially breathed air for 10 minutes followed by pure oxygen (O_2) for 20 minutes, and then air for a final 10 minute period using a sequential re-breathing circuit. Simultaneously, SO_2 values measured with a pulse oximeter ear clip and finger clip were recorded. The validity of the dual wavelength method was demonstrated by comparing the values to those from the ear clip pulse oximeter.

Chapter 5: Participants attended eight separate visits over the course of a day. Levels of bulbar conjunctival redness, red blood cell velocity and blood oxygen saturation were measured on a vessel of interest.

Chapter 6: Participants attended three separate visits during an allotted 60 minute session. Bulbar conjunctival redness, red blood cell velocity and blood oxygen saturation were measured on a vessel of interest, pre-insertion, just after insertion and, 10 minutes after insertion of a topical ocular decongestant. Significant differences between the three measures were assessed and correlations between the three parameters were reported.

Chapter 7: Participants were measured 8 times over the course of a day with their contact lenses in place. Bulbar conjunctival redness, red blood cell velocity and blood oxygen saturation were measured.

Results

Chapter 3: Results for the simulated videos demonstrated a very good concordance between the estimated and actual velocities supporting its validity. The mean relative error for the modified Dynamic Time Warping (DTW) method is 6%.

Chapter 4: The intraclass correlations (ICCs) between the three locations at each time point were 0.93, 0.56 and 0.86 respectively. Measurements across 5 consecutive frames showed no significant difference for all subjects (ICC = 0.96). The ICCs between the two methods at each time point were 0.45, 0.10 and 0.11 respectively. a) There was no significant difference in SO_2 between the three locations measured using the dual wavelength method for all subjects. There was also no significant difference between the three locations at any of the time points for the dual wavelength method. b) In response to isocapnic hyperoxic provocation using the dual wavelength method, blood oxygen saturation was increased from control values and subsequently recovered after withdrawal of hyperoxia. Blood oxygen saturation values recorded from the ear clip and finger clip of the pulse oximeter also showed an increase from control values and subsequently recovered after withdrawal of hyperoxia. SO_2 comparison between the dual wavelength method and the ear-clip pulse oximeter method did not show a significant difference. The interaction between the two methods and time on SO_2 was not significant.

Chapter 5: From baseline, the group mean redness and oxygen saturation did not change significantly over time. There was a significant difference in the group mean red blood cell

velocity values over time. There was no significant difference between age strata for all three measures.

Chapter 6: After drop instillation redness values decreased significantly. There was no change in red blood cell velocity and oxygen saturation over time. There was a moderate significant correlation between SO_2 and red blood cell velocity just after drop insertion.

Chapter 7: When comparing the study and control groups, no significant difference in redness or SO_2 over time was found. RBC velocity over time was found to be significantly different between groups. When comparing the two study groups (SH vs. hydrogel) no significant difference across either measure over time was found.

Conclusions

Chapter 3: Signal displacement estimation through the DTW algorithm can be used to estimate mean red blood cell velocity. Successful application of the algorithm in the estimation of RBC velocity in conjunctival vessels was demonstrated.

Chapter 4: The application of the dual wavelength method was demonstrated and optical density ratios can be used in a reliable manner for relative oxygen saturation measurements. This valid method promises to enable the study of conjunctival O_2 saturation under various experimental and physiological conditions.

Chapter 5: The results of this study support the theory of metabolic regulation. The lack of any significant change across time for redness and oxygen saturation along with significant changes in red blood cell velocity substantiates this notion.

Chapter 6: This study supports the literature regarding metabolic regulation of the microvasculature during the use of various stimuli. The results demonstrated that oxygen saturation levels remain stable even when a significant decrease in ocular redness is measured. The novel techniques used in this experiment demonstrated the expected action of the decongestant further contributing to their application and validity.

Chapter 7: In summary, the participants in the study group were habitual contact lens wearers that had lower RBC velocities when compared to the control group supporting the notion that contact lenses initiate a hypoxic response. The lack of change in SO_2 in either group supports the theory of metabolic regulation.

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Dedication

I dedicate this work to my husband Michael and my family. I am thankful and very fortunate to have each and every one of you in my life. Without your support I would not be where I am today. It’s the little things that count! Thank you. ∞

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1. Introduction

1.1 Systemic Circulation

The segment of the cardiovascular system which carries oxygenated blood away from the heart, delivers it to the body, and returns deoxygenated blood back to the heart is the systemic circulation.[1]

Oxygenated blood is circulated to the tissues via the arteries in this system. As the oxygenated blood circulates through the body, the oxygen diffuses into cells surrounding the capillaries and at the same time, carbon dioxide diffuses into the blood from the capillary cells.[1] The veins are responsible for returning the deoxygenated blood back to the heart.

1.1.1 Arteries[1]

Systemic arteries are subdivided into two types; muscular and elastic. The larger arteries (more than 1cm in diameter) are generally elastic and the smaller ones (between 0.1-10mm) tend to be muscular. Systemic arteries transport blood to the arterioles, and then to the capillaries, where oxygen, carbon dioxide and other gases are exchanged.

When oxygenated blood leaves the left ventricle of the heart, it enters the systemic circulation. The systemic circulation begins with the aorta, a massive and thick-walled artery. Numerous major arteries are given off from the aortic arch and go on to supply the upper body. Branches that stem from the diaphragm supply the lower parts of the body.

1.1.2 Capillaries[1]

The capillary network is where important exchanges happen in the circulatory system. Capillaries are the smallest of the body's blood vessels, measuring 5-10 μm. The capillaries have walls that are composed of a single layer of cells (the endothelium). This layer is so thin that molecules such as oxygen, water and lipids can pass through them by diffusion and enter the tissues. Waste products such as carbon dioxide can diffuse back into the blood for removal from the body.

The capillaries are the most numerous of the blood vessels and receive blood from the arteries. Capillaries join tissue with arterioles for the movement of oxygen and nutrients to the cells.

The network of capillaries that supply an organ is termed a capillary bed. The more metabolically active the organ, the greater number of capillaries it requires to supply it with nutrients.[2] The capillary bed only carries a fraction of the amount of blood it could, although this percentage can be increased through regulatory mechanisms, inducing smooth muscle relaxation and constriction of the smaller arterioles downstream.[2]

The capillaries do not include smooth muscle in their walls and therefore molecule exchange is passive.[3] If signalling molecules are released (e.g. endothelin for vasoconstriction or nitric oxide for vasodilation), they will act on the smooth muscle cells in the walls of larger vessels.[3]

1.1.3 Venules[1]

The systemic cycle is completed when the deoxygenated blood is collected by the venules, flows into the veins, into the inferior and superior vena cavae and back to the right heart. Blood re-enters the systemic circulation after it is re-oxygenated via the pulmonary circulation.

1.1.4 Veins[1]

The venous system collects the de-oxygenated blood and then branches into two major veins: the superior vena cava and the inferior vena cava. The right atrium of the heart is where these two major veins enter the systemic circulation.

1.2 Circulation of the Head and Neck

The upper systemic circulatory loop circulates blood to the head and neck regions.[1,4,5] This loop originates at the aortic arch and includes the brachiocephalic artery, left common carotid and left subclavian artery.[1,4,5] Blood leaves the head and neck via the subclavian vein and jugular vein.[1,4,5]

1.2.1 Blood Supply[1,4,5]

The right common carotid artery and the right subclavian artery are the first branches off of the brachiocephalic artery. The next branch (vertebral artery) provides blood to the right upper chest,

right arm, neck, and head. The vertebral artery then feeds into the basilar artery and the posterior cerebral artery and is responsible for providing most of the brain with oxygenated blood.

The left common carotid artery branches into the internal carotid (ICA) and the external carotid arteries (ECA) which supply the brain and the neck and face respectively.

1.2.2 Blood Brain Barrier

The circulatory system is protected by the blood-brain barrier (BBB) which is a semi-permeable membrane that controls the capillary leak potential.[6] In most parts of the body endothelial cells line the capillaries.[4,5] Endothelial tissue allows substances to move easily between the inside and the outside of the vessel via the small spaces that are between each individual cell.[4,5] Conversely, in the brain, substances cannot pass out of or into the bloodstream since the endothelial cells fit tightly together to create a tight junction.[4,5] Other transport methods, such as active transport, assist in the transport of some molecules (e.g. glucose) out of the blood.[6]

1.2.3 Ophthalmic Artery[4,8]

The major artery of the head and neck that helps supply the brain is the internal carotid artery.[7] One of the branches of the internal carotid artery is the ophthalmic artery. The ophthalmic artery supplies the eye and other structures in the orbit. Along with the optic nerve, the ophthalmic artery enters the orbit via the optic canal.[9] The branches of the ophthalmic artery are subdivided into the orbital and ocular groups.

1.2.4 The Ocular Group[4,8]

The ocular group is responsible for the distribution of vessels to the eye and its surrounding muscles. The ocular group includes the posterior ciliary arteries (long and short) and branches into two systems: the retinal supply and the uveal tract/ciliary body.

1.2.4.1 The Retinal Supply[4,8]

The central retinal artery enters inferior to the optic nerve 10mm posterior to the back of the eyeball in conjunction with the venous drainage exit and the central retinal vein. It pierces the optic nerve close to the eyeball, sending terminal branches over the internal surface of the retina

and is a major blood supply to the larger part of it. The branches develop to form continuous loops of arterioles flowing into venules with very few retinal anastomoses. Avascular areas form around the arterioles because of sufficient oxygen availability from freely diffusing oxygen.

The retinal arteries distribute blood to the inner two-thirds of the retinal depth while the remaining outer layers (e.g. photoreceptors) are nourished by the choroidal supply.[9]

The retinal areas are supplied by the uveal tract since they are in close proximity.[10]

1.2.4.2 Ciliary Body/Uveal Tract

The ciliary body and uveal tract supply blood to the iris, ciliary body, choroid, optic nerve head and give way to the short and long posterior ciliary arteries.[4,8]

The short posterior ciliary arteries (SPCA) pass forward around the optic nerve to the posterior part of the eyeball, pierce the sclera around the entrance of the optic nerve, and supply the choroid (up to the equator of the eye) and ciliary processes.[4,8] The SPCA's branch into the anterior and posterior choriocapillaris.[9]

The long posterior ciliary arteries are arteries of the head and supply the iris, ciliary body and choroid.[4,8] They pierce the posterior part of the sclera at some little distance from the optic nerve, and run forward, along either side of the eyeball, between the sclera and choroid, to the ciliary muscle, where they divide into two branches and form the major circle of iris with the anterior choriocapillaris that branch from the SPCA's.[9]

1.2.4.3 Anterior Ciliary Arteries[4,8]

The anterior ciliary arteries (ACAs) originate from the ophthalmic artery and supply the conjunctiva and sclera.[11]

The ACAs travel to the anterior portion of the eye alongside the extraocular muscles. The ACAs and the extraocular muscles form a vascular area underneath the conjunctiva, and eventually meet with the sclera a short distance from the cornea.

1.2.4.4 Conjunctival blood supply

The circulation of the conjunctiva can be divided into two portions, the peripheral and the central conjunctiva. The peripheral conjunctiva is supplied by the posterior conjunctival branches of the

peripheral arterial arch.[4,8] The central bulbar conjunctiva/limbal area is supplied by the anterior conjunctival branches of the anterior ciliary arteries (superficial) and the long posterior ciliary arteries (deep).[11] The anterior ciliary arteries branch toward the cornea from the ophthalmic artery to form a superficial plexus, the episcleral arterial circle[12].

As described by Bloch[13], there are two branches from the ophthalmic artery that supply the central bulbar conjunctival/limbal portions of the eye. One branch is responsible for superficial circulation and the other, deeper circulation. Both branches form the anterior ciliary arteries.[4,8] These arteries advance into the corneo-scleral junction (episcleral arterial circle) and then help form the intra-ocular arterial circle along with the long posterior ciliary arteries[14]. Many sets of capillaries branch from the episcleral circle at this point then run parallel to the corneo-scleral margin. From this point, the plexus divides into its connected superficial and deep layers responsible for the circulation[15].

The posterior component is responsible for supplying blood to the iris and the ciliary body [12].

The superficial component is a branch that forms the episcleral arterial circle[12]. Branching from the circle are 2 types of vessels, the first set of vessels form limbal arcades and the second set of vessels supplies the superficial conjunctiva.[4,8]

There are also communicating vessels that connect the intra-ocular arterial circle with the episcleral arterial circle, allowing for communication routes in the anterior eye[12].

1.3 Structure of Ocular Vessels

The arteries and veins have the same basic structure. There are three layers, from the inside to the outside (endothelium, smooth muscle cells, adventitia).[4,8] Conversely, the capillaries are only one cell layer thick.[1]

Capillaries consist of little more than a layer of endothelium and occasional connective tissue.[4,8] The endothelium in the capillaries is associated with a basement membrane which contains pericytes.[4,8] The pericytes get activated to stabilize the vessel.[4,8]

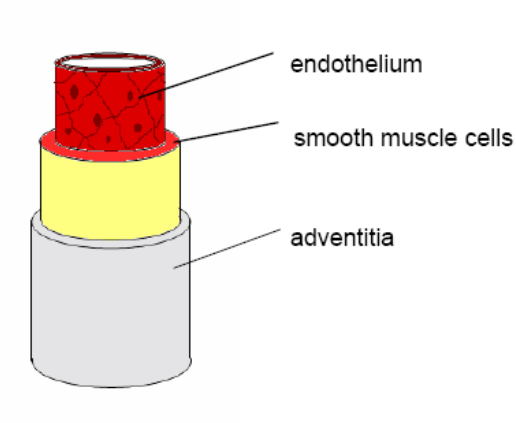

Figure 1-1: Structure of a blood vessel (Re-printed with the permission of Dr. Edward Gilmore)[16]

1.3.1 Tunica intima (the thinnest layer)

The tunica intima is a single layer of simple squamous endothelial cells joined by an intercellular matrix, surrounded by a thin layer of sub endothelial tissue connected with a number of circularly arranged elastic bands called the internal elastic lamina.[2]

There are three types of endothelial cells that line the interior of blood vessels depending on the location and function of the vessel.[2,4,8]

1.3.1.1 Continuous endothelial cells

Continuous endothelial cells are the most prevalent type found in the walls of arterioles, capillaries and venules of skeletal, smooth and cardiac muscle, skin mesentery, connective tissue, retina, brain, lung and lining of major conduit vessels.[2] These endothelial cells are characterized by occluding tight junctions and provide an impermeable barrier to large molecules.[2]

1.3.1.2 Fenestrated endothelial cells

Fenestrated endothelial cells are found mainly in exchange vessels of secretory and excretory organs and are characterized by numerous small “openings” that are between 50 and 80 nm wide.[2]

1.3.1.3 Discontinuous endothelial cells

Discontinuous endothelial cells are characterized by relatively large "openings" and are highly permeable. The capillary bed of the liver is an example of where these cells may be found.[2,4,8]

1.3.2 Tunica media (the thickest layer)

Circularly arranged elastic fibre that separates the second and third layer is the external elastic lamina.[2,4,8] The tunica media may (especially in arteries) be rich in vascular smooth muscle, which controls the calibre of the vessel.[2,4,8]

1.3.3 Tunica adventitia

This outer layer is entirely made of connective tissue and contains numerous fibroblasts.[2,4,8] It also contains nerves that supply the muscular layer, as well as nutrient capillaries in the larger blood vessels.[2,4,8]

1.4 Blood Flow

The flow of blood in the cardiovascular system and the study of it through vessels and vascular networks occupies an important place in physiology. Blood flow (Q) is equal to a change in pressure (ΔP) with respect to resistance (R).[17] The pressure difference is generally expressed as the difference between the arterial pressure and venous pressure and the resistance is the resistance to flow that is related in large part to the size of the valve opening.[18-20] Blood flow is readily described partially by Ohm's law and partially by Hagen-Poiseuille's law since Ohm's law is only accurate for Newtonian fluids.[17-21] The combination and use of the two laws help our understanding of the vast arrays of physiological phenomena. Blood is a non-Newtonian fluid whose viscosity varies with flow rate or rate of shear and its flow can be described as laminar only in the small vessels; elsewhere it is turbulent.[18-20]

$$Q = [\Delta P/R]^{18\text{-}20} \qquad \text{where,} \qquad R = [(8\upsilon L)/(\Pi r^4)]^{18\text{-}20}$$

and, υ = dynamic viscosity

L = the length of the tube/vessel

r = is the radius

It is important to note that with Poiseuille's equation, blood flow changes with the fourth power of the radius and therefore increases in blood flow can be achieved with small increases in vessel radius.

There are several rheological complexities that remain present given the non-Newtonian nature of blood and therefore caution should be used when interpreting results that are present in the literature since important assumptions are made about the vascular network "system" when using these formulas.[22]

There has been some on-going success in quantifying the rheological behaviour of blood in vitro but much work needs to be done about the physiological implications of rheological alterations.[18-20]

1.4.1 Blood Pressure

Blood pressure is the force exerted on the walls of blood vessels by the circulating blood and generally refers to arterial pressure.[4,5,22] As blood moves through arteries, arterioles, capillaries, and veins, the pressure of the blood decreases.[4,5,22]

Systolic pressure is the peak pressure in the arteries, occurring near the beginning of the cardiac cycle and diastolic pressure is the lowest pressure which occurs at the resting phase of the cardiac cycle.[4,5,22]

A healthy adult human typically has a resting blood pressure of approximately 120 mmHg systolic and 80 mmHg diastolic.[4,5] Arterial pressure values are not static and undergo natural variations throughout the day (circadian rhythm) and from heartbeat to heartbeat.[4,5] Blood pressure measures can also change in response to stress, nutritional factors, drugs, or disease.[4,5]

1.5 Ocular Blood Flow Measurement Techniques

Blood flow measurement of any circulatory path can be quite difficult since blood is a non-Newtonian fluid and its viscosity constantly changes.[23] Non-Newtonian fluids have distinctive characteristics, such as pulsatile flow.[23,24] The elastic nature of the vessels allows them to change

their shape and diameter in order to accommodate changes in circulation. When measuring blood flow, there are many factors that have to be considered. Some of the factors are patient history, surrounding conditions, temperature, blood pressure, medications, state of the red blood cells (e.g. shape, concentration). Those factors can dramatically change or influence the rate of flow.[25]

There are a few articles demonstrating the usefulness of using the measurement of bulbar conjunctival blood flow as a representation of the cerebral blood flow,[26] and microcirculation.[27] Most articles dealing with the measurement of blood flow in the conjunctiva do not measure it quantitatively. The techniques reported are qualitative or involve relative quantitative techniques and include photography[28], cinephotography[29], biomicroscopy[23,27] and laser Doppler flowmetry.[26]

Blood flow measurement of the retina is much more common. There are numerous publications that deal with various aspects of quantitatively and qualitatively measuring the blood flow of the posterior eye.[30-37] These techniques available for these tasks are sometimes used in combination to aid in the complete understanding of ocular hemodynamics.

1.5.1 Scanning Laser Ophthalmoscopy

Scanning laser ophthalmoscopy is used to measure erythrocyte and leukocyte velocity in the retina and is often used for measurements in perimacular retinal capillaries. The scanning laser ophthalmoscope is a confocal laser instrument with good spatial resolution and is capable of producing images with good contrast.[30,31] Reflected light intensities exiting the eye via the pupil pass through a confocal aperture and proceed to a detector.[30,31] Any light scattered outside the focal plane is blocked by the aperture.[30,31]

1.5.2 Colour Doppler Imaging

Colour Doppler Imaging (CDI) is used to measure extraocular blood velocities in primarily the central retinal artery, posterior ciliary arteries and the ophthalmic artery. CDI includes ultrasound techniques that combine b-scan grayscale imaging of tissue structure, and colour representation of blood velocity computed from Doppler-shifted reflections.[32]

1.5.3 Pulsatile Ocular Blood Flow

Pulsatile ocular blood flow is a representation of the pulsatile component of arterial inflow to the eye achieved from the intraocular pressure pulse.[33,36] Subsequently, the volume and the IOP are highest during systole and lowest during diastole.[33-36] Methods have been used to measure the pulse wave of the rhythmic changes in IOP during the cardiac cycle.[35,36] The ocular pulse and the amplitude of the IOP pulse wave are recorded for use in calculating the change in ocular volume and consequently the pulsatile component of blood flow.[33] Since the greater percentage of ocular blood flow is part of the choroidal circulation, this technique is regarded as a representation of choroidal blood flow and not the retinal circulation.

1.5.4 Laser Doppler Velocimetry and Flowmetry

Bi-directional laser Doppler velocimetry (LDV) is used to measure blood velocity in the main retinal vessels. This method provides data on a single vessel and therefore no information about the perfusion of the rest of the eye.[33] The velocity of the red blood cells is calculated from an analysis of Doppler shifts observed in the light that is scattered by them in real time.[38] This velocity is then combined with a measure of the vessel diameter (measured by micrometry) to obtain an estimation of flow.[37] An instrument that used this method is the Canon Laser Doppler Flowmeter 100 (CLBF). The CLBF uses a tracking device and the Bi-directional Laser Doppler Velocimetry principle to monitor changes in retinal blood vessel diameter, velocity and blood flow.[39-42] Since the CLBF is also capable of recording retinal vessel diameter it is therefore, able to derive blood flow in real units.[40]

Laser Doppler flowmetry (LDF) is somewhat similar to laser Doppler velocimetry, but it is able to calculate blood flow rather than just blood velocity. This method provides information on capillary blood flow at a specific location over a specific period in arbitrary units.[33] The Heidelberg Retina Flowmeter (HRF) is an example of this technique using confocal scanning laser Doppler flowmetry to provide a 2-dimensional map of the retinal micro-circulation.[30,31,33] The HRF provides a non-invasive, continuous measure of microcirculatory blood flow by recording the frequency change that light undergoes when reflected by moving red blood cells within the capillaries.[41] The possible parameters that can be measured and quantified are blood flow, blood velocity and blood volume. Due to limitations with this device, the HRF is only capable of providing relative measurements of blood flow at the same sites over time.[43,44]

Previous studies have also utilized this method for the anterior surface, measuring relative blood flow values in the capillaries of the bulbar conjunctiva.[45]

1.5.5 Fluorescein Angiography

Angiographic techniques allow for the visualization of anatomic structures by the passage of sodium fluorescein and therefore, quantification of blood flow in the retina.[46] Initially these angiograms were used to gather qualitative characteristics and information about the vascular anatomy and integrity within the eye. More recently the approaches include an attempt to obtain a level of quantitative information. Even though these techniques cannot measure blood flow, they do however provide valuable information on the effect of ocular disease in the retina.[46]

1.5.6 Retinal Vessel Diameters

The above techniques are limited by the fact that they are only capable of directly measuring blood velocity and not blood flow. As an example, if information about vessel diameter is absent, results showing an increase in blood velocity should be interpreted with caution. The apparent increase in velocity could foreseeably be as a result of an increase in blood flow or conversely a resultant vasodilation of the vessel.[47,48] Therefore, in order to determine blood flow, vessel diameter quantification is crucial. Many techniques have been used for this purpose and they include fundus camera-based techniques using photography and simultaneous image analysis.[47,48]

1.5.7 Laser Interferometry

Interferometry measures changes in corneo-retinal distance during the cardiac cycle and is used in the hemodynamic assessment of the choroid. As blood enters the eye (systole) choroidal blood volume increases and axial length decreases.[49,50] When a change in axial length occurs, the interference pattern (produced from a laser light directed along the pupillary axis and reflected from the cornea and the fundus) changes.[49,50] The change in the interference pattern represents flow, but the relationship between flow and pulsation is not known and therefore is only convenient for studies of intra-individual blood flow changes rather than comparing different groups.[49,50]

1.5.8 Video Cameras and Cell Tracking

Video recordings of red blood cell flow through capillary networks offer an enormous amount of information relating to the microcirculation and image analysis[51,52] of those videos have been used as a method to monitor and calculate blood cell velocity.[51,53-57]

With this method, recordings of capillary networks are made using video microscopy. Various capillary networks have been used including the rat extensor digitorum longus muscle[56], cheek pouch retractor muscle of hamsters[58] and the retina[55]. Although types and models between groups vary, the images are recorded using a video camera/recorder with multiple magnifications. An addition to the system usually includes an interference filter (approximately 421nm) that is used to enhance red blood cell (RBC) contrast. Video processing is then performed with various software systems to track specific RBC sequences. One group in particular developed an automated method to track RBCs as they traversed capillary networks and then analyzed the recordings using a space-time imaging technique.[51,58]

These methods provide a means to study changes in flow patterns over time and in response to various stimuli or disease states. In addition, by varying the length of time corresponding to each processed image, various characteristics of RBC flow within a network (e.g. local or global changes) can be determined.

1.6 Blood Flow Regulation

Regulation of blood flow (homeostasis) is necessary to adapt to different internal and external conditions. Circulation provides transport to a large variety of molecules, including oxygen and cells such as leukocytes.[33,34] The regulation of blood flow also compensates for varying perfusion pressures, adapts to metabolic activity and helps to maintain a constant ocular temperature.[34]

Blood flow in an organ is regulated by perfusion pressure and local resistance to flow.[4,34] Local resistance is controlled by the calibre of the vessels. Among other things, the autonomic nervous system, circulating hormones and the endothelial cell layer participate in this regulation.[33,34]

Adequate blood flow and oxygen levels are maintained by the organs' strong capacity to autoregulate.[33,34]

1.6.1 Retinal Blood Flow Regulation

In the retina, the regulation of blood flow is determined by perfusion pressure and the diameter of retinal vessels.[8,34] Local myogenic responses, metabolic factors and endothelial derived substances influence vascular resistance.[8,59] Retinal blood flow is regulated through modifications of vascular resistances (i.e. changes in the contractile state of retinal arterioles).[8,34] Retinal blood flow is fairly constant despite moderate variations in perfusion pressure and therefore, the partial pressure of O_2 (PO_2) is maintained at constant values in the inner retinal tissue during moderate reductions of perfusion pressure.[8,34,59]

1.6.2 Choroidal Blood Flow Regulation

The conjunctiva is supplied by the long posterior ciliary arteries, branching from the ophthalmic artery and is part of the choroidal circulation.[11] In contrast to retinal blood flow, choroidal blood flow is regulated and changes very little during sudden increases or decreases in blood pressure.[34,59] Choroidal blood flow is innervated by the autonomic nervous system (sympathetic activity) and through consequent vasoconstriction is able to maintain constant blood flow.[4,5]

Vessels are controlled by the vascular endothelial cells, which release vasoactive factors.[4,5] These factors include nitric oxide, which induces vasodilation, and endothelin-1, which induces vasoconstriction.[4,5] Unlike in the retina (because of the blood-retina-barrier), these and other circulating molecules have direct access to smooth muscle cells and pericytes in the choroidal circulation because they can escape the fenestrations in the vessels.[33,34]

When the perfusion and oxygen extraction of the vascular network is maximized, a drop in arterial pressure and flow lower the tissue PO_2 and lead to relaxation of the larger arterioles and restoration of blood flow.[4,5] Whether due to elevated metabolism or low blood flow, it has been suggested that a low PO_2 in the tissue favours autoregulation.[4,5]

Although there is not a substantial amount of work examining blood flow in the bulbar conjunctiva, there has been work on the study of the blood flow in other parts of the

microcirculation.[60] There are 4 main aspects to the control of blood flow- metabolic, nervous, myogenic and humoral regulation[61].

