

# **EVALUATION OF AN OPHTHALMIC VISCOSURGICAL DEVICE UTILIZING A GLYCOSAMINOGLYCAN**

**By**

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## **ABSTRACT**

# **EVALUATION OF AN OPHTHALMIC VISCOSURGICAL DEVICE UTILIZING A GLYCOSAMINOGLYCAN**

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### **Background**

An ophthalmic viscosurgical device (OVD) is a clear gel like chemical substance that has viscous and elastic properties and is biocompatible. It is used in ophthalmic surgery to create and maintain space, to protect the delicate corneal endothelial cells as well as to assist the surgeon in manipulating the ocular tissues during the course of the surgery. OVD's are made from various sources which include hyaluronic acid, chondroitin sulphate and hydroxypropylmethylcellulose. Hyaluronic acid OVD's are the most popular and its chemical composition lends it to have several favorable characteristics making it very successful as an OVD. These include being a non-Newtonian fluid enabling the change of its viscosity with various pressures as required while being injected through a thin cannula in the eye as well as being stationary in the anterior chamber of the eye. Its elastic properties ensure the protection of the corneal endothelial cells by absorbing the mechanical trauma created by the surgical instruments during the operation. It can either be a cohesive device, used for space creation and maintenance, or a dispersive device, used to protect the corneal endothelial cells, depending on the molecular weight of the OVD. A transient rise in the intraocular pressure (IOP) is expected with the use of an OVD but should return to normal within 24 hours after surgery. A cohesive OVD was developed and the scope of this thesis included the various preclinical tests in order to ensure its safety for human use.

## **Aims**

The aim of this study was to test and evaluate a buffered hyaluronic acid (BHA) in order to determine its effect on *in vivo* and *in vitro* systems as part of preclinical testing as required by ISO 15798 for OVD's. Firstly the cytotoxic potential of the BHA was determined by exposing human dermal fibroblasts to different concentrations of the test substance. Next, the sensitization potential was determined by the Guinea pig Maximization test followed by the determination of the IOP pattern that can be expected postoperatively in comparison to a currently existing OVD on the market. Lastly, an eye irritation test was conducted on New Zealand white rabbits in order to determine the eye irritation potential of the OVD.

## **Materials and Methods**

Human dermal fibroblasts were used in order to determine the cytotoxic potential of the BHA at various concentrations and cells were subjected to the test substance for 1 and 24 hours. The colorimetric MTT and Neutral red assays were performed as well as the qualitative Crystal violet assay. For the skin sensitization test intradermal injections of the test substance were made into the dorsal skin region of thirty Hartley Guinea pigs. The animals were left for 14 days after which the skin was challenged with the test substance again. The IOP pattern was determined by the injection of the test and control substance into the right and left eyes of six New Zealand white rabbits, and the IOP was then evaluated at different time intervals. For the eye irritation test, 0.1ml of the test substance was instilled into the lower conjunctival sac of six New Zealand white rabbits and was allowed to spread over the surface of the eye. The eyes were then evaluated with a slit lamp and a handheld ophthalmoscope.

## **Results and Conclusion**

The test substance was seen to promote cell proliferation rather than cell damage. The overall test results indicated that the test substance was not cytotoxic. The test substance produced a 5.3% incidence in the skin sensitization test and is therefore not regarded as a skin sensitizer. A similar intraocular pressure pattern was seen in both the test and control OVD when injected into the eyes of white New Zealand rabbits and the peak IOP rise was found to be at 4 hours  $\pm$  1 hour for both substances. No positive scores were seen in the eye irritation test and it was concluded that the test substance is not an eye irritant.

## Declaration

I, Marilize van der Walt, hereby declare that this research dissertation is my own work and has not been presented for any degree at another University;

Signed: .....

Date: .....

Department of Anatomy, School of Medicine, Faculty of Health Sciences,  
University of Pretoria

South Africa

## LIST OF ABBREVIATIONS

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|           |                                                                                |
|-----------|--------------------------------------------------------------------------------|
| AH        | Aqueous humor                                                                  |
| AUCC      | Animal use and care committee                                                  |
| BCOP      | Bovine Corneal Opacity and Permeability assay                                  |
| BHA       | Buffered hyaluronic acid                                                       |
| CEC       | Corneal endothelial cells                                                      |
| CS        | Chondroitin sulphate                                                           |
| CV        | Crystal Violet                                                                 |
| FCA       | Freund's complete adjuvant                                                     |
| HA        | Hyaluronic acid                                                                |
| HCE-T TEP | HCE-T transepithelial permeability assay,                                      |
| HET-CAM   | Hen's egg Test-Chorioallantoic Membrane assay                                  |
| HPMC      | Hydroxypropylmethylcellulose                                                   |
| ICCVAM    | Interagency Coordinating Committee on the Validation of<br>Alternative Methods |
| ICE       | Isolated chicken eye assay                                                     |
| IOP       | Intraocular pressure                                                           |
| ISO       | International standards organization                                           |
| MDCK FL   | MDCK Fluorescein leakage assay                                                 |
| MTT       | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide                   |
| NR        | Neutral red                                                                    |
| OECD      | Organization for Economic Cooperation and Development                          |

|     |                                 |
|-----|---------------------------------|
| OVD | Ophthalmic viscosurgical device |
| TM  | Trabecular meshwork             |

## **CHAPTER 1**

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# CHAPTER 1

## INTRODUCTION

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An Ophthalmic viscosurgical device (OVD) is a clear, gel-like chemical substance with viscous and elastic properties that is biocompatible. Ophthalmic viscosurgical devices are well known surgical aids used in ophthalmic surgery in order to create and maintain space, to protect the corneal endothelial cells and to assist with the manipulation of tissues during surgery. They differ with regard to their composition, characteristics and rheological profiles.

OVD's are widely used in cataract surgery throughout the world. The OVD is injected into the anterior chamber of the eye after the corneal incision was made and then serves to protect the corneal endothelial cells while the ultrasonic vibrations of the phaco tip are breaking up the cataractous lens of the eye. After the lens has been emulsified, the viscosurgical device as well as the cataractous lens is aspirated from the eye, fresh OVD is injected into the anterior chamber and the artificial lens is put into place. The remaining OVD is then again aspirated from the eye at the end of surgery in order to prevent a rise in the IOP.

The rheological profile of the OVD is determined by its molecular weight, concentration, viscosity and elasticity. These characteristics will determine whether an OVD is a cohesive or a dispersive, that is, whether it will be used to create and maintain space or for the protection of the corneal endothelial cells. The scope of surgeries performed using OVD's has, since the introduction of the first OVD in 1979, been expanded to a larger variety as well as to different types of surgeries. Depending on the condition of the structures of the eye, different characteristics in OVD's are required. For instance in compromised corneas, surgeons are advised to use a dispersive OVD for its maximum corneal protection whereas in the case of floppy iris syndrome, a cohesive OVD is recommended for its space creating and maintaining ability (Goldberg, 2007).

An ISO standard has been set to which all OVD's must adhere to and it serves as a tool whereby OVD's can be compared and tested. The standard specifies the values for the different characteristics of OVD's, such as pH, viscosity, elasticity and molecular mass distribution to

name only a few, it also stipulates the different preclinical tests an OVD must undergo in order to ensure the safety of the OVD before clinical testing on humans can commence.

Before any new medical product can be released into the field of medicine it must be tested thoroughly in order to determine its effect *in vitro* as well as *in vivo* in order to ensure the safety of the end user, the patient. The scope of this thesis was to determine the effects of a potential OVD on test cultures as well as animal models as is required by the ISO 15798, specifically for OVD's.

## **HYPOTHESIS OF THE STUDY**

Hyaluronic acid is a substance found naturally in the body in structures such as the eyes and skin. The hyaluronic acid OVD being evaluated is not cytotoxic, is not a sensitizing agent or irritant and will not produce adverse IOP during its installation into the eye.

## **STUDY OBJECTIVES AND AIMS**

The OVD consists of a buffered hyaluronic acid and was designed as a cohesive OVD for the purpose of creating and maintaining space in the eye. The following aims were met as part of the preclinical evaluations done in order to determine its safety on *in vivo* and *in vitro* systems:

1. To give an accurate overview of the history, different types of OVD's, their properties and sources, and complications associated with OVD surgery pertaining to intraocular pressure
2. To test the cytotoxicity potential of the buffered hyaluronic acid OVD on dermal fibroblasts
3. To determine the sensitization potential of the OVD by the guinea pig maximization test for delayed hypersensitivity
4. To compare the postoperative intraocular pressure pattern of the buffered hyaluronic acid with an existing OVD on the market using New Zealand white rabbits
5. To use the Draize eye test in order to determine the eye irritation potential of the OVD in New Zealand white rabbits

## CHAPTER 2

### LITERATURE REVIEW

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#### INTRODUCTION

Cataract surgery is one of the most common eye surgeries performed in the world today. A cataract is a partial or complete clouding of the lens of the eye that gives rise to a blurry or distorted vision and is especially seen in the elderly (Maltese *et al.*, 2006). Statistics in 2011 shows that 33% of all global visual impairment is caused by cataracts, that is 33% out of a population of 285 million people (World health Organization, <http://www.who.int/mediacentre/factsheets/fs282/en/>). Cataracts are caused by intrinsic factors such as aging of the crystalline lens of the eye and congenital diseases and extrinsic factors such as smoking, eye trauma, diabetes, the use of corticosteroids, malnutrition, exposure to ultraviolet sunlight, several diseases and various medications (Allen *et al.*, 2006; Wong *et al.* 2002). Cataracts are removed by a technique called phacoemulsification during which the cataractous lens is fragmented and emulsified utilizing high intensity ultrasound energy and is then aspirated from the eye and replaced with an artificial intraocular lens (Maltese *et al.*, 2006). During this procedure, all the structures surrounding the anterior chamber of the eye are at risk of being damaged, especially the delicate corneal endothelial cells responsible for the health and maintenance of the cornea (Gomes *et al.*, 2004). To protect the cells of the eye during surgery an ophthalmic viscosurgical device (OVD) is injected into the anterior chamber of the eye that then serves as a barrier or “cushion” to absorb mechanical insult by the surgical instruments (Arshinoff, 2004; Maltese *et al.*, 2006). An OVD is a clear, gel-like chemical substance with viscous and elastic properties; it’s biocompatible and is used to create and maintain space, protect the delicate corneal endothelial cells and serves as an aid to assist the surgeon in separating tissues while maneuvering the surgical instruments in the eye (Goldman *et al.*, 2007). It also creates even pressures in the anterior and posterior chambers that greatly facilitate the surgical procedure (Arshinoff *et al.*, 2005).

Before 1979 when the first ophthalmic viscosurgical device was introduced, the severe irreparable damage to the corneal endothelial cells during surgeries performed in the anterior chamber of the eye was considered as just part of the procedure. Mediums such as air, saline

and autologous serum were used with very little success (O'hEineachain, <http://www.eurotimes.org/07feb/pdf/UnderstandingOVDs.pdf>). With the introduction of the first viscosurgical device by Balazs *et al.* however, the endothelial cell loss during surgery was shown to be reduced to only a third of the total loss as when compared to not using an OVD (Arshinoff, 2004). OVD's are now considered fundamental instruments during ophthalmic surgery (Arshinoff, 2004).

## HISTORY OF OVD'S

In 1934 Karl Meyer and John Palmer isolated a substance from cow's eye which they later termed "hyaluronic acid" because it was isolated from the hyaloid of the eye and it contained uronic acid (O'hEineachain, <http://www.eurotimes.org/07feb/pdf/UnderstandingOVDs.pdf>). That same year a Hungarian polymer chemist, Dr Endre Balazs isolated hyaluronic acid from umbilical cord and rooster combs (Scholtz, 2007). In 1958 he was the first to suggest that hyaluronic acid should be used in ophthalmic surgery and in 1977 Miller and Stegman did their first experimental intraocular lens implants on rabbits. In the same year, the first 1% methylcellulose (inexpensive alternative to hyaluronic acid as an OVD) OVD was tested by Fechner (Scholtz, 2007).

In 1979 Balazs presented his new patent concerning the first purified high molecular weight hyaluronic acid OVD at a symposium on ocular surgery and coined surgery involving viscosurgical devices "viscosurgery" (Scholtz, 2007). He then sold his new patent to Pharmacia (now Pfizer) and two Swedish rheologists, Ove Wik and Hege Bothner Wik, at Pharmacia then developed the first OVD called Healon (O'hEineachain, <http://www.eurotimes.org/07feb/pdf/UnderstandingOVDs.pdf>). The first cataract surgeries were performed that year by Drs Robert Stegmann and David Miller after which the product was finally launched in 1980. The product gained FDA approval in January 1983 and the development and initiation of Healon paved the way for numerous OVD's to hit the market after its initial success for its use in cataract surgeries. Hereafter the number of uses of an OVD in ophthalmic surgery expanded beyond cataract surgery only (Scholtz, 2007).

Following in Healon's footsteps, OVD's consisting of other polymers such as chondroitin sulphate, hydroxypropylmethylcellulose and combination OVD's consisting of chondroitin sulphate and hyaluronic acid soon made their appearance into the ophthalmic markets

(O'hEineachain, <http://www.eurotimes.org/07feb/pdf/UnderstandingOVDs.pdf>). Each of these, now more than 80 OVD's, had a different composition, different characteristics and "feel". It therefore became important to set a standard to which the different OVD's could be contrasted and compared especially as different OVD's are used for different types of surgeries. The standardization of OVD's came about as an ISO standard set out by Dr John Alpar and Dr John Emes wherein they stipulated rules and regulations for the safety, performance, requirements and standardization of the different OVD's. Their main goals with the standard was to define "requirements for minimum safety standards, and to mandate disclosure or rheologic performance characteristics in a standard fashion" and to include features such as the "accurate chemical description, required preclinical and clinical evaluations, sterilization, product packaging, product labeling and the information required to be supplied by the manufacturer." (O'hEineachain, <http://www.eurotimes.org/07feb/pdf/UnderstandingOVDs.pdf>). These standards now enabled all surgeons to compare the different OVD's based on performance and specific characteristics so that they can make an informed decision on choosing the best OVD for the specific surgery that they need to perform.

## **SOURCES OF OVD'S**

Three main compositions for OVD's include the natural polymers, Sodium hyaluronate (hyaluronic acid) and chondroitin sulphate and a semi-synthetic polymer, Hydroxypropylmethylcellulose (Arshinoff, 2004; Maltese *et al.*, 2006).

### **Hyaluronic acid**

Hyaluronic acid (HA) a natural glycosaminoglycan that consists of alternating  $\beta$ 1-3 glucuronicidic and  $\beta$ 1-4 glucosaminidic bonds (Chong *et al.*, 2005). Figure 1 shows the structure of the repeating disaccharide unit.