1.6.3 Metabolic Control

Local control is maintained by each individual tissue, based on the particular metabolic need of the tissue.[8,59,62] Anatomically, blood enters the capillary bed via an arteriole and leaves via a venule. The network of arterioles branches (metarterioles) and then lead into the capillaries. The arteries and arterioles have a strong muscular coat whereas metarterioles have a weaker one.[63] The arteries and arterioles are also innervated by the sympathetic nervous system and therefore their contraction is limited to the strength of the signal transmitted by the nervous system.[8,59,62] The arterioles and the capillaries are sparsely innervated, and therefore contraction is locally controlled (i.e. the levels of oxygen, carbon dioxide, hydrogen ions, electrolytes, etc., present in the tissue).[62]

Local control can be short term or long term. As detailed by Guyton[4], the short term control is based on 2 theories and makes up about 75% of the regulation. First, the vasodilator theory, that suggests with increased tissue metabolism, there is less blood flow, oxygen and nutrients available.[4,5] In a situation where this occurs, a vasodilator substance (e.g. carbon dioxide, adenosine, histamine, potassium, hydrogen, etc.) will be formed,[4,5] causing dilation. The second theory is the oxygen demand theory, where a resultant dilation also results due to a lack of oxygen and nutrients.[4,5]

Because the short term local control can cause either rapid spikes or dips in blood flow, an autoregulatory response is needed to maintain blood flow.[4,8,62] There are again, 2 processes to describe the homeostasis that occurs after these immediate responses. The first is a metabolic theory and the second is a myogenic theory.[4,5] The metabolic theory maintains that since there is a rise in blood flow, more nutrients and vasodilator substances will be cleared out of the tissue and therefore dilation will decrease and homeostasis will occur.[64] The myogenic theory maintains that when dilation occurs, the vessel wall stretches.[4,5] When the vessel wall is stretched the smooth muscle is signalled to contract and therefore, bring blood flow back to homeostasis. The triggered contraction from the smooth muscle also acts as a protective mechanism to prevent damage from occurring to the vessels due to over stretching.[4,5]

Aside from the short term control that occurs when the metabolic needs of the tissue change, there has to be some long-term, regular control.[23] Long term control accommodates approximately 25% of the regulation. Long term control will compensate for any metabolic changes (increase or decrease) in the tissue.[24,65] If arterial pressure drops, then the long term regulation will accommodate by either increasing the size or even the number of vessels present in the tissue.[24,65] Because of the constant fluctuations in pressure, vessel size or number, the vasculature within the tissue is constantly changing.[23,66,67] It is also important to note that if a tissue becomes ischemic or has an increased metabolic need, initiation of new vessel growth will occur, triggered by angiogenesis factors.[4,8] These factors develop in the tissues and promote the growth of new vessels.

1.6.4 Nervous Control

Nervous control is utilized in situations where there is a requirement to increase or decrease blood flow. Nervous control can affect larger areas of the systemic circulation.[68] The parasympathetic and sympathetic systems act on the vascular smooth muscle cells to aid in the blood flow demand requirement.[4,5,66,67] When activated, the parasympathetic system causes the smooth muscle cells to relax, allowing the vessel to vasodilate via endothelial derived relaxing factors.[4,5,66,67] Conversely, when the sympathetic system is activated, it causes the smooth muscle cells to constrict the vessel via α-adrenergic receptors.[66,67]

The complexity of the nervous system does not end in its control of blood flow and circulation. It also controls impulses to the heart and brain which can either excite or inhibit various centres of the body.[4] Its complexity is beyond the scope of this introduction and will not be discussed in detail.

1.6.5 Myogenic Control

Myogenic control keeps blood flow constant irrespective of changes in arterial pressure. This control is maintained through the vasoconstrictor fibres of the sympathetic system.[4] The vasoconstrictor fibres within the vessel emit constant impulses that control the vasomotor tone, that is, the constant, slight contraction of the vessels.[63] Norepinephrine is the substance that is secreted from the fibres and then travel throughout the body to induce vasoconstriction.[63]

Distension on the wall of a vessel causes the smooth muscle cells to contract, inducing vasodilation.[4,63]

1.6.6 Humoral Control

Humoral control involves various types of factors that are dissolved in the blood, which cause a local increase, decrease or an overall change in flow.[4] The vascular endothelium is the source of most of these substances and thus it remains important in blood flow regulation.[63] There are many important humoral factors, some of which cause vasodilation or vasoconstriction, or even both, depending on the situation.

Some of the important vasodilators include nitric oxide, prostacylin, bradykinin, histamine, serotonin, prostaglandins and, potassium, magnesium, sodium and hydrogen ions.[4,5]

Some of the important vasoconstrictors include endothelin-I, vasopressin, angiotensin, Norepinephrine, epinephrine and, calcium and hydrogen ions.[4,5]

1.6.6.1 Nitric Oxide

Nitric oxide (endothelium derived relaxing factor) is biosynthesised endogenously by many cells in the body however, its production by vascular endothelium is particularly important in the regulation of blood flow. The endothelium (inner lining) of blood vessels uses nitric oxide to signal the surrounding smooth muscle cells to relax, thus resulting in vasodilation and increasing blood flow.[4,5,69] Nitric oxide (NO) contributes to vessel homeostasis by inhibiting the contraction and growth of vascular smooth muscle cells, the aggregation of platelets, and the adhesion of leukocytes to the endothelium.[4,5] Since NO has a very important role in vascular function, its abnormal production which is apparent in different disease states, can adversely affect many vascular functions.[4,5]

1.6.6.2 Endothelin

Endothelins are proteins that cause blood vessel constriction and are mainly produced in the endothelium. Among the strongest vasoconstrictors known, endothelins have a primary role in vascular homeostasis and are implicated in vascular diseases of several organ systems, including the heart, and brain and the circulatory system.[69,70]

There are four isoforms (ET-1, -2, -3, -4) with varying areas of expression and two key receptor types, ET_A and ET_B.[71]

ET_A receptors are located in the smooth muscle tissue of blood vessels, and binding of endothelin to ET_A increases vasoconstriction leading to an increase in blood pressure.[4,69,71]

ET_B receptors are primarily found on the endothelial cells lining the interior of the blood vessels. NO is released when endothelin binds to these receptors.[4,69,71]

Both ET_A and ET_B receptors are present in the nervous system where they may play a role in the mediation of neurotransmission and vascular functions.[72]

1.7 Oxygen Saturation

Healthy blood circulation provides tissues with a vital amount of oxygen required for cell function. Oxygen in the blood stream is transported by hemoglobin contained in a red blood cell.[5,73,74] A hemoglobin molecule consists of four units, and each unit has a heme that can be bound to oxygen.[5] Hemoglobin is bound to oxygen in high oxygen partial pressure, while it releases oxygen in low oxygen partial pressure.[73,74] The content of hemoglobin that binds to oxygen is represented as the degree of the oxygen saturation (SO_2); 100% oxygen saturation represents that all hemoglobin molecules bind to the maximum oxygen.

The study of oxygen transport through the circulation requires the assessment of hemodynamics and oxygenation of individual RBC's within microvessels. Since approximately 98% of oxygen carried by blood is bound to the red blood cells (RBC's) (with the remaining 2% dissolved in the plasma), a relatively accurate estimate of the quantity of oxygen carried in the blood can be obtained from measurements of hemoglobin oxygen saturation.[73]

Current applications of the measurement of oxygen saturation are based on many years of research. Measurements of oxygen consumption date as far back as 1932 and were recorded in the human hand, and in the ear of experimental animals.[75]

Measurement of retinal blood oxygen saturation has been used to provide a microcirculatory assessment of the hemodynamic state of the retina.[58,76-80] Common methods to measure oxygen

saturation in the retinal vessels are based on quantitating the differences between oxyhemoglobin and deoxyhemoglobin light absorption spectra at different wavelengths.[57,58,80]

1.7.1 Lambert-Beer Law

The Lambert-Beer Law is an empirical relationship that relates the absorption of light to the properties of the material through which the light is traveling. This law demonstrates that light transmission through a solution diminishes logarithmically as the concentration of the solution and the distance through it increases.[75]

$$I = I_o * 10^{(-\varepsilon * d * C)}$$

where, I is the intensity of light transmitted through blood, Io is the original intensity of light, ε is a negative constant, d is the thickness and C is the concentration.[75]

By rearranging the equation, it is evident that ε is proportional to log (I_o/I).

$$\varepsilon = (1/C * d)(\log (I_o/I))$$

where, ε is a specific extinction coefficient describing the portion of light that is lost due to absorption per unit distance and per unit concentration in a specific medium.[75]

Since optical density is also defined by the same proportion (log (I_o/I)), saturation is a function of optical densities and the properties of oxygenated and deoxygenated hemoglobin.[75]

Simplified, oxygen saturation is equal to the percentage of oxygenated hemoglobin within total hemoglobin. However, we must take into account the extinction coefficients of the heterogeneous mixture of oxy- and deoxygenated hemoglobin. The specific extinction coefficients of oxy- and deoxygenated hemoglobin are detailed elsewhere.[81] Using two different wavelengths, one that is isosbestic (the extinction coefficients for oxy- and deoxygenated hemoglobin are identical) and one that is oxygen sensitive, allows for the determination of oxygen saturation.[38,75,81] Taking these principles into account, the relationship between oxygen saturation and the ratio of optical densities (OD/OD_{ISO}) is linear.[75]

1.7.2 Optical Density Measurements

The principle of using the relationship between oxygen saturation and the ratio of optical densities has only been applied to the retina. Images of the eye are obtained with two wavelengths (oxygen sensitive and isosbestic). Various groups have used different oxygen sensitive/isosbestic combinations and some of them include 600nm/570nm[82], 600nm/569nm[80], 436nm/420nm[57] and, 431nm/420nm[73]. From these images, the apparent OD can be calculated on desired vessel segments by obtaining the minimum intensity value inside the vessel and the average intensity outside the vessel.[58,73,80-82] The optical density ratio (ODR) for a segment of a particular vessel is calculated by the following series of previously derived equations (using 600nm/570nm as the two wavelengths)[58,80]:

$$ODR = OD_{600}/OD_{570}$$

$$OD_{600} = \log_{10} [(I_{out})/(I_{in})]$$

$$I_{out} = 1/M \Sigma I_{out}$$

and, $I_{in} = 1/M \Sigma I_{in}$ where M is the number of points (from 1 to M)

$$OD_{570} = \log_{10} [(I_{out})/(I_{in})]$$

$$I_{out} = 1/M \Sigma I_{out}$$

and, $I_{in} = 1/M \Sigma I_{in}$ where M is the number of points (from 1 to M)

1.7.3 Oxygen Saturation Calculation

ODR bears an inverse relationship to SO_2, and therefore oxygen saturation is simply calculated by ODR^{-1}.[82]

1.8 Oxygen Saturation Measurement Techniques in the Eye

The methods used to estimate hemoglobin oxygen saturation are based on the assumptions about the relationship between light transmittance and oxygen saturation.[57,58,73,80]

1.8.1 Reflectance Pulse Oximetry

Pulse oximetry has become one of the most commonly used tools in the clinical setting for assessing patients' blood oxygenation status. It is a simple, non-invasive tool that measures the percentage of haemoglobin in the blood that is saturated with oxygen by using a probe to transmit light through the tip of a finger or the ear lobe. Most pulse oximeters typically have a pair of small light-emitting diodes (LEDs) facing a photodiode through a translucent part of the patient's body, usually a fingertip or an earlobe.[83,84] One LED is red, with wavelength of 660 nm, and the other is infrared, 905, 910, or 940 nm.[83,84] Absorption at these wavelengths differs significantly between oxyhemoglobin and its deoxygenated form, therefore from the ratio of the absorption of the red and infrared light the oxy/deoxyhemoglobin ratio can be calculated.[83,84] The absorbance of oxyhemoglobin and deoxyhemoglobin is the same (isosbestic point) for the wavelengths of 590 and 805 nm.[83,84]

A blood-oxygen monitor displays the percentage of arterial hemoglobin in the oxyhemoglobin configuration. Acceptable normal saturation ranges are from 95 to 100 percent, although values down to 90% are common.[83,84]

1.8.2 Wavelength Comparisons

1.8.2.1 Photographic Systems

Retinal oximetry was initiated when a study that digitized fundus photographs taken with two illuminating wavelengths was published.[85] This study estimated relative retinal vessel oxygen saturation by using ratio analysis and recording the OD at oxygen-sensitive and insensitive wavelengths.[85] Because there are many factors that influence the relationship between oxygen saturation and retinal vessel reflectance and therefore vary across individuals, this approach requires calibration for absolute units of oxygen saturation. This method was extended by Laing et al[78] and showed that oxygen saturation was linearly related with the ratio over physiological and hypoxic ranges of saturation.[82] A three-wavelength oximetry method was used by Delori[86]

and allowed them the ability to calculate absolute oxygen saturation values in single vessel segments. Reflectance spectroscopy is a popular method for saturation measurement and has been reported using two,[57,76,80,87] three,[86] and four wavelengths.[88]

1.8.2.2 Digital Systems

Further advancements to these wavelength methods include the development of digital imaging systems.[79,80] With these systems, the oxygen-sensitive and insensitive images are obtained and then analyzed by computers that use equations similar to those described in section 1.8.2 and 1.8.3.

1.8.3 Imaging Spectrometry

Imaging spectroscopy uses light spectra reflected from and transmitted through a blood column and separately applies those reflected spectra to algorithms to calculate oxygen saturation.[89-92] The internally reflected spectrum is dependent on oxygen saturation and experimentally determined reflectance spectra of reduced and saturation whole blood.[89] The transmitted spectrum is dependent on the background reflectance, oxygen saturation and experimentally determined transmission spectra (determined by measuring saturated and reduced whole blood at different concentrations and thicknesses).[89] At wavelengths between 510nm and 586nm, only transmitted light is affected by tissue layer thickness and hematocrit (the proportion of blood volume occupied by red blood cells).[89-91,93] Any effects of tissue layer thickness and hematocrit on internal reflectance are not taken into consideration as long as wavelengths between those ranges are used.[92]

A system developed by Schweitzer[92] obtains reflectance spectra of single fields from a line of tissue. A logarithmic scale is then used to plot the intensity of light reflected at each wavelength from the tissue sample. Once image quality is established,[94] the system is designed to optimize the values of many unknowns, such as oxygen saturation.[91]

1.8.4 Multi-Spectral Confocal Scanning Laser Ophthalmoscope

A scanning laser ophthalmoscope acquires images at four wavelengths across a single video frame. There are three diode laser modules (635, 670 and 830nm) and one argon laser (488nm) that are made coaxial by a set of mirrors. The light is detected by a photodiode detector.

Transmittance through a vessel is calculated by using the image intensity from the background tissue. Irradiance from the background tissue is determined from points surrounding the vessel of interest and from computer software that estimates the background in absence of the vessel. If a central vessel reflex is present, a polarizer is used to decrease it, if there is no reflex present then the lowest image intensity value within the vessel is used.[95,96] The ratio between the intensities of the background and the vessel is used to calculate transmittance and ultimately oxygen saturation.[75,97,98]

1.9 Conjunctival Hyperemia

Ocular hyperemia is commonly known as "red eye" and is defined by the dilation of blood vessels on the anterior portion of the eye, giving rise to a reddish appearance[99]. Red eye is an attribute that has been of some considerable importance to patients and practitioners, since it is complicated and not well understood.

The conjunctiva is a transparent, vascular mucous membrane that surrounds the anterior portion of the globe, not including the cornea. The conjunctiva is attached to the episclera, the anterior portion of the sclera.[100]

Conjunctival hyperemia can be classified anatomically. The various locations of conjunctival hyperemia are bulbar, limbal and palpebral. The underlying initiators for hyperemia are different at each anatomical location. Some of these initiators include contact lens use[101,102], chemicals, coughing, diurnal variations[103-105], the presence of foreign bodies, abrasions, fatigue, disease[106], medications, hormonal levels, diet, age, mechanical irritants, allergens[107], environmental factors[108], an unstable tear film[109] and dry eye. Each initiator may result in different reactions, ranging from quite mild to severe[110].

1.10 Grading of Hyperemia

In research and in the clinic, ocular redness has been evaluated by means of subjective grading since it is simple to do and non-invasive. However, it is important that the grading system that is used possesses reliability, repeatability, accuracy and is also easy to use.

1.10.1 Subjective Grading

There are many types of subjective grading systems that are used, but there is not one that is standard.[111] Some subjective systems require the clinician to use a biomicroscope to examine, judge and compare the redness based on a scale or a series of pictures.[112,113] Some of the subjective methods include questionnaires, numeric scales and descriptive scales, but they tend to have less sensitivity when it comes to detecting meaningful change.[114]

Even though the use of scales tends to be less time consuming and inexpensive, these have a decreased reliability due to the intra- and inter-observer inconsistencies.[115,116] Some methods use redness grading scales based on a 4 point scale, 1 being mild. So, a 1 on the scale to one observer may be a 1.5 or 1.75 on the scale to another observer.

There are other redness measurement methods that segment the eye into different quadrants and are based on the number of blood vessels within those quadrants.[117]

Although the numeric scales use a global language (numbers) and are more readily used than descriptive scales and questionnaires, they are still regarded as having a lower sensitivity when it comes to detecting meaningful change.[114] A more objective method of measurement would be advantageous.

1.10.2 Objective Redness Measurement

Other methods of redness measurement are based on objective means with the expectation of being more reliable.[118] Unfortunately these methods are sometimes more expensive and time consuming and can be regarded as less clinically-friendly.

Some of the examples of objective measurement methods used include: Photographic imaging based on a region of interest on the ocular surface[119], image analysis[120-122], digital processing and analysis[123] and photometry[45,120].

One method in particular involved automated measurement of scales and image analysis.[120] This study developed an automated method of redness measurement by comparing redness and blood vessel appearance from several images to the redness values assigned by clinicians, on a 100-point scale, in a web survey.

A user-friendly method to measure redness in a quick, reliable manner with objective results is the photometric measurement.[45]

1.10.3 Colour Science and Colourimetry

There are 3 attributes that can be used to describe a colour; saturation, hue and, luminance.[124,125] Saturation is best described in terms of the amount of white present in a colour. The more of white present in a colour, the less saturated the colour. The absence of white in a colour is a completely saturated colour. The second component is hue. The colour itself, for example red, is the hue. So if pink is a mixture of red and white, then pink and red have the same hue, but different saturations. The third component is luminance, the measure of brightness.[124,125]

1.10.4 The Chromaticity System

There are various colour systems that have been used to measure the luminance, hue and saturation of colour.[124,126] The system of interest is objective and is called the CIE system (The Commission Internationale de L'Éclairage).[127]

The CIE system is a measurement system for colour and is represented in a chromaticity diagram.[124-126] The system is based on the spectral power distribution parameters (SPD). The SPD is a classification of the power of light at each specific wavelength viewed by the eye.[124-126] Once the data about light are known, then colour can be analysed quantitatively. SPD can be measured by a spectroradiometer and the luminance and saturation of any colour can be derived.[125]

The CIE system was developed so that the visible spectrum could be expressed in a quantitative way.[127]

The colour system in which part of the upcoming experiments are based on, is the CIE L' u' v' colour space diagram.[124-126] The variable u' has been used to represent and measure ocular redness in previous experiments.[45,111,118,120,128]

1.11 Ocular Pharmacology and Therapeutics

Irritants cause vasodilation, redness and oedema in the eyelid margins, palpebral, orbital and bulbar conjunctiva.[121,129] One of the responses of the vasculature to irritation is the dilation of blood vessels.[121,129,130] The primary vasodilatory substances are those responsible for acute conditions associated with external irritants and allergens (e.g. histamine) and those that are biological mediators (e.g. prostaglandins), involved in a more chronic vasodilatory and inflammatory response.[131] The histamine and prostaglandin-mediated systems can be manipulated with topical pharmaceuticals to decrease redness and change the calibre of the anterior blood vessels.[132]

1.11.1 Anti-Allergy and Decongestant Agents

Histamine, prostaglandins, leukotrienes and various cytokines get released from mast cells during an allergic response and can cause uncomfortable symptoms or even life-threatening complications.[132]

1.11.1.1 Type I Hypersensitivity Reactions

Type I reactions are anaphylactic and Immunoglobulin E-mediated (IgE).[132,133] These reactions occur when an antigen (e.g. pollen, drug) is re-introduced to someone who has previously been exposed to it.[133] The initial exposure causes the production and attachment of IgE antibodies to mast cells after which, a re-introduction to that specific antigen causes their degranulation.[132]

The disruption and degranulation of mast cells cause the release of large quantities of inflammatory mediators (e.g. histamine, prostaglandins, leukotrienes etc.).[133] Histamine activates the H_1 receptors on the blood vessels causing vasodilation.[132] When vessels dilate, they become leaky and fluid escapes causing swelling.[132]

The ocular diseases that are manifested by type I reactions include seasonal allergic conjunctivitis, vernal conjunctivitis, atopic keratoconjunctivitis and giant papillary conjunctivitis.[133-136]

1.11.2 Ocular Drug Entry

The pharmacokinetics of a drug determines the rate at which the drug is absorbed by the system.[137] Drugs affecting blood vessels alter the existing cell function by among other things either mimicking a neurotransmitter (e.g. phenylephrine binds to an α-adrenergic site) or blocking the action of a neurotransmitter.[132,138] Drugs will bind to specific sites or receptors such as proteins on a cell surface, active sites on enzymes or within cells. Drug binding and action depends on the concentration of the drug, as well as its molecular structure and the effectiveness of the delivery.[132,138]

There are a few ways in which an ocular drug can enter the eye. One way is through the cornea to the aqueous humour.[132,133] Only lipid-soluble drugs are able to pass through the tissue, but since the stroma is made up largely of water, the molecule entering must be bi-phasic.[132,138] A second way that drugs can enter the eye is through the systemic circulation via the conjunctival and lid vasculature or the nasolacrimal drainage system.[132,133] If absorption takes place, the drug enters the blood stream, but is eliminated systemically.[132,138] Because of systemic absorption, the ocular effect could be lessened. Drainage in the nasolacrimal system usually includes absorption of some of the drug into the blood from the vascularised lining of the nasolacrimal duct.[132,138] This occurrence could account for systemic toxicity.[132]

Topical ophthalmic pharmaceuticals are mainly water soluble formulations and are miscible with the aqueous phase of the tear film and penetrate into the eye.[132,138] The properties of the drug in water determine its corneal penetration, with the net ionic charge on the drug being the most important determinant.[132,133] The pH and the time the drug is in contact with the ocular surface are also factors that determine the permeability of the drug and ultimately its penetration.[132,138]

The conjunctival tissue contains numerous secretory cells and glands that could have a substantial impact on the net absorption of a drug and therefore, drug penetration into the conjunctival tissue is twice as high as it is in the cornea.[132,138]

Topical ocular drugs are absorbed via the conjunctiva or cornea and enter the anterior ocular tissue and aqueous humor. The effects of the drug are anterior and do not move more posterior than the crystalline lens.[132] The ultimate removal of the drug is achieved via the uveo-scleral pathway and the anterior ciliary veins.[132,138]

1.11.3 Countering vasodilation

Histamine and prostaglandin-mediated systems can be manipulated with topical pharmaceuticals to decrease redness and change anterior uveal blood flow.[132,138]

1.11.3.1 Decongestants

Decongestants can manage exposure to dry or dusty work places, exposure to chemicals and fumes and strain from dry environments.[132] Over use of decongestants can cause a rebound effect and can reduce ocular sensitivity to allergens and irritants.[139]

Decongestant activity has a direct α-adrenergic action or histamine H_1-blocking action on the superficial conjunctival vasculature.[132,138] Adrenergics will stop vasodilation and/or cause vasoconstriction of the vessels and mydriasis by interacting with the α_1-receptors on the blood vessels.[132,138] Drug interaction with the receptors will help counter vasodilation and other signs and symptoms of a mild inflammatory condition.

The vasoconstricting effect of these adrenergic agonists makes them useful topical decongestants.[132,138] Some common decongestants include phenylephrine and imidazole compounds.

Phenylephrine is an α-adrenergic receptor agonist used primarily as a decongestant, as an agent to dilate the pupil and to increase blood pressure.[139] It is useful since it is quite effective at low concentrations while causing little to no pupil dilation.

Imidazole compounds such as, naphazoline, tetrahydrozoline and oxymetazoline are also sympathomimetic agents with α-adrenergic activity.[138] They can act as vasoconstrictors with a rapid action in reducing swelling when applied to mucous membranes. They act on α-receptors in the arterioles of the conjunctiva to produce constriction, resulting in decreased congestion.[132,138]

1.11.3.2 Antihistamines, mast cell stabilizers

Many people suffer from allergies. These are caused by an excessive response of the body to allergens such as the pollen released by grasses and trees. An allergic reaction indicates an excessive release, by the body, of histamines.[132,138] Antihistamines are used for the reduction of ‘simple’ ocular irritation and relief from these allergic or inflammatory ocular conditions.[132,138]

Several H_1-receptor antagonists are formulated for topical, ocular use. Ophthalmic antihistamines have dual action: 1) to block histamine from the H_1 receptors and; 2) prevent the release of other mediators from cells involved in allergic reactions (e.g. mast cells which are found in several types of tissues and contain many granules rich in histamine).[138]

There are many compounds used for antihistamine action including, azelastine hydrochloride, cromolyn sodium, epinastine hydrochloride and diphenhydramine.[132]

1.11.3.3 Corticosteroids and Non-steroidal anti-inflammatory drugs (NSAIDs)

Synthetic topical drugs with corticosteroid-like effect are used in a variety of conditions and can be used in the treatment of various inflammatory conditions (hyperemia, cellular infiltration, vascularisation).[131] Corticosteroids can be warranted if rapid relief is needed to control non-specific inflammatory and immunologic diseases of the eye.[132]

The corticosteroidal benefits on ocular inflammation are: a decrease in capillary permeability and cellular exudation, an inhibition of mast cell degranulation, a suppression of lymphocyte proliferation, an inhibition of phospholipase-A synthesis (resulting in a decreased synthesis of prostaglandins and leukotrienes) and an inhibition of a cellular-mediated immune responses.[131]

NSAIDs are “aspirin-like”/salicylate drugs with analgesic, antipyretic and, in higher doses, anti-inflammatory effects.[131] The term "non-steroidal" is used to distinguish these drugs from steroids, which (among a broad range of other effects) have a similar eicosanoid-depressing, anti-inflammatory action.[131]

Most NSAIDs act as non-selective inhibitors of the enzyme cyclooxygenase, inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes.[131] Cyclooxygenase is essential in the formation of prostaglandins and thromboxanes from arachidonic acid.[138] Prostaglandins act (in addition to other things) as mediators in the process of inflammation. In the eye, prostaglandins mediate inflammation by disrupting the blood-aqueous humor barrier causing vasodilation, increased vascular permeability, leukocytosis and changes to intraocular pressure.[131]

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2. Rationale

Our ability to assess ocular circulation has evolved over many decades from a subjective physical description of the visible vessels to direct and indirect quantitative measurement of a number of ocular hemodynamic parameters. Today, the techniques available for hemodynamic assessment each examine a unique facet of the ocular circulation. These methods have the potential to contribute greatly to our understanding of normal hemodynamics with the goal of adding to our understanding of altered hemodynamic states, such as those found in disease. Each ocular hemodynamic technique provides contributions to the understanding of ocular physiology with the potential to provide signs for diagnosis and treatment.

By measuring red blood cell velocity, oxygen saturation and ocular surface redness in the conjunctival vessels we are able to study the changes that occur in the circulation and the levels of oxygen delivery and saturation over many states and conditions. By establishing baseline levels of these measurements with healthy individuals, it will give us a basis of comparison to situations which affect this baseline.

A major objective of this work was to design a system that could track individual red blood cells as they travel through capillaries, thereby allowing analysis of hemodynamic and oxygenation parameters of individual cells over time and position. A high magnification camera was used to capture videos that would allow for the assessment of ocular capillary hemodynamics. In addition, videos were taken with specific single wavelength interference filters to allow for the calculation and assessment of oxygen saturation values. Method validation was achieved through simulation videos and a unique isocapnic hyperoxic stimulus. By using the combination of these two techniques, we can provoke changes in ocular hemodynamics by using various stimuli in healthy groups of volunteers, to try to obtain a greater understanding of the physiology of blood flow and its regulation.

The first aim of this thesis was to develop and validate a method capable of producing high magnification videos for accurate red blood cell velocity calculation. Once a camera capable of this task was decided upon, proper set up (camera and subject) and video acquisition techniques needed to be fine tuned in order to achieve videos with a sufficient amount of resolution for pre- and post-processing. The goal to estimate red blood cell velocity would ultimately be achieved

through the use of various processing techniques (pre-processing and registration, vessel tracking and velocity estimation). Validation of the estimation technique was accomplished using simulation videos with known velocities and comparing those velocities with the ones estimated by the algorithms (Chapter 3). An additional aim was to establish and validate a method to estimate hemoglobin oxygen saturation levels in the vessels on the anterior ocular surface (Chapter 4). Common methods to measure oxygen saturation in the retinal vessels are based on quantitating differences between oxyhemoglobin and deoxyhemoglobin light absorption spectra at different wavelengths. These principles were modified for assessments on the anterior ocular surface. The hypotheses of Chapter's 3 and 4 were that the hemodynamic and oxygen saturation measurement methods would accurately reflect real velocities and real changes in saturation as demonstrated by the validation techniques.

Having developed the two methods that can accurately assess red blood cell velocity and oxygen saturation, the second aim of this thesis was to define the magnitude of change, if any, of conjunctival red blood cell velocity and oxygen saturation in a group of clinically normal subjects. Red blood cell velocity, oxygen saturation and ocular redness measurements were acquired every hour over the course of a day. The measurements acquired in these healthy individuals were used to facilitate our understanding of ocular hemodynamics (Chapter 5). In addition, these measures were compared in two age strata. The hypothesis was that there would be no change over time for all three measures in the group of healthy individuals. When comparing strata, it was expected that subjects in the older age stratum would have increased levels of redness, and decreased red blood cell velocities when compared to the younger age stratum. It was expected that the blood oxygen saturation levels in the older aged stratum would be lower than the younger aged stratum.

The next aim of this thesis was to demonstrate the validity of the two established methods and at the same time, to examine the characteristics of the RBC velocity, oxygen saturation levels and ocular redness levels in clinically normal subjects when a topical vasoconstrictor was instilled onto the eye (Chapter 6). Measurements were acquired before and after the instillation of a vasoconstrictor. The change in red blood cell velocity, oxygen saturation and ocular surface redness was calculated and compared between all time points. The hypothesis was that the

vasoconstrictor would cause the red blood cell velocity and ocular surface redness to decrease while oxygen saturation remained stable.

The fourth aim of this thesis was to evaluate the effect of conventional contact lens wear on red blood cell velocity, oxygen saturation and ocular surface redness in subjects with a history of contact lens wear compared to subjects that did not have a history of contact lens wear. With the advent of newer contact lens materials on the market, these techniques could possibly show whether ocular health, specifically corneal health, has improved with these new lenses of choice. Red blood cell velocity, oxygen saturation and ocular redness measurements were acquired every hour over the course of a day for both groups. The variations over time and between subject groups were characterized (Chapter 7). The hypothesis of this section was that the subjects who wore contact lenses on a regular basis would have a decreased red blood cell velocity, decreased oxygen saturation and an increased ocular surface redness when compared to the group of subjects that did not wear contact lenses.

3. Validation of the estimation of average red blood cell velocity in conjunctival vessels using simulation videos

3.1 Overview

Purpose: Measurement of retinal blood flow has been used to provide a microcirculatory assessment of the hemodynamic state of the retina. To date, there have been no studies that have looked at measuring real-time blood velocity in bulbar conjunctival vessels. The objective was to design, develop and validate a system that would non-invasively quantify the red blood cell velocity in the conjunctival capillaries. A tool was developed to automatically analyze video sequences of conjunctival capillaries, digitally imaged with high enough magnification to resolve movement of the blood within the vessel, with the future prospect of gaining a comprehensive understanding of the conjunctival circulation in health and disease. **Methods:** Simulations representing moving RBCs within a vessel and the random variation of each cell in terms of speed, shape and intensity were created in order to evaluate the performance of the algorithm. Gaussian noise was added to simulate disturbances and random misalignment between frames. Videos were pre-processed and registered to improve image quality and then the algorithm was set up to automatically recognize the vessels within the sequences. For each vessel, a signal that correlated to blood cell position was extracted from each frame, and the inter-frame displacement was estimated through a modified dynamic time warping (DTW) algorithm. This provided the red blood cell velocity over time in each point of the vessels. Thus, from these estimates, the mean red blood cell velocity for each vessel was easily evaluated. The true mean velocity from the simulation with the one estimated by the algorithm was compared and the system accuracy was determined. **Results:** Results for the simulated videos demonstrate a very good concordance between the estimated and actual velocities. The mean relative error for the modified DTW method is 6%. **Conclusions:** Signal displacement estimation through DTW algorithm can be used to estimate mean red blood cell velocity. Successful application of the algorithm in the estimation of RBC velocity in conjunctival vessels was demonstrated. Signal displacement estimation demonstrated very good concordance to the actual velocities supporting its validity.

This technique promises to enable the study of conjunctival hemodynamics under various experimental and physiological conditions.

3.2 Introduction

The eye is a unique structure because it is one of the few locations in the body where we can non-invasively monitor capillary blood flow. This advantage provides a unique opportunity to study hemodynamics. What is also advantageous is that the reactivity of the vessel walls in the bulbar conjunctiva are similar to the reactivity of the vessels in connective tissue elsewhere in the body (unlike the skin).[1] There is a lack of information regarding the anterior surface and its hemodynamics in healthy people. This lack is in part due to the unavailability of real-time research tools and methods to non-invasively quantify micro vascular parameters (in health and disease).

There are many factors that are responsible for the regulation and control of ocular blood flow (e.g. metabolic demands, ocular perfusion pressure etc.) and the examination of the interactions of these factors, alone and in concert, will give us insight into the potential hemodynamic alterations that occur during disease or various altered states.[2]

The measurement of retinal blood flow provides a microcirculatory assessment of the hemodynamic state of the retina and studies of this nature are much more common in the literature.[3-12] There are publications that deal with various aspects of quantitatively and qualitatively measuring the blood flow of the posterior eye. There are techniques[10,11,13-20] available for these tasks, and there is usually more than one method used at one time to aid in the complete understanding of ocular hemodynamics. These methods, detailed in Chapter 1, are used to visualize and measure directly or calculate indirectly a variety of aspects related to ocular blood flow.

In the retina, the estimation of the velocity of red blood cells (RBCs) from in vivo microscopy videos is generally divided into two categories. One category is where the vessel calibre is large enough so that the blood cells are able to move within the vessel both along its axis and in its perpendicular direction (large artery hemodynamics).[21] This situation demands precise tracking of each RBC movement in order to estimate both components of the movement.[22] The second

category involves small calibre micro vessels. Since the calibre of these vessels is relatively small (5-10 μm), red blood cells, which are a mere 6-8 μm in diameter,[23] are only capable of lining up and moving along the vessel in a single-file. The estimation of RBC velocity and movement through a capillary network is achieved through the image analysis of video recordings. Several techniques currently exist for measuring various parameters in the retina (e.g. RBC velocity, lineal density, supply rate) using frame-by-frame analysis of the videos[24,25] or automated techniques.[26-30]

To date, there have been no studies that have looked at measuring real-time blood velocity in bulbar conjunctival vessels. It has been suggested that the appearance of blood that flows through the arterioles on the surface of the human body is the same as that which flows through the arterioles in the brain, lung, intestines, skeletal muscle, kidney, ureter and bladder.[1] The blood that flows through the capillaries of the bulbar conjunctiva is a valid sample and comparable to that of the composition of the blood in all of the capillaries in the body.[1,2]

There are many advantages of *in vivo* observations of red blood cell hemodynamics in the bulbar conjunctiva. These advantages include that there is no treatment or preparation required for the surface of the eye in order to study the moving particles, the possibility of adequate optical resolution of the flowing blood, maximum contrast between the red blood and the white sclera and the tissue under investigation is bathed by its own isotonic fluid (tears). The development of newer techniques and their correct use, combined with a careful analysis and consideration of the results provides the potential for assessing ocular blood flow in humans.

In biomicroscopy videos that are taken in a clinical setting, the cells within the capillaries are barely recognizable (low magnification and resolution), and this makes the task of accurate tracking quite difficult. Using a camera that offers high enough magnification to clearly resolve movement of the blood within the vessel with good resolution, the task of estimating RBC velocity at specific points and across vessels over time becomes less intimidating. By coupling good quality videos with specifically designed algorithms to automatically estimate RBC velocity profiles, we are able to measure a detail of the circulation.

Therefore the purpose of this study was to design, develop and validate a system that would non-invasively quantify the red blood cell velocity in the conjunctival vessels and could meaningfully

be interpreted by way of a standardized model. A tool that was previously developed was used to automatically analyze video sequences of conjunctival vessels, digitally imaged with high enough magnification to resolve movement of the blood within the vessel, with the future prospect of gaining a comprehensive understanding of the conjunctival circulation in health and disease.

3.3 Materials and Methods

3.3.1 Simulated Vessels

In order to evaluate the performance of the algorithm, a simulation representing moving RBCs within a vessel was created. After drawing an arbitrary line representing the vessel center line, the line was populated by circles representing a Gaussian intensity pattern. Their diameters and mean intensity were drawn from normal distributions. The video was generated by moving each cell separately along the vessel center line with a speed chosen (for each frame and for each cell) from a normal distribution. When a particular cell would fall out of the end of the vessel it was eliminated from the video and similarly, when there was a place for a cell at the beginning of the vessel, a new one was inserted. The image background had a constant intensity with the addition of noise, with a standard deviation proportional to the image intensity. An example of a generated frame is shown in Figure 3-1.

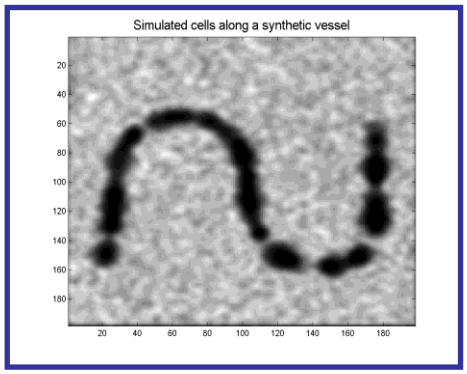

Figure 3-1: Single frame from simulation video of red blood cell movement

3.3.1.1 Pre Processing

Image sequences were converted to AVI files so that the algorithm was able to analyze them. Automatic analysis of the simulated conjunctival videos required that the information carried by each pixel was kept consistent throughout the entire sequence (i.e. if a pixel represented a point of a particular vessel, it had to do so in every frame of the video). In addition, imaged intensities of similar objects (RBC) in similar positions were required to be as comparable as possible.

A few issues with the videos that had to be resolved were the illumination and small movements. Movements cause subsequent frames in the sequence to be misaligned. Due to these movement effects, the same position within a vessel had the possibility of being imaged in different positions in different frames. The uneven illumination made the same red blood cell appear quite different. Given a video composed of several frames, the illumination showed a distinctive pattern (highest in the center of the frame and decreased smoothly toward the borders, with a central symmetry). Assuming that each frame was considered an independent entity, the luminance pattern for each frame was estimated separately, as well as for each pixel of coordinates, in order to remove the uneven pattern. After correcting the illumination, the geometric transformation among frames was corrected. It was assumed that the misalignment of the frames was due to translations and therefore, the estimation of the horizontal and vertical shift between two frames was estimated as the peak of their phase correlation.

Once pre-processing of the videos was complete, the algorithm then had to indentify the vessel structures. The different segments of vessels were automatically extracted from the video image by taking the mean of all the registered frames. A previously developed algorithm[31] was used for vessel recognition and is described in full elsewhere.[32] After vessel structure recognition the algorithm was able to estimate RBC velocity.

3.3.1.2 Velocity Estimation

The goal of velocity estimation was to treat the grey level intensity of each vessel as a signal, and not to track the single red cells (Figure 3-2). For each vessel, a signal that correlated to blood cell position was extracted from each frame, and the inter-frame displacement was estimated through

a modified DTW (dynamic time warping) algorithm that was able to calculate a point-wise estimation of velocity.

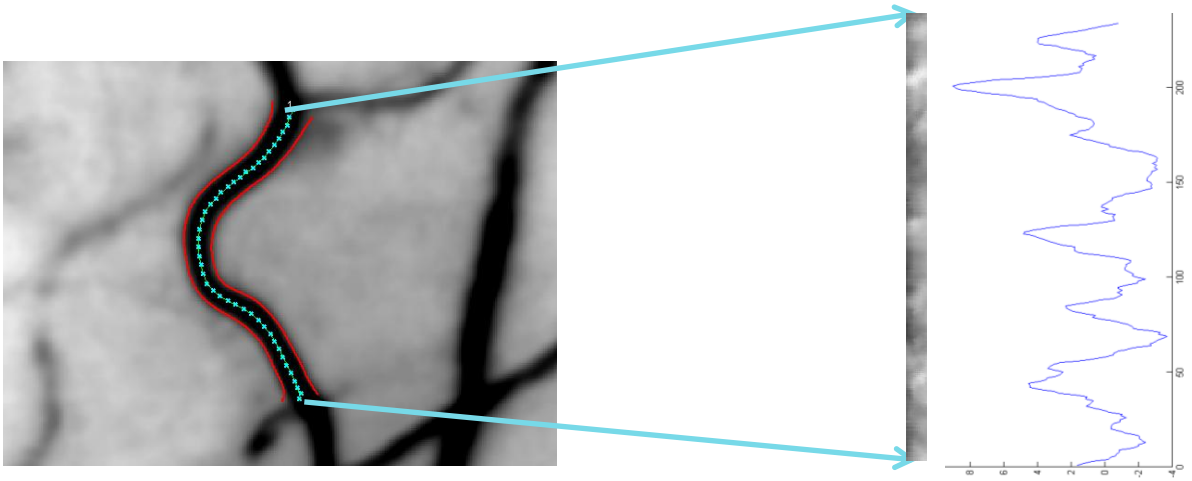

Figure 3-2: Example of gray level intensity extraction of a vessel segment

The grey level values were considered on a vessel axis and it was assumed that the valleys in the intensities corresponded to the presence of RBCs, whereas the high intensities corresponded to the plasma. The average displacement of RBC between two frames of a video was then estimated by the shift of two, on-axis, grey level signals. This estimation is sometimes achieved by using a simple correlation algorithm, but due to the different velocities and intensities of each cell among the different frames, the signals were warped and unmanageable.[31-34] In order to deal with these irregular cell dynamics in the capillary vessels, a modification of an algorithm used in the speech recognition community, dynamic time warping, was used.[33,34] The intention was to estimate the local displacement of two signals and therefore, the only assumption made was that no RBC suddenly appeared or disappeared within a vessel.

Dynamic time warping (DTW) tries to evaluate the local translation of two signals (warping).[32] This is particularly useful in dealing with objects that can distort their shape or vary their relative distances like red blood cells. In order to estimate the velocity of the RBCs it was necessary to evaluate the translations they undergo between subsequent frames. Different frames showed the same sequence of cells, but displaced along vessels. The signals extracted appeared as time-shifted versions of one another (i.e. the higher the velocity, the larger the shift). Therefore, velocity estimation became a problem of phase displacement estimation (Figure 3-3). The DTW

algorithm found the path that minimized the distance between the grey-level intensities of two pixel points from frame to frame (Figure 3-4).

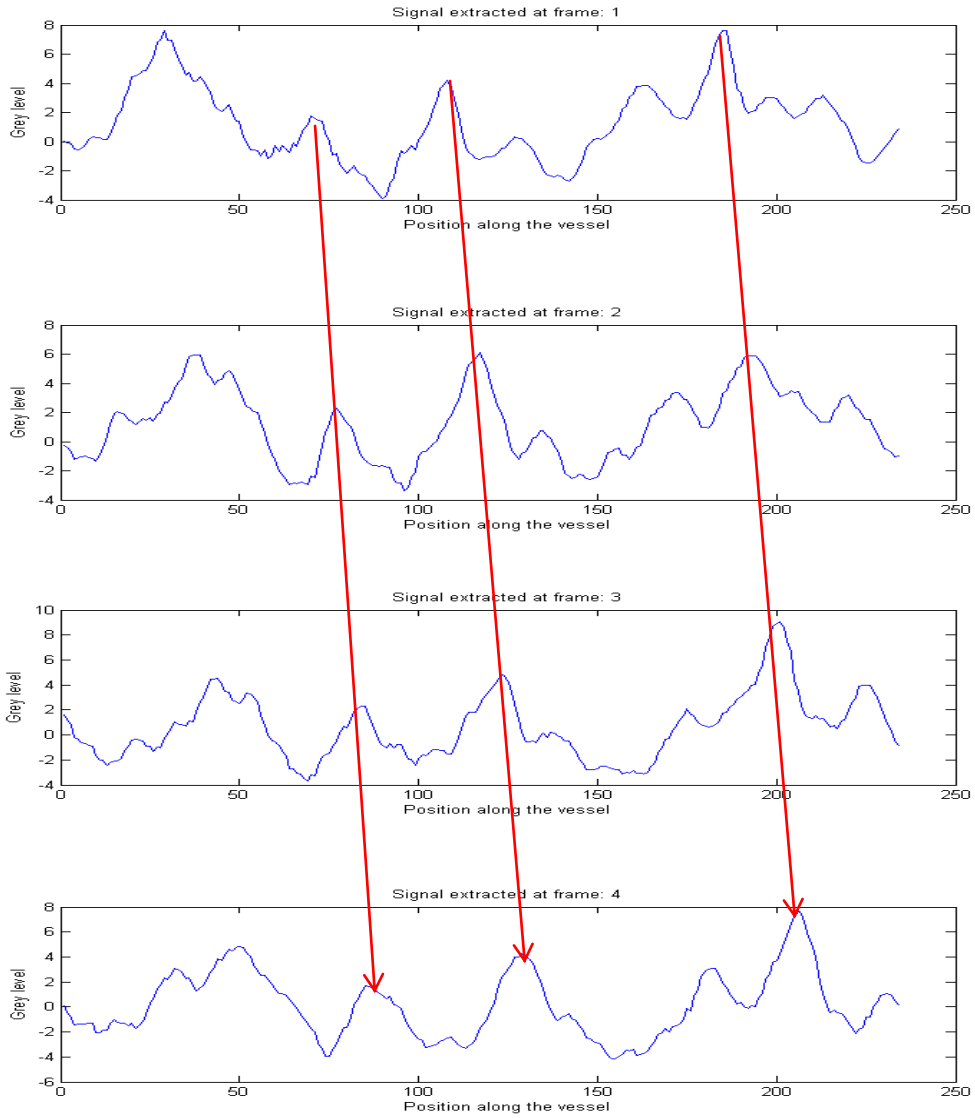

Figure 3-3: Phase displacement estimation

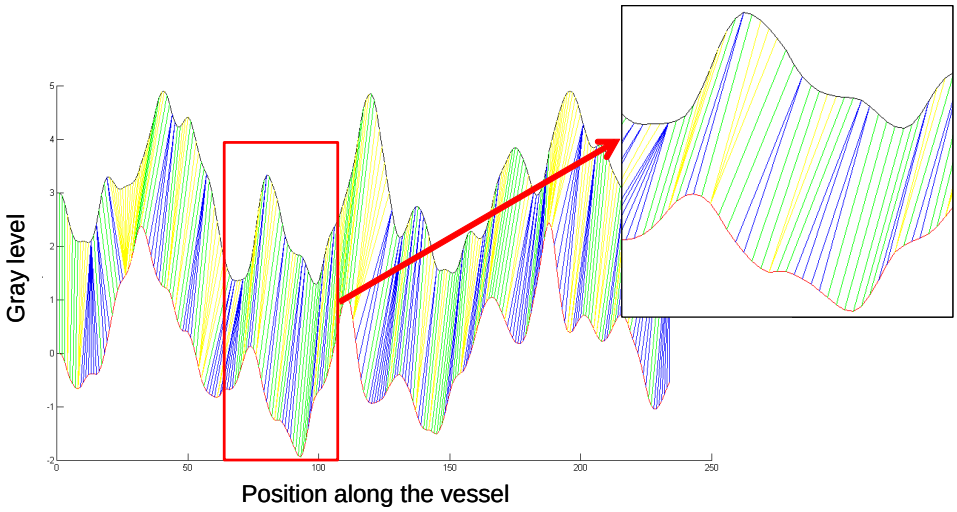

Figure 3-4: The connection of two pixel-point signals using the DTW algorithm

There were other conditions that the algorithm verifies[34,35] as well as the addition of a memory boost to allow multiple-frame path computations and again, these are detailed in the literature.[32,34,35]

An attractive advantage of the DDTW is that it can give an estimation of the mean velocity for the entire vessel, the instantaneous velocity for the entire vessel and the instantaneous local velocity. This advantage gives us the opportunity to study the variation in the velocity both along the vessel and in time. Using a graticule for calibration (Figure 3-5), the red blood velocity was calculated in micrometers per second (detailed below).

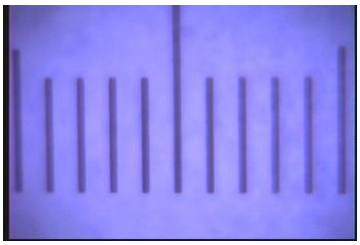

Figure 3-5: Image of graticule ruler used for calibration

It was calculated that 1mm measured on the graticule corresponded to 660 pixels and therefore, 1 micron corresponded to 1.5 pixels. Having a frame rate of 7 frames per second resulted in a calibration factor of 10.71. The calibration factor was applied to initial results in frames/second to achieve meaningful units of microns/second.

3.4 Results

Results for the simulated videos consisting of the mean of 10 different simulated videos for each velocity are shown in Fig. 3-6, demonstrating a very good concordance between the estimated and actual velocities. The variance of the estimates is so small that it is not visible in the plot, and is smaller than the dimension of the plot marker. The mean relative error for the modified DTW method is 6%.

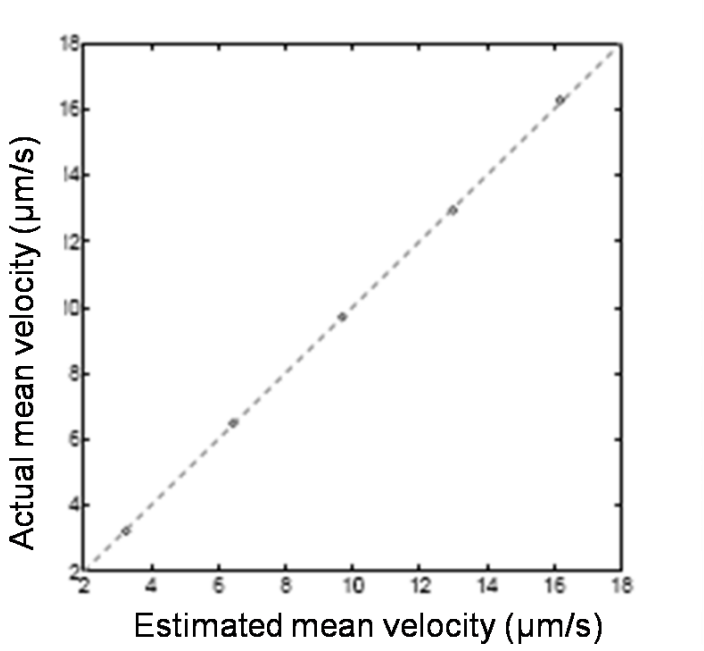

Figure 3-6: Concordance between estimated and actual velocities in the simulation videos

3.5 Discussion

A novel method for tracking RBCs and estimating RBC velocity in conjunctival vessels has been established. By using a DTW algorithm and a simulation video displaying a vessel with RBC movement, the estimation of RBC velocity is possible. One goal of this study was to assess the validity of the DTW method. By estimating velocity within a simulated vessel and comparing those values to the actual velocities this goal was achieved.

Current literature regarding ocular circulation measurements pertains to the retina. [3-12] To our knowledge this chapter introduces the only direct method available using image processing that is capable of non-invasive red blood cell velocity measurement in conjunctival vessels. Literature involving conjunctival vessel changes or responses relate to morphometry.[2,36] Devices and techniques used for circulation measurements in the retina have several disadvantages including the lack of meaningful units, indirect methods, high expense and requirement of skilled technicians, invasive techniques such as dilation and dye injections and difficulty in data interpretation.[37]

This chapter demonstrated the validity of the DTW algorithm by using a simulated vessel with a series of velocities. The low variance of the estimates and low mean relative error demonstrate the accuracy of this technique. This validation technique would be a valuable tool to demonstrate the accuracy in future methods and cross-functionally.

3.6 Conclusion

Signal displacement estimation through DTW algorithm can be used to estimate mean red blood cell velocity. Successful application of the algorithm in the estimation of RBC velocity in conjunctival vessels was demonstrated. Signal displacement estimation demonstrated very good concordance to the actual velocities supporting its validity. This technique promises to enable the study of conjunctival hemodynamics under various experimental and physiological conditions.

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4. Measurement of oxygen saturation in conjunctival vessels

4.1 Overview

Purpose: Measurement of retinal oxygenation saturation has been used to provide vital clinical information about the metabolic state of the retina. To date, there have been no non- invasive studies that have looked at the oxygen saturation in bulbar conjunctival vessels. The objective was to: a) design and develop a method in order to non-invasively quantify the changes in blood oxygen saturation (SO_2) in the conjunctival vessels and demonstrate reliability of the measures and, b) demonstrate the application of the method by showing a response to an isocapnic hyperoxic provocation and compare those values to the results from an instrument. **Methods:** a) The sample consisted of 6 males and 6 females of average age 27.1 (range 19-34 years). Conjunctival vessels were imaged with dual wavelengths using a Zeiss slit lamp at 32x magnification. Images were obtained through narrow band interference filters with O_2-sensitive and O_2-insensitive peak transmissions (440nm and 570nm, respectively). Images were imported into the image processing application ImageJ and optical densities (OD) were calculated from vascular segments using the average reflected intensities inside and outside the vessels. Optical density ratios (OD_{440}/OD_{570}) were used to calculate relative oxygen saturation values. Video images of the bulbar conjunctiva were recorded at three times of the day. Three different areas of one vessel were analyzed using the dual wavelength method for each subject at each time point. The same area was also measured using this method for five consecutive frames. Measurement repeatability was assessed over location at each time and across consecutive frames. b) The sample consisted of 5 subjects (mean age 29.6; range 24-34 years). Subjects initially breathed air for 10 minutes followed by pure oxygen (O_2) for 20 minutes, and then air for a final 10 minute period using a sequential re-breathing circuit (Hi-OxSR, Viasys). Simultaneously, SO_2 values measured with a pulse oximeter ear clip and finger clip were recorded. The validity of the dual wavelength method was demonstrated by comparing the values to those from the ear clip pulse oximeter. **Results:** The intraclass correlations (ICCs) between the three locations at each time point were 0.93, 0.56 and 0.86 respectively. Measurements across 5 consecutive frames showed no significant difference for all subjects ($p=0.60$; ICC = 0.96). The ICCs between the two methods at each time point were 0.45, 0.10 and 0.11 respectively. a) There was no significant

difference in SO_2 between the three locations measured using the dual wavelength method for all subjects (p=0.31). There was also no significant difference between the three locations at any of the time points for the dual wavelength method (p=0.37). b) In response to isocapnic hyperoxic provocation using the dual wavelength method, blood oxygen saturation was increased from control values (mean ± SD) of 94.0 ± 1.5% to 96.7 ± 1.3% (p=0.04) and subsequently recovered after withdrawal of hyperoxia to 94.8 ± 0.7% (p=0.03). Blood oxygen saturation values recorded from the ear clip and finger clip of the pulse oximeter also showed an increase from control values of 95.5 ± 1.1% to 98.3 ± 1.1% (p<0.01) and 97.3 ± 1.1% to 98.8 ± 0.5% (p=0.02) and subsequently recovered after withdrawal of hyperoxia to 96.3 ± 1.3% (p<0.00) and 97.3 ± 1.1% (p=0.01) respectively. SO_2 comparison between the dual wavelength method and the ear-clip pulse oximeter method did not show a significant difference (p=0.54). The interaction between the two methods and time on SO_2 was not significant (p=0.18). **Conclusions:** The application of the dual wavelength method was demonstrated and optical density ratios can be used in a reliable manner for relative oxygen saturation measurements. This valid method promises to enable the study of conjunctival O_2 saturation under various experimental and physiological conditions.

4.2 Introduction

Healthy blood circulation provides tissues with a vital amount of oxygen required for cell function.[1] Oxygen in the blood stream is transported by hemoglobin in the red blood cells. A hemoglobin molecule consists of four units, and each unit has a heme that can be bound to oxygen. Hemoglobin is bound to oxygen in high oxygen partial pressure, while it releases oxygen in low oxygen partial pressure.[1] The content of hemoglobin that binds to oxygen is represented as the degree of the oxygen saturation.