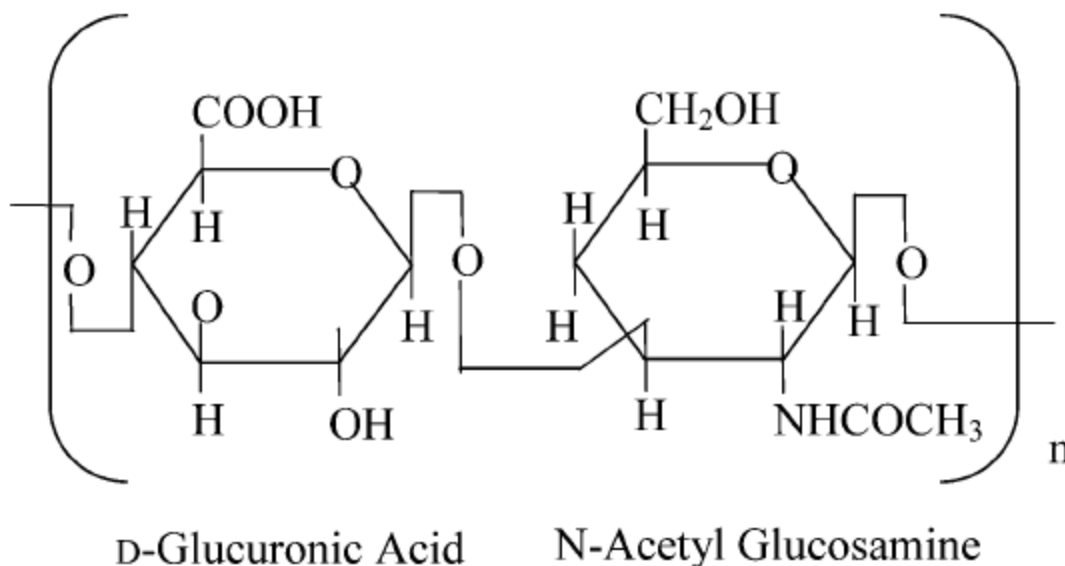


**Figure 1.** Repeating unit of hyaluronic acid comprising of D-glucuronic acid and N-acetylglucosamine joined alternately by beta 1–3 and beta 1–4 glycosidic bonds (Chong *et al.*, 2005).

The hydrogen and glycosidic bonds between the HA molecules provides for its “rigidness” and entangled structure that forms networks while in solution (Chong *et al.*, 2005). These entangled networks display Non-Newtonian behavior, discussed later, at concentrations as low as less than 1 mg per liter (Armstrong *et al.*, 1997b; Chong *et al.*, 2005). The behavior and performance of the OVD during surgery is based on the molecular weight, concentration and intermolecular connections with the solvent. (Metabolic engineering of hyaluronic acid production, <http://web.aibn.uq.edu.au/cssb/Projects/HA.html>).

Of the glycosaminoglycan family, which also includes chondroitin sulphate, heparin and dermatin sulphate, hyaluronic acid is the only polymer that forms non-covalent bonds with surface proteins (Chong *et al.*, 2005; Metabolic engineering of hyaluronic acid production, <http://web.aibn.uq.edu.au/cssb/Projects/HA.html>). This mucoadhesive property as well as HA’s hygroscopic and osmotic effects can be attributed to its negative charge (Chong *et al.*, 2005; Maltese *et al.*, 2006). Its hygroscopic characteristic also lends HA to bind water molecules to form a gel that results in its large hydrodynamic volume that accounts for its functions in the body (Armstrong *et al.*, 1997b; Price *et al.*, 2007; Wysenbeek *et al.*, 1988). The higher the

molecular weight of the HA the higher the water binding capacity will be (Metabolic engineering of hyaluronic acid production, <http://web.aibn.uq.edu.au/cssb/Projects/HA.html>).

HA is the primary substance found in all soft tissue extracellular matrixes of the body like the eye, synovial fluid, the umbilical cord and the intracellular space of the epidermis (Becker *et al.*, 2009; Chong *et al.*, 2005; Gura *et al.*, 1997; Maltese *et al.*, 2006). In the eye the highest concentration of HA is found in the vitreous humor and the trabecular angle. The endothelial cells on the posterior surface of the cornea are covered with a thin layer of HA on their surface and contains CD44 (a transmembrane cell surface adhesion molecule) and the receptor for HA-mediated motility (RHAMM) (Chong *et al.*, 2005; Madsen *et al.*, 1989; Maltese *et al.*, 2006; Price *et al.*, 2006). The binding of HA to these receptors on the endothelial cell surface permits the protection of these cells when the OVD binds to them during ophthalmic procedures.

HA can either be extracted from rooster combs or can be synthesized by bacterial fermentation. The latter is the more preferred method as bacterial fermentation has the advantages of less protein impurities that needs to be removed as well as no animal rights issues are raised with the use of bacteria in sourcing HA (Chong *et al.*, 2005). Another advantage of bacterial fermentation is that the molecular weight of the HA can be controlled and monitored while in the protein purification process of animal HA, the molecular weight is dramatically reduced (Becker *et al.*, 2009; Metabolic engineering of hyaluronic acid production, <http://web.aibn.uq.edu.au/cssb/Projects/HA.html>).

HA is produced by Lancefield group A and C streptococci in their extracellular shell which serves protection and aids in the migration of these bacteria into tissues (Chong *et al.*, 2005). The bacteria arranges themselves in pairs or chains surrounded by a large extracellular capsule as can be seen in figure 2 (Chong *et al.*, 2005).

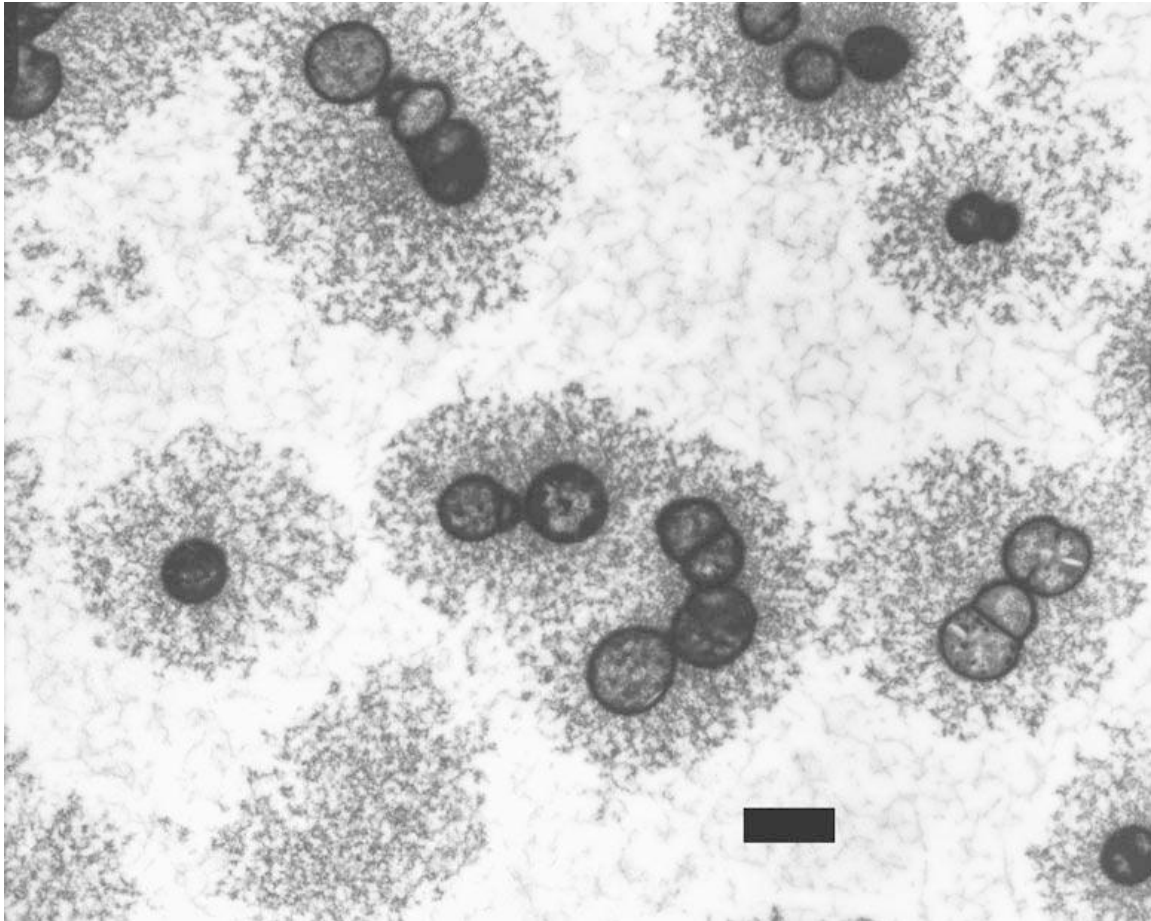


**Figure 2.** *Streptococcus equi zooepidemicus* (Chong *et al.*, 2005). HA can be seen as a mucoid capsule around the bacteria (Metabolic engineering of hyaluronic acid production, <http://web.aibn.uq.edu.au/cssb/Projects/HA.html>).

The enzyme that produces HA, HA synthase, has a life span of 2-4 hours and therefore only synthesizes one chain of HA during its life time (Metabolic engineering of hyaluronic acid production, <http://web.aibn.uq.edu.au/cssb/Projects/HA.html>). In order to control the chain length and therefore molecular weight of the HA produced, culture conditions are extremely important and much research has been done to perfect these conditions in order to increase chain length (Armstrong *et al.*, 1997a; Armstrong *et al.*, 1997b; Chong *et al.*, 2005; Metabolic engineering of hyaluronic acid production, <http://web.aibn.uq.edu.au/cssb/Projects/HA.html>; Stoolmiller *et al.*, 1969; Weigel *et al.*, 1997). It was found that optimal HA chain lengths are observed at a temperature of 37°C and at a pH of 7 and interestingly enough at less favorable levels of glucose uptake (Armstrong *et al.*, 1997b; Chong *et al.*, 2005). This principle of competing for substrate is exploited in that more HA with a higher molecular mass can be

produced with just the correct amount of glucose to sustain suboptimal conditions (Chong *et al.*, 2005; Metabolic engineering of hyaluronic acid production, <http://web.aibn.uq.edu.au/cssb/Projects/HA.html>). The optimal glucose concentration range is between 10 – 60g/liter. HA can be produced under aerobic or anaerobic conditions while dynamic agitation is required (Armstrong *et al.*, 1997b). The focus in the metabolic engineering of HA is thus more on increasing the lifespan and glucose uptake of the enzymes than it is increasing the amount of enzymes (Metabolic engineering of hyaluronic acid production, <http://web.aibn.uq.edu.au/cssb/Projects/HA.html>).

In the body HA serves as a carrier wherever cell migration and proliferation takes place; HA is thus found where cells are developing and growing, tissue repair is taking place and in normal cellular functions of the epithelial cell surface (Chong *et al.*, 2005; Gomes *et al.*, 2004). HA is also frequently seen in cancers where its pro-angiogenic nature together with vascular endothelial growth factor enhances tumor angiogenesis (Price *et al.*, 2007). Because of its nature it also serves as lubrication and protection of synovial and eye structures (Chong *et al.*, 2005; Gomes *et al.*, 2004).

Some commercial uses for HA include being a carrier for drug delivery, as HA binds to surface cells via their CD44 receptors and therefore increases the contact time that the drug has with the tissue. This mucoadhesive property is exploited in the treatment of “dry eye” where HA is incorporated in artificial eye drops (Becker *et al.*, 2009; Gomes *et al.*, 2004; Gura *et al.*, 2001; Wysenbeek *et al.*, 1988). In this regard HA has also shown to protect the corneal and conjunctival surface as well as have antioxidant effects when used together with the cytotoxic preservative benzalkonium chloride (Debbasch *et al.*, 2002; Nepp *et al.*, 2001; Yokoi *et al.*, 1997). It is also used in the cosmetic market where crosslinked HA is injected as a dermal filler into the skin to fill up and replace lost soft connective tissue and thereby reduces and obliterates wrinkles (Price *et al.*, 2007). It is also widely used as a synovial fluid supplement in the joints and has been used in cochlear implant surgery to coat the electrodes used during surgery (Becker *et al.*, 2009; Price *et al.*, 2007). As HA is one of the major components found naturally in the vitreous humor of the eye, it is most attractive as an OVD above other synthetic polymers, moreover HA does not differ between species and is therefore non-allergenic (Price *et al.*, 2007). HA has been shown to protect the corneal endothelial cells against free radical damage caused by phacoemulsification during ophthalmic surgery and also promotes migration,

proliferation and wound healing of the corneal epithelium (Maltese *et al.*, 2006; Sakuma *et al.*, 2000). Examples of HA OVD's include the Healon® range of products, Provisc® and Biolon®.

### **Chondroitin Sulphate**

Chondroitin sulphate (CS) is found in the cornea, cartilage and other connective tissues (Arshinoff, 2004). As stated, CS belongs to the same family of glycosaminoglycans as HA, but differs from HA in that it has an extra negative charge per repeating unit and contains *N*-acetylgalactosamine in the place of *N*-acetylglucosamine (Arshinoff, 2004). Because of its extra negative charge, it is inclined to bind more to the positively charged endothelial cells of the cornea (Arshinoff, 2004). CS is extracted from shark fin cartilage and is therefore from animal origin (Scholtz, 2007). This is important as it has to undergo vigorous purification processes in order to remove protein impurities. CS, unlike HA, is a Newtonian fluid implying that its composition does not change with an increase in pressure (Arshinoff, 2004). For this reason CS is included with HA in OVD formulations to obtain dispersive OVD's like DiscoVisc® and Viscoat®.

### **Hydroxypropylmethylcellulose**

Hydroxypropylmethylcellulose (HPMC) is a synthetic polymer made from methylcellulose found commercially in wood pulp and cotton (Scholtz, 2007). The polymer can easily be manufactured but is hard to purify for ophthalmic use and is also hydrophilic in nature (Arshinoff, 2004; Scholtz, 2007). These types of OVD's are very useful in that it can undergo autoclaving sterilization and has a shelf life of two years when stored at room temperature without its chemical composition being altered whereas other types of OVD's need to be kept cool for its composition not to be altered (Arshinoff, 2004). HPMC, like HA, is also a non-Newtonian fluid. An example of a HPMC OVD commercially available as a 2% solution is OccuGel® and Cellugel® (Arshinoff, 2004).

## **PROPERTIES OF OVD'S**

There are numerous OVD's available today and each one differs in its composition, concentration and chain lengths (Arshinoff *et al.*, 2005). Each of these factors greatly influences how the OVD will perform during surgery. Rheology (the study of the deformation and flow of

materials) is the field of science used to describe the OVDs' characteristics (Stedman, 2000). This is extremely helpful in assisting the surgeon in optimally pre-setting the phaco machine for a specific OVD and procedure (O'hEineachain, <http://www.eurotimes.org/07feb/pdf/UnderstandingOVDs.pdf>).

These physical properties (viscosity and elasticity) and types of OVD's will now be discussed.

## Viscosity

Viscosity can be defined as a measure of the resistance that develops when a fluid is flowing. Viscosity is the result of its cohesiveness, that is, the attraction and repulsion forces between the molecules of the fluid (Stedman, 2000). The viscosity is inversely proportional to the flow rate when the fluid is under pressure i.e. the greater the viscosity the slower the fluid will move when pressure is applied to it; thus greater pressures are required for higher viscosity fluids in order for it to move (Brown *et al.* 2006). Because the viscosity is caused by the attractive or repulsion forces between the molecules in the fluid, more entangled network polymers (high molecular weight) will account for a higher viscosity.

Two types of fluids can be distinguished based on their viscosity profile. A Newtonian fluid is one of which the viscosity does not change with increasing pressure e.g. water. A non-Newtonian fluids' viscosity changes with a change in pressure e.g. HA. With increasing pressure the viscosity of HA is decreased and it flows more easily, while with less pressure applied it has a higher resistance to flow; this principle of changing viscosity with increasing shear force is called pseudoplasticity as it is typical of plastics however the fluids does not become solid like plastics do at rest (Arshinoff, 2004; Buratto *et al.*, 2003; Maltese *et al.*, 2006). This principle is exploited in Non-Newtonian OVD's in that at high shear rates (when the OVD is injected into the eye through a thin cannula) the viscosity (resistance) is low and therefore flows more easily while at low shear rates (when the OVD is stationary in the eye such as during capsulorhexis, phaco probe entry) has a high viscosity as to create and maintain space in the anterior chamber (Buratto *et al.*, 2003; Maltese *et al.*, 2006). In the mid-shear section (while instruments are being used or when the artificial lens is inserted into the eye) the OVD should exhibit the potential to facilitate the maneuvering of the instruments without it causing damage to any of the corneal endothelium (Vajpayee *et al.*, 2005).