Pulse oximetry has become one of the most commonly used tools in the clinical setting for assessing patients' blood oxygenation status.[2-5] It is a simple, non-invasive tool that measures the percentage of haemoglobin in the blood that is saturated with oxygen by using a probe to transmit light through the tip of a finger or the ear lobe. Most pulse oximeters that are commonly used in medical practice utilize the difference of oxy- and deoxyhemoglobin absorptions at two wavelengths; red and near-infrared. Pulse oximetry is a well recognized and commonly used method of measuring blood oxygen saturation and therefore, it is usually preferred over invasive

measurements from arterial lines.[6] It is important that the monitors used to make these measurements produce accurate, responsive and reliable results. A study has shown that the use of finger probes results in output delays of up to approximately 15 seconds when compared to readings taken with an ear-clip probe.[5] When rapid changes in SO_2 are expected, it is preferable to use an ear lobe probe when using pulse oximetry.[5]

Retinal blood oxygen saturation has previously been used to demonstrate the metabolic state of the retina.[2,7-14] Reflectance spectroscopy dates back to the late 60s[15,16] and is a common method that can measure oxygen saturation in the retinal vessels. This method is based on quantitating the differences between oxyhemoglobin and deoxyhemoglobin light absorption spectra at different wavelengths.[2,7] Much progress has been made since then to improve the approach.[11-13,17-19] One of the drawbacks of this technique is the restriction on the number and locality of measurements at one single cross section of one or two vessels. In more recent literature newer and more diverse multispectra imaging techniques using a scanning laser ophthalmoscope have been used.[20,21]

SO_2 can be manually calculated since there is a linear dependence of the SO_2 in a (retinal) vessel to the ratio of the logarithmic contrast of the vessel versus its surrounding at two specific wavelengths, the optical density ratio (ODR).[8-10,22] Using this relationship, the SO_2 can be calculated from two images at two wavelengths. Recently, an automated method that is based on the above principle and uses an image splitter was developed for use on the retina. [12]

Retinal vascular reactivity studies reported in the literature have typically used gas delivery systems to induce a change in vascular parameters. These systems usually utilize a reservoir bag and one-way valves to essentially counteract the mixing of inspired and expired gases (a non-re-breathing system). Since hyperoxia naturally stimulates hyperventilation (faster or deeper respiration), an uncontrolled and unpredictable reduction of the partial pressure of arterial carbon dioxide (PCO_2) occurs.[23,24] A reduction in the PCO_2 tends to exaggerate a vasoconstrictive consequence. Therefore a vasoconstrictive effect that may otherwise be attributed to hyperoxia when using non-rebreathing circuits can most likely represent a combined effect of increased PO_2 and decreased PCO_2 levels.[25] Previous studies have acknowledged this probable exaggerated effect and have experimented in many ways in the attempt to correct for it.[1,26-29] A useful system

that maintains a constant PCO_2 level (isocapnia) uses a sequential re-breathing circuit that has a feedback loop in order to balance a reduction in PCO_2 caused by hyperventilation.[25,30]

The information regarding the anterior surface of the eye and its hemodynamics in healthy people is limited. One study looked at the microvascular abnormalities in the conjunctival circulation of paediatric diabetic patients and revealed a significant change when compared to healthy patients.[31] However, despite being aware of the significance of circulation *in vivo* we know little of the factors influencing this on the ocular surface in healthy people and consequently in those with vascular anomalies.

The quest to connect intellectual ideas with scientific facts is often pursued by researchers and clinicians. Validity is the degree to which a study accurately assesses the specific idea a researcher attempts to measure.[32,33] The concept of validity is often discussed in connection with measurement reliability. However, a reliable measure is not necessarily valid. That is, a reliable measure can measure something consistently, but not necessarily what it is supposed to measure. A measurement method is valid if it is measures or calculates actual, real values consistently.[32,33]

In this study, we establish and validate a new, simple, filter-based method for bulbar conjunctival vessel oximetry. The reliability of the dual wavelength method was tested across digital video frames and between locations on a single vessel for a series of subjects. The application of the dual wavelength method was demonstrated using an isocapnic hyperoxic provocation and responses were compared and analyzed for significance over time. The validity of the new method was tested by comparing results to those obtained from the ear-clip digital pulse oximeter that were recorded simultaneously.

4.3 Materials and Methods

4.3.1 Sample

Ethics clearance was obtained through the Office of Research Ethics at the University of Waterloo before commencement of the study and the tenets of the Declaration of Helsinki were followed. Eligible subjects signed an informed consent document before enrolment in the study.

4.3.1.1 Reliability

The sample consisted of 6 males and 6 females of average age 27.1 (range 19-34 years). Subjects with any cardiovascular or respiratory disorders were excluded from the study.

4.3.1.2 Isocapnic Hyperoxic Provocation (Validity)

The sample consisted of 3 males and 2 females of average age 29.6 (range 24-34 years). Subjects with any cardiovascular or respiratory disorders were excluded from the study.

4.3.2 The Pulse Oximeter

The pulse oximeter is a non-invasive instrument that indirectly measures the oxygen saturation of a patient's blood (Figure 4-1)[3]. It was attached to a monitor for easy viewing of the subjects' oxygenation. A sensor containing small light-emitting diodes (LEDs) facing a photodiode was clipped onto the subject's earlobe and finger-tip, and a light containing both red and infrared wavelengths was passed from one side to the other. Absorption at these wavelengths differs significantly between oxyhemoglobin and its deoxygenated form, therefore from the ratio of the absorption of the red and infrared light the oxy/deoxyhemoglobin ratio can be calculated by the instrument.[3] The machine displayed the percent hemoglobin molecules in the blood stream bound with oxygen molecules (oxygen saturation).

Figure 4-1: Digital Pulse Oximeter and Finger and Ear-Clip

4.3.3 Dual Wavelength Method

Conjunctival vessels were imaged with two narrow band interference filters with specific wavelengths using a Zeiss slit lamp at 32x magnification. Images were obtained with filters having O_2-sensitive and O_2-insensitive peak transmissions (440nm (Figure 4-2) and 570nm (Figure 4-3), respectively). Images were imported into the image processing application ImageJ.[34] A particular vessel in the region of interest was individually analyzed for each subject at their visit. For each image of each subject, a transparency was placed over the computer screen. The image was opened up in ImageJ and the vessel of interest was tagged and marked on the transparency to ensure the exact spot on the conjunctiva was measured for each subject's visit. Optical densities (OD) were calculated from the pre-determined vascular segment using the average reflected intensities inside (3 points) and outside the vessel (6 points, 1 vessel diameter on each side of the vessel). Optical density ratios (ODR, OD_{440}/OD_{570}) were used to calculate relative oxygen saturation values with the following formula.

OD Optical Density
$I_{(out)}$ Intensity outside the vessel
$I_{(in)}$ Intensity inside the vessel
p_k Point in/out of vessel (centerline, right or left boundary)

$$OD_{\lambda,\,seg}(i) = \log_{10}\left(\frac{I_{out}}{I_{in}}\right) \qquad \text{where, } I_{(x)} = \frac{1}{n}\left[\sum I_{(x)} \cdot p^k\right]$$

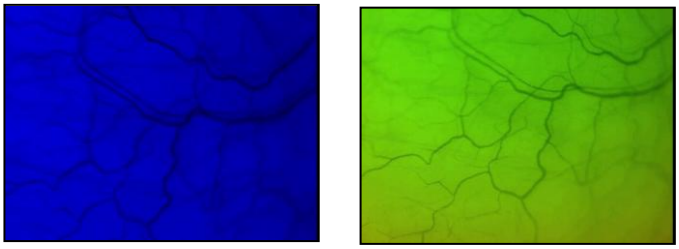

Figure 4-2 and Figure 4-3: Images of conjunctival vessels obtained with narrow band interference filters; O2-sensitive (440nm) and O2-insensitive (570nm) peak transmissions, respectively

4.3.4 Gas Delivery System

The sequential re-breathing system (Figure 4-4) that is described elsewhere[35,36] was made up of a fresh gas reservoir and an expiratory gas reservoir that were connected to the patient by one-way valves. A single PEEP valve connected the inspiratory and expiratory branches. When the gas in the inspiratory limb was depleted, the interconnection between the branches enabled the exhaled gas to be re-breathed. To complete the assembly of the system, a gas reservoir was added to the expiratory port of a commercial 3-valve oxygen delivery system (Hi-OxSR, ViasysHealthcare, Yorba Linda, CA).

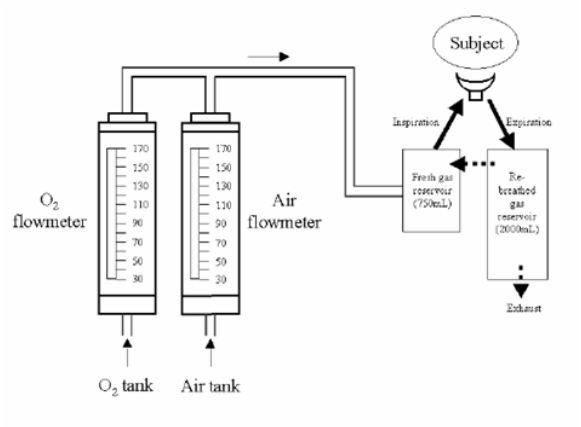

Figure 4-4: Schematic diagram showing the components of the sequential re-breathing system (Re-printed with the permission of Dr. Edward Gilmore)[37]

4.3.5 Procedures

4.3.5.1 Repeatability

Each subject attended 1 session of approximately 15 minutes. At the start of the session, the subject was seated for 5 minutes prior to commencing the study. The blood oxygen saturation levels were monitored with an ear clip probe attached to a digital pulse oximeter while an investigator simultaneously imaged the conjunctival vessels with the dual wavelength method. Video images of one particular area on the bulbar conjunctiva were recorded for each subject for 1 minute each at three times of the day (9:00 am, 12:00 pm and 3:00 pm).

Once the vessel of interest was decided upon, 3 different areas of the vessel, within 10 pixels of each other, were analyzed using the dual wavelength method for each subject at each time point. Also, the same area was measured for five consecutive frames. Measurement repeatability was assessed across frames and between locations.

As an additional test of between method concordance, intraclass correlation coefficients were calculated[64] between the gold standard ear SO2 and the dual wavelength method.

4.3.5.2 Isocapnic Hyperoxic Provocation (Validity)

Each subject attended 1 session of approximately 60 minutes. At the start of the session, the subject was seated for 5 minutes prior to commencing the study. The blood oxygen saturation levels were monitored with an ear clip probe and a finger probe attached to a digital pulse oximeter while an investigator simultaneously imaged the conjunctival vessels with the dual wavelength method. Measurements were recorded at 5 minute intervals. Subjects initially breathed air for 10 minutes followed by an isocapnic hyperoxic stimulus (O_2) for 20 minutes, and then air for a final 10 minute period using the sequential re-breathing circuit. The $PETCO_2$ levels were kept at a steady state.

The flow of O_2 was set and monitored by an experienced investigator. Tidal gas concentrations were continuously sampled from the mouthpiece using a rapid response critical care gas analyzer (Cardiocap 5, Datex-Ohmeda, USA). All data were downloaded to an electronic data acquisition system (S5 Collect, Datex-Ohmeda, USA).

A rapid response critical care gas analyzer (Cardiocap 5, Datex-Ohmeda, USA) was used to quantify the relative concentrations of O_2 and CO_2 in both the inspired and expired gases on a breath-by-breath basis. The inspired and end-tidal concentrations of O_2 and CO_2 were downloaded to a personal computer every 5 seconds (S5 Collect software, Datex-Ohmeda, USA) and are displayed in Table 4-1.

Table 4-1: Group mean and SD of inspired O_2, expired O_2, Inspired CO_2, end-tidal CO_2, SO_2 (dual wavelength, pulse oximeter – ear and finger clip) as a function of provocation.

	Inspired O_2 (SD)	Expired O_2 (SD)	Inspired CO_2 (SD)	End-Tidal CO_2 (SD)	SO_2 (Dual Wavelength) (SD)	SO_2 (Pulse Oximeter – Ear clip) (SD)	SO_2 (Pulse Oximeter – Finger clip) (SD)
Air	19.73 (0.80)	14.8 (0.68)	4.33 (4.91)	38.73 (2.49)	93.96 (1.46)	95.47 (1.10)	97.33 (1.11)
Pure O_2	93.72 (2.62)	88.08 (4.70)	7.84 (7.67)	38.16 (1.89)	96.68 (1.34)	98.28 (1.08)	98.76 (0.48)
Air	20.6 (3.76)	18.13 (5.83)	8.13 (7.20)	38.53 (2.17)	94.81 (0.77)	96.27 (1.30)	97.33 (1.13)

4.4 Results

4.4.1 Repeatability

There was no significant difference in SO_2 between the three locations for the dual wavelength method ($F(2, 22)=1.25$, $p=0.31$, Figure 4-5). The intraclass correlations between the three locations were $r(34)=0.93$, $r(34)=0.56$ and $r(34)=0.86$ for the three times points 9am, 12pm and 3pm respectively. The interaction between location and time was not significant ($F(4, 44)=1.09$, $p=0.37$, Figure 4-6) for the dual wavelength method. There was no significant difference across 5 consecutive frames ($F(4, 28)=0.70$, $p=0.60$, Figure 4-7) and the intraclass correlation was $r(38)=0.96$.

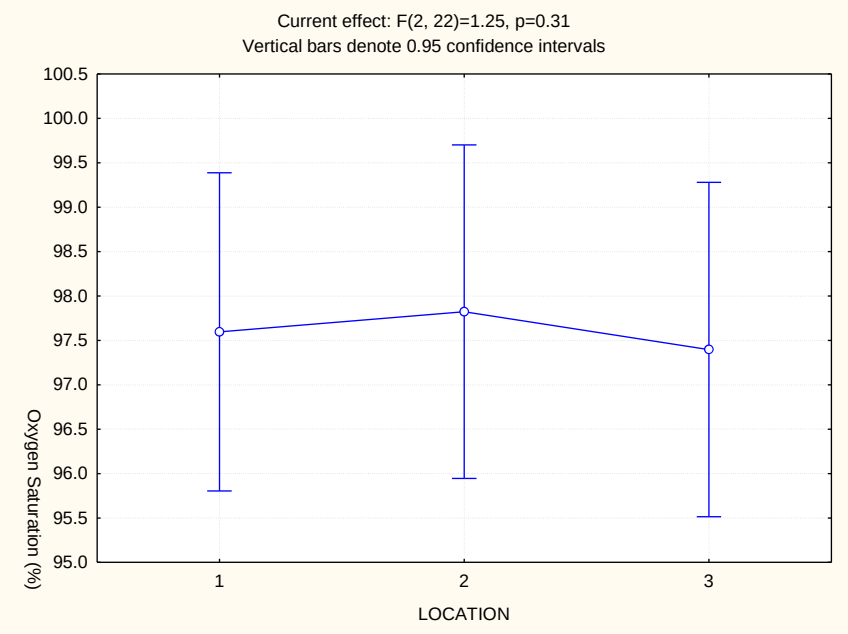

Figure 4-5: SO_2 at three nearby locations

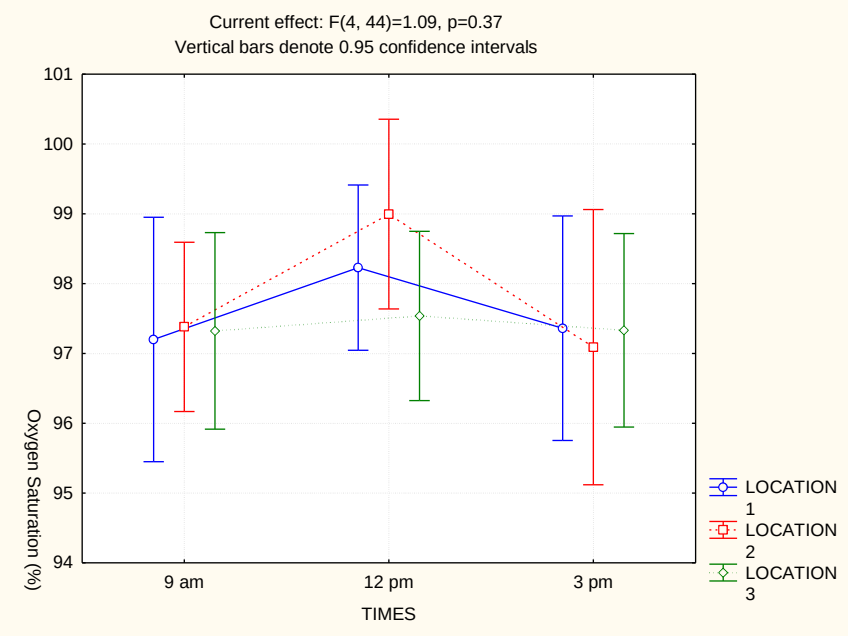

Figure 4-6: Interaction between location and time on SO_2

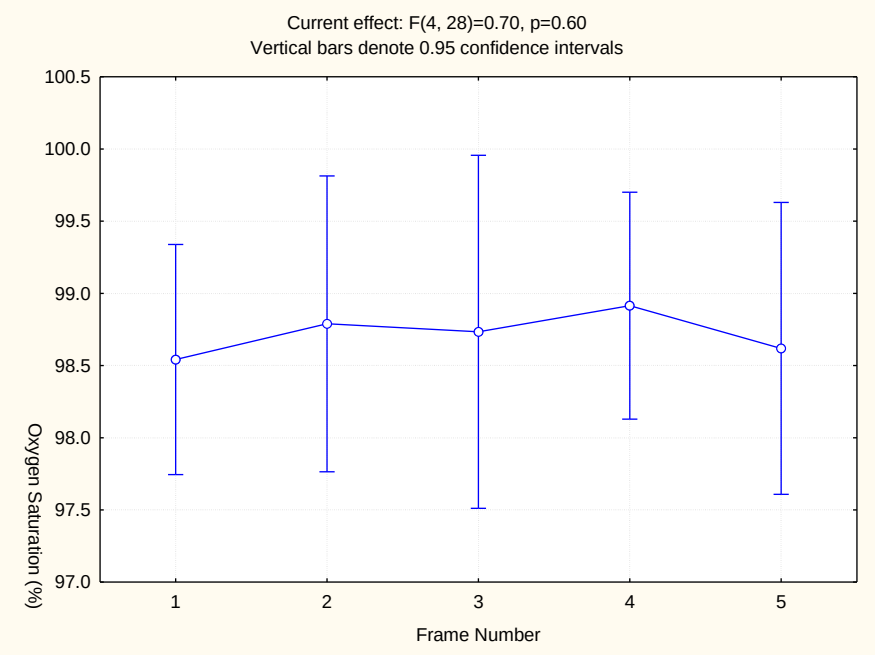

Figure 4-7: SO_2 across 5 consecutive frames

4.4.2 Isocapnic Hyperoxic Provocation

There was a significant increase in oxygen saturation on initiation of hyperoxia for all three measurement methods. SO_2 returned to initial levels when hyperoxia was discontinued ($p > 0.05$ from baseline; Figures 4-8, 4-9 and 4-10).

In response to isocapnic hyperoxic provocation using the dual wavelength method, blood oxygen saturation increased from control values (mean ± SD) of 94.0 ± 1.5% to 96.7 ± 1.3% and subsequently decreased after withdrawal of hyperoxia to 94.8 ± 0.7% ($F(2,8)=5.77$, $p=0.03$). Post hoc analysis using Tukey's post hoc test indicated the increase from baseline (Air) during oxygen provocation (O_2) was significant ($p=0.03$). Blood oxygen saturation values from the ear clip pulse oximeter increased from 95.5 ± 1.1% to 98.3 ± 1.1% during hyperoxia and subsequently decreased to 96.3 ± 1.3% after withdrawal of hyperoxia ($F(2,8)=35.65$, $p=0.0001$). Post hoc analysis using Tukey's HSD post hoc test for significance indicated the increase from baseline (Air) during oxygen provocation (O_2) was significant, ($p=0.0003$), as well as the

decrease from O_2 after withdrawal of hyperoxia (p=0.001). Blood oxygen saturation values from the finger clip pulse oximeter increased from 97.3 ± 1.1% to 98.8 ± 0.5% during hyperoxia and subsequently decreased to 97.3 ± 1.1% after withdrawal of hyperoxia (F(2,8)=11.90, p=0.004). Post hoc analysis using Tukey's post hoc test indicated the increase from baseline (Air) during oxygen provocation (O_2) was significant, (p=0.01), as well as the decrease from O_2 after withdrawal of hyperoxia (p=0.01).

There was no significant difference between the dual wavelength and ear-clip pulse oximeter method (F(1, 11)=0.40, p=0.54, Figure 4-11). The interaction between the two methods (dual wavelength and ear-clip pulse oximeter) and time on SO_2 was not significant (F(2, 22)=1.83 , p=0.18, Figure 4-12). The intraclass correlations between the two methods were r(22)=0.45, r(22)=0.10 and r(22)=0.11 at 9am, 12pm and 3pm, respectively.

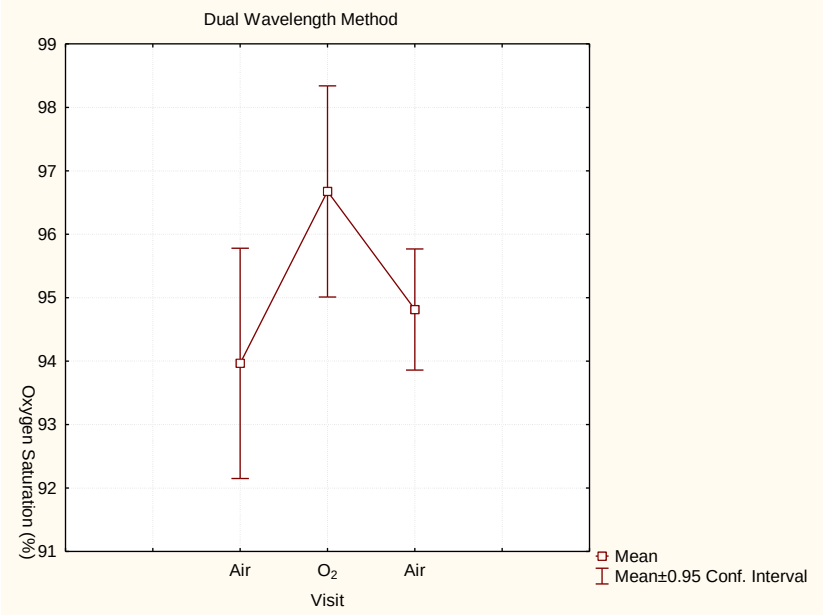

Figure 4-8: Oxygen saturation response to isocapnic hyperoxic provocation using the dual wavelength method.

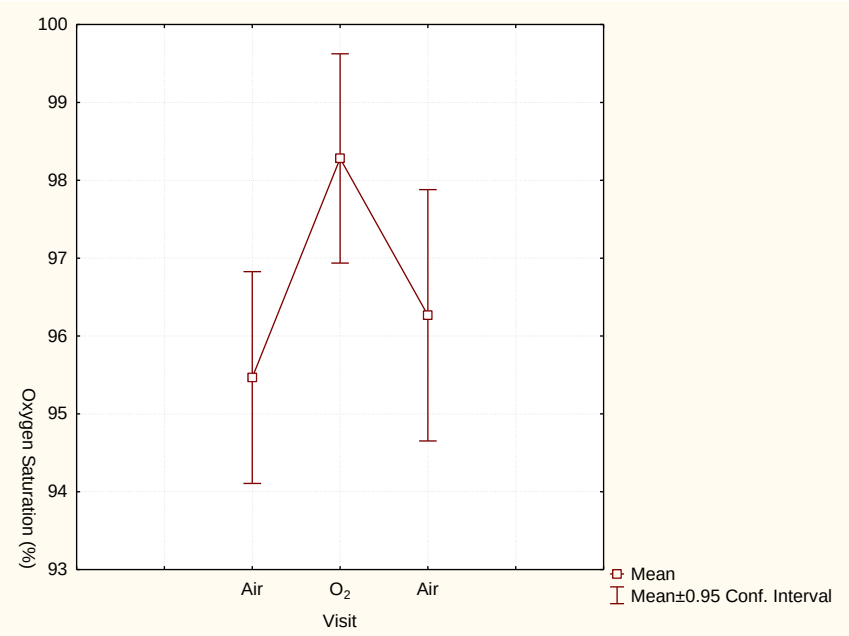

Figure 4-9: Oxygen saturation response to isocapnic hyperoxic provocation using the pulse oximeter method with the ear clip probe.

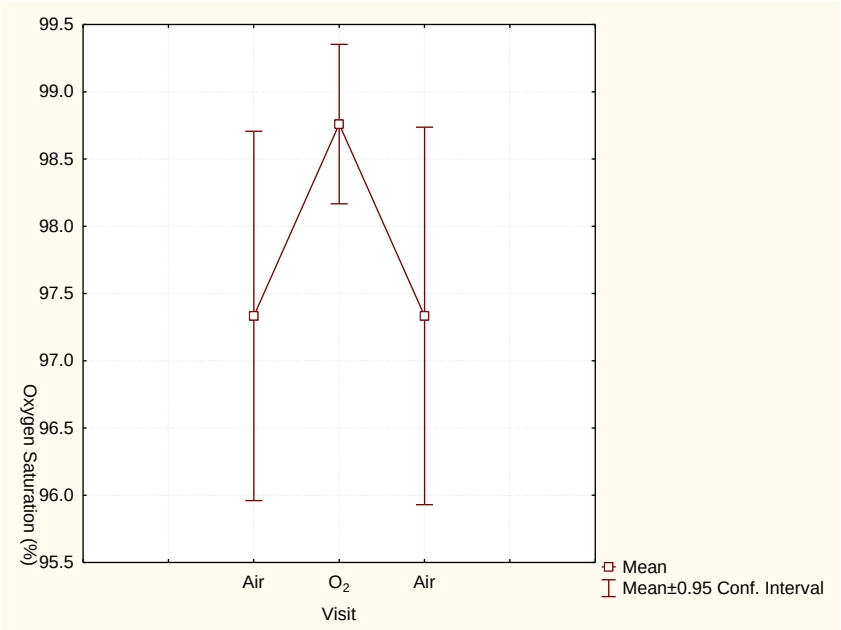

Figure 4-10: Oxygen saturation response to isocapnic hyperoxic provocation using the pulse oximeter method with the finger clip probe.

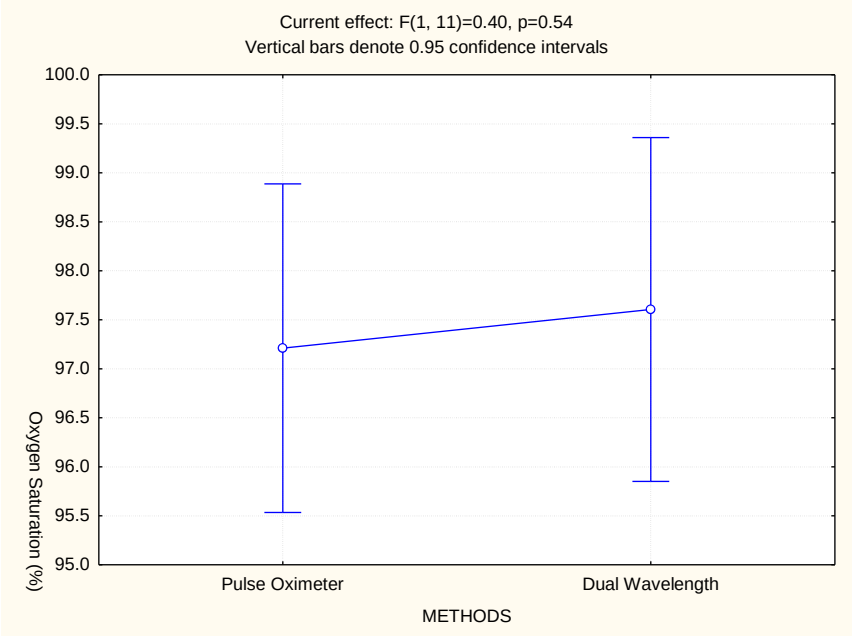

Figure 4-11: Comparing the dual wavelength method with the ear-clip pulse oximeter method

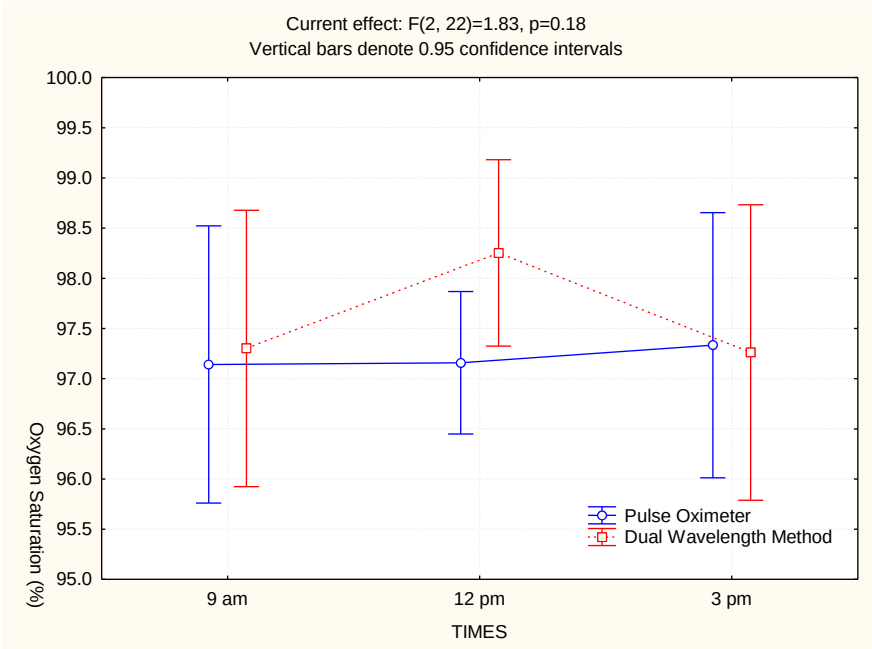

Figure 4-12: Interaction between the two methods and time on SO_2

4.5 Discussion

A novel imaging method for measuring conjunctival vessel SO_2 has been established. By using two narrow band interference filters having O_2-sensitive and O_2-insensitive peak transmissions and a slit lamp camera ("dual wavelength method"), a novel extension to previous work on the measurement of SO_2 was demonstrated.[2,8-10] The intraclass correlations between locations measured and across consecutive frames with this method demonstrated high levels of repeatability.[38]

One goal of this experiment was to assess the reliability of the dual wavelength method. By measuring multiple locations within a vessel and across several consecutive frames, testing of this goal was possible. No significant difference in SO_2 between the three locations measured over time using this method or across consecutive frames was found. The intraclass correlations between the three locations at the three time points were 0.93, 0.56 and 0.86 and across consecutive frames was 0.96. Therefore, the ICC levels demonstrated an excellent degree of reliability.[38]

Verification of the SO_2 measurements was dependent on the accuracy of the external pulse oximetry SO_2. Clinical studies have demonstrated that the accuracy of pulse oximeters under steady state conditions is within ±2%, as long as the measures fall between the range of 100 to 70% saturation.[39,40] We chose to use an ear probe as our comparison method, rather than the finger probe, because of its ability to respond quickly and accurately to a changing systemic arterial saturation.[5] Confidence regarding the accuracy of the SO_2 measurement relied on the agreement achieved when it was compared to the ear clip pulse oximeter measurement. When comparing the two methods using repeated measures ANOVA, no significant difference was found. There was also no significant difference between the methods at either of the time points.

The intraclass correlations for the two methods were 0.45, 0.10 and 0.11 for the three times points. Since both methods demonstrated good reliability, the low ICC values indicate that there is variability between methods which may possibly be related to gas exchange delay times due to proximity of the eye to the lungs. [52]

There have been a number of studies that have used a hyperoxic stimulus to investigate vascular reactivity in the retina.[1,8,22,25,26,43-45] These studies investigated a variety of parameters including oximetry,[8,22,43] vessel diameter,[25,44] and various characteristics of hemodynamics using a variety of techniques.[1,26,45] The technique utilized in this study used the dual wavelength method to measure SO_2 in conjunctival vessels. Not only did the aforementioned studies measure vascular reactivity in the retina, neither of them used an isocapnic hyperoxic stimulus to demonstrate their techniques. Using augmented O_2 concentrations has been shown to reduce the partial pressure of arterial carbon dioxide, as reflected by a change in end-tidal CO_2 concentration.[23,46] It has been previously shown in the cerebral vasculature[47], during respiration[30] and in the retina[48] that the gas delivery system used in this study stabilizes end-tidal CO_2 during hyperoxic provocation and therefore exclusively reflects vascular reactivity responses to O_2. Studies administering augmented O_2 to assess vascular reactivity generally found higher degrees of vasoconstriction, and were as a result of the compounded effect of increased arterial O_2 and diminished CO_2.[49-51]

The dual wavelength method was demonstrated using a sequential re-breathing technique. Using this technique, elevated levels of O_2 were administered while controlling $P_{ET}CO_2$ and SO_2 with three different methods was measured; dual wavelength method, ear-clip pulse oximetry and finger-clip pulse oximetry. As shown in Figures 4-8, 4-9 and 4-10, there was a significant increase in oxygen saturation on initiation of hyperoxia with all three methods and a return to initial values when hyperoxia was discontinued (recovery).

There have been previously published studies that have investigated SO_2 in the retina using a hyperoxic stimulus.[8,22,43] This is the first time that the SO_2 in conjunctival vessels has been quantified during hyperoxic provocation. These SO_2 values and subsequent increases in SO_2 during hyperoxia reported in the present study are comparable to those of previous studies involving vessels of the retina[37,43,52]. As expected, the dual wavelength method used to measure SO_2 in the conjunctival vessels demonstrated a significant increase during hyperoxic provocation and a subsequent decrease after withdrawal of the stimulus, similar to the ear and finger pulse oximetry methods. The pulse oximeter is a commonly used tool in the clinical setting for assessing patients' blood oxygenation status and is regarded as a valid instrument.[2-5] The values from the dual wavelength method and the ear clip pulse oximeter were compared to assess the validity of the dual wavelength method. When the two methods were compared, no significant

difference was found (p=0.54, Figure 4-11). This demonstrates that the dual wavelength method offers good construct validity.[32]

Ten minutes after hyperoxia was discontinued (recovery) mean SO_2 levels for all three methods were not significantly different from baseline (p>0.05). A significant difference between hyperoxia and recovery of SO_2 was demonstrated with the finger probe pulse oximeter method. Differences in recovery response times between methods are unknown and again, may possibly be due to gas exchange delay times.[52] This hypothesis needs further testing.

It has been demonstrated that a finite time is required for O_2 to reach the ocular vasculature due to a physiological delay of gas exchange in the lungs and lung-to-eye circulation time.[52] The exact mechanism that manages the reduction in vessel diameter during hyperoxia in order to maintain oxygen regulation is not known but various biochemical factors may be responsible.[53] The factors include endothelin-1 released from endothelial cells,[54-56] physical changes to red blood cells,[57-59] superoxide generation and nitric oxide.[60-62] In addition to a reduction of vessel diameter, oxygen supply during hyperoxia can be controlled by a change in wall shear rate via an up-stream flow-induced mechanism that initiates a secondary diameter response.[63]

4.6 Conclusions

This is a novel study that utilized an isocapnic hyperoxic stimulus to provoke conjunctival vascular reactivity while controlling $P_{ET}CO_2$ to demonstrate the dual wavelength method.

The application of the dual wavelength method was demonstrated and optical density ratios can be used in a reliable manner for relative oxygen saturation measurements. This valid method promises to enable the study of conjunctival O_2 saturation under various experimental and physiological conditions.

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5. Oxygen saturation, red blood cell velocity and bulbar conjunctival redness in conjunctival vessels

5.1 Overview

Purpose: The aim of this experiment was to examine variations in ocular redness levels, red blood cell velocities and oxygen saturation levels over time in clinically healthy participants. An ancillary question was to examine these measures in an older age stratum as well as a younger age stratum and to compare differences between the groups. **Methods:** The sample consisted of 14 healthy participants with average age of 34.7 years (range 20-61 years). Participants attended eight separate visits over the course of a day. Levels of bulbar conjunctival redness, red blood cell velocity and blood oxygen saturation were measured on a vessel of interest. **Results:** From baseline, the group mean redness and oxygen saturation did not change significantly over time ($p > 0.05$). There was a significant difference in the group mean red blood cell velocity values over time ($p = 0.01$). There was no significant difference between age strata for all three measures. **Conclusions:** The results of this study support the theory of metabolic regulation. The lack of any significant change across time for redness and oxygen saturation along with significant changes in red blood cell velocity substantiates this notion.

5.2 Introduction

A function of the circulatory system is to provide tissues with the required amount of oxygen and nutrients vital for cell function.[1,2] Ocular circulation assessment has evolved from a subjective physical description of the visible vessels to direct and indirect quantitative measurement of a number of ocular hemodynamic parameters. Today, the techniques available for hemodynamic assessment (outlined in Chapter 1) examine various aspects of the ocular circulation. These methods have the potential to contribute greatly to our understanding of normal hemodynamics.

Regardless of our understanding of the significance of circulation, we know little of the factors (and interaction of these factors) influencing this on the ocular surface. Quantitative and non-invasive studies on blood flow and blood oxygen saturation in the *in vivo* human capillary flow

have been limited.[3-6] By measuring red blood cell velocity, oxygen saturation and ocular surface redness in the conjunctival vessels we are able to study the changes that occur in the circulation and the levels of oxygen delivery and saturation over many health states and conditions.

Regulation of blood flow is necessary to adapt to different internal and external conditions. This regulation also compensates for varying perfusion pressures, adapts to metabolic activity and helps to maintain a constant ocular temperature.[7] By and large, red blood cell velocity is regulated by the cardiac output, which is mainly controlled by the autonomic nervous system and circulating hormones.[7] The resistance of local vessels is controlled by the vascular endothelial cells. The endothelial cells achieve desired changes in resistance by releasing vasoactive molecules such as endothelin-1 (vasoconstriction) and nitric oxide (vasodilation). The retina is regulated mainly by the neural and glial cells.[8] The choroidal circulation is regulated by the autonomic nervous system and circulating hormones.[9,10] The optic nerve head is regulated by endothelial cells and circulating hormones.[11]

The mechanisms underlying vessel resistance are not completely understood. Although constriction and dilation are regulated by the autonomic nervous system, circulating hormones and vasoactive molecules, other mechanisms may also be involved. It has been demonstrated that organs and local tissue require specific levels of blood oxygen saturation for optimal function and therefore blood flow must be adequately regulated to achieve this.[7,12] Therefore, required oxygen levels are maintained by the organs' strong capacity to regulate.[7,12-15]

The capacity of an organ (e.g. brain) to function optimally in a physiological manner has been demonstrated to decline with age.[16,17] The age-related physiological declines that occur include, reductions in growth and insulin-like growth factors, increases in vascular resistance, reductions in distensibility within vessels, and compromises to the endothelium-dependent vascular relaxant mechanisms.[18-20] It has been demonstrated in the cerebral vessels that the cerebral oxygen reserve remains fairly stable because the vessels regulate regional blood flow to accommodate changes in perfusion and arterial oxygenation[21,22] but, it has also been demonstrated that with age the vessels lose some of their ability to maintain this reserve.[16,17] As a result, oxygen extraction from capillary blood must increase to maintain cerebral oxygen metabolism and tissue function, and this increased oxygen extraction is reflected as a decrease in measured regional cerebral vessel SO_2.[23]

As age increases, the systemic vascular resistance increases and contributes to the disturbed capacity of local blood vessel dilation.[24] The result of disturbed vasodilation can contribute to reductions in ocular flow,[24,25] as well as limit the ability of the vessels to increase flow in response to physiological challenges such as hypoxemia.[24] In addition, aging has been demonstrated to reduce aqueous inflow and outflow,[26] decrease the mobility of the ciliary muscle,[27] decrease the optical performance of the eye,[28] increase ocular surface redness,[29] and gradually enlarge the optic nerve head cup and thin the neuroretinal rim.[30,31]

The aim of this study was to characterize conjunctival red blood cell velocity, oxygen saturation and bulbar conjunctival redness in a group of clinically normal subjects. An additional initiative was to examine these measures in two age strata.

5.3 Hypothesis

It was expected that there would be no changes in ocular surface redness, red blood cell velocity and blood oxygen saturation over time in the healthy individuals. In the comparison between age strata, it was expected that the older age stratum would have increased levels of redness, and decreased red blood cell velocities when compared to the younger age stratum. It was expected that the blood oxygen saturation levels in the older aged stratum would be lower than the younger aged stratum.

5.4 Materials and Methods

5.4.1 Sample

Ethics clearance was obtained through the Office of Research Ethics at the University of Waterloo before commencement of the study. Eligible subjects signed an informed consent document before enrolment in the study.

The eligibility of participants was determined at a screening appointment. The sample consisted of 14 healthy participants with an average age of 34.7 years (range 20-61 years). The participants were also evenly divided into two age strata (between 18-30 yoa and 31-62 yoa) with average

age of 25.1 (range 20-28 years) and 45.6 (range 32-61 years) and assessed for differences between them. Subjects with any vascular disorders were excluded from the study.

5.4.2 Redness Measurements

For the measurements of conjunctival hyperemia, chromaticity (u') values were taken by the SpectraScan PR650 Spectrophotometer (Figure 5-1). The photometer and a modified slit-lamp were mounted onto a table-top device.

The PR650 measures luminance and chromaticity. It measures the absolute intensity at each wavelength and then calculates the equivalent CIE value. The modified slit-lamp mount contained the PR650, and illumination source, targets for the participant to focus on and a chin rest.

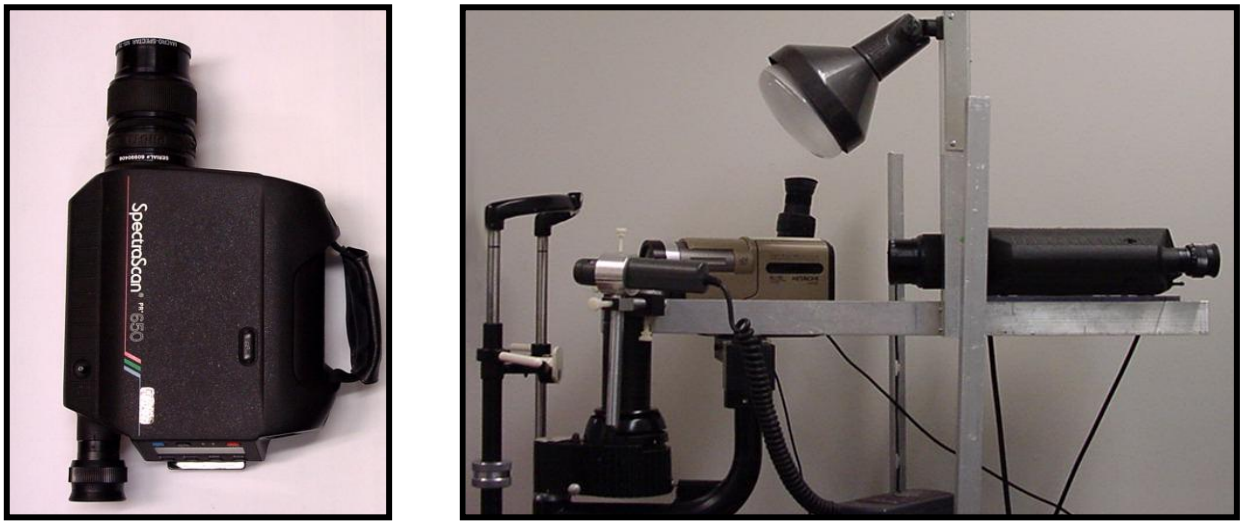

Figure 5-1: a) SpectraScan PR650 Spectrophotometer; b) Photometer and illumination set up

5.4.3 Blood Oxygen Saturation Measurements

Please refer to chapter 4.3.3 of this thesis for SO_2 measurement description.

5.4.4 Red Blood Cell Velocity Measurements

Videos were taken with the Handy Alpha, Hyper Micro Color CCD Camera with LED lighting (Plumnet Co. Ltd.©, Japan) which was mounted on a modified slit-lamp (Figure 5-2). The magnification of the camera (Figure 5-3) was altered by adding on 2 adapters. The modified slit lamp mount contained the Handy Alpha camera, a chin rest and a velcro strap that wrapped around the subject's head. The AV cable supplied with the camera was connected to a desktop computer via a USB video capture card. Video images were then displayed on the computer screen.

Figure 5-2: Set-up of the high magnification camera

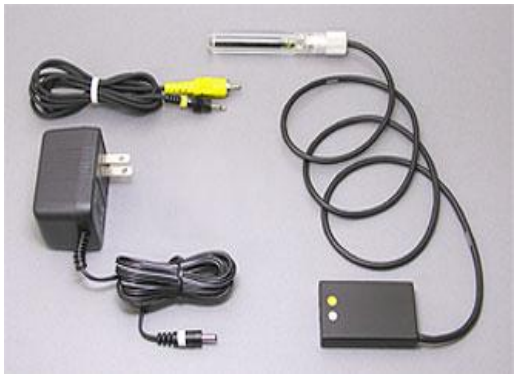

Figure 5-3: Handy Alpha camera

Conjunctival vessels of healthy individuals were digitally imaged at 30 frames per second with high enough magnification (2 adapters, approximately 144x magnification[32]) to clearly resolve

movement of the blood within the vessel. The videos were then coded in AVI format with 7 frames per second, 720x480 pixels, 24 bits per pixel. For each video one vessel was selected and the cell shift along the vessel center line was manually evaluated to estimate their mean RBC velocity.

5.4.5 Procedures

At 8 measurement sessions, 1 hour apart, the first occurring at 9:00 am, redness, SO_2 and RBC velocity video data were acquired. Each measurement was recorded under controlled illumination.

The participant sat at each instrument and looked at a target to their left or right. This was for the temporal measurements of either the right or the left eye respectively (depending on which eye was randomly assigned the study eye). The examiner positioned the instrument on the temporal bulbar conjunctiva and focused on the "vessel of interest" (VOI). Participants remained in the building during the entire experiment.

5.4.5.1 Redness Measurement Procedures

The participants' temporal conjunctiva was viewed with the slit lamp, a vessel was chosen as the VOI and was used for all subsequent measurements.

The chromaticity (CIEu') of a small fixed area surrounding the participant's VOI was measured with the photometer under controlled illumination. The light was turned on just prior to the measurement and turned off just after.

The participant sat at the photometer and looked at the target (Figure 5-4). The examiner looked through the eye piece and positioned a black measuring spot (approximately 5 mm diameter) on the temporal bulbar conjunctiva on top of the VOI. The measuring spot was also aimed approximately 2mm from the limbus. There were five measurements of redness taken on both eyes at each visit and averaged. To ensure consistent results, no adjustments to the eye piece were made. Also, the lateral position and the illumination of the instrument remained unchanged.

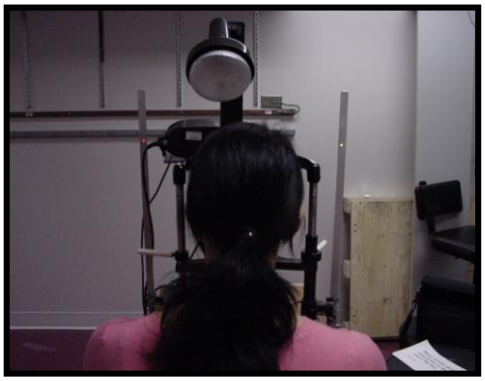

Figure 5-4: Participant set up at the photometer with viewing targets to the left and right (red and green light).

5.4.5.2 Blood Oxygen Saturation Procedures

The participants' VOIs were recorded with the slit-lamp under controlled illumination. The participant sat at the slit lamp and looked at one of the targets. The examiner positioned the slit lamp so they were able to focus on the temporal bulbar conjunctiva and the VOI. The investigator imaged the VOI with the dual wavelength method detailed in Chapter 4. The video images of the VOI on the bulbar conjunctiva were recorded for each participant for 1 minute each at all visits during the day.

5.4.5.3 Red blood cell velocity measurement procedures

The participant sat at the slit lamp mount and looked at a specific target. The examiner positioned the camera on the temporal bulbar conjunctiva and focused on the VOI. While monitoring the video on the computer screen, the examiner recorded several minutes of video in order to ensure good quality and an ample number of frames. To ensure consistent results the participant's head was strapped in with a Velcro strip across the back of their head and they were instructed to be conscious of minimizing any small movements.

Videos were imported into the video processing utility VirtualDub (v1.8.8, Cambridge, MA) and cropped into image sequences of manageable size. The image sequences were converted to AVI files. All files were pre-processed as described below and velocity was estimated.

5.4.5.3.1 Pre Processing and RBC Velocity Estimation

Please refer to chapter 3.3.1.1 and 3.3.1.2 of this thesis for pre processing and RBC velocity estimation description.

5.4.5.4 Data Analysis

The group of 14 healthy participants was assessed for differences across the 8 visits for all three measures using repeated measures ANOVA (Statistica 7; Statsoft, Tulsa, OK). The group of 14 participants was also divided into two age strata (7 participants between 18-30 yoa and 7 participants between 30-62 yoa) and assessed for differences between them across the 8 visits for all three measures using a mixed ANOVA.

5.5 Results

5.5.1 Redness

The effect of age, time and their interaction on redness is shown below. The redness of the younger and older strata ranged from 0.267 ±0.004 to 0.269 ± 0.004 and 0.272 ± 0.008 to 0.276 ± 0.007, respectively. The redness over time ranged from (mean ± SD) 0.270 ± 0.006 to 0.272 ± 0.007.

There was no effect of age ($F(1, 12)=3.57$, $p>0.05$; Figure 5-5). The redness over time was not significant ($F(7.91)=1.31$, $p>0.05$; Figure 5-6) . The interaction between age and time on redness was not significant ($F(7,84)=0.87$, $p>0.05$; Figure 5-7).

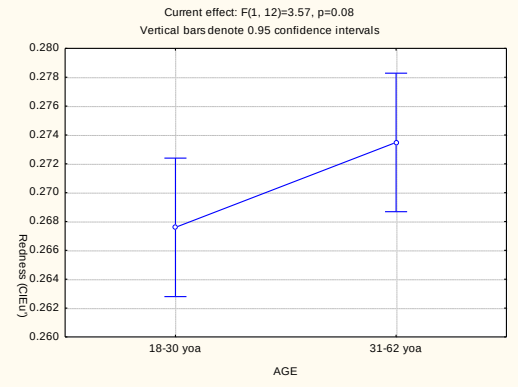

Figure 5-5: Bulbar conjunctival redness sorted by age

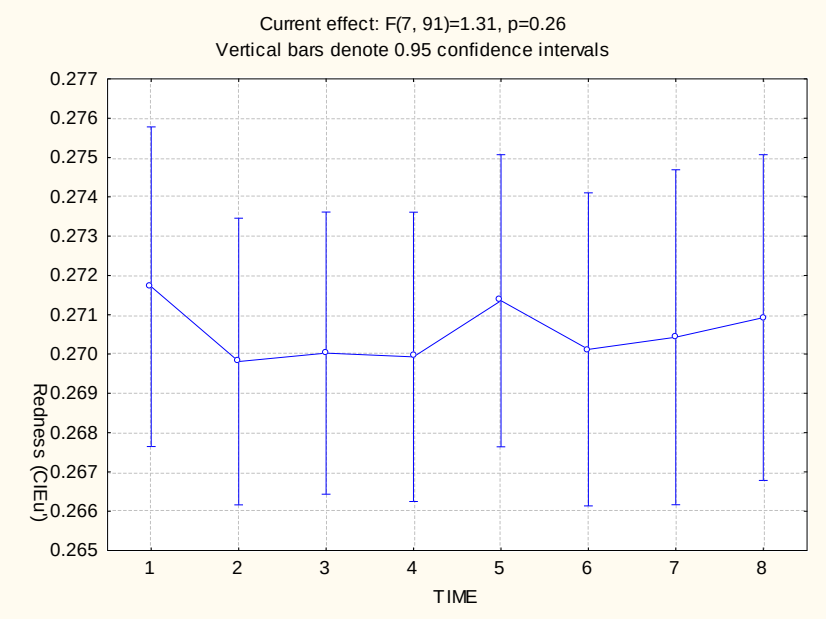

Figure 5-6: Redness over time

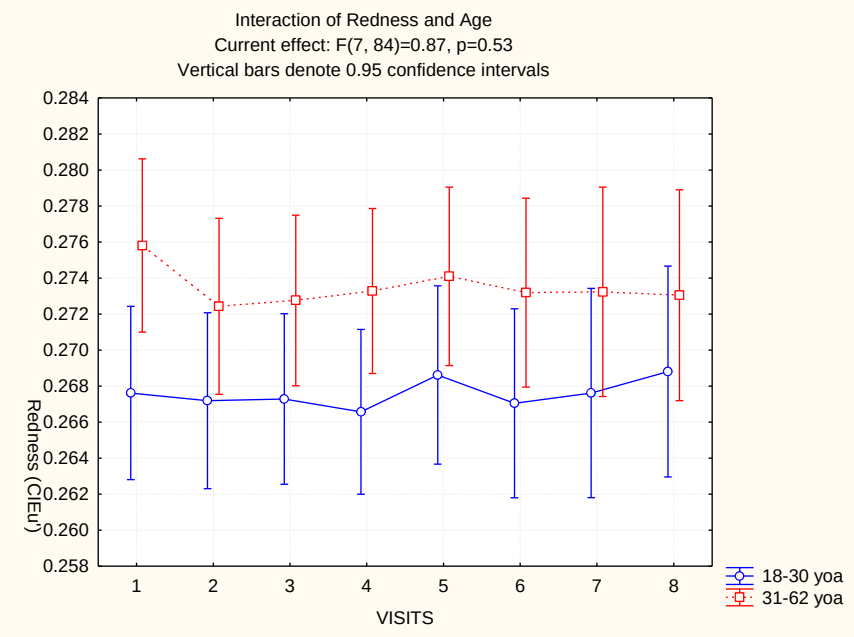

Figure 5-7: Interaction between age and time on redness

5.5.2 Oxygen Saturation Measurements

The effect of age, time and their interaction on oxygen saturation is shown below. The oxygen saturation of the younger and older strata ranged from 97.1 ± 1.3 to 98.7 ± 2.2 and 98.3 ± 1.4 to 99.7 ± 2.0, respectively. The group mean SO_2 values over time (mean ± SD) ranged from 98.2 ± 2.2 to 99.2 ± 2.1.

There was no main effect of age ($F(1,12)=4.14$, $p>0.05$; Figure 5-8). The SO_2 over time was not significant ($F(7,91)=0.60$, $p>0.05$; Figure 5-9). The interaction between age and time was not significant ($F(7,84)=0.61$, $p>0.05$; Figure 5-10).