The protection of the endothelial cells by the OVD is the major concern during ophthalmic surgery as to avoid any detrimental effects to these vision sustaining cells. It is assumed that a varying degree of the endothelial cells will be damaged or lost during these types of surgeries and as these cells does not possess a regenerative capability, the prevention of damage to them are of the utmost concern (Balazs, 2009; Gomes *et al.*, 2004; Maltese *et al.*, 2006; Vajpayee *et al.*, 2005). In this regard, the flow dynamics of an OVD is of great importance as the OVD itself exerts force on the cells while in direct contact with the posterior surface of the cornea during surgery. The two main forces to be taken into account are: a compression (perpendicular) force and shear force (parallel to the cells' plane), see figure 3 (Hammer *et al.*, 1984). In clinical practice the viscosity of the OVD is responsible for negating both these forces to a certain extend. The compression force is better distributed throughout a more viscous (cohesive) OVD while the high parallel shear force is better accounted for by a less viscous (dispersive) OVD. A very high viscosity OVD would damage the cells by exerting a too high shear force whereas a very low viscosity would not provide adequate protection against the compression force.

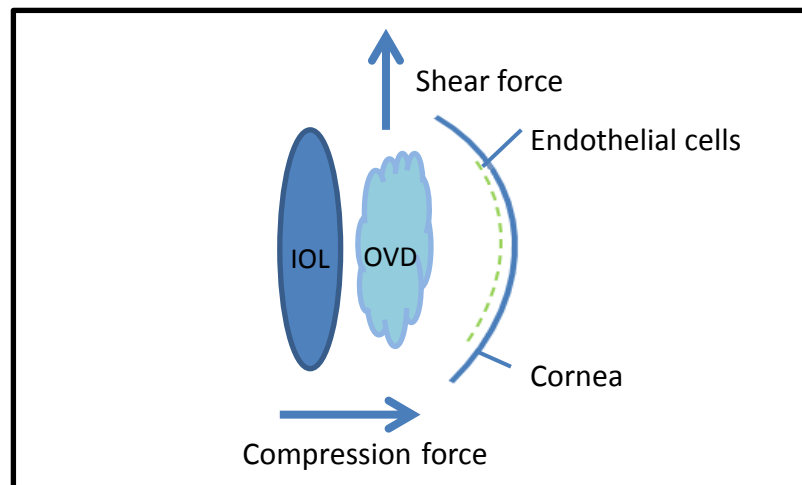


**Figure 3.** The two forces involved in cataract surgery (Hammer *et al.*, 1984).

Hammer *et al.* showed the clinical importance of the correct viscosity by comparing sodium hyaluronate, chondroitin sulphate and methylcellulose at different concentrations (Hammer *et al.*, 1984). They concluded that little endothelial damage was seen with an intermediate viscosity substance as opposed to a balanced salt solution (low viscosity) where no protection was provided to the cells while a very viscous solution showed to be the most damaging to the

corneal endothelial cells. Their study also confirmed the pseudoplastic behavior of HA and HPMC and the Newtonian behavior of CS.

Another important protective feature of OVD's related to viscosity is the protection of the eye structures against free radicals released by the phacoemulsification process during cataract surgery (Bissen-Miyajima, 2008). OVD's inhibit the formation of free radicals during surgery and in a study done by Bissen-Miyajima, it was shown that between a dispersive HPMC, dispersive HA and a cohesive HA OVD, the degree of cell loss due to free radical formation was the least when the dispersive HA OVD was used (Bissen-Miyajima, 2008). A possible reason for this could be its superior retention capabilities because of its binding to the tissues of the eye thereby providing a protective layer on top of the cells.

Viscosity ultimately depends on the molecular weight, the concentration of the substance and the temperature (Buratto *et al.*, 2003). The higher the molecular weight the more entangled the network that forms will be and that will give rise to a higher viscosity. The lower the molecular weight (molecular size), the higher the concentration of the polymer must be for a viscoelastic network to form in order to obtain a higher viscosity (Becker *et al.*, 2009). The concentration of the OVD determines the minimum viscosity of the OVD and is an indication of the limit of the injectability at which it can be injected through a cannula. The product of the concentration and the intrinsic viscosity determines the nature of the entangled network that will form and the more entangled the chain the stiffer the conformation, which will give rise to a more cohesive OVD (Becker *et al.*, 2009).

OVD's are compared in terms of viscosity according to their zero shear viscosity at room temperature, that is, the viscosity the OVD has at rest when stationary in the eye (Arshinoff *et al.*, 2005; Buratto *et al.*, 2003). OVD's are used at room temperature and should be compared at this temperature as the viscosity is inversely proportional to temperature and thus will be lower at high temperatures and higher at low temperatures (Arshinoff, 2004). This is also a direct indication of the space creation and stabilizing ability of the OVD (Arshinoff *et al.*, 2005).

## **Elasticity**

Elasticity refers to the ability of a substance to return to its original form after being under pressure, be it compression, stretch forces or any other kind of distortion (Stedman, 2000).

Long chain polymer molecules are more elastic than short chain polymers – in other words – the higher the molecular weight the more “elastic” the OVD will be. The elasticity is clinically important as it reflects the ability of the OVD to “absorb” the physical trauma induced by the instruments used by the surgeon during the procedure (Arshinoff, 2004).

## **TYPES OF OVD'S**

The types of an OVD's are dependent on its molecular weight (MW). An OVD with an MW of above one million Dalton is called a cohesive viscosurgical device whereas below one million Dalton it is classified as a dispersive device (Arshinoff, <http://aspx.apacrs.org/apacrs-publication/Default2.aspx?id=555#top>).

### **Cohesive**

A cohesive OVD has a viscous gel like structure and is famous for its ability to create and maintain space in the anterior or posterior chamber of the eye during surgery (Goldman *et al.*, 2007). Its cohesiveness is a result of its long entangled molecules (high molecular weight) and because of the forces that exist between these molecules the device tends to be aspired from the eye as “one blob” of gel as the molecules stick together (from here “cohesion”) (Arshinoff *et al.*, 2005). This feature of cohesive devices makes it easy to remove the OVD at the end of surgery (Stahl, 2011). The longer the chains of the molecules the more entangled the structure the better the space filling ability of the device will be (Buratto, 2003; Goldman *et al.*, 2007; O'hEineachain, <http://www.eurotimes.org/07feb/pdf/UnderstandingOVDs.pdf>, 2003). Cohesive devices can further be divided into super viscous cohesives (OVD's with a very high zero-shear viscosity above 1 million mPaS) and viscous cohesives (Arshinoff, 2004). Examples of cohesive OVD's include Provisc® and Amvisc® and the Healon® range of products.

### **Dispersive**

Because of its lower molecular weight a dispersive device is a lot less viscous than the cohesive device because its molecules have a much shorter chain length (Goldman *et al.*, 2007). It is aspired with more difficulty and tends to remain in the eye as the molecules are inclined to separate and disperse when removed (Arshinoff, 2004). A dispersive device is mainly used to serve as protection for the corneal endothelial cells during phacoemulsification because of its

high retentive ability. It is also used to divide spaces into viscoelastic protected areas during surgeries where complications arise (O'hEineachain, <http://www.eurotimes.org/07feb/pdf/UnderstandingOVDs.pdf>). Dispersives can also be divided into medium viscosity dispersives and very low viscosity dispersives (HPMC products are an example of these dispersives), (Arshinoff, 2004). Viscoat®, Ocucoat® and Vitrax® are some of the well-known names marketed as dispersive OVD's.

A combination of a cohesive and dispersive OVD is usually used during surgery, as both functions of space creation and protection of the delicate endothelial cells are required and a technique called the “soft shell technique” has been developed incorporating both types of OVD's (Arshinoff, <http://aspx.apacrs.org/apacrs-publication/Default2.aspx?id=555#top>). The soft-shell technique starts with the injection of the dispersive OVD into the anterior chamber at the beginning of the surgery, followed by an injection of a cohesive OVD beneath the dispersive OVD thereby displacing the dispersive “upward” towards the posterior corneal surface to the endothelial cells (Arshinoff, 2004). Next the capsulorhexis is performed and phaco begins, extracting the cohesive OVD while leaving the dispersive as a protective layer against the corneal endothelial cells. After the removal of the lens and lens capsule, the IOL is inserted. Older methods included that the OVD's were injected in opposing order than above before lens insertion. The cohesive was injected first to stabilize and maintain the anterior chamber and the dispersive was then injected beneath the cohesive providing a “maneuverable” environment for the intraocular lens to be placed and deployed in the eye. At the end of surgery both OVD's were then aspirated (Arshinoff, 2004). This technique thus exploits the best qualities of each of the cohesive and dispersive devices in the same procedure.

### **Viscoadaptive**

A third class of OVD exist called the viscoadaptive OVD's. These OVD's, for example Healon5®, contains properties of both cohesive and dispersive devices. The create space (cohesive) and protect the endothelial cells (dispersive), these properties can be attributed to their ability to adapt themselves to the change in flow rate during phacoemulsification (Goldman *et al.*, 2007). Its composition lends it to become more rigid under high pressure vacuum and remains fluid-like under lower vacuum pressures (O'hEineachain, <http://www.eurotimes.org/07feb/pdf/UnderstandingOVDs.pdf>). One drawback of these types of

OVD's is that they are difficult to remove as the tangled networks fracture when aspirated (O'hEineachain, <http://www.eurotimes.org/07feb/pdf/UnderstandingOVDs.pdf>).

The ultimate soft shell technique was developed to incorporate the use of viscoadaptives. In the ultimate soft shell technique balanced salt solution replaces the function of the cohesive OVD: a viscoadaptive is injected to fill 75% of the anterior chamber of the eye and then balanced salt solution is injected over the surface of the lens capsule (Arshinoff, 2004; O'hEineachain, <http://www.eurotimes.org/07feb/pdf/UnderstandingOVDs.pdf>). By using this method the difficulty in removing the fracturing viscoadaptive from the posterior chamber is accounted for as the balanced salt solution now occupies this space (Arshinoff, 2004).

The difficulty in the removal of the types of OVD's from the anterior chamber was investigated in a study done by Vajpayee *et al.* where Viscoat® (dispersive OVD made of HA and CS), Healon GV® (viscoadaptive made of HA) and Healon5® (viscoadaptive made of HA) were compared (Vajpayee *et al.*, 2005). Viscoat® proved to be more difficult to remove in comparison to Healon GV® and Healon5® while Healon® GV was easier removed Healon5®. Dispersives and viscoadaptives fracture while being aspirated from the eye and the pieces have to be individually "searched for" by the surgeon, but as seen in this study viscoadaptives are still easier to remove than dispersive devices (Goldman *et al.*, 2007). In another comparative study done by Oshika *et al* in 2006 that included a viscoadaptive OVD (Healon5®), a cohesive OVD (Provisc®) and two dispersive OVD's (DiscoVisc® and Viscoat®), it was found that the cohesive was completely removed whereas the viscoadaptive was retained in 80% of the eyes and the dispersive OVD's were retained in 100% of the eyes (Oshika *et al.*, 2006).

### **OVD's and Intraocular pressure**

A transient rise in the intraocular pressure (IOP) post operatively is assumed after the use of an OVD. The reason for this is believed to be the mechanical blockage of the trabecular meshwork (TM) by the OVD thereby obstructing the normal outflow pathway of the aqueous humor of the eye (Rainer *et al.*, 2007). The extent to which an OVD will cause an obstruction in the TM is influenced by its molecular weight, volume, the presence of inflammation at the time of surgery and the integrity of the TM (Tosi *et al.*, 2005). Once left in the eye, a low molecular weight polymer will drain easier than one with a high molecular weight because the high molecular weight OVD first have to be degraded by the enzyme hyaluronidase (found naturally in the eye)

before it can drain from the eye (Englert *et al.*, 1999). For this reason surgeons are advised to remove all, or as much as possible, of the OVD at the end of surgery.

A rise in the IOP postoperatively, especially above 30 mm Hg, may cause damage to the optic disc, corneal epithelial oedema and may cause pain. This is a concern as most patients are discharged the same day of the cataract surgery and are not monitored post-operatively (Rainer *et al.*, 2007). Unnecessary damage to the internal ocular structures as a result of an unsupervised rise in IOP could be prevented by the thorough removal of the OVD at the end of surgery. It was estimated that 10.6% of patients that were discharged required intervention the day following surgery where the majority of these patients were due to a rise in IOP (Thirumalai *et al.*, 2007). It was suggested that patients be made aware of the possible symptoms that may occur with a rise in IOP (Thirumalai *et al.*, 2007).

Because of the importance of the removal of the OVD after surgery, several removal techniques have been developed to assist the surgeon in thoroughly removing the OVD from the anterior chamber.

### **Removal techniques**

The Rock and roll technique, the two compartment technique and the bimanual irrigation/aspiration technique are examples of these techniques (Vajpayee *et al.*, 2005). The rock and roll technique entails the irrigation/aspiration handpiece being moved in circles in the anterior chamber and then the application of slight pressure to the different sides of the IOL optic (Goldman *et al.*, 2007). The two compartment technique removes OVD from both the anterior and posterior chambers (Goldman *et al.*, 2007). The bimanual irrigation/aspiration technique utilizes two separate cannulas for irrigation and aspiration instead of one. The anterior chamber is stabilized because the irrigation and aspiration are monitored (Al-Muammar, 2009). Another technique, called the empty-bag technique, reduces the need of the two compartment technique by a balanced salt solution containing bag being placed beneath the anterior chamber (Goldman *et al.*, 2007). The improvement of the techniques of phacoemulsification plays an enormous role in the protection of the anterior structures of the eye after surgery.

## Conclusion

Cataract surgery is one of the most common surgeries performed in the world today. OVD's have been developed as an aid in ophthalmic surgery in order to create and maintain space, to protect the corneal endothelial cells and to facilitate the maneuvering of instruments by the surgeon during surgeries like cataract surgery. An ISO standard has been set according to which all OVD's must adhere and serves as a tool whereby OVD's can be compared and tested. OVD's can consist of different sources of which HA, CS and HPMC are the most common materials used. The performance of an OVD is greatly dependent on several characteristics, such as its molecular weight, concentration, chain length, viscosity and elasticity. Rheology is the field of science which concerns itself with the study of the flow of materials and is used to classify and measure the different properties of OVD's. An OVD can either be cohesive or dispersive depending on its molecular weight. Cohesive OVD's are mainly used to maintain and create space while dispersives are used to protect the delicate endothelial cells of the eye. Viscoadaptives are OVD's that adapts to the flow rate during surgery and can serve as a cohesive or dispersive depending on the stage of surgery. OVD's cause a rise in the IOP postoperatively by mechanically blocking the TM, which is prevented by removing all, or as much as possible, of the OVD at the end of surgery. Several removal techniques have been developed to ensure the clearance of the OVD, namely the rock and roll, two compartment and the bimanual irrigation/aspiration technique. OVD's will continue to remain a very important tool in most ophthalmic surgeries in the modern world.

## CHAPTER 3

# TESTING THE CYTOTOXICITY POTENTIAL OF AN HYALURONIC ACID OPHTHALMIC VISCOSURGICAL DEVICE ON DERMAL FIBROBLASTS

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## INTRODUCTION

The corneal endothelial cells (CEC) are located at the most posterior part of the cornea as a layer of flattened polygonal shaped cells that is responsible for the health of the cornea (Bourne, 2003; Young *et al.*, 2000). The cornea consists of 5 main layers namely, the outer layer of stratified non-keratinised squamous epithelium, a specialized layer of corneal stroma called Bowman's membrane, the substantia propria consisting of highly organized dense collagenous tissue arranged in thin lamellae in which fibroblasts and sometimes lymphocytes are found, a supportive basement membrane called Descemet's membrane and finally the flattened layer of delicate CEC (Young *et al.*, 2000).

The cornea is a clear living lens through which light must move in order for vision to take place. The diverse structural arrangement of the collagen fibrils and cells in the cornea is of such that no blood vessels are present and crystalline placement of the fibrils permits this tissue to be optically transparent (Bourne, 2003). Any disruption in the crystalline structure, by any means such as an accumulation of fluid or damage to the fibrils, would have a detrimental effect on the optical clarity of the cornea (Bourne, 2003). The CEC accounts for the hydration and the nourishment of the cells of the cornea replacing the functions of a blood supply (Bourne, 2003; Young *et al.*, 2000). Its functionality permits it to serve as a permeable barrier that actively pumps ions from the substantia propria of the cornea into the aqueous humor, thereby osmotically moving water from the layers of the cornea. Conversely it also serves as a barrier to block excess fluid moving from the aqueous humor into the corneal stroma thereby adding to a state of relative dehydration and thereby optical clarity (Bourne, 2003; Stedman, 2000). If damage to the CEC occurs and it loses its barrier function the cornea swells and becomes opaque (Bourne, 2003). The CEC also secrete collagen throughout life that is deposited as Descemet's membrane (Bourne, 2003). Interestingly enough the collagen deposited at birth has a banded pattern, whereas the collagen deposited throughout life does not have a banded pattern, although in certain disease or stress situations the collagen deposited by the cells are

banded and an abnormal posterior layer is then formed, called the posterior banded layer (Bourne, 2003).

The use of ophthalmic instruments during ocular surgery accounts for most of the damage created to the CEC and the amount of trauma introduced during surgery. It was found that there is a decrease in the corneal cell density every year for at least ten years after cataract extraction surgery (Bourne, 2003). The endothelial reduction rate after cataract surgery is 1% per year in comparison to 0.5% for the non-operated eye. A lower cell density may result in a thickening of the cornea as there are less endothelial cells to uphold the barrier function (Bourne, 2003). The critical cell density for cells to still function as a barrier without corneal swelling taking place should not be less than 500-1000cells/mm<sup>2</sup> (Van den Bruel *et al.*, 2011).

It was with the realization of the critical function and role of these endothelial cells that ophthalmic viscosurgical devices (OVD's) came to have such an influential effect on the scene of ophthalmic surgery. Hyaluronic acid OVD's have been shown to protect the CEC during cataract surgery (Garg *et al.*, 2008). The CEC are subjected to many insults during cataract surgery of which phacoemulsification, mechanical damage by the surgical instruments, the artificial lens being placed in the eye, and exposure to harmful chemical substances contained in the anesthetics are some of the main factors (Garg *et al.*, 2008; Glasser *et al.*, 1986; Lockington *et al.*, 2010).

Phacoemulsification, the technique used in modern days to remove cataracts, in itself, has a very detrimental effect on the CEC that is caused by mechanical damage, thermal damage, air bubbles forming and oxidative tissue damage because of the formation of reactive oxygen species (Murano *et al.*, 2009; Wirbelauer., 2000). Numerous studies have shown the protective effect of hyaluronic acid OVD's in that it "absorbs" the free radicals that forms during the procedure and protects the cells against air bubbles and counteracts the harmful effects of preservatives used to clean the instruments with (Cutler Peck *et al.*, 2009; Debbasch *et al.*, 2002). In a study done by Camillieri *et al.* in 2004, hyaluronic acid was shown to decrease free radical formation by approximately 58-60% during phacoemulsification, it decreased the mean changes in corneal thickness by 76-80% and also decreased the CEC loss by 51-61% (Camillieri *et al.*, 2004). Several animal studies have shown the protective effects of OVD's while inserting the artificial lens provided that the CEC are coated with enough OVD to effectively protect them (Glasser *et al.*, 1986).

Some complications have been associated with the use of OVD's in intraocular surgery; these include mild to moderate intraocular inflammation, a transient rise in the intraocular pressure and corneal edema (Madhavan *et al.*, 1998; Nguyen *et al.*, 1992). The OVD is in contact with the delicate structures of the eye for less than two hours depending on the type of surgery, the reasons behind inflammation and edema because of the OVD is sometimes unclear. The possibility of the toxicity of an OVD because of the various compounds used to manufacture an OVD may be a valid reason (Glasser *et al.*, 1986). The international organization for standardization (ISO) is an international organization that develops and prepares standards like ISO 15798 for OVD's that defines and sets requirements with regard to its intended performance, design attributes, preclinical and clinical evaluation, sterilization, product packaging, product labeling and information that must be supplied by the manufacturer for the device (ISO 15798, 2001). According to this standard each OVD should undergo a cytotoxicity test to determine the effects thereof on live cells as part of the preclinical evaluation of the OVD before it can go to the next phases of clinical testing.

Cytotoxicity testing involves the testing of a chemical or substance for its potential to cause damage or destruction to cells. There are several ways in which damage to cells can be evaluated which involve the assessment of cell damage by vital stains, cytosolic enzyme release, cell growth and cloning efficiency (Bourdeau *et al.*, 1990). When organ specific cell cultures are used more specific end point results can be monitored e.g. evaluating specific cell functions (e.g. phagocytosis in macrophages) (Bourdeau *et al.*, 1990).

Primary or permanent cell cultures (cell lines) or cell strains can be used. A primary cell culture is defined as "a culture started from cells, tissues or organs taken directly from organisms" (Bourdeau *et al.*, 1990). A primary culture may be regarded as such until it is successfully subcultured for the first time. It then becomes a cell line: "The term, cell line, implies that cultures from it consist of numerous lineages of cells originally present in the primary culture." (Bourdeau *et al.*, 1990). Cell lines are classified as continuous or finite where finite cell lines have a life span of approximately 40-50 divisions and a continuous cell line are normally coupled with viruses, chemical or physical vehicles so that it has an infinite lifespan (Bourdeau *et al.*, 1990). A cell strain "derives either from a primary culture or a cell line by the selection or cloning of cells having specific properties or markers and the properties or markers must persist during subsequent cultivation" (Bourdeau *et al.*, 1990).

Fibroblasts are spindle shaped cells which are routinely used in cell culture studies because changes in their morphology are easy to observe and evaluate, they are readily available and are relatively easy to culture. When the cells are exposed to a toxic substance, blebbing and vacuolization will be visible under a light microscope (Bordeau *et al.*, 1990).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay utilizes the mitochondrial succinic dehydrogenase enzyme to form an insoluble blue formazan product that accumulates in viable cells from a yellow MTT substrate (Chiba *et al.*, 1998). The amount of color formation is not only an indication of viable cells but also of any cell proliferation that took place over time. Another quantitative colorimetric method, the Neutral Red (NR) assay, is aimed at the lysosomes of viable cells. Lysosomes contain many digestive enzymes inside a membranous sack under normal circumstances (Solomon *et al.*, 2002). When cell damage occurs, the lysosomal membrane is usually one of the first structures to be damaged with a consequent release of the digestive enzymes from the cell. The NR assay utilizes this principle in that the weak cationic dye binds to anionic membranes such as the lysosomal membrane. When cell damage occurs the lysosomal membrane is damaged and loses its integrity and consequently its function and charge. The vital dye will only bind to an intact membrane and a color formation is thus an indication of the undamaged or viable cells (Chiba *et al.*, 1998). The Crystal Violet (CV) stain is used to non-specifically stain proteins in a cell and gives an indication of the growth rate reduction (or proliferation) as indicated by the amount of color that was absorbed by the cells (Chiba *et al.*, 1998). The integrity and number of cells can thus be derived from this assay and an evaluation of the general morphology of the cells can be seen.

As part of this study, a buffered hyaluronic acid (BHA) was evaluated according to ISO 15798 in order to determine its cytotoxic potential. Because of its contact with the CEC during ocular surgery, the determination of every possible toxicological aspect is of great importance. The study was conducted on human fibroblasts in order to evaluate the effects of this substance on the cells in order to ensure its safety during ocular surgery.

## **MATERIALS**

The BHA and human dermal fibroblasts were supplied by Southern Medical (Pty) Ltd, Keratinocyte basal medium (CC-3103) and Fetal bovine serum (14-401F) were obtained from Whitehead scientific, Brackenfell, South Africa. Poly-D-Lysine (P2659), Trypan blue (T6146),

MTT (1-(4,5-Dimethylthiazol-2-yl)-3,5 diphenylformazan) , Crystal Violet (61135) and Neutral Red (57993) were obtained from Sigma-Aldrich (Pty) Ltd, Midrand, South Africa. Culture flasks (250ml T75) and 24 well-plates were obtained from Lasec SA, Cape Town, South Africa.

## **METHODS**

### **Preparation of cell cultures**

The culture medium was supplemented with 10% Fetal bovine serum and were used throughout the study. Primary cultures were allowed to reach confluence, aseptic technique was strictly adhered throughout the study. After the second passage a cell count and viability assay (Trypan blue) was performed. A uniform volume of 1ml of the cell suspension (50 000 cells) was seeded into each well of a 24 well-plate previously coated with poly-D-lysine. The cells were then incubated for 28 hours at 5% CO<sub>2</sub> and 37°C to allow attachment of the monolayer of cells to the culture plates. The cells were monitored for attachment to the culture flasks with a light microscope before commencing the test.

### **Preparation of test product**

The test product, BHA, was made up at concentrations used for OVD's in the market ranging from 0.8% to 2.3%. The BHA dilutions of 1:10 and 1:100 were freshly made up with the cell culture medium for each of the concentrations and were allowed to stand for 24 hours at 2-8°C. The undiluted samples were also kept at 2-8°C. After this period the dilutions were vortexed vigorously and all samples were then incubated at 37°C for 1 hour.

### **Replacement of culture medium with test product**

The culture medium was then replaced with 2 ml of each test substance. Control cells were covered with culture medium only. The cells were incubated at 37°C and 5% CO<sub>2</sub> for a period of 1 hour and 24 hours. Each of the tests was done in triplicate.

### **Cell culture assays**

At the end of the incubation period a combined NR, MTT and CV assay was performed as follows. The assays were started by adding 100µl of a 0.05% Neutral red solution dissolved in double distilled water into each of the wells. The cells were incubated at 37°C and 5% CO<sub>2</sub> for 1 hour after which 100µl of a 0.1mg/ml MTT solution was added to each well and incubated for a further 2 hours. The medium was removed and the cells were fixed with 200µl of a 1% acetic acid and 1% formaldehyde solution in double distilled water for 15 minutes. The Neutral red was extracted first by adding 200µl of destaining solution consisting of 1% glycolic acetic acid and 50% ethanol solution in double distilled water, the dissolved Neutral red was transferred into a 96 well plate. Next 200µl of DMSO was added to the cells in order to dissolve the developed formazan crystals. The plates were placed on a cell shaker (IKA Schuttler MTS 2) for 20 minutes after which the formed color solution were transferred into a 96 well plate. The color formation was measured with a spectrophotometer (EL900 plate reader) at 570nm. Two hundred microliters of (0.1% (w/v) Crystal violet solution (in 200mM formic acid, pH 3.5) was then added to each of the wells and left to stain the cells for 30 minutes. The solution was then removed and the cells were washed with double distilled water and left to dry. Photographs were taken with a light microscope (Zeiss Axiocam MRc5). The crystal violet color was extracted with 10% acetic acid in double distilled water and transferred into a 96 well plate. The color formation was read with a spectrophotometer (EL900 plate reader) at 570nm.

### **Statistical analysis**

Data were statistically analyzed by using a student's t-test. The means, standard deviations and p-value, with the p-value calculated at a confidence level interval of 95%, were used to analyze the data.

## **RESULTS**

No contamination was seen during the study period. A confluent monolayer of spindle shaped cells was seen attached to the bottom of the multiwall plates at the beginning of the study. Each test was done in triplicate and the absorbancy values were calculated to obtain the average value for each concentration. The undiluted samples of BHA absorbed all the culture medium

because of the osmotic capability of hyaluronic acid. Therefor these results were excluded from the study.

### **MTT cell proliferation assay**

**Table 1.** Results for 1:10 dilution after 1 hour of exposure to the BHA.

| <b>MTT 1:10 1 HOUR</b> |                 |                |                 |                 |                 |                |
|------------------------|-----------------|----------------|-----------------|-----------------|-----------------|----------------|
| <b>Concentration</b>   | <b>0.8%</b>     | <b>1.0%</b>    | <b>1.2%</b>     | <b>1.8%</b>     | <b>2.3%</b>     | <b>Control</b> |
|                        | 503.6295        | 72.21527       | 75.59449        | 194.1176        | 86.85857        | 100            |
|                        | 92.06897        | 88.16092       | 75.74713        | 69.77011        | 108.8506        | 100            |
|                        | 89.32292        | 110.9375       | 119.5313        | 131.1198        | 122.526         | 100            |
| <b>Average</b>         | <b>228.3405</b> | <b>90.4379</b> | <b>90.29097</b> | <b>131.6692</b> | <b>106.0784</b> | <b>100</b>     |
| <b>SD</b>              | 238.4113        | 19.46128       | 25.32298        | 62.17559        | 17.99459        | 0              |
| <b>p-value</b>         | <b>0.404</b>    | <b>0.443</b>   | <b>0.543</b>    | <b>0.428</b>    | <b>0.59</b>     |                |

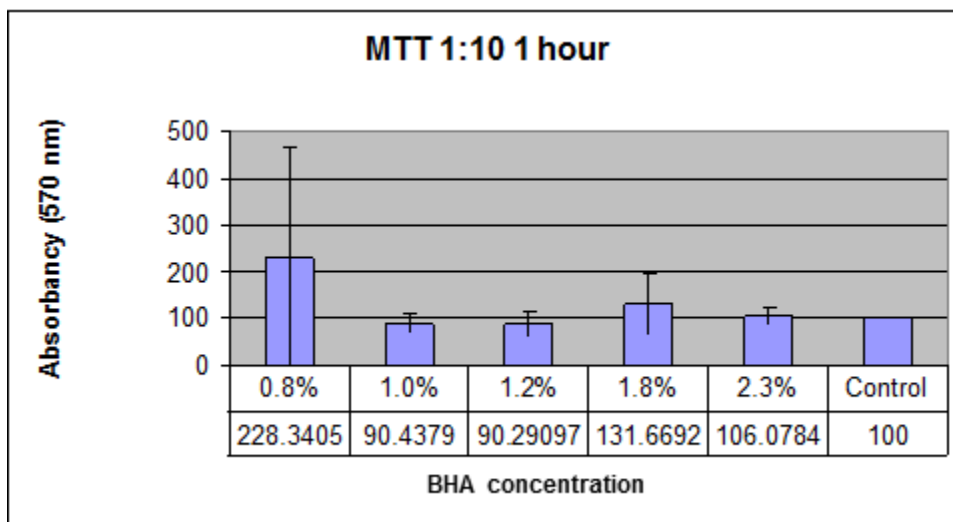


**Figure 1.** Cell survival/proliferation of cultured human fibroblasts as a percentage of the control, after incubation period of 1 hour in different concentrations (0.8%-2.3%) of BHA at a 1:10 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