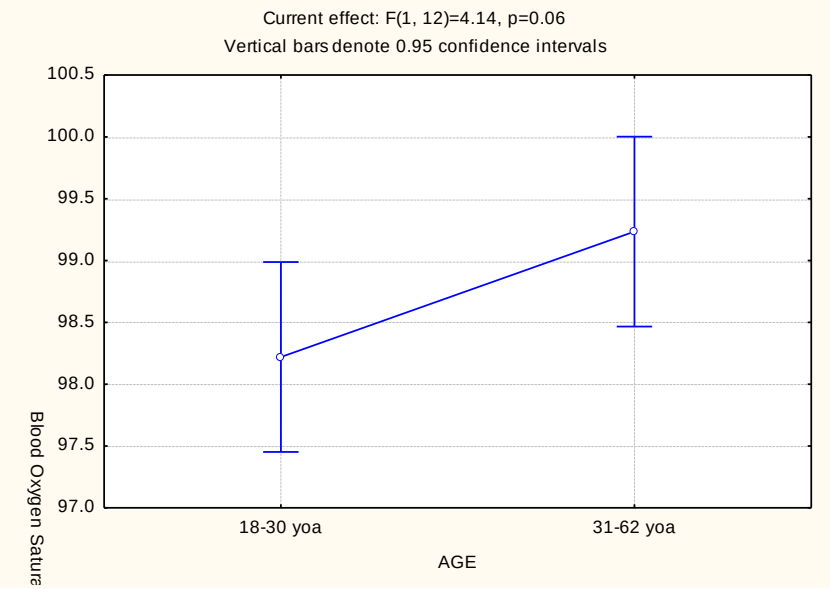

Figure 5-8: Blood oxygen saturation sorted by age

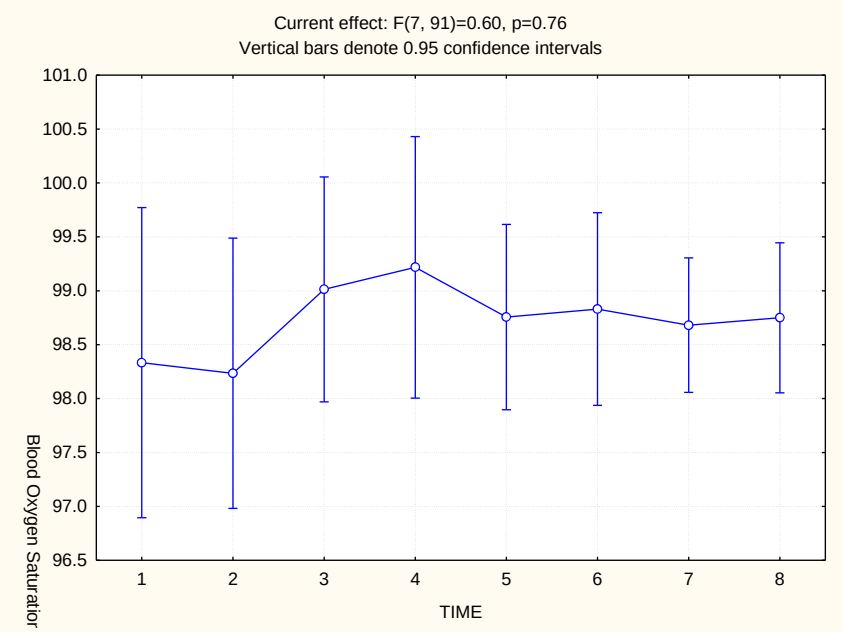

Figure 5-9: Blood oxygen saturation over time

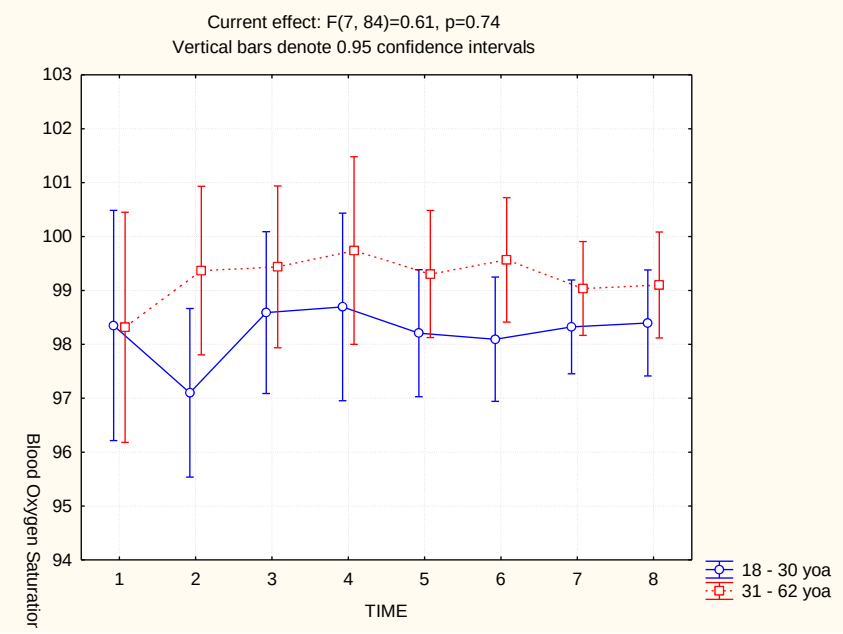

Figure 5-10: Interaction between age and time on SO_2

5.5.3 Red blood cell velocity

The effect of age, time and their interaction on RBC velocity is shown below. The RBC velocity in the younger and older strata ranged from 18.9 ± 7.9 to 49.0 ± 15.3 and 17.8 ± 14.1 to 45.8 ± 14.9, respectively. The group mean velocity values over time (mean ± SD) ranged from 21.8 ± 23.0 to 38.8 ± 23.6.

There was no main effect of age ($F(1,5)=2.83$, $p > 0.05$; Figure 5-11). The RBC velocity over time was significant ($F(7,42)=3.17$, $p=0.01$; Figure 5-12). The velocity was less in the first few measurements and higher in the last few measurements. The significance was between the eighth measure and the first and fourth measures (Tukey HSD; $p = 0.002$ and $p = 0.02$, respectively). The interaction between age and time was not significant ($F(7,35)=0.46$, $p>0.05$; Figure 5-13).

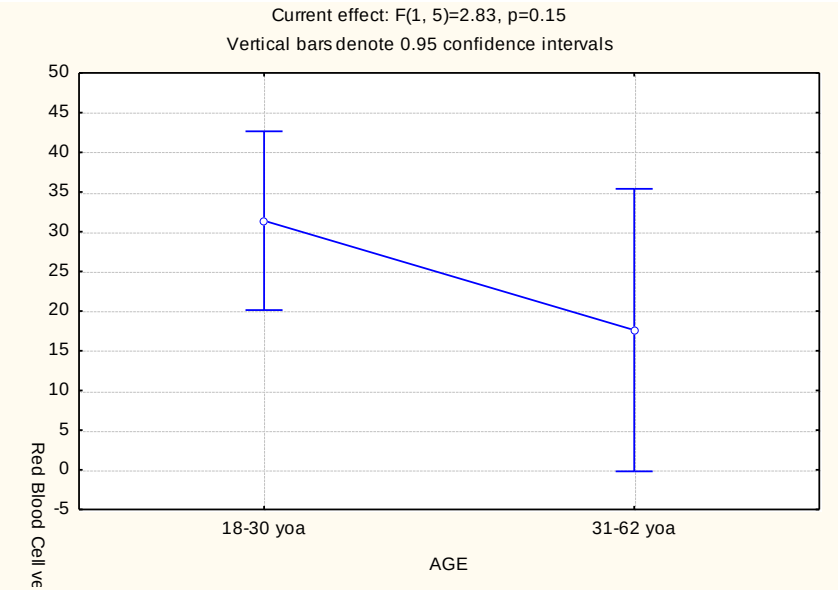

Figure 5-11: Red blood cell velocity sorted by age

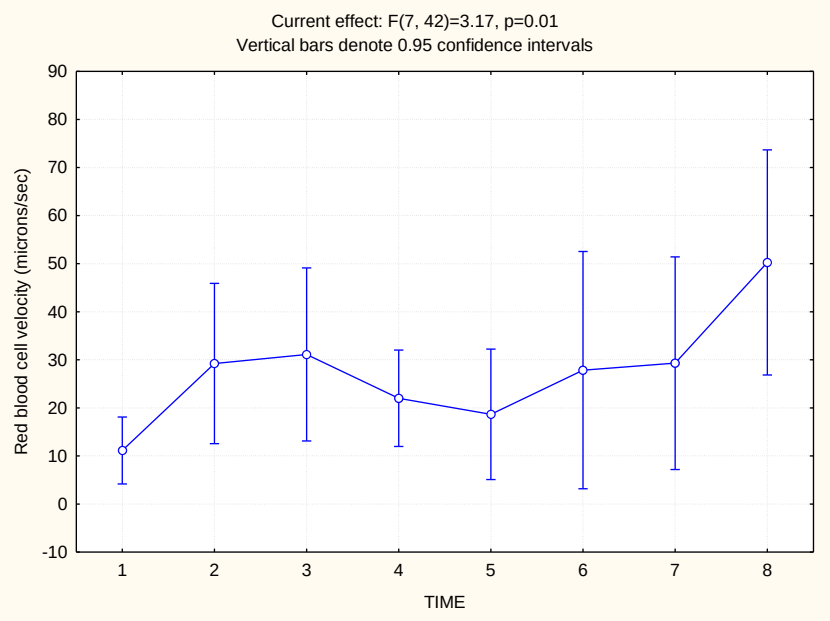

Figure 5-12: Red blood cell velocity over time

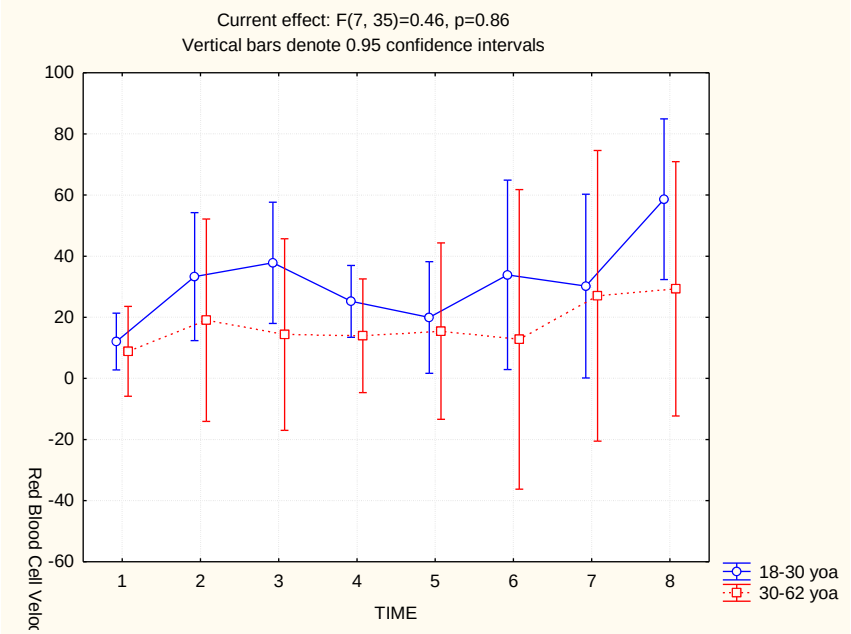

Figure 5-13: Interaction between age and time on RBC velocity

5.6 Discussion

The use of novel methods for the measurement of red blood cell velocity and oxygen saturation within the conjunctival vessels on the surface of the eye has been demonstrated. The present study investigated conjunctival redness, oxygen saturation and RBC velocity in a group of healthy participants. The change in redness and oxygen saturation over time in the group of healthy participants (n = 14) was not significant. We did however find a change of RBC velocity.

Since the participants in this particular study were not suffering from vascular dysregulation, it is likely that their vessels were under normal, local, regulatory control and the lack of any significant change in oxygen saturation over time was expected. Oxygen saturation was hypothesized not to vary significantly since cerebral vessels have been previously shown to regulate regional blood flow to accommodate changes in perfusion and arterial oxygenation in order to keep SO_2 levels within a specific range.[21,22] The results of this study demonstrated that over time, SO_2 did not change, supporting this theory of vascular regulation.

A primary function of the vasculature is homeostatic regulation during ordinary instances and especially during times when various stimuli are applied to the system.[7,33,34] The results of this study support this notion. If regulation does not occur according to the needs of the tissue it is referred to as vascular dysregulation.[7] During the regulation of any parameter, several systems are involved in such a way that the dysfunction of one of the systems would lead to compensation by another. The regulations of different parameters are frequently interconnected. Blood flow, for instance, is involved in the regulation of oxygen supply, transport of nutrients and regulation of temperature and volume. The vascular system is significantly regulated on different levels such as in large or small arteries, capillaries and veins.[35] Vascular dysregulation merely means that this local adaptation does not meet the requirements of the body or its corresponding tissues. This insufficiency may be local, such as damage to the endothelial cells in the vessel wall (e.g. atherosclerosis), may be secondary to an underlying disease (e.g. diabetes) or, may be a natural tendency to react differently to various stimuli (e.g. cold temperatures).

A previous study has shown significant changes in ocular surface redness across time.[36] These significant changes were accounted for by a cascade of electro-chemical reactions within the body[37,38], the metabolic effects of hypoxia during sleep[39], cytokines and mediators[40] that cause

inflammatory and immune-mediated reactions that contribute to ocular inflammation[41] and, several neurotransmitters, hormones and chemicals that have potent effects on the vessels within the eye that cause either vasodilation or, vasoconstriction.[1] The previous study was different from this present study in that the number of hours each participant slept and the food they ate was tightly controlled. These differences could account for the lack of significance found in this experiment. Although we did not expect or find significance, our redness values were comparable in range.[36,42] The measurement area of redness in this study was different than the SO_2 and RBC velocity measure in that the former includes a total area of 19.6 mm^2 versus a single point along the vessel of interest (VOI). The area includes the VOI as well as deeper and additional vessels within that 19.6mm^2 area. The latter two measures were an estimation within the VOI alone. When trying to understand the interconnection of the regulation between the three measures, this must be taken into account. Although "global" redness (redness in an area of 19.6mm^2) may be expected to change significantly over time (and has previously been demonstrated), the redness as a function between the ratio of vessel to background for one vessel was not hypothesized to change significantly. This notion will help in the understanding of the changes or lack of changes in the measures of SO_2 and RBC velocity.

There are no experimental comparisons of the blood velocity or oxygen saturation on the anterior ocular surface. The values obtained for velocity and SO_2 were comparable to previous studies done on vessels of the cheek pouch of a hamster[5,43], retinal vessels[44,45] and cerebral vessels[34]. Blood flow comparisons are somewhat difficult because of circulation differences within specific ocular regions.[46] Moreover, change in velocity across measurements was not expected to occur since in normal eyes, autoregulation keeps blood flow relatively constant.[47-51] The eye requires constant perfusion during dynamic changes in blood pressure, intraocular pressure and ocular perfusion pressures. Since the results demonstrate a significant change in RBC velocity over time, it can be assumed that there were changes occurring with respect to the oxygen demands on the tissue and the vessels had to accommodate the blood flow in order to maintain a specific level of oxygenation.[21,22] The blood flowing through the majority of the vessels (specifically the VOI) that were imaged on the conjunctival surface consist of RBCs that traverse the vessel column in single file. The diameter of the column itself is quite narrow, to the point that the RBCs cannot line up side by side. Because of regulation, the vessel was not expected to increase

or decrease significantly in calibre. The expectation for the lack of a significant change in calibre of the vessel supports the hypothesis of a lack in change of redness over time (in the VOI).

When the groups were sorted by age and compared, a few interesting effects were found. There were no significant differences found between age strata for either of the variables. However, there was a strong trend towards significant differences in redness and oxygen saturation. Both trends indicated higher levels of redness and higher levels of circulating oxygen for the older age stratum. On average, the redness was 2.2% higher and the SO_2 was 0.89% higher for all time points for the older cohort. The trend for the older age stratum to have a greater level of redness is consistent with a study that found redness increases with each decade in life by approximately 0.1 units on the McMonnies grading scale.[29] It was speculated that the gradual increase in redness with age may be attributable to a reduction in arteriolar wall muscle tone.[29]

Again, although not significant, our results showed a trend for RBC velocity to be lower in the older age stratum. On average, the RBC velocity was 30.0% higher across all points for the younger age stratum. Under normal, healthy circumstances, the cerebral oxygen reserve remains fairly stable because the cerebral vessels regulate regional blood flow to accommodate changes in cerebral perfusion and arterial oxygenation[21,22] but with age, the cerebral vessels lose some of their ability to maintain this reserve of oxygen, which declines on average by 5% to 10% between the ages of 40 and 75.[16,17] Such regional decreases in blood flow may result from disturbed vasodilatation of local blood vessels due to increased vascular resistance. In any case, the result of disturbed vasodilation is a limited ability to increase flow in response to challenges like hypoxemia. As a result, oxygen extraction from capillary blood must increase to maintain cerebral oxygen metabolism and tissue function, and this increased oxygen extraction is reflected as a decrease in measured regional cerebral SO_2.[23] The trend for the older age stratum to have a greater level of oxygen may be indicative of the inability of the tissue to extract the available oxygen. Conversely, a lower level of available oxygen in the younger aged stratum may be indicative of good and/or sufficient levels of oxygen extraction by the tissue. The lower RBC velocity demonstrated in the older age stratum coincides with the higher levels of SO_2. With high levels of available oxygen, RBC velocity would not be required by the system to increase.

The systemic increase of vascular resistance that arises with age may also be involved in the reduction of ocular flow.[24] Increasing age inevitably causes widespread physiological declines

that reduce functional capacities and increase susceptibility to disease via reductions in growth and insulin-like growth factors, increases in vascular resistance, reduction of distensibility within vessels, and compromises the endothelium-dependent vascular relaxant mechanisms.[18-20] These declines essentially include many alterations within the eye. For example, ageing reduces aqueous inflow and outflow,[26] decreases the mobility of the ciliary muscle,[27] decreases the optical performance of the eye,[28] and gradually enlarges the cup and thins the neuroretinal rim.[30,31] The consequence of these numerous effects of aging on the eye is to concentrate diseases such as cataract, glaucoma, and age-related macular degeneration within older age patients.[52]

In summary, these results are for a small cohort of healthy adults and further testing would be required to discern the exact relationship between the variables and the aging eye. These results support the theory of vascular regulation under normal circumstances. In healthy individuals it was demonstrated that although significant variations in RBC velocity were recorded, levels of SO_2 remained stable.

It is recognized[1,2,24,35] that aging, disease, or a prolonged vascular stress alters the normal vessel anatomy or reactivity in various ways and therefore furthering these findings in a much larger cohort of healthy individuals is essential.

5.7 Conclusions

In summary, the participants in this particular study did not suffer from any known causes that would contribute to vascular dysregulation and therefore it is plausible that their vessels were under normal, local, regulatory control. The results of this study support the theory of metabolic regulation. The lack of any significant change across time for redness and oxygen saturation along with significant changes in red blood cell velocity substantiates this notion.

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6. Conjunctival redness, red blood cell velocity and oxygen saturation in participants after instillation of a topical ophthalmic decongestant

6.1 Overview

Purpose: The aim of this experiment was to examine the ocular redness levels, red blood cell velocities and oxygen saturation levels in clinically healthy participants when a topical ophthalmic decongestant was instilled onto the eye and to demonstrate the validity of the use of two novel techniques. **Methods:** The sample consisted of 7 healthy participants with an average age of 35 years (range 25-61 years). Participants attended three separate visits during a 60 minute session. Bulbar conjunctival redness, red blood cell velocity and blood oxygen saturation were measured on a vessel of interest, pre-instillation, just after insertion and, 10 minutes after insertion of a topical ocular decongestant. Significant differences between the three measures were assessed and correlations between the three parameters were reported. **Results:** From baseline, the group mean redness values increased slightly from 0.268 ± 0.004 to 0.271 ± 0.004 at drop instillation. The group mean red blood cell velocity and oxygen saturation values changed from 35.2 ± 46.9 µm/s and 98.1 ± 2.6 % at baseline to 28.8 ± 17.0 µm/s and 98.6 ± 1.6 % at drop instillation, respectively. After instillation redness values decreased to 0.265 ± 0.01 ($p=0.01$). The red blood cell velocity and oxygen saturation were 31.3 ± 28.1µm/s and 97.4 ± 2.6 % at the post measurement, respectively. These two measures did not change significantly ($p > 0.05$). There was a moderate significant correlation between SO_2 and red blood cell velocity just after drop insertion ($r = 0.79$). **Conclusions:** This study supports the literature regarding regulation of the microvasculature during the use of various stimuli. The results demonstrated that oxygen saturation levels remain stable even when a significant decrease in ocular redness is measured. The novel techniques used in this experiment demonstrated the expected action of the decongestant further contributing to their application and validity.

6.2 Introduction

The circulation in the eye is regulated differently in various local tissues but the common goal is to provide these tissues, regardless of location, with a required amount of oxygen and nutrients essential for cell function.[1-4] A better understanding of normal vascular physiology of the eye and its changes during provocation or disease is required. By studying the effects of various agents on normal microcirculatory hemodynamics necessary insight into vascular reactivity may be provided.

The availability of research tools to study and quantify various hemodynamic parameters on the ocular surface is limited. The novel techniques presented in Chapter 3 and Chapter 4 of this thesis have the potential to provide the necessary means to explore vascular physiology and reactivity on the ocular surface.

There are only 2 easily accessible areas for non-invasive *in vivo* microcirculation research in human participants: the nail fold capillary bed and the conjunctival microcirculation.[5-8] Vascular reactivity can characterize a hemodynamic response of the vasculature and has been demonstrated in these areas by various stimuli such as hyperoxia[9-12], hypercapnia[12,13], the use of topical vasoactive agents[14,15] and hypoxia[16].

Topical decongestants are over the counter products that are sometimes used to initiate vasoconstriction of ocular surface vessels in order to relieve itching and redness due to pollen, ragweed, grass, animal hair, and dander.[17,18]

Decongestant activity has a direct α-adrenergic action or histamine H_1-blocking action on the superficial conjunctival vasculature.[18,19] Adrenergics will stop vasodilation and/or cause vasoconstriction of the vessels and mydriasis by interacting with the α_1-receptors on the blood vessels.[17,18] Drug interaction with the receptors will help offset vasodilation and other signs and symptoms of a mild inflammatory condition.[17,18] Some common decongestants include phenylephrine and imidazole compounds.

Imidazole compounds such as, naphazoline, tetrahydrozoline and oxymetazoline are sympathomimetic agents with α-adrenergic activity.[18] They can act as vasoconstrictors with a

rapid action in reducing swelling when applied to mucous membranes. They act on α-receptors in the arterioles of the conjunctiva to produce constriction, resulting in decreased congestion.[18,19]

The aim of this experiment was to examine the RBC velocity, oxygen saturation levels and ocular redness levels in clinically healthy subjects when a topical ophthalmic decongestant[14,15] was instilled onto the eye. By using the novel techniques to quantify the expected changes in the vasculature after the instillation of a topical vasoconstrictor the utility and validity of these techniques will be demonstrated. Correlations between variables were also tested before and after vasoconstrictor use.

6.3 Hypothesis

The hypothesis of this study is that after the instillation of a topical vasoconstrictor onto the ocular surface, a decrease in ocular redness and RBC velocity with no change in oxygen saturation levels would be demonstrated. A decrease in ocular redness would demonstrate the action of the topical vasoconstrictor and the lack of change in SO_2 during the action of the vasoconstrictor would demonstrate metabolic regulation.

6.4 Materials and Methods

6.4.1 Sample

Ethics clearance was obtained through the Office of Research Ethics at the University of Waterloo before commencement of the study. Eligible participants signed an informed consent document before enrolment in the study.

The eligibility of participants was determined at a screening appointment. The sample consisted of 7 healthy participants with an average age of 35 years (range 25-61 years). Subjects with any vascular disorders were excluded from the study.

6.4.2 Materials

The tetrahydrozoline topical ophthalmic decongestant used was Visine® Original and is available over the counter. Visine contains tetrahydrozoline 0.05%, a direct acting sympathomimetic or nonselective α-adrenergic agonist.

6.4.3 Redness Measurements

Please refer to chapter 5.4.2 of this thesis for redness measurement description.

6.4.4 Blood Oxygen Saturation Measurements

Please refer to chapter 4.3.3 of this thesis for blood oxygen saturation measurement description.

6.4.5 Red Blood Cell Velocity Measurements

Please refer to chapter 5.4.4 of this thesis for RBC velocity measurement description.

6.4.6 Procedures

The participants attended three separate visits during the 60 minute session. At each session, redness, dual wavelength images and red blood cell velocity videos were taken on the VOI. The variables were measured at baseline, immediately following drop instillation (Visine®) and 10 minutes later.

6.4.6.1 Redness Measurement Procedures

Please refer to chapter 5.4.5.1 of this thesis for redness measurement procedure description.

6.4.6.2 Blood Oxygen Saturation Measurement Procedures

Please refer to chapter 5.4.5.2 of this thesis for SO_2 measurement procedure description.

6.4.6.3 Red blood cell velocity measurement procedures

Please refer to chapter 5.4.5.3 of this thesis for RBC velocity measurement procedure description.

6.5 Results

6.5.1 Redness

The group mean redness (CIEu') increased slightly from 0.268 ± 0.004 to 0.271 ± 0.004 at drop instillation and significantly decreased to 0.265 ± 0.006 at the post measurement (Figure 6-1). These variations in redness over time were statistically significant (F(2,12)=6.18, p=0.01). The significance was between the drop and the post drop measures (Tukey HSD; p = 0.01).

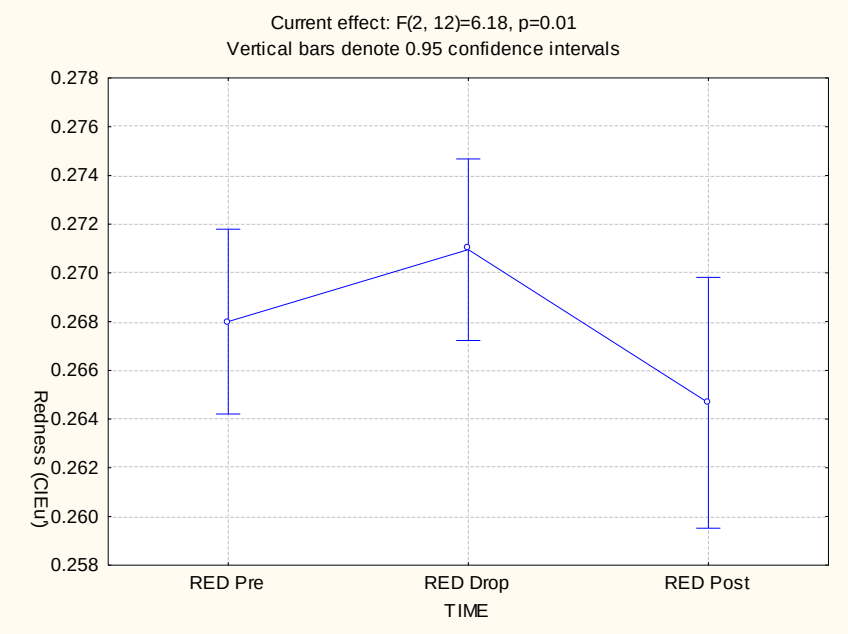

Figure 6-1: Redness over time

6.5.2 Oxygen Saturation Measurements

The group mean oxygen saturation was constant over time (Figure 6-2). SO_2 was 98.1 ± 2.6 %, 98.6 ± 1.6 % and 97.4 ± 2.6 % at each time point. These variations in SO_2 across visits were not statistically significant (F(2,12)=0.77, p>0.05).

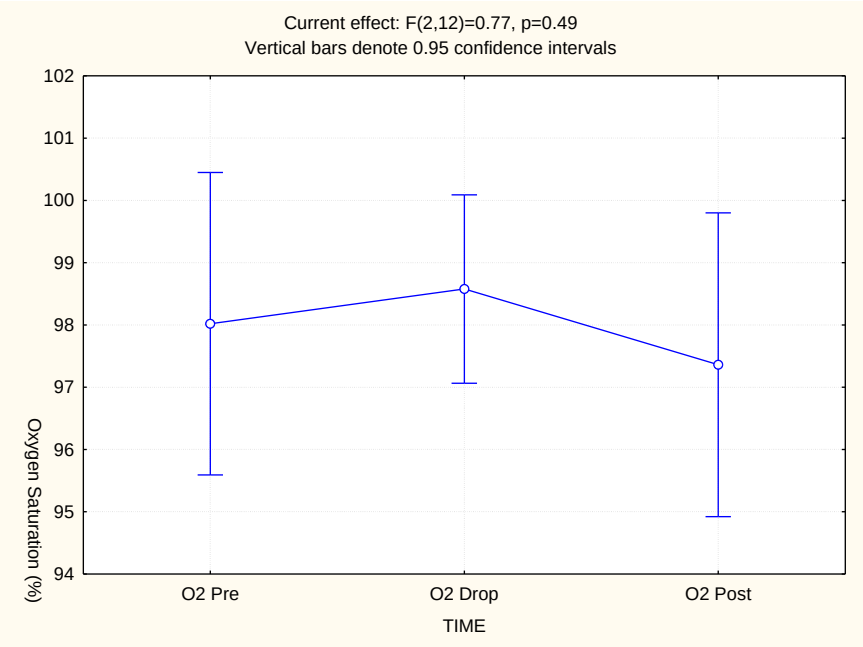

Figure 6-2: Blood oxygen saturation over time

6.5.3 Red blood cell velocity

Red blood cell velocity was 35.2 ± 46.9 µm/s, 28.8 ± 17.0 µm/s and 31.3 ± 28.1µm/s at each time point (Figure 6-3). These variations in RBC velocity across visits were not statistically significant ($F(2,12)=0.18$, $p>0.05$).