**Table 2.** Results for 1:10 dilution after 24 hours of exposure to BHA.

| <b>MTT 1:10 24 HOURS</b> |                 |                 |                 |                 |                 |                |
|--------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|
| <b>Concentration</b>     | <b>0.8%</b>     | <b>1.0%</b>     | <b>1.2%</b>     | <b>1.8%</b>     | <b>2.3%</b>     | <b>Control</b> |
|                          | 152.7644        | 100.8413        | 95.79327        | 123.4375        | 131.4904        | 100            |
|                          | 72.6145         | 107.1565        | 80.24809        | 129.9618        | 100.1908        | 100            |
|                          | 87.17557        | 93.12977        | 96.33588        | 196.1832        | 98.77863        | 100            |
| <b>Average</b>           | <b>104.1848</b> | <b>100.3759</b> | <b>90.79241</b> | <b>149.8608</b> | <b>110.1533</b> | <b>100</b>     |
| <b>SD</b>                | 42.69646        | 7.02494         | 9.135681        | 40.24876        | 18.49197        | 0              |
| <b>p-value</b>           | <b>0.874</b>    | <b>0.932</b>    | <b>0.156</b>    | <b>0.098</b>    | <b>0.396</b>    |                |

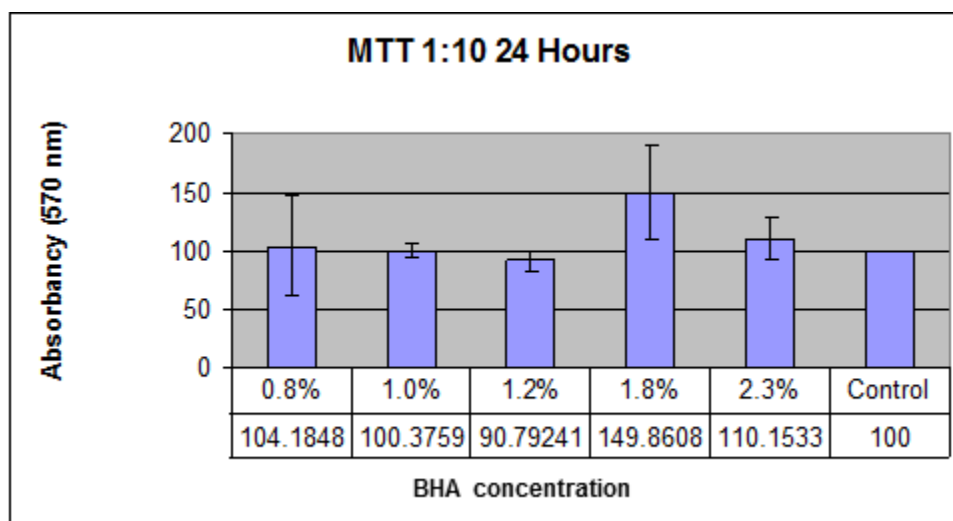


**Figure 2.** Cell survival/proliferation of cultured human fibroblasts as a percentage of the control, after incubation period of 24 hours in different concentrations (0.8%-2.3%) of BHA at a 1:10 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

**Table 3.** Results for 1:100 dilution after 1 hour of exposure to BHA.

| <b>MTT 1:100 1 HOUR</b> |                 |                 |                 |                 |                 |                |
|-------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|
| <b>Concentration</b>    | <b>0.8%</b>     | <b>1.0%</b>     | <b>1.2%</b>     | <b>1.8%</b>     | <b>2.3%</b>     | <b>Control</b> |
|                         | 88.96848        | 93.98281        | 88.39542        | 117.0487        | 93.55301        | 100            |
|                         | 180.2395        | 102.2455        | 89.67066        | 89.07186        | 93.56287        | 100            |
|                         | 85.92814        | 86.97605        | 89.22156        | 85.47904        | 111.5269        | 100            |
| <b>Average</b>          | <b>118.3787</b> | <b>94.40145</b> | <b>89.09588</b> | <b>97.19987</b> | <b>99.54759</b> | <b>100</b>     |
| <b>SD</b>               | 53.59458        | 7.643329        | 0.646845        | 17.28321        | 10.37439        | 0              |
| <b>p-value</b>          | <b>0.585</b>    | <b>0.273</b>    | <b>0</b>        | <b>0.793</b>    | <b>0.943</b>    |                |

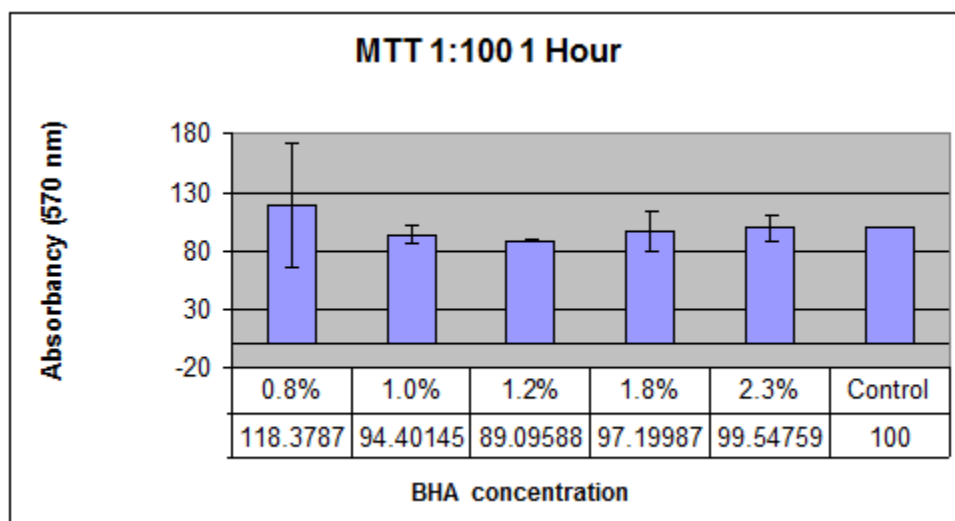


**Figure 3.** Cell survival/proliferation of cultured human fibroblasts as a percentage of the control, after incubation period of 1 hour in different concentrations (0.8%-2.3%) of BHA at a 1:100 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

**Table 4.** Results for 1:100 dilution after 24 hours of exposure to BHA.

| <b>MTT 1:100 24 HOURS</b> |                 |                 |                 |                 |                 |                |
|---------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|
| <b>Concentration</b>      | <b>0.8%</b>     | <b>1.0%</b>     | <b>1.2%</b>     | <b>1.8%</b>     | <b>2.3%</b>     | <b>Control</b> |
|                           | 80.87291        | 79.20411        | 176.6367        | 124.0051        | 100.3851        | 100            |
|                           | 87.45247        | 102.7883        | 116.4766        | 104.3093        | 99.61977        | 100            |
|                           | 102.2942        | 94.46694        | 95.27665        | 96.35628        | 90.55331        | 100            |
| <b>Average</b>            | <b>90.20653</b> | <b>92.15312</b> | <b>129.4633</b> | <b>108.2236</b> | <b>96.85273</b> | <b>100</b>     |
| <b>SD</b>                 | 10.97299        | 11.96114        | 42.20612        | 14.23397        | 5.468862        | 0              |
| <b>p-value</b>            | <b>0.197</b>    | <b>0.319</b>    | <b>0.319</b>    | <b>0.374</b>    | <b>0.375</b>    |                |

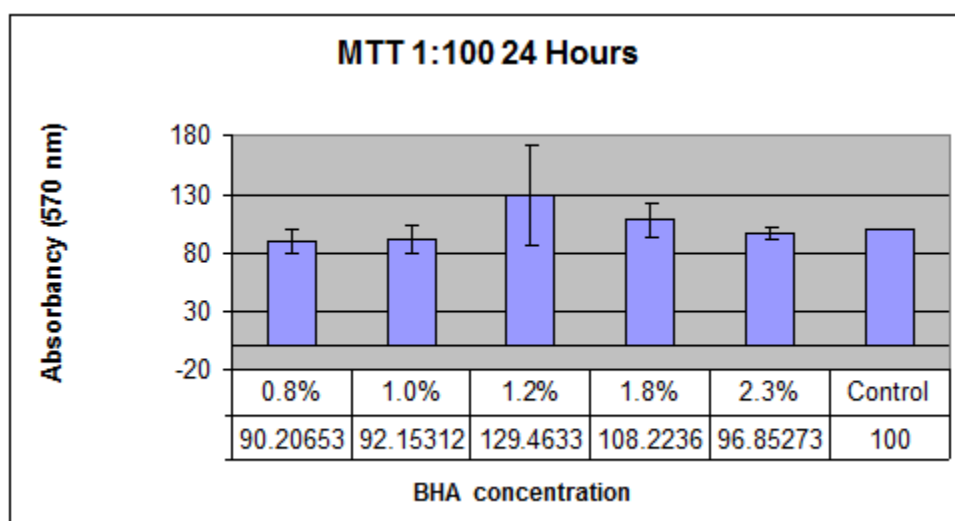


**Figure 4.** Cell survival/proliferation of cultured human fibroblasts as a percentage of the control, after incubation period of 24 hours in different concentrations (0.8%-2.3%) of BHA at a 1:100 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

From these results it can be conducted that:

- All proliferating rates of cells at all concentrations, except 1.2% at a 1:100 dilution, did not differ from that of the control cells ( $p > 0.05$ ).
- The test and control cells were metabolically active and viable

## Neutral red assay

Cell viability as measured by the Neutral red assay was as follows:

**Table 5.** Neutral red results for 1:10 dilution after 1 hour of exposure to BHA.

| <b>Neutral Red 1:10 1 HOUR</b> |                 |                 |                 |                 |                 |                |
|--------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|
| <b>Concentration</b>           | <b>0.8%</b>     | <b>1.0%</b>     | <b>1.2%</b>     | <b>1.8%</b>     | <b>2.3%</b>     | <b>Control</b> |
|                                | 103.4965        | 104.1958        | 90.67599        | 101.3986        | 105.8275        | 100            |
|                                | 84.70825        | 86.51911        | 89.53722        | 90.54326        | 92.35412        | 100            |
|                                | 94.19643        | 100             | 103.3482        | 92.1875         | 108.4821        | 100            |
| <b>Average</b>                 | <b>94.13373</b> | <b>96.90497</b> | <b>94.52047</b> | <b>94.70979</b> | <b>102.2212</b> | <b>100</b>     |
| <b>SD</b>                      | 9.394282        | 9.235842        | 7.666212        | 5.850731        | 8.647646        | 0              |
| <b>p-value</b>                 | <b>0.34</b>     | <b>0.592</b>    | <b>0.283</b>    | <b>0.192</b>    | <b>0.751</b>    |                |

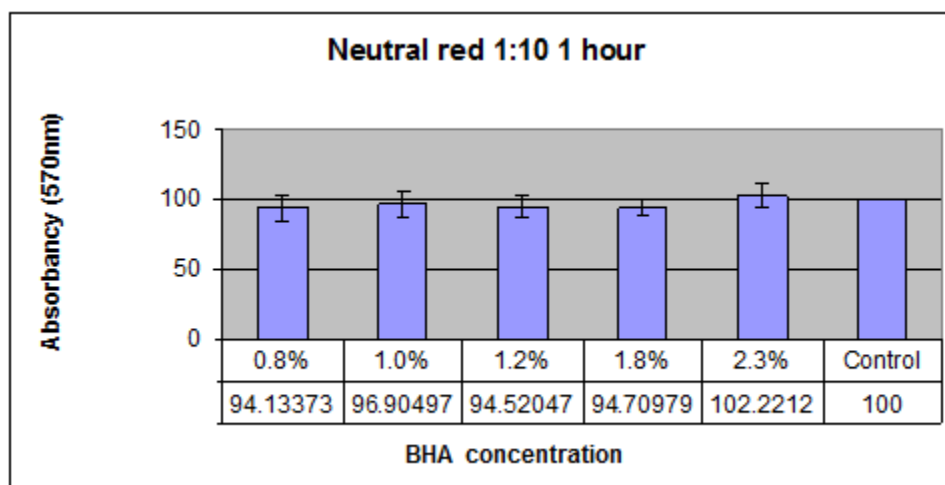


**Figure 5.** Cell viability of cultured human fibroblasts as a percentage of the control, after incubation period of 1 hour in different concentrations (0.8%-2.3%) of BHA at a 1:10 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

**Table 6.** Neutral red results for 1:10 dilution after 24 hours of exposure to BHA.

| <b>Neutral Red 1:10 24 HOURS</b> |                 |                 |                 |                 |                 |                |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|
| <b>Concentration</b>             | <b>0.8%</b>     | <b>1.0%</b>     | <b>1.2%</b>     | <b>1.8%</b>     | <b>2.3%</b>     | <b>Control</b> |
|                                  | 117.765         | 93.40974        | 104.5845        | 102.5788        | 104.5845        | 100            |
|                                  | 100             | 108.7533        | 111.4058        | 110.6101        | 111.1406        | 100            |
|                                  | 87.95812        | 96.33508        | 96.0733         | 93.4555         | 109.9476        | 100            |
| <b>Average</b>                   | <b>101.9077</b> | <b>99.49937</b> | <b>104.0212</b> | <b>102.2148</b> | <b>108.5576</b> | <b>100</b>     |
| <b>SD</b>                        | 14.99473        | 8.146518        | 7.681757        | 8.583091        | 3.492084        | 0              |
| <b>p-value</b>                   | <b>0.837</b>    | <b>0.92</b>     | <b>0.416</b>    | <b>0.679</b>    | <b>0.013</b>    |                |

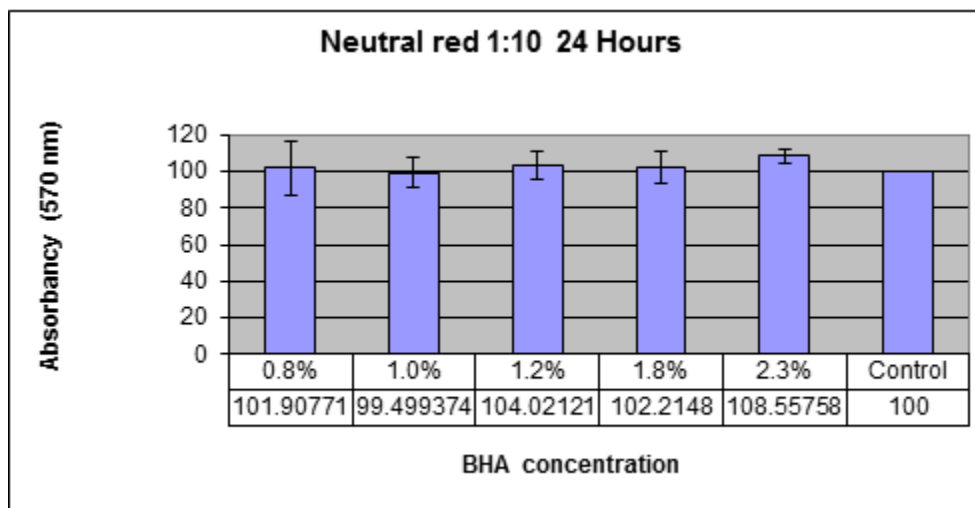


**Figure 6.** Cell viability of cultured human fibroblasts as a percentage of the control, after incubation period of 24 hours in different concentrations (0.8%-2.3%) of BHA at a 1:10 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

**Table 7.** Neutral red results for 1:100 dilution after 1 hour of exposure to BHA.

| <b>Neutral Red 1:100 1 HOUR</b> |                 |                 |                 |                |               |                |
|---------------------------------|-----------------|-----------------|-----------------|----------------|---------------|----------------|
| <b>Concentration</b>            | <b>0.8%</b>     | <b>1.0%</b>     | <b>1.2%</b>     | <b>1.8%</b>    | <b>2.3%</b>   | <b>Control</b> |
|                                 | 100.6711        | 108.0537        | 103.8031        | 95.30201       | 108.5011      | 100            |
|                                 | 93.20843        | 100.9368        | 122.9508        | 109.1335       | 110.7728      | 100            |
|                                 | 91.20879        | 85.71429        | 90.29304        | 88.64469       | 93.95604      | 100            |
| <b>Average</b>                  | <b>95.02944</b> | <b>98.23493</b> | <b>105.6823</b> | <b>97.6934</b> | <b>104.41</b> | <b>100</b>     |
| <b>SD</b>                       | 4.987072        | 11.41216        | 16.40978        | 10.45165       | 9.124356      | 0              |
| <b>p-value</b>                  | <b>0.159</b>    | <b>0.802</b>    | <b>0.581</b>    | <b>0.721</b>   | <b>0.45</b>   |                |

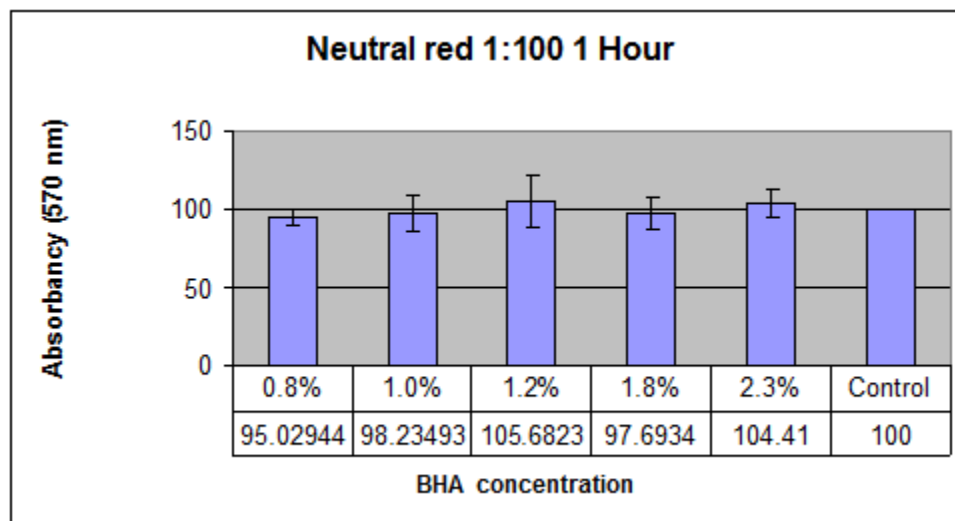


**Figure 7.** Cell viability of cultured human fibroblasts as a percentage of the control, after incubation period of 1 hour in different concentrations (0.8%-2.3%) of BHA at a 1:100 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

**Table 8.** Neutral red results for 1:100 dilution after 24 hours of exposure to BHA.

| <b>Neutral Red 1:100 24 HOURS</b> |                 |                 |                |                |                |                |
|-----------------------------------|-----------------|-----------------|----------------|----------------|----------------|----------------|
| <b>Concentration</b>              | <b>0.8%</b>     | <b>1.0%</b>     | <b>1.2%</b>    | <b>1.8%</b>    | <b>2.3%</b>    | <b>Control</b> |
|                                   | 121.2209        | 111.3372        | 111.9186       | 104.0698       | 108.1395       | 100            |
|                                   | 39.16877        | 45.21411        | 47.48111       | 69.64736       | 45.71788       | 100            |
|                                   | 113.5279        | 101.5915        | 111.1406       | 96.28647       | 104.244        | 100            |
| <b>Average</b>                    | <b>91.30586</b> | <b>86.04761</b> | <b>90.1801</b> | <b>90.0012</b> | <b>86.0338</b> | <b>100</b>     |
| <b>SD</b>                         | 45.31559        | 35.697          | 36.98046       | 18.05143       | 34.9689        | 0              |
| <b>p-value</b>                    | <b>0.756</b>    | <b>0.536</b>    | <b>0.669</b>   | <b>0.392</b>   | <b>0.527</b>   |                |

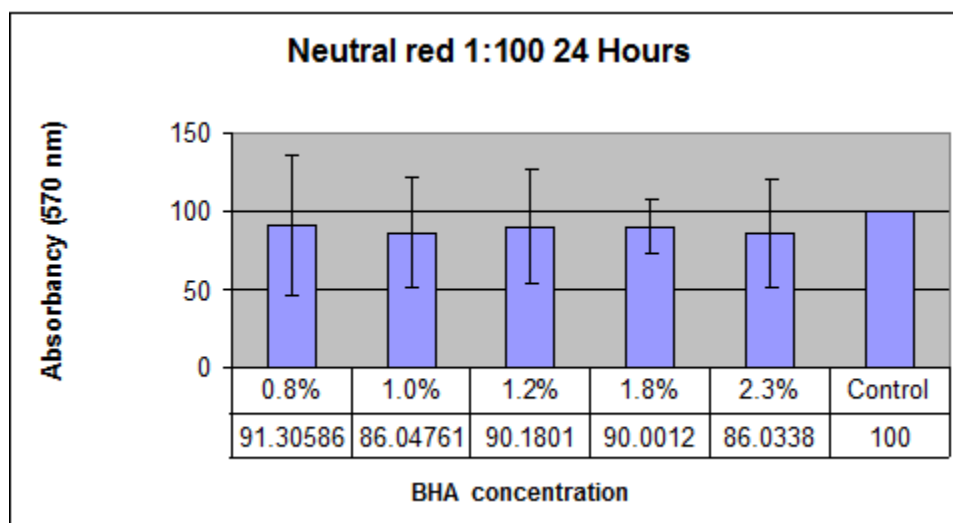


**Figure 8.** Cell viability of cultured human fibroblasts as a percentage of the control, after incubation period of 24 hours in different concentrations (0.8%-2.3%) of BHA at a 1:100 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

From these results it can be conducted that:

- The lysosomal cellular activity of all cells did not differ from the control cells except at 2.3% at a 1:10 dilution at 24 hours after exposure to the OVD ( $p > 0.05$ ).

## Crystal Violet assay

**Table 9.** Crystal violet results for 1:10 dilution after 1 hour of exposure to BHA.

| <b>Crystal violet 1:10 1 HOUR</b> |               |                |                 |                 |                 |                |
|-----------------------------------|---------------|----------------|-----------------|-----------------|-----------------|----------------|
| <b>Concentration</b>              | <b>0.8%</b>   | <b>1.0%</b>    | <b>1.2%</b>     | <b>1.8%</b>     | <b>2.3%</b>     | <b>Control</b> |
|                                   | 156.2212      | 168.51         | 148.6943        | 155.4531        | 111.2135        | 100            |
|                                   | 93.63057      | 103.6943       | 91.97452        | 102.293         | 134.0127        | 100            |
|                                   | 100.9081      | 103.5187       | 108.6266        | 78.54711        | 94.09762        | 100            |
| <b>Average</b>                    | <b>116.92</b> | <b>125.241</b> | <b>116.4318</b> | <b>112.0978</b> | <b>113.1079</b> | <b>100</b>     |
| SD                                | 34.22983      | 37.47215       | 29.15433        | 39.37937        | 20.02486        | 0              |
| <b>p-value</b>                    | <b>0.44</b>   | <b>0.308</b>   | <b>0.384</b>    | <b>0.623</b>    | <b>0.32</b>     |                |

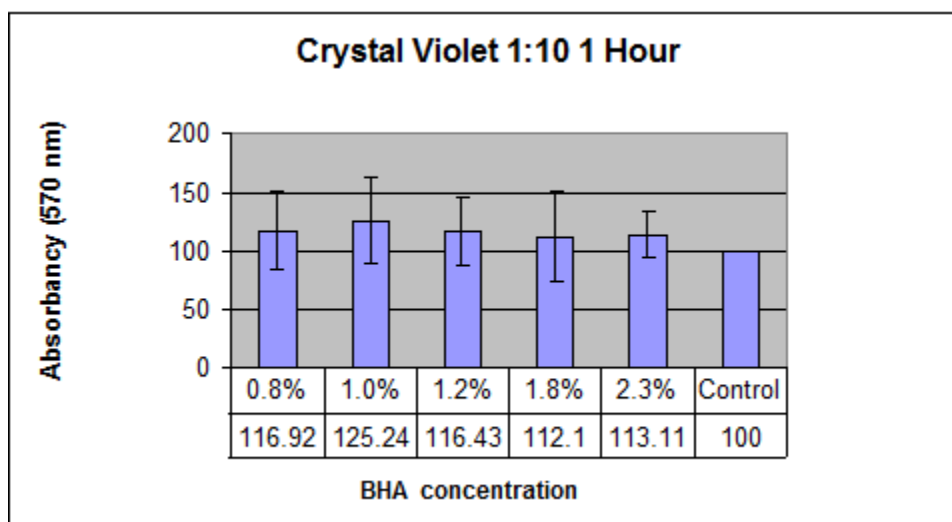


**Figure 9.** Cell number of cultured human fibroblasts as a percentage of the control, after incubation period of 1 hour in different concentrations (0.8%-2.3%) of BHA at a 1:10 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

**Table 10.** Crystal violet results for 1:10 dilution after 24 hours of exposure to BHA.

| <b>Crystal violet 1:10 24 HOURS</b> |                 |                 |                |                 |                 |                |
|-------------------------------------|-----------------|-----------------|----------------|-----------------|-----------------|----------------|
| <b>Concentration</b>                | <b>0.8%</b>     | <b>1.0%</b>     | <b>1.2%</b>    | <b>1.8%</b>     | <b>2.3%</b>     | <b>Control</b> |
|                                     | 108.1967        | 107.8454        | 115.5738       | 115.2225        | 101.171         | 100            |
|                                     | 103.3449        | 131.6032        | 118.4544       | 105.3057        | 129.0657        | 100            |
|                                     | 103.1579        | 107.3684        | 99.15789       | 126.2105        | 128.5263        | 100            |
| <b>Average</b>                      | <b>104.8998</b> | <b>115.6057</b> | <b>111.062</b> | <b>115.5796</b> | <b>119.5877</b> | <b>100</b>     |
| SD                                  | 2.856701        | 13.85631        | 10.40941       | 10.45697        | 15.9516         | 0              |
| <b>p-value</b>                      | <b>0.041</b>    | <b>0.123</b>    | <b>0.14</b>    | <b>0.061</b>    | <b>0.101</b>    |                |

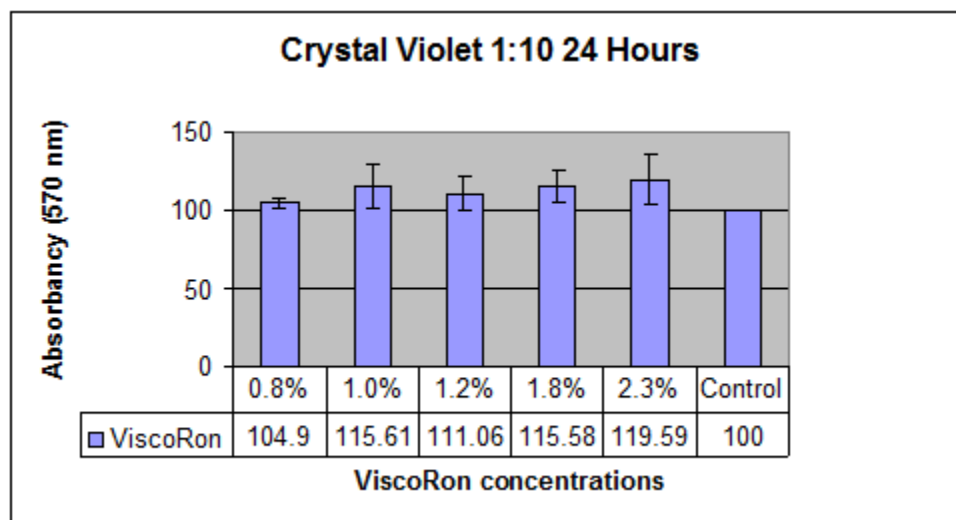


**Figure 10.** Cell number of cultured human fibroblasts as a percentage of the control, after incubation period of 24 hours in different concentrations (0.8%-2.3%) of BHA at a 1:10 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

**Table 11.** Crystal violet results for 1:100 dilution after 1 hour of exposure to BHA.

| <b>Crystal violet 1:100 1 HOUR</b> |                 |                 |                 |                 |                 |                |
|------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|
| <b>Concentration</b>               | <b>0.8%</b>     | <b>1.0%</b>     | <b>1.2%</b>     | <b>1.8%</b>     | <b>2.3%</b>     | <b>Control</b> |
|                                    | 79.17981        | 99.05363        | 118.9274        | 117.9811        | 117.0347        | 100            |
|                                    | 81.57895        | 79.47368        | 78.77193        | 90.52632        | 95.96491        | 100            |
|                                    | 99.30556        | 91.2037         | 94.90741        | 84.49074        | 118.0556        | 100            |
| <b>Average</b>                     | <b>86.68811</b> | <b>89.91034</b> | <b>97.53558</b> | <b>97.66605</b> | <b>110.3517</b> | <b>100</b>     |
| SD                                 | 10.99268        | 9.85384         | 20.20633        | 17.85029        | 12.46981        | 0              |
| <b>p-value</b>                     | <b>0.104</b>    | <b>0.151</b>    | <b>0.843</b>    | <b>0.832</b>    | <b>0.224</b>    |                |

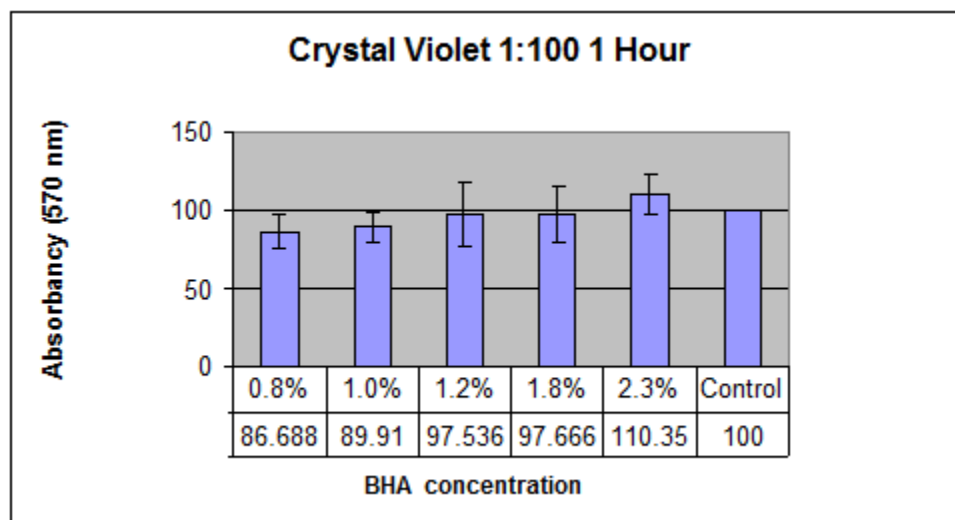


**Figure 11.** Cell number of cultured human fibroblasts as a percentage of the control, after incubation period of 1 hour in different concentrations (0.8%-2.3%) of BHA at a 1:100 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

**Table 12.** Crystal violet results for 1:100 dilution after 24 hours of exposure to BHA.

| <b>Crystal violet 1:100 24 HOURS</b> |                |                 |                |                 |                 |                |
|--------------------------------------|----------------|-----------------|----------------|-----------------|-----------------|----------------|
| <b>Concentration</b>                 | <b>0.8%</b>    | <b>1.0%</b>     | <b>1.2%</b>    | <b>1.8%</b>     | <b>2.3%</b>     | <b>Control</b> |
|                                      | 131.6847       | 122.4111        | 126.5842       | 100.1546        | 110.3555        | 100            |
|                                      | 99.09794       | 110.9536        | 110.567        | 92.65464        | 88.2732         | 100            |
|                                      | 124.4924       | 110.4061        | 106.8528       | 96.82741        | 103.934         | 100            |
| <b>Average</b>                       | <b>118.425</b> | <b>114.5903</b> | <b>114.668</b> | <b>96.54554</b> | <b>100.8542</b> | <b>100</b>     |
| SD                                   | 17.1197        | 6.778587        | 10.48552       | 3.757897        | 11.35872        | 0              |
| <b>p-value</b>                       | <b>0.136</b>   | <b>0.02</b>     | <b>0.073</b>   | <b>0.186</b>    | <b>0.903</b>    |                |

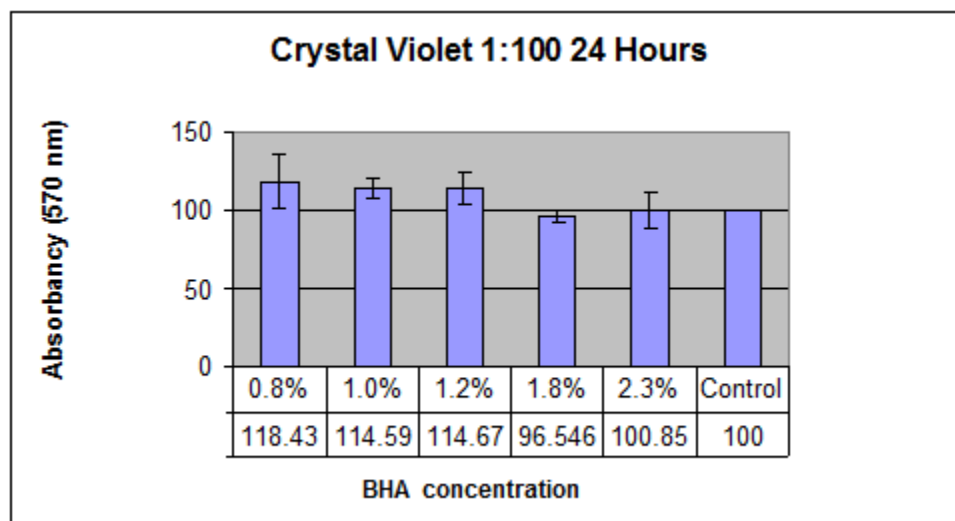


**Figure 12.** Cell number of cultured human fibroblasts as a percentage of the control, after incubation period of 24 hours in different concentrations (0.8%-2.3%) of BHA at a 1:100 dilution. Values are mean  $\pm$  standard deviation of 3 replicates.