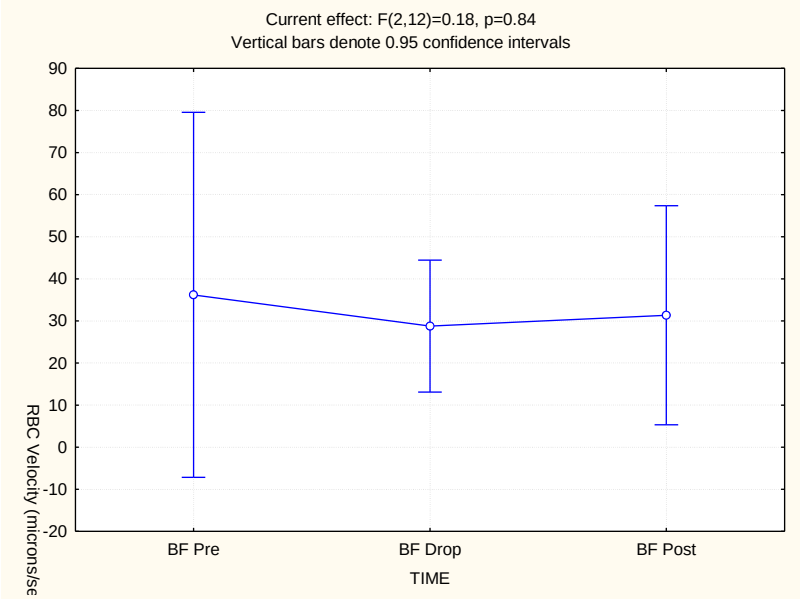

Figure 6-3: Red blood cell velocity over time

6.5.4 Correlations

A correlation matrix for all variables is displayed in Table 6-1. There was a significant correlation found between SO_2 and red blood cell velocity upon drop insertion ($r = 0.79$; $p<0.05$) and between redness pre and post drop insertion ($r=0.80$; $p<0.05$). The correlation[20] between redness and red blood cell velocity post drop was $r = 0.51$.

Partial correlations between two variables were completed on correlation values above an r of |0.50| since correlation values below this level are considered weak.[21,22] When redness was controlled at the "drop" time point r between SO_2 and red blood cell velocity remained the same ($r = 0.79$; $p<0.05$, Table 6-2). When SO_2 was controlled at the "post drop" time point r increased from 0.51 to 0.59 between redness and RBC velocity (Table 6-3).

Table 6-1: Correlation matrix between all variables at all time points

	Redness Pre	**Redness Drop**	**Redness Post**	**SO2 Pre**	**SO2 Drop**	**SO2 Post**	**RBC vel. Pre**	**RBC vel. Drop**	**RBC vel. Post**
Redness Pre	1.0	0.39	0.80	0.54	0.23	0.29	0.04	0.54	0.66
Redness Drop		1.0	0.26	0.37	-0.26	-0.21	-0.30	-0.13	0.13
Redness Post			1.0	0.15	0.10	-0.14	-0.20	0.40	0.51
SO2 Pre				1.0	0.32	0.68	-0.19	0.17	0.17
SO2 Drop					1.0	0.07	0.33	0.79	0.07
SO2 Post						1.0	0.18	0.07	0.3
RBC vel. Pre							1.0	0.65	0.61
RBC vel. Drop								1.0	0.61
RBC vel. Post									1.0

Table 6-2: Partial correlation matrix between SO2 and RBC velocity (redness controlled)

Controlled Variable: Redness (Drop)		
	SO2	**RBC Velocity**
SO2	1.0	**0.79**
RBC Velocity		1.0

Table 6-3: Partial correlation matrix between redness and RBC velocity (SO_2 controlled)

Controlled Variable: SO_2 (Post Drop)		
	Redness	**RBC Velocity**
Redness	1.0	**0.59**
RBC Velocity		1.0

6.6 Discussion

After applying a topical ophthalmic decongestant to the ocular surface a significant group mean reduction in redness post-insertion of the drop over time was demonstrated. The red blood cell velocity and SO_2 values did not change over time. No high[20] correlations between any variables occurred.

Given that the goal of the vasculature is to maintain a level of homeostatic regulation when a stimulus is applied to the system,[1,23,24] the lack of a significant change in SO_2 was not surprising. When the vascular system encounters a stimulus or is under duress, the vessels accommodate in such a way to aid in the maintenance of a stable oxygenation level.[25,26] The action of the vasoconstrictor was demonstrated with a significant reduction in redness over time. Despite the

reduction in redness (vasoconstriction), the oxygen saturation levels did not change demonstrating vascular regulation.[27]

It was hypothesized that after the instillation of the topical decongestant there would be a significant decrease in redness due to the constriction of the vessels and this was demonstrated. Decongestant activity has previously shown to have a direct α-adrenergic action on the conjunctival vasculature and causes vasoconstriction of the vessels by interacting with the α_1-receptors of the blood vessels.[18,19] The reduction in redness demonstrated the action of the decongestant.

It was hypothesized that a decrease in red blood cell velocity would be demonstrated given the vasoconstrictive action of the decongestant. By constricting the vessels, the vascular resistance increases.[28] With an increase in vascular resistance, a decrease in red blood cell velocity was hypothesized. It was demonstrated in this experiment that there was a significant decrease in redness and was likely as a result of the decrease in the calibre of the vessels. As mentioned in Chapter 5, the measurement area of redness in this study was different than the SO_2 and RBC velocity measure in that the former includes a total area of 19.6 mm^2 versus a single point along the vessel of interested (VOI). The area includes the VOI as well as deeper and additional vessels within that 19.6mm^2 area. The latter two measures were an estimation within the VOI itself. Although it was completely expected (and demonstrated) that redness would decrease "globally" with the use of the topical decongestant, it is possible that the amount the VOI itself decreased in diameter was not enough to increase resistance to a level where a significant decrease in RBC velocity would be measured. This is plausible since the current diameter of the vessel is already quite small and a further constriction may not be possible.

The correlation between SO_2 and RBC velocity just after drop instillation was significant ($r = 0.79$). This correlation is quite interesting since a function of the vasculature is to continually maintain specific levels of oxygenation via changes in vessel calibre and velocity/flow.[1,28-30] Therefore, it is expected that these two variables should be highly correlated. After removing the contribution of redness (partial correlation) on the correlation between SO_2 and RBC velocity just after drop instillation, the correlation value did not change. This suggests that redness does not contribute any variance to the correlation between SO_2 and RBC velocity. Again, the definitive effect of redness on the SO_2 and RBC velocity measures in the VOI is difficult to

discern since the redness measure does not only involve the VOI but between those two variables at that particular instant, no effect was evident.

The correlation between redness and RBC velocity post drop insertion was 0.51 ($p>0.05$). This suggests that when redness levels are low (brought on by the action of the vasoconstrictor) a correlation to RBC velocity is apparent. This notion seems somewhat plausible in that with a smaller calibre, the effect on RBC velocity would be greater because of an increase in vascular resistance. After removing the contribution of SO_2 on the original correlation between redness and RBC velocity, the r value increased to 0.59. An increase in the strength of the correlation suggests that SO_2 was masking some of the correlation which exists in its absence.

6.7 Conclusions

This study supports the literature regarding metabolic regulation of the microvasculature during the use of various stimuli. The results demonstrated that oxygen saturation levels remain stable even when a significant decrease in ocular redness is measured. The novel techniques used in this experiment demonstrated the expected action of the decongestant further contributing to their application and validity.

Many disciplines have investigated changes in blood flow and blood oxygen saturation levels while using various stimuli to assist in the pathological understanding of vascular reactivity. The bulbar conjunctiva provides an ideal, readily accessible, microvascular bed for *in vivo* research. In future, these techniques can possibly be used to noninvasively study vascular changes in the eye in both health and disease.

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7. Oxygen saturation, red blood cell velocity and bulbar conjunctival redness in participants who wear contact lenses

7.1 Overview

Purpose: Ocular redness is the principle clinical sign of an inflammatory response affecting the anterior segment of the eye. The aim of this experiment was to examine ocular redness, red blood cell velocity and oxygen saturation in participants who were habitual soft contact lens wearers. An ancillary question was to compare these outcomes in silicone (SH) and non-silicone hydrogel wearers. **Methods:** The sample consisted of 11 healthy participants (control group) who did not wear contact lenses with an average age of 34.5 years (range 24-57 years) and 11 healthy participants (study group; 6 SH and 5 hydrogel) who habitually wore contact lenses with an average age of 32.3 years (range 23-55 years). Participants were measured 8 times over the course of a day. Bulbar conjunctival redness, red blood cell velocity and blood oxygen saturation were measured. **Results:** When comparing the study and control groups, no significant difference in redness or SO_2 over time was found ($p > 0.05$). RBC velocity over time was found to be significantly different between groups ($p = 0.04$). A significant effect between the interaction of group and time on redness and RBC velocity was demonstrated ($p = 0.02$ and $p = 0.02$, respectively). When comparing the two study groups (SH vs. hydrogel) no significant difference across either measure over time was found ($p > 0.05$). A significant difference in the interaction between group and time on RBC velocity was found ($p = 0.05$). **Conclusions:** In summary, the participants in the study group were habitual contact lens wearers that had lower RBC velocities when compared to the control group supporting the notion that contact lenses initiate a hypoxic response. The lack of change in SO_2 in either group supports the theory of vasoregulation.

7.2 Introduction

Oxygen plays a vital role in supporting the metabolic activity that maintains corneal health. An adequate amount of available oxygen is one of the primary requirements for healthy eyes and

consequently, successful contact lens wear.[1] Various changes in the cornea associated with hypoxia have been observed by clinicians since the advent of contact lens wear.[1] Symptoms are reported to be complex and dynamic and have included redness, dryness and discomfort.[1] Long-term daily wear of contact lenses can cause ocular changes including corneal swelling, vascular changes, refractive changes and corneal epithelial changes.[2]

It has been reported and hypothesized that ocular inflammation associated with contact lens wear is associated with oxygen availability and produces key signs and symptoms that include redness, swelling, and pain.[1,3] Cytokines, growth factors, and matrix degrading enzymes play an important role in ocular inflammation.[4] Acute red eye is one form of an ocular inflammatory response that produces significant patient discomfort, pain, and photophobia with corneal hypoxia being at least a contributing factor and possibly further stimulating the response.[2,5-7] Prolonged hypoxia may result in infiltration and new vessel growth.[2] The ocular redness response to contact lens wear is therefore of considerable importance.

The development of new vessels into the cornea (corneal neovascularization) from the limbal vasculature is stimulated by hypoxia.[8,9] This vascularization as a result of contact lens wear was proposed to initiate inflammation and the release of various inflammatory cells.[10]

The surfaces of the contact lens interact with the palpebral conjunctiva, corneal surface and bulbar conjunctiva and thus, in addition to the metabolic effects of the contact lens on the corneal surface, there is also the potential for mechanical or toxic effects. Mechanically the lens may exert excessive negative pressure on the cornea[11] through altered surface properties or produce localized dehydration-type corneal staining.[12] Toxic adverse effects are produced through the unwanted accumulation of corneal epithelial by-products between the contact lens and cornea.[13]

The adverse effects described all have the potential to produce an inflammatory response of the anterior ocular segment ultimately causing vasodilation and the circulation of inflammatory mediators, initially leading to hyperemia.[14] It has been hypothesized that if the majority of the redness is limbal and localized, the contributing factor is of corneal origin and if the redness is diffuse the contributing factor is bulbar.[15]

Silicone hydrogel (SH) contact lenses transmit more oxygen to the eye than conventional hydrogel lenses,[16] significantly lowering the risk of hypoxia-related complications.[17,18] The

clinical performance of SH lenses is better compared to lenses with a lower oxygen permeability (low Dk) on a number of physiological markers.[17,19] Improvements in ocular health have been reported in all corneal layers as well as in the conjunctival response to lens wear.[20-23] Silicone hydrogel lenses should therefore sustain or improve the corneal health of contact lens patients and are being used progressively more as the preferred lens for new patients and for refitting existing patients.[24,25]

A few studies have compared SH lenses with low Dk disposable hydrogels and have demonstrated that SH lenses allow for healthier corneas which may provide greater resistance to inflammation or infection.[26-29] Therefore, the risk of corneal inflammatory rates should theoretically be reduced in those wearing SH lenses compared to traditional hydrogels.[27-29] Recent reports have acknowledged that although SH lenses have eliminated lens-induced hypoxia for the majority of wearers and ocular health benefits of silicone hydrogel lenses have increased the length of time these lenses can be worn overnight, the risk of infection is similar to that found with other soft lens types. [30-33] A consensus amongst practitioners is that a comprehensive understanding of how SH lenses interact with the corneal surface, upper eyelid and the tear film, and the risk factors contributing to infection and inflammatory responses is needed.[30-33]

By measuring red blood cell velocity, oxygen saturation and ocular surface redness in the conjunctival vessels of habitual contact lens wearers, we are able to study the changes that occur in the circulation and the levels of oxygen delivery and saturation.

This study is novel in that these three parameters have never been measured in contact lens wearers. By comparing these values to those of individuals who do not wear contact lenses, we will perhaps be able to elucidate the effect that a contact lens has on these ocular surface parameters.

7.3 Hypothesis

The hypothesis of this study was that the participants who wore contact lenses on a regular basis would have an increased red blood cell velocity, decreased oxygen saturation and an increased ocular surface redness when compared to the group of subjects that did not wear contact lenses.

7.4 Materials and Methods

7.4.1 Sample

Ethics clearance was obtained through the Office of Research Ethics at the University of Waterloo before commencement of the study. Eligible subjects signed an informed consent document before enrolment in the study.

The eligibility of participants was determined at a screening appointment. The sample consisted of 11 healthy participants (control group) who did not wear contact lenses with an average age of 34.5 years (range 24-57 years) and 11 healthy participants (study group) who habitually wore contact lenses with an average age of 32.3 years (range 23-55 years). Lens type and care regimen used by each study participant are shown in Table 7-1. Participants in the study group wore their contact lenses for on average 11.2 hours/day, 5.7 days/week (silicone hydrogel sub sample: 12.1 hours/day, 6.3 days/week; hydrogel sub sample: 10.2 hours/day, 5.0 days/week). Subjects with any vascular disorders were excluded from the study.

Table 7-1: Lens type and care regimen used by each study participant

Study Participant (Silicone Hydrogel Group)	**Lens Brand**	**Lens Care System**
1	Focus Night & Day	Opti-Free Express
2	Acuvue OASYS	Clearcare
3	Acuvue OASYS	Opti-Free Express
4	Acuvue OASYS	Clearcare
5	Acuvue OASYS	Opti-Free Express
6	Acuvue OASYS	Opti-Free Express
Study Participant (Hydrogel Group)	**Lens Brand**	**Lens Care System**
1	Proclear	Clearcare
2	Acuvue Moist Dailies	n/a
3	Acuvue 2	Clearcare
4	Biomedics Dailies	n/a
5	Acuvue Moist Dailies	n/a

7.4.2 Redness Measurements

Please refer to chapter 5.4.2 of this thesis for redness measurement description.

7.4.3 Blood Oxygen Saturation Measurements

Please refer to chapter 4.3.3 of this thesis for blood oxygen saturation measurement description.

7.4.4 Red Blood Cell Velocity Measurements

Please refer to chapter 5.4.4 of this thesis for RBC velocity measurement description.

7.4.5 Procedures

The participants' temporal conjunctiva was viewed with the slit lamp and a vessel was chosen as the "vessel of interest" (VOI) and was used for all subsequent measurements. 8 measurement sessions, 1 hour apart, began at 9:00am. The study group was instructed to wear their habitual contact lenses on the day of measurements.

7.4.5.1 Redness Measurement Procedures

Please refer to chapter 5.4.5.1 of this thesis for redness measurement procedure description.

7.4.5.2 Blood Oxygen Saturation Measurement Procedures

Please refer to chapter 5.4.5.2 of this thesis for SO_2 measurement procedure description.

7.4.5.3 Red blood cell velocity measurement procedures

Please refer to chapter 5.4.5.3 of this thesis for RBC velocity measurement procedure description.

7.4.5.4 Data Analysis

The control group and study group were assessed for differences across the 8 visits for all three measures using a mixed ANOVA (Statistica 7; Statsoft, Tulsa, OK).

7.5 Results

7.5.1 Control and Study Group Comparisons

7.5.1.1 Redness Measurements

The mean redness over time (mean ± SD) ranged from 0.270 ± 0.006 to 0.272 ± 0.006 for the control group and 0.271 ± 0.007 to 0.274 ± 0.010 for the study group. There was no effect of group on redness ($F(1,20)=0.67$, $p>0.05$; Figure 7-1). The interaction between group and time on redness was significant ($F(7,140)=2.52$, $p=0.02$; Figure 7-2). The differences were between visit 1 and visit 4, 6 and 7 of the control group (Tukey HSD; $p=0.05$, $p=0.05$ and $p=0.03$ respectively) and between visit 1 and 7; visit 2 and 7, 8; visit 3 and 5 and; visit 5 and 6, 7, 8 of the study group (Tukey HSD; $p=0.05$; $p=0.02$, $p=0.04$; $p=0.01$; $p=0.01$, $p=0.001$, $p=0.001$ respectively).

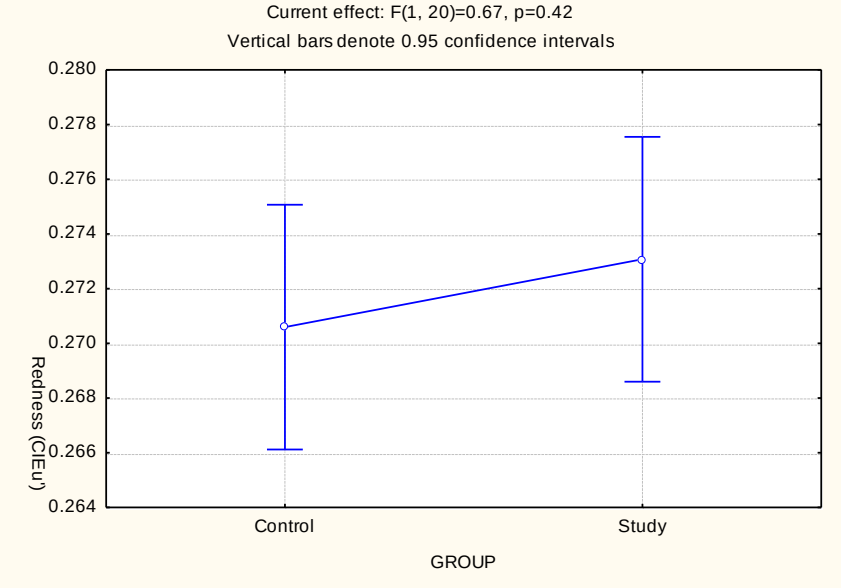

Figure 7-1: Redness sorted by group

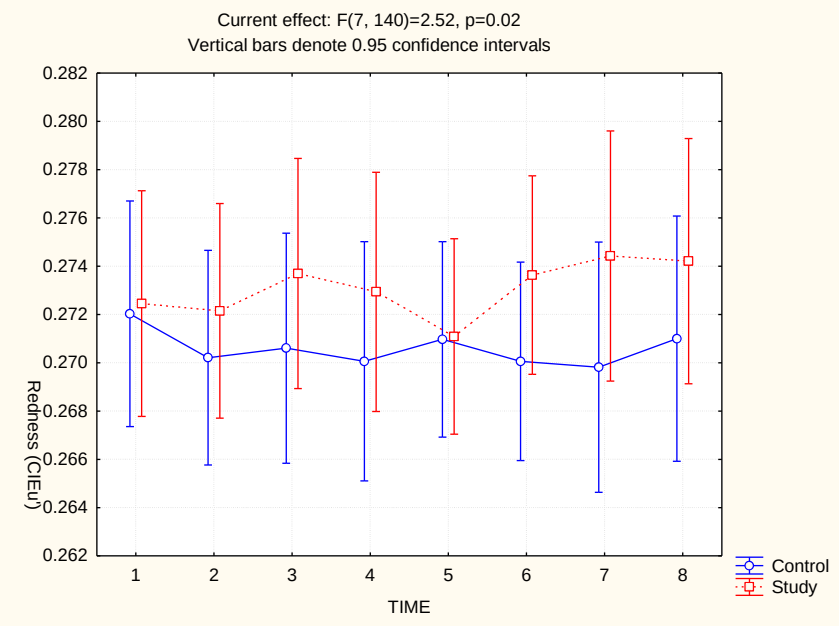

Figure 7-2: Interaction between group and time on redness

7.5.1.2 Oxygen Saturation Measurements

The mean oxygen saturation over time (mean ± SD) ranged from 98.0 ± 2.5 to 99.2 ± 2.2 for the control group and 98.5 ± 1.0 to 99.5 ± 1.6 for the study group. There was no effect of group on SO_2 ($F(1,20)=0.87$, $p>0.05$; Figure 7-3). The interaction between group and time on SO_2 was not significant ($F(1,140)=1.15$, $p>0.05$; Figure 7-4).

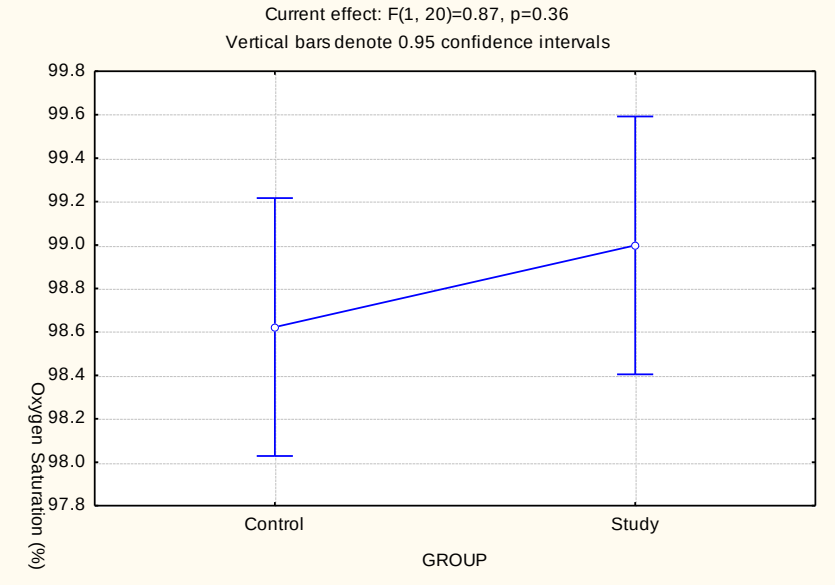

Figure 7-3: SO_2 sorted by group

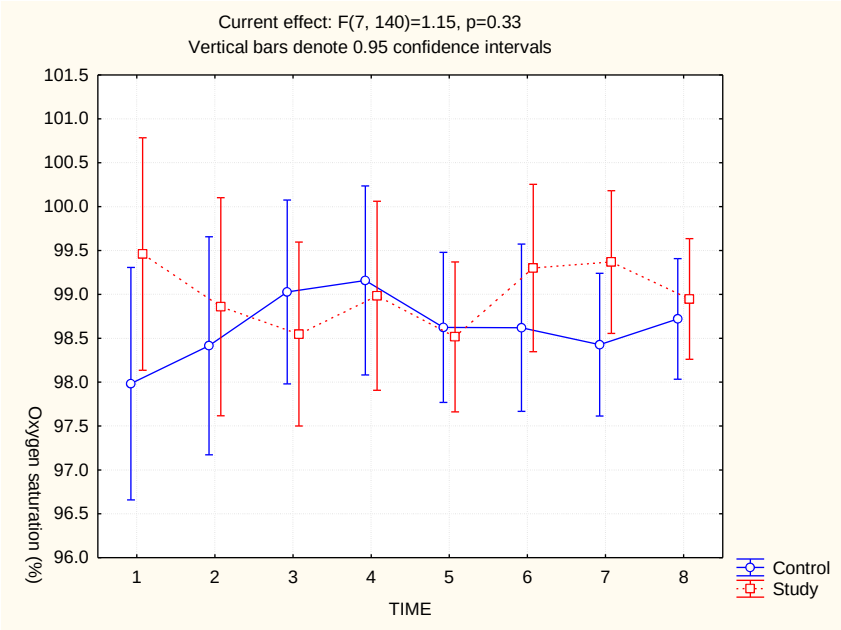

Figure 7-4: Interaction between group and time on SO_2

7.5.1.3 Red Blood Cell Velocity

The mean RBC velocity over time (mean ± SD) ranged from 16.4 ± 10.8 to 44.6 ± 14.9 for the control group and 10.2 ± 14.5 to 27.8 ± 23.1 for the study group. There was an effect of group on RBC velocity ($F(1,11)=5.77$, $p=0.04$; Figure 7-5). The interaction between group and time was significant ($F(7,77)=2.68$, $p=0.02$; Figure 7-6). The differences were between visit 8 and visit 1, 4 and 5 of the control group (Tukey HSD; $p=0.0002$, $p=0.03$ and $p=0.01$ respectively) and between visit 8 of the control group and visits 2, 3, 4, 5, and 7 of the study group (Tukey HSD; $p=0.01$, $p=0.01$; $p=0.02$, $p=0.02$ and $p=0.003$ respectively).

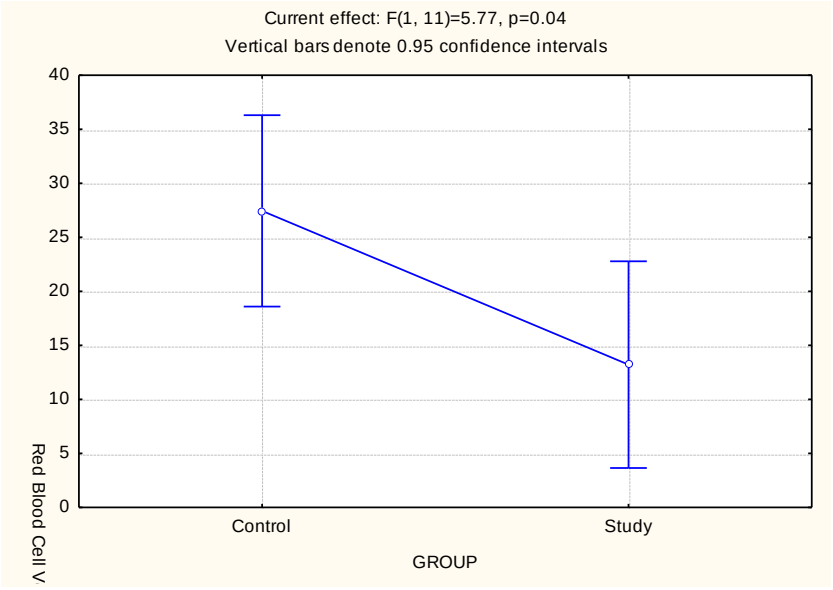

Figure 7-5: RBC velocity sorted by group

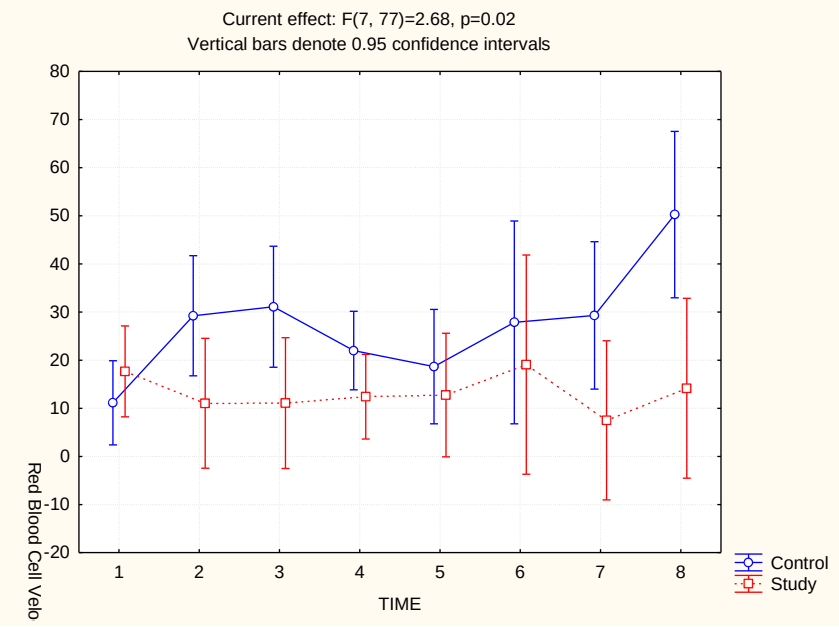

Figure 7-6: Interaction between group and time on RBC velocity

7.5.2 Study Group Comparisons sorted by lens type

7.5.2.1 Redness Measurements

The mean redness over time (mean ± SD) ranged from 0.272 ± 0.006 to 0.276 ± 0.008 for the SH group and 0.270 ± 0.009 to 0. 272 ± 0.001 for the hydrogel group. There was no effect of group ($F(1,9)=0.28$, $p>0.05$; Figure 7-7). The interaction between group and time on redness was not significant ($p > 0.05$; Figure 7-8).