From the results it can be conducted that all cells did not differ from that of the control except at the 0.8% concentration for the 1:10 dilution at 24 hours as well as at 1.0% for the 1:100 dilution at 24 hours.

## DISCUSSION

OVD's are one of the most important tools used in ocular surgery; it is used to create and maintain space, to assist the surgeon with manipulating tissues and to protect the CEC from damage. It comes into direct contact with the CEC responsible for the maintenance of the cornea. CEC have a poor regenerative capability and therefore all measures must be put in place in order to ensure the safety of products coming into contact with these cells (Van den Bruel *et al.*, 2011). In this study five different concentrations of a hyaluronic acid OVD were tested on human fibroblast cells in order to determine the cytotoxic potential of the OVD.

The same dilutions were used as the study conducted by Madhavan *et al.*, dilutions of 1:10 and 1:100 were made for each of the concentrations used for OVD's namely, 0.8%, 1.0%, 1.2%, 1.8% and 2.3% (Madhavan *et al.*, 1998). Three quantitative assays, MTT, NR and CV were done to assess the cell proliferation, cell viability and cell number respectively. CV, also a qualitative assay, was done to determine the general morphology of the cells. These tests were done on fibroblasts, known for their ease of handling and definable morphology in cytotoxicity studies, but also because fibroblasts are present in the cornea (Givens *et al.*, 1990; Young, 2000).

For the MTT cell proliferation assay at a 1:10 dilution of the test substance, all the cells were comparable with the control cells and at a 1:100 dilution only the 1.2% differed significantly from the control at 1 hour, but increased with 45,246% at 24 hours after exposure to the test substance, see figures 3 and 4. A possible reason for the discrepancy at 1 hour after exposure may be the beginning cell density at this concentration was too low as can be seen in the crystal violet photo documentation. All cells showed proliferative capacity with no detrimental effects to be observed because of the test substance.

The results for the NR assay in this study indicated that there were no significant differences between the test and control cells for any of the concentrations at both dilutions except for the 2.3% at 24 hours for the 1:10 dilution, see figure 14. The significant difference at this concentration is due to the high proliferation rate of the cells at this concentration ( $108.56\% \pm 3.492084$ ). At this concentration there were significantly more cells that could take up the viability dye than that of the control cells because of cell proliferation at 24 hours for the 1:10 dilution (see MTT cell proliferation assay). No other cellular damage was seen in the cells.

For the CV assay there was a significant difference between the test and control cells of the 0.8% at 24 hours at a 1:10 dilution. There was a decrease in cell number from 1 hour to 24 hours but there were still more cells present than in the control cells ( $104.90 \pm 2.86$ ), see figure 15. These results also correlate with the MTT cell proliferation assay where there was also a cell proliferation decrease at 1:10 for the 0.8% at 24 hours. The reason for these reductions are unclear at this concentration, nevertheless the results are still indicative of a non-toxic effect of hyaluronic acid at this concentration. There was also a significant difference for the 1.0% cells at the 1:100 dilution at 24 hours, there were considerably more cells present at 24 hours after exposure to the test substance ( $114.59 \pm 6.78$ ), see figure 16. With regards to the staining of the cells with crystal violet, a very noteworthy increase in cell number could be seen at a 2.3% when comparing the test and control cells at all times. These two results correlate with a study done by Givens *et al* in 1990 that found that hyaluronic acid promotes the proliferation of human corneal fibroblasts (Givens *et al.*, 1990).

From the morphology of the cells stained with Crystal violet, intact oval nuclei can be distinguished in most cells, cells are spindle shaped with no indication of cell membrane damage, collagen deposition took place at some of the cells exposed to the BHA indicative of normal cellular function and metabolism.

In order for the cells to survive in a cell culture the following is required: the correct temperature, a good substrate for attachment and the correct culture medium (Ryan, 2008). The medium provides the essential nutrients and growth factors, maintains the correct osmolality and pH and also provides a mean whereby vital gas exchange ( $\text{CO}_2$  and  $\text{O}_2$ ) can take place. The hygroscopic nature of hyaluronic acid permits it to absorb any fluid, especially water, into its matrix like structure. Therefore, as could be seen in this study, the culture medium of all the undiluted hyaluronic acid test samples (0.8%-2.3%) was absorbed by the polymer. The results of these samples could thus not be further used in the study. This finding corresponds with other studies in which it was found that hyaluronic acid promotes cell proliferation and migration (Givens *et al.*, 1990; Gomes *et al.*, 2004).

Except for the differences found and explained, the overall outcome of the study showed that hyaluronic acid at all five concentrations (0.8%-2.3%) were not cytotoxic at a 1:10 or 1:100 dilution at 1 or 24 hours. These results correlate with several studies that have shown the advantageous effect of hyaluronic acid on cell cultures. In a study done by Nguyen *et al.*

several OVD's namely Healon® (1% Hyaluronic acid), Amvisc® (3% hyaluronic acid) and Viscoat® (4% chondroitin sulphate and 3% Hydroxypropylmethylcellulose) were found not to be cytotoxic when tested on bovine CEC (Nguyen *et al.*, 1992). All of the OVD's in their study displayed a protective effect on the CEC and this was confirmed in another study done by Glasser *et al* in 1986. In examining the morphological features in the living CEC after the installation of 1% hyaluronic acid it was also concluded that the substance is not cytotoxic to these cells (Glasser *et al.*, 1986).

Interestingly enough in study done by Bahn *et al.* 1% hyaluronic acid showed to have an advantageous effect on the rejection of corneal homografts: the rejection was somewhat less in grafts that were pre-coated with 1% hyaluronic acid (Bahn *et al.*, 1986). Possible reasons for this was postulated to be that hyaluronic acid can block cell surface antigens, inhibit macrophage phagocytosis, inhibit neutrophil adhesion and locomotion, and inhibit active rosette forming T lymphocytes and lymphocyte mediated cytotoxicity (Bahn *et al.*, 1986).

Hyaluronic acid is also used in several ophthalmic preparations intended to deliver drugs to the surface of the eye – it protects the eye against dryness by increasing tear film stability and thereby reducing grittiness and burning on the surface of the eye (Debbasch *et al.*, 2002).

Cytotoxicity studies such as this one can be used to investigate the suspected cytotoxic effect of any OVD destined to reach the ophthalmic market. With these simple colorimetric and qualitative tests, cell morphology and cell growth can be determined and be indicative of the possible toxic effects of OVD's. These tests are less expensive and less hazardous than *in vivo* testing. There is however no *in vitro* method that will ever replace the true effect of a substance in a live organism that can mimic the reactions of the true hormonal and cellular components and interactions in the body. Therefore the effect of the potential OVD, BHA, were also tested in two *in vivo* systems, the rabbit and guinea pig, in three separate tests to further determine the safety of the BHA.

## CONCLUSION

The purpose of this study was to evaluate the effect of the ophthalmic viscosurgical device, BHA, on human fibroblasts. Colorimetric and morphological evaluations were done to assess the cytotoxic potential of BHA and from the results it can be concluded that BHA does not have a detrimental effect on fibroblast cell cultures and is therefore not regarded as cytotoxic.