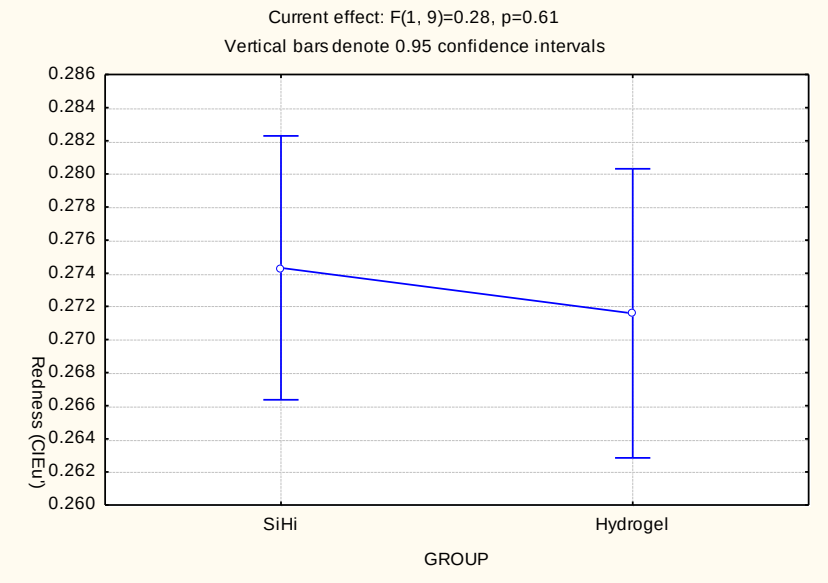

Figure 7-7: Redness sorted by group

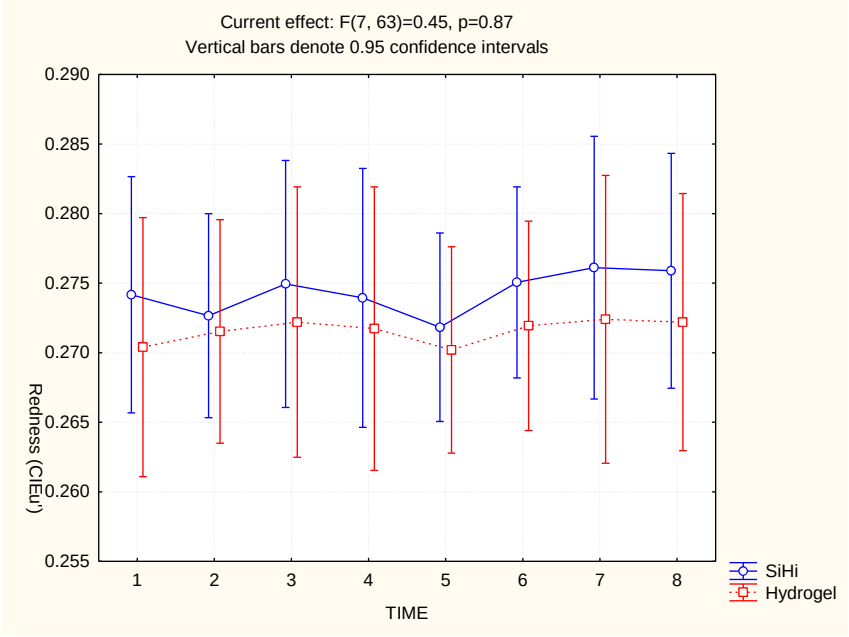

Figure 7-8: Interaction between group and time on redness

7.5.2.2 Oxygen Saturation Measurements

The mean oxygen saturation over time (mean ± SD) ranged from 98.6 ± 1.6 to 99.8 ± 1.6 for the SH group and 98.1 ± 2.0 to 99.9 ± 1.4 for the hydrogel group. There was no effect of group ($F(1,9)=0.47$, $p>0.05$; Figure 7-9). The interaction between group and time on redness was not significant ($F(7,63)=0.85$, $p>0.05$; Figure 7-10).

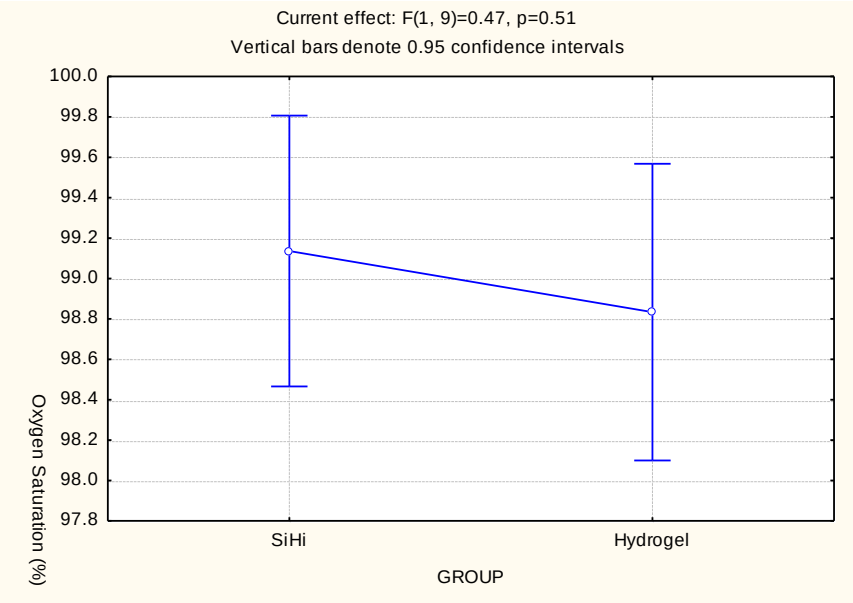

Figure 7-9: SO_2 sorted by group

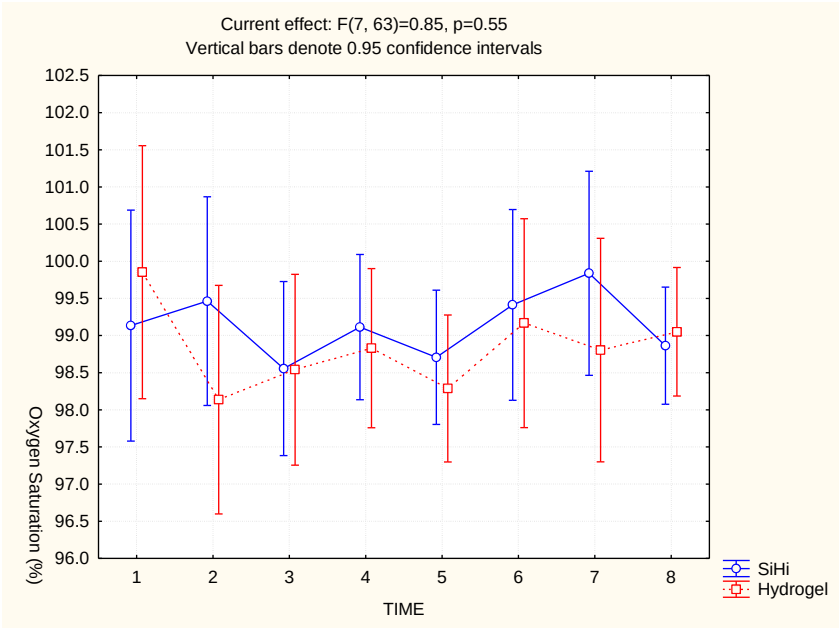

Figure 7-10: Interaction between group and time on SO_2

7.5.2.3 Red Blood Cell Velocity

The mean RBC velocity over time (mean ± SD) ranged from 9.3 ± 11.1 to 15.5 ± 17.5 for the SH group and 8.1 ± 6.4 to 40.2 ± 28.8 for the hydrogel group. There was no effect of group ($F(1,4)=0.11$, $p>0.05$; Figure 7-11). The interaction between group and time on RBC velocity was significant ($F(7,28)=2.33$, $p=0.05$; Figure 7-12). The significance was between the sixth and the seventh time points of the hydrogel group (Tukey HSD; $p=0.04$).

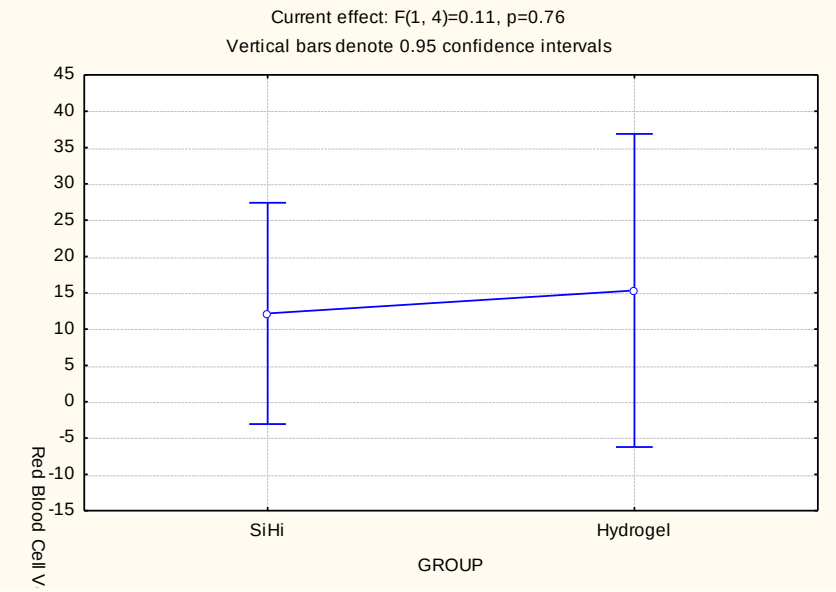

Figure 7-11: RBC velocity sorted by group

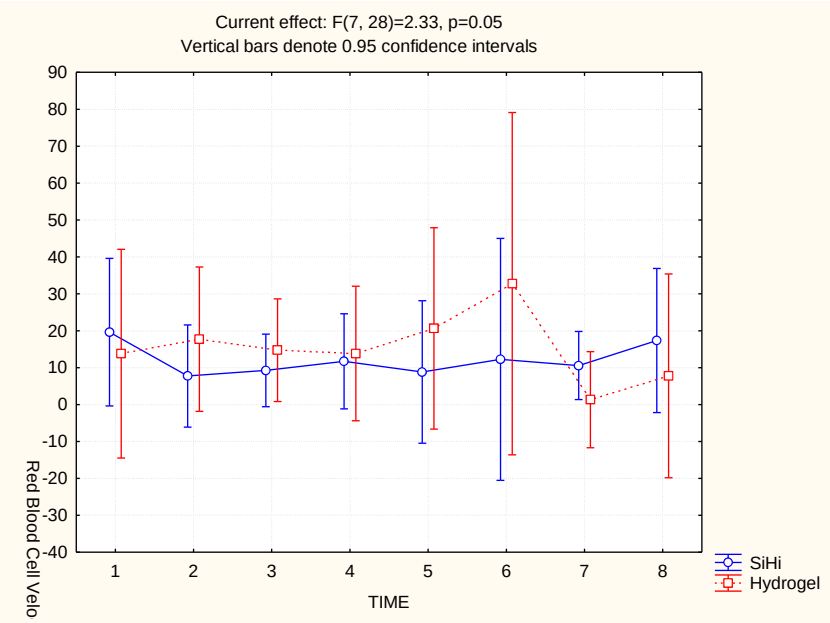

Figure 7-12: Interaction between group and time on RBC velocity

7.6 Discussion

The present study investigated conjunctival redness, RBC velocity and oxygen saturation in a group of participants who were not contact lens wearers and a group who were habitual contact lens wearers.

The measurements in this present study were of the bulbar conjunctival vessels. There is a lack of information regarding the effects of contact lens wear on the bulbar conjunctiva. However, it has been demonstrated that there is a strong association between the hypoxic effects of contact lens wear and limbal hyperemia.[34-38] Vessel dilation associated with contact lens wear has been attributed to a decrease in blood velocity and flow, potentially induced by changes in blood rheology and metabolic demands.[39] The chronic level of vessel dilation in soft lens wearers is cause for concern, as persistent dilation of the limbal vessels may be a precursor to new vessel growth.[40]

Normally, new blood vessel growth is tightly synchronized and usually limited to wound healing.[41] The process is initiated by the release of various growth factors and vasoactive molecules.[42] It is the balance of these naturally occurring stimulators and inhibitors that is thought to tightly control the normal metabolism of the vasculature.[43] When this balance is upset, as is possible in contact lens wear, endothelial cells of the blood vessels are stimulated to proliferate, migrate, and ultimately differentiate.

Red blood cell velocity in this experiment was significantly lower in the study group and on average, was 36.7% lower than the control group across time. It is sometimes difficult to interpret measurements of blood velocity/flow primarily because of the various types of instruments used for flow measurement and the differences in the circulation between specific ocular regions.[46] It could be plausible that the contact lens interfered with the availability of oxygen at the ocular surface and therefore the changes that occurred could have been as a result of the oxygen demands on the tissue. With the interference of available oxygen because of lens wear, the vessels would have to accommodate the RBC velocity in order to maintain adequate levels of oxygenation to the tissue. Contact lens wear has been demonstrated to be one of the initiators in corneal neovascularization.[21,27-29,36] It has also been documented that these new vessels can "empty" and vascularization can decrease when oxygen levels increase again.[32] The significant decrease in red blood cell velocity that was demonstrated could have occurred as a result of vessel filling (ghost vessels). With a greater number of vessels (i.e. additional ghost vessels) supplying the same blood volume, RBC velocity would be expected to decrease. Vascularization is associated with hypoxic stress, and vessels have been shown to "refill" when hypoxia is reapplied after having discontinued lens wear.[2]

Since a decrease in RBC velocity is attributed to vessel dilation in contact lens wearers[39] (potentially induced by changes in blood rheology and metabolic demands) and a significant decrease in RBC velocity in the study group was shown, an increase in redness was expected. However, no significant difference in redness between groups was demonstrated. Although a difference was not found statistically, if the trend evident in Figure 7-1 is correct and there is a difference that was not revealed because of the lower power to detect differences in the variables then a higher level of bulbar redness in the study group would be evident.

There were no significant differences in oxygen saturation between the control and study groups or in their interaction. A function of the vasculature is to maintain a constant level of regulation during ordinary instances and especially during the times when various stimuli are applied to the system, such as during contact lens wear.[42,44,45] It was demonstrated that even with a significant difference in RBC velocity between groups, SO_2 levels remained constant and thus supports vascular regulation. This regulatory mechanism is necessary to maintain structure and function of the tissue when a contact lens is in place.

It has been reported that when compared to traditional hydrogels, SH's provide sufficient oxygen, enabling healthier corneas to effectively decrease the effects of hypoxia and decreasing complications associated with acute and chronic oxygen deprivation.[21,48,49] Because of high oxygen transfer and in addition to high tear elimination rates that enhance the removal of potentially antigenic postlens debris, SH lenses are thought to provide a greater resistance to inflammation and infection.[26,50] However regardless of the minimal effects due to hypoxia, clinical and adverse complications continue to be reported. These events seem to be related to inflammation, infection, trauma, or mechanical disturbances.[48] There has also been a lack of information relating to effects imposed on the bulbar conjunctiva as a result of SH lens wear. The studies in the literature refer to increased or decreased redness in the region of the limbus. Since there are no studies in the literature that directly measure and compare bulbar redness oxygen saturation levels and RBC velocities in SH and hydrogel wearers it is difficult to form any conclusions.

There have been a few ocular response studies that have directly compared SH with low Dk disposable hydrogels.[27-29] With the exception of one of the studies, they all utilized SH lenses for up to 30 days continuous wear and low Dk lenses on a 7-day wear schedule and reported a higher rate of corneal inflammatory events in the SH group (although the differences compared to low Dk lenses were not statistically significant). A meta-analysis by Szczotka-Flynn et al[33] revealed that SH lenses typically worn for up to 30 days extended wear doubles the risk of corneal inflammatory events when compared with low Dk lenses when typically worn for 7 days of extended wear. It was concluded in that study that the increase in risk associated with corneal inflammatory events cannot be definitively linked to SH lens materials since the effect of material on the outcome is confounded by length of wear.[33] Although the participants in the SH

group of this present study reported wearing their lenses on average 1.9 hours/day and 1.3 days/week more than the hydrogel group, this most likely had no effect on the outcome of the measures. It has also been reported that long-term clinical performance of SH worn for 6- or 30-nights continuously was similar and that the clinical markers of hypoxia were low in both groups (regardless of wear schedule), with respect to the prevention of lens spoilage, the improvement of corneal physiology and the subjective symptoms of comfort and vision.[51]

A paper by Stapleton et al[32] re-iterated the points about ocular physiological improvements that are documented with the use of highly oxygen transmissible SH materials including relatively low severity levels of corneal inflammatory events. The authors go on to state that many of the infiltrates involved in the inflammatory events are "asymptomatic" and possibly caused by a normal ocular response to the environment. It should be noted that the results of this present study regarding redness, SO_2 and RBC velocity measurements are different to the studies in the literature[2,21,28,32,32,48,52] in that the measurements were taken on the bulbar conjunctiva, whereas the literature reports inflammatory events occurring in the limbal and corneal regions. Although decreases in limbal redness[53,54] and inflammatory response in the cornea[36] are reported, perhaps more attention should be focused on the bulbar area of the conjunctiva since this present study indicates trends for redder eyes, higher levels of circulating oxygen and lower RBC velocities, possibly suggesting an ocular response, whether inflammatory, mechanical or toxic.

Although differences were not found statistically, if the trends evident in Figures 7-7, 7-9 and 7-11 are correct, and there are differences that were not revealed because of the lower power to detect differences in the variables then, higher levels of bulbar redness, higher circulating levels of oxygen and lower RBC velocities in the SH group would be evident. These trends, if found could possibly indicate a greater level of inflammation[28,32-33] or increased mechanical effects[48] and an impaired ability to extract available oxygen compared to hydrogel wearers.

Again, if the trends are correct, the results of the present study in general (control vs. study) demonstrated that the study group had increased ocular surface redness and decreased RBC velocities when compared to the control group. Both groups did not demonstrate any significant changes in SO_2 over time. These findings at the very least suggest influences of hypoxia related changes in the study group and support the theory of regulation. The effects, beneficial or not, imposed on the rest of the vasculature and the bulbar conjunctiva by new materials remains to be

elucidated. Since adverse complications continue to be reported with the use of newer materials and may possibly be related to inflammation, infection, trauma, or mechanical disturbances, [48] furthering these findings in a much larger cohort of healthy individuals would prove beneficial.

7.7 Conclusions

In summary, the participants in the study group were habitual contact lens wearers that had lower RBC velocities when compared to the control group supporting the notion that contact lenses initiate a hypoxic response. The lack of change in SO_2 in either group supports the theory of metabolic regulation.

7.8 References

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8. General Discussion

Research related to ocular blood flow and hemodynamics represents a significant opportunity for us to make discoveries relating to the basic mechanisms involved in the healthy eye and more so in underlying diseases and disorders, whether ocular or systemic. The eye is a unique structure because it is one of the few locations in the body where we can non-invasively monitor blood flow in the microvasculature. The blood that flows through the arterioles of the bulbar conjunctiva represents a valid sample of the mechanical composition of the blood in all of the arterioles in the body and, in addition, the reactivity of the vessel walls are similar to the reactivity of the vessels present in the connective tissue. In healthy eyes, autoregulation keeps blood flow constant, only increasing or decreasing it in response to metabolic demands. However, a better understanding of the normal vascular physiology of the eye and its changes in disease is required. We do not yet know many of the mediators of blood flow in the various ocular beds, or what role they may play in disease. There is a lack of information surrounding the anterior surface and its hemodynamics in healthy people. This lack is in part due to the unavailability of real-time research tools and methodology to non-invasively quantify microvascular parameters.

The image analysis approach to the assessment of RBC dynamics enables us to visualize and quantify a number of important hemodynamic parameters simultaneously. Since the methods are based on the analysis of recorded video images, simultaneous results can be obtained from other capillaries in the same field of view. Ultimately we can have the means of evaluating oxygen supply and oxygen extraction in individual capillaries and capillary networks.

One of the major objectives of this work was to design a system that could track individual red blood cells as they travel through capillaries, thereby allowing analysis of hemodynamic and oxygenation parameters of RBC's over time and position. In Chapter 3, we established a high magnification camera system that was used to capture videos for the assessment of ocular capillary hemodynamics. Using signal displacement estimation through DTW algorithms, mean red blood cell velocity was estimated. In order to evaluate the performance of the algorithm, a simulation representing moving RBCs within a vessel was created. Successful application of the algorithm in the estimation of RBC velocity in conjunctival vessels was demonstrated. Signal

displacement estimation demonstrated very good concordance to the actual velocities supporting its validity.

Chapter 4 detailed the second application of this technique. Utilizing two specific narrow band interference filters with O_2-sensitive and O_2-insensitive peak transmissions during video capture allowed us the ability to calculate and assess haemoglobin oxygen saturation values. This technique had been used and demonstrated in the retina, but was novel to the anterior surface. Using an extension to this technique and applying it to the anterior surface, we were able to measure conjunctival vessel SO_2. The intraclass correlations between location measured and across consecutive frames on a single vessel for a series of subjects confirmed the reliability of the method. The application of the dual wavelength method was subsequently demonstrated with the use of a sequential re-breathing technique. Using this technique, we administered elevated levels of O_2 while controlling $P_{ET}CO_2$ and measured SO_2 with three different methods; dual wavelength method, ear-clip pulse oximetry and finger-clip pulse oximetry. A significant increase in oxygen saturation on initiation of hyperoxia with all three measurement methods was demonstrated with a subsequent return to initial values when hyperoxia was discontinued. The values from the dual wavelength method and the ear clip pulse oximeter were compared to assess the validity of the dual wavelength method. When the two methods were compared, no significant difference was found. This study was novel in that it utilized an isocapnic hyperoxic stimulus to provoke conjunctival vascular reactivity while controlling $P_{ET}CO_2$. By controlling the compounded vasoconstrictive effects when a reduction in systemic PCO_2 occurs, the utility of the dual wavelength method was demonstrated with significant changes between effect and recovery. The ability to determine changes in SO_2 in normal eyes has the potential of an application in disease and before and after interventions to aid in the understanding of the effects of metabolism or blood flow changes on vascular function. These measurements were proven to be straight forward to obtain in vessels of small diameter.

Ch.	Outcome Measure	N (avg. age)	Visits (f)	Between Group (Hyp./Rslt/Trend) ; p-value			Over Time (Hyp./Rslt)			Interxn b/w grp & time on OM (Hyp./Rslt)		
5	Redness	14 (34.7) (18-30: 25.1; **31-62: 45.6**)	8 (hour)	↑	↔	↑		↔			↔	
	SO_2			↓	↔	↑		↔			↔	
	RBC v			↓	↔	↓		0.01			↔	
6	Redness	7 (35.0)	3 (prior to insertion, just after, **10 min. after**)	↓	0.01	↓						
	SO_2			↔	↔	↔						
	RBC v			↓	↔	↔						
7	Redness	11 control (34.5): 11 **c/l** (32.3)	8 (hour)	↑	↔	↑					0.02	
	SO_2			↓	↔	↑					↔	
	RBC v			↓	0.04	↓					0.02	
7	Redness	6 SH/ **5 Hydrogel**	8 (hour)		↔	↓					↔	
	SO_2				↔	↓					↔	
	RBC v				↔	↑					↔	

Table 8-1: Summary of results from chapters 5-7

A summary of the following results are shown in Table 8-1. The results in the "between group" column display (if applicable) the hypothesis, statistical result and trend relative to the **group** (in bold and underline font) in the "N" column. Having developed the two methods that accurately assessed red blood cell velocity and oxygen saturation, the purpose of Chapter 5 was to characterize the change in conjunctival red blood cell velocity, oxygen saturation and bulbar conjunctival redness in a group of clinically normal subjects. An ancillary question was to examine these measures in an older age stratum as well as a younger age stratum and to compare differences between the groups. The results of this study support the theory of regulation. The lack of any significant change across time for redness and oxygen saturation along with significant changes in red blood cell velocity substantiates this notion. We were not able to show

significant differences between age strata and this was most likely due to the small size of our cohorts used. If the trends between the age strata are real, those in the older stratum were shown to have redder eyes with lower RBC velocities. It is known that aging, disease, or a prolonged vascular stress alters the normal vessel anatomy or reactivity in various ways and these results indicate a loss of vascular reactivity in older patients. Furthering these findings in a much larger cohort of healthy individuals would prove beneficial.

Chapter 6 of this thesis demonstrated the validity of the two measurement techniques after the instillation of a topical vasoconstrictor onto the ocular surface of clinically healthy individuals. This study also supports the literature regarding regulation of the microvasculature during the use of various stimuli. The results demonstrated that oxygen saturation levels remain stable even when a significant decrease in ocular redness is measured. The novel techniques used in this experiment demonstrated the expected action of the decongestant further contributing to their application and validity.

Chapter 7 evaluated the effect of conventional contact lens wear on red blood cell velocity, oxygen saturation and ocular surface redness in subjects with a history of contact lens wear compared to subjects that did not have a history of contact lens wear. Although no significant difference for redness between groups was found, there was a trend showing that the study group had consistently redder eyes than the control group across measures. This finding, if real, was consistent with previous studies in the literature. RBC velocity was found to be significantly different between groups with the study group demonstrating lower velocities across all measures suggesting the occurrence of vessel dilation, growth and/or vessel filling (ghost vessels). The participants in the study group were habitual contact lens wearers that had lower RBC velocities when compared to the control group supporting the notion that contact lenses initiate a hypoxic response. The lack of change in SO_2 in either group supports the theory of vasoregulation.

Chapter	Measure	n
5 (18-30 vs. 31-62)	Redness	434
	SO_2	158
	RBC velocity	2598
6 (Visine)	Redness	-
	SO_2	128
	RBC velocity	351
7 (Non-C/L vs. C/L)	Redness	352
	SO_2	872
	RBC velocity	-
7 (SiHi vs. Hydrogel)	Redness	1860
	SO_2	468
	RBC velocity	1090

Table 8-2: Sample power calculations

As alluded to in previous experimental chapters and raised by members of my examining committee, a summary of power sample calculations for non-significant factors are shown in Table 8-2. A post-hoc power analysis (G*Power 3.0.10; Universitat Kiel, Germany) was computed using paired t-test comparisons (α-level of 0.05). If the respective comparisons (as per each study) are statistically different, Table 8-2 shows that there is a requirement for either a very large n or, they really aren't real effects.

8.1 Future Work

Future work should investigate the vascular reactivity response of healthy patients in substantially larger groups. Further to the confirmation of significant findings relating to vascular regulation, studies involving patients with vascular disorders or diseases should be investigated. Under normal circumstances, the blood oxygen saturation remains fairly stable because the vessels regulate regional blood flow to accommodate changes in perfusion and arterial oxygenation (acute changes). During cases of vascular dysregulation (chronic changes), regulation does not occur according to the needs of the tissue. Dysregulation occurs when there is an underlying disease present or even as a result of an inborn tendency to respond differently to a variety of stimuli (e.g. changes in temperature). The occurrence of a disturbed autoregulation will lead to an unstable blood flow and since several systems are involved in regulation, the clear dysfunction of one of the systems would lead to compensation by another. Investigation of vascular reactivity in patients with vascular anomalies (e.g. diabetes) or with the use of various stimuli (e.g. different contact lens materials) may also provide more functional information about the pathophysiology of the microvasculature on the anterior ocular surface.