## CELL CULTURE PHOTODOCUMENTATION

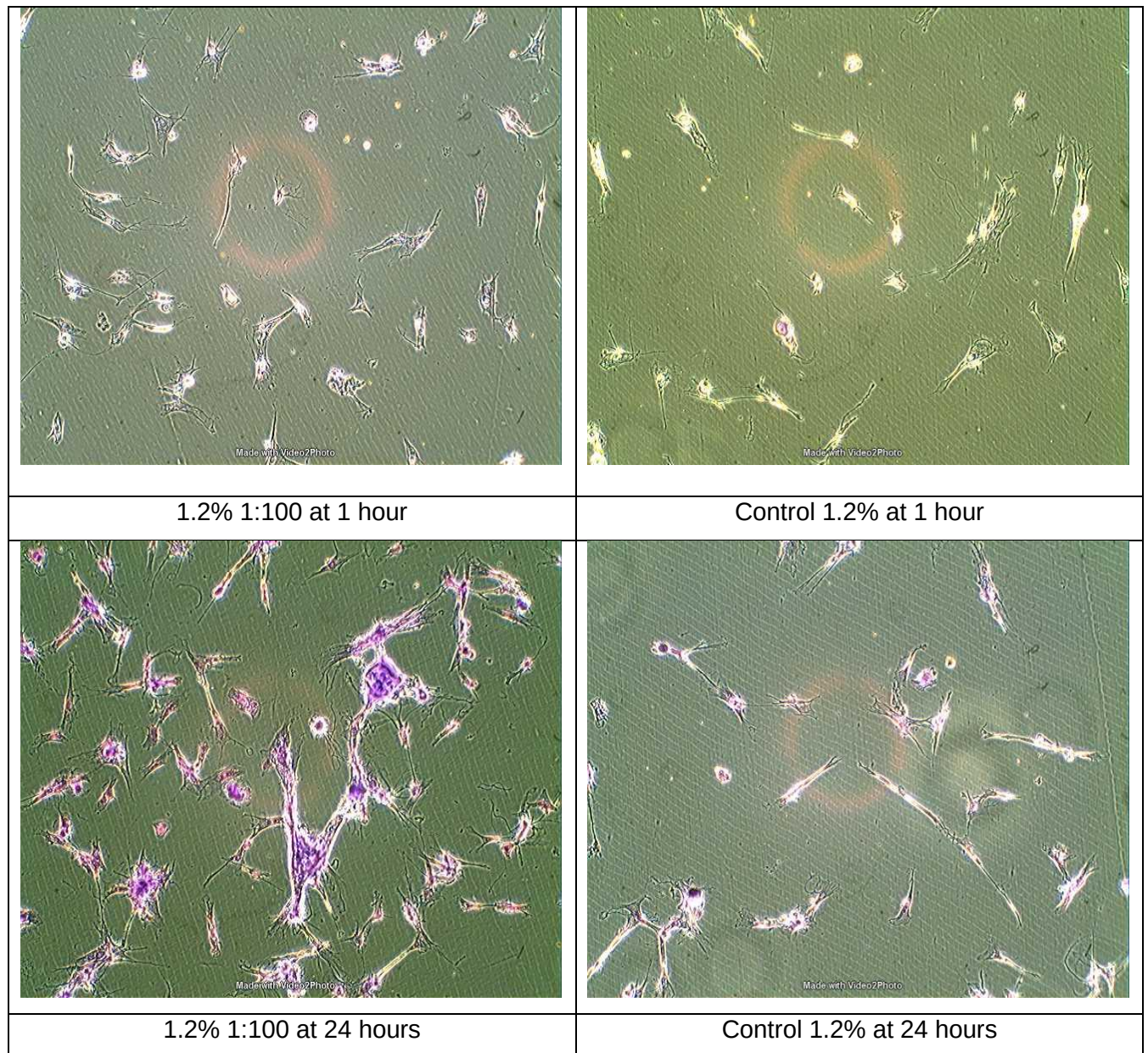


**Figure 13.** Crystal violet stains of the test and control cells of 1.2% of a 1:100 dilution at 1 and 24 hours.

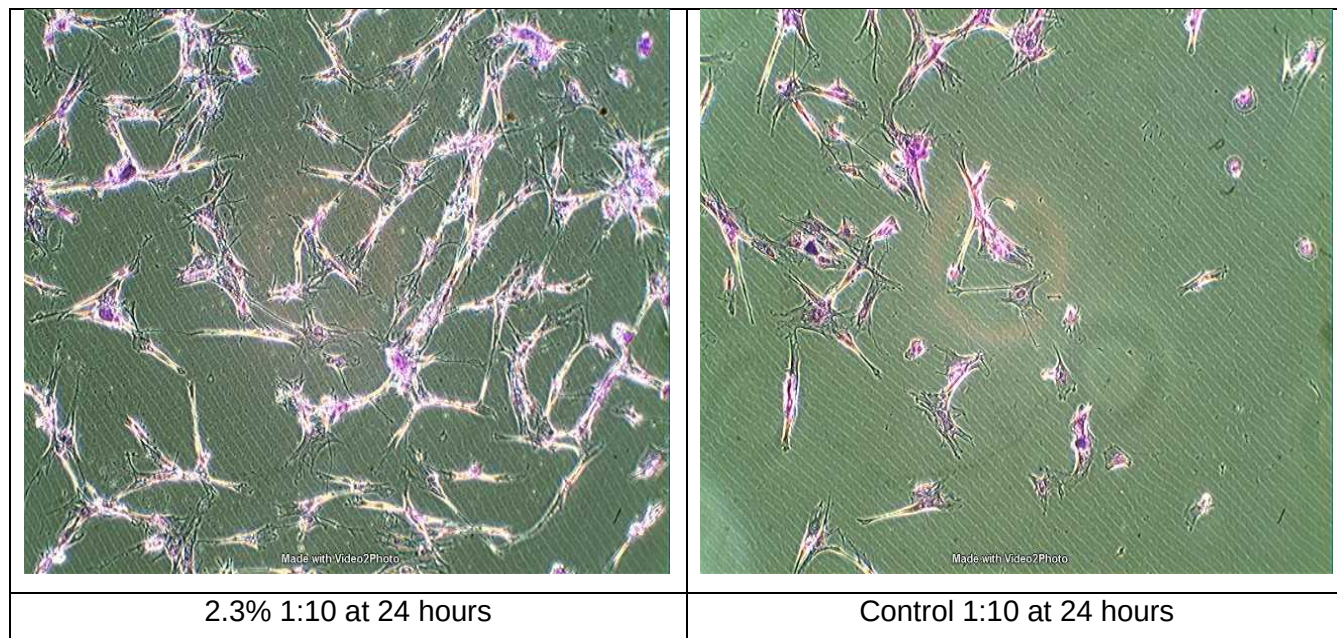


**Figure 14.** Crystal violet stains of the test and control cells of 2.3% of a 1:10 dilution at 24 hours.

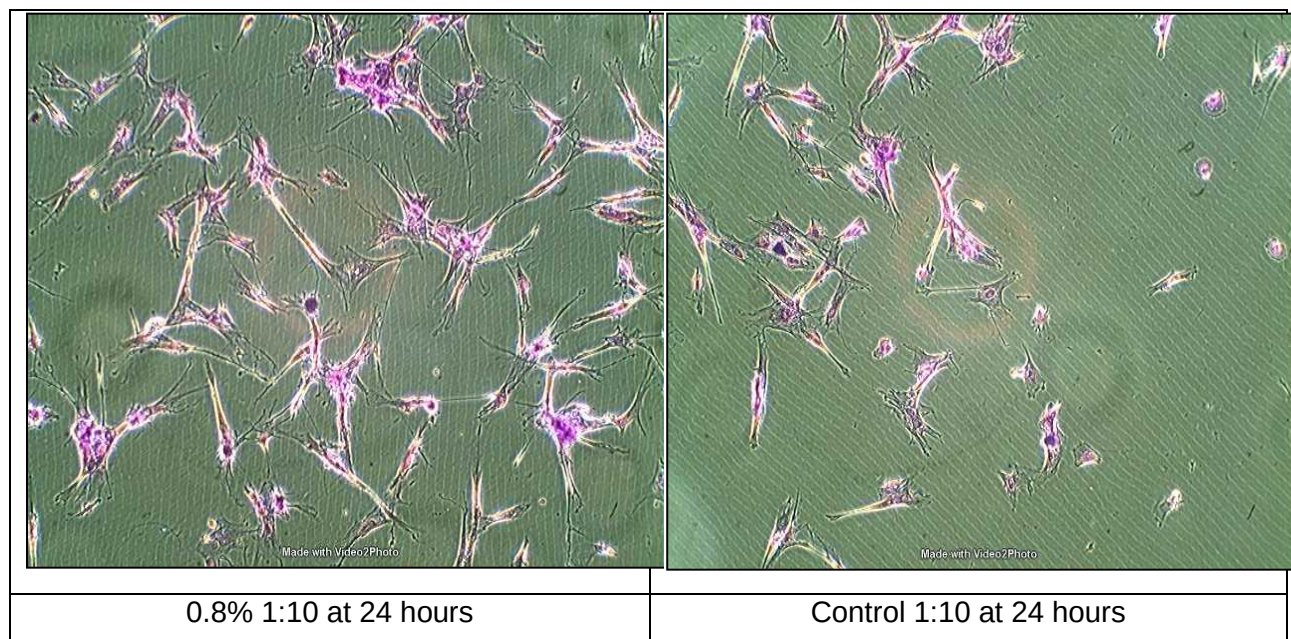


**Figure 15.** Crystal violet stains of the test and control cells of 0.8% of a 1:10 dilution at 24 hours.

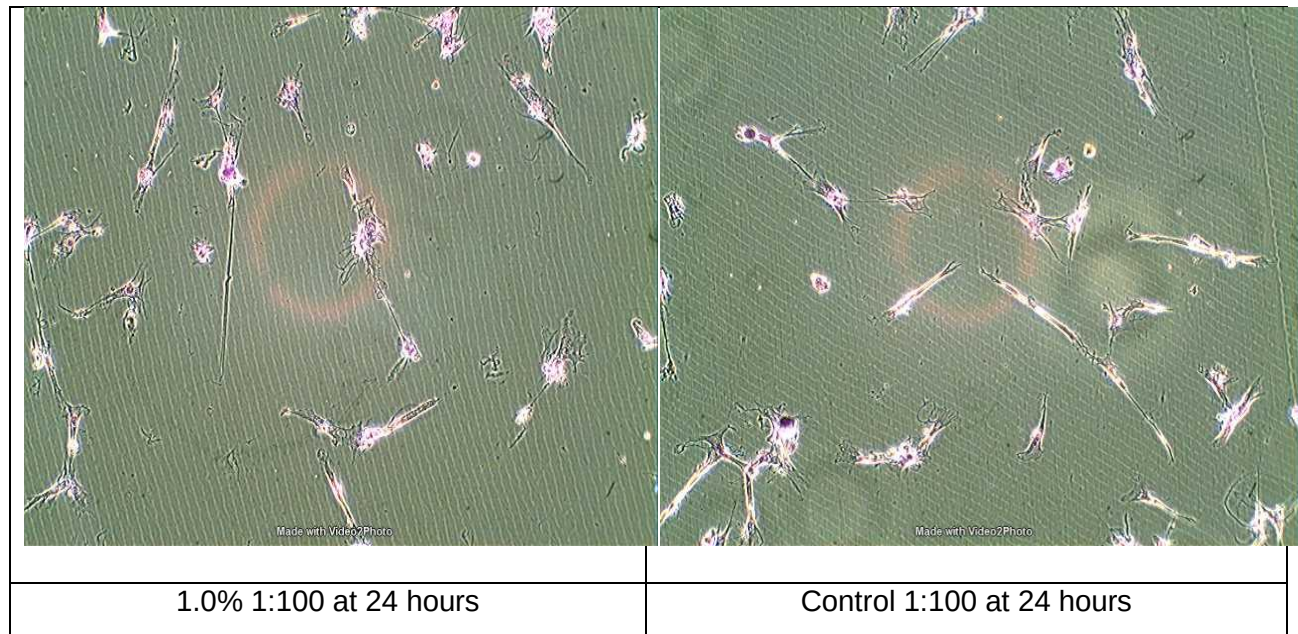


**Figure 16.** Crystal violet stains of the test and control cells of 1.0% of a 1:100 dilution at 24 hours.

## CHAPTER 4

# THE GUINEA PIG MAXIMIZATION TEST FOR TESTING DELAYED HYPERSENSITIVITY

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### INTRODUCTION

According to the European Union, before any new substance can be approved for human use, it must be tested for its potential to cause skin sensitization. According to this legal requirement if a substance is found to cause notable skin sensitization it should be labeled with a St Andrew's cross and must contain the R43 risk phrase 'May cause sensitization by skin contact' (Basketter *et al.*, 1995a).

Sensitization tests exploits the hypersensitivity type I (IgE mediated) cascade elicited in the skin when it is introduced to an allergen. In short, the cascade works as follows: the allergen enters the body and binds to antibodies presented on the B cells (antigen presenting cells) of the immune system. The antigen is internalized by the B-cells and is then presented on the MHC II receptor of the B cell. T helper cells class II ( $T_H2$ ) then binds to the MHC II receptor via its T-cell surface receptor and CD4 marker thereby activating the B cell to differentiate into IgE-secreting plasma cells (see figure 1). The first type of differentiated plasma cell is called a memory B cell; it presents the antibodies for the specific type of allergen on its surface so as to be prepared for re-exposure to the allergen. The second type of differentiated plasma cell secretes allergen specific IgE antibodies that then bind to mast cells and basophils in the blood. The mast cells are then sensitized and presents these antibodies, bound to its IgE specific Fc receptors ( $Fc\epsilon RI$ ), on its surface (see figure 2). When the body is exposed to the allergen again, the allergen crosslinks with these presented antibodies on the Fc receptors of mast cells and basophils. This activates the release of pharmacologically active mediators (vasoactive amines) causing an "allergic reaction" (Kindt *et al.*, 2007). Based on this principle sensitization tests can utilize different animal models and expose them to repeated dosages of an allergenic test substance thereby eliciting an immune response, whereafter the immune reaction can be measured and quantified as contact dermatitis (Robinson *et al.*, 1996).

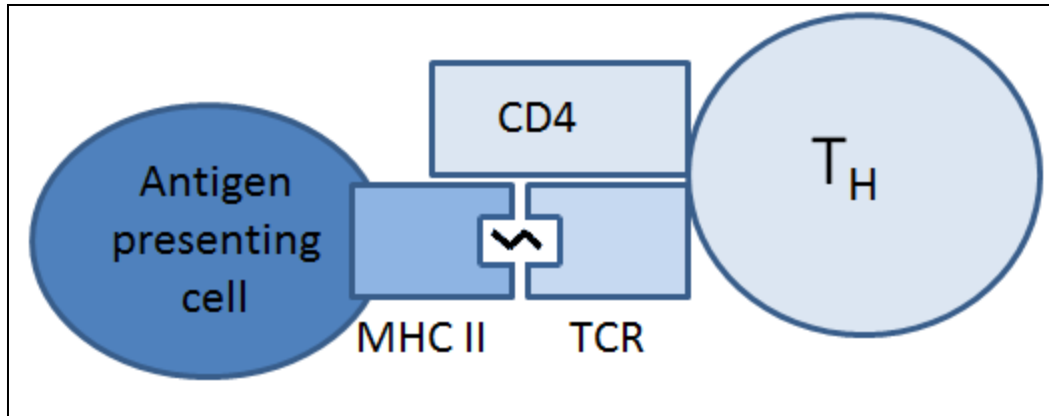


**Figure 1.** An antigen presenting cell (e.g. macrophages, dendritic cells, monocytes, Langerhans cells) presenting an antigen (↯) on its MHC II receptor in contact with a T helper cell ( $T_H$ ) receptor (TCR) containing a CD4 marker (Roitt, 1997).

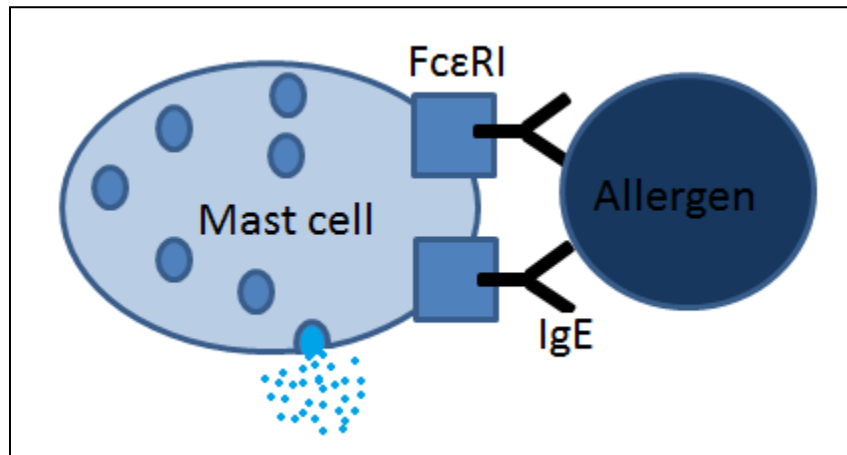


**Figure 2.** A sensitized mast cell presenting the antibody (IgE) on its IgE specific Fc receptors (FcεRI). The crosslinking of the allergen elicited the release of pharmacologically active mediators (vasoactive amines) causing an “allergic reaction” (Kindt *et al.*, 2007).

Several methods exist for identifying the skin sensitization potential of a substance of which seven methods have been incorporated in regulatory guidelines (Botham *et al.*, 1991). These include the Magnusson and Kligman Maximization Test, the Draize test, Freud's Complete Adjuvant Test, Maurer Optimization Test, Buehler Test, Open Epicutaneous Test and the Split Adjuvant Test (Botham *et al.*, 1991). The two most preferred skin sensitization tests, the Magnusson and Kligman Maximization test and the Buehler test, are extensively described by the European Commission (EC, 1993b) and the Organization for Economic Cooperation and Development (OECD) guidelines for skin sensitization (Basketter *et al.*, 1995a).

Of the two methods the Magnusson and Kligman test is considered the more sensitive test method as it includes the use of the sensitizer, Freund's complete adjuvant (FCA), which allows the more accurate detection of a skin sensitizing substance (Momma *et al.*, 1997). Both these methods use guinea pigs which has been historically the animal of choice for the evaluation of allergic contact dermatitis (Steiling *et al.*, 2001). As scientists' understanding of the mechanisms of skin sensitization grew, newer methods could be developed to provide an alternative to the use of guinea pigs (Steiling *et al.*, 2001). The Mouse Ear Swelling Test (MEST) and the Murine Local Lymph Node Assay (LLNA) are two examples of these tests utilizing mice. In some instances these tests provide inadequate results and then still require verification by the guinea pig tests again proving the adequacy of these tests (Steiling *et al.*, 2001).

## THE GUINEA PIG MAXIMIZATION TEST

The Guinea pig maximization test (Magnusson and Kligman method) consists of two phases, the induction phase (intradermal injection step followed by a topical application step), and a challenge phase (topical application step). A rechallenge phase follows if the challenge phase results are inconclusive or positive results were seen in the control animals (Botham *et al.*, 1991). The rechallenge phase should in theory present an opportunity to detect weak sensitizers more accurately as it should result in skin reactions being more evident either in incidence or intensity (Prinsen *et al.*, 1997).

During the induction phase a series of intradermal injections are made with the test substance in combination with FCA which serves as non-specific stimulator of the immune system that enhances the ability of the immune system to react to sensitizing agents upon re-exposure (Botham *et al.*, 1991; Steiling *et al.*, 2001). The initial intradermal induction phase is followed by a topical application phase where an occlusive patch is applied to the injection sites for 48 hours followed by a rest period of 14 days. The challenge phase, another topical application for 48 hours, then follows. When no skin irritation is observed prior to the second topical application phase (challenge phase) the test is further intensified by applying a penetration/irritation enhancer substance (usually sodium lauryl sulphate) in order to provoke a mild to moderate inflammatory reaction to the skin (Prinsen *et al.*, 1997; Steiling *et al.*, 2001). The reason for this substance is to enhance the percutaneous penetration of the test material and thereby increase the bioavailability of the test material (Robinson *et al.*, 1994; Steiling *et al.*, 2001). The skin

reactions are evaluated by a scoring system grading the extent of the sensitization reaction that took place. Based on the scores obtained the potential of a substance to induce a sensitizing reaction can be determined.

In terms of animal numbers the maximization test generally requires 30 animals, 20 in the test group, and 10 control animals. Despite the recommended number of animals by the OECD guidelines, it is generally agreed that less animals can be used to prove skin sensitization when a substance is expected to be a hazardous substance (Maurer, 2007; Steiling *et al.*, 2001). In this case, considering animal rights issues and if no other *in vitro* method can be used, using less animals should be considered. Provided that not less than 10 animals in the test group and 5 animals in the control group are used, studies concluded that fewer animals will have little effect on the outcome of the study (Botham *et al.*, 1991; Steiling *et al.*, 2001).

Hyaluronic acid is a glycosaminoglycan consisting of two basic sugar units namely glucuronic acid and *N*-acetylglucosamine (Price *et al.*, 2007). The sugars are arranged into long chains of over 30 000 repeating units to form large macromolecules which make up large amounts of the extracellular matrix of the skin (Prince *et al.*, 2007). Hyaluronic acid is found right throughout the body but especially the eye, umbilical cord and synovial fluid. Hyaluronic acid has a vast majority of applications in the cosmetic world (eye lotions etc.) and in the medical field (such as drug delivery) (Becker *et al.*, 2009). The utilization as an ophthalmic viscosurgical device (OVD) is one of the most important uses in the medical field as OVD's changed the course of ophthalmic surgery forever. As an OVD, one of its many functions is to serve as a space filler in the anterior chamber during the removal of cataracts and also to protect the delicate corneal endothelial cells (Prince *et al.*, 2007). Hyaluronic acid is usually mixed with a suitable buffer agent (such as phosphate buffered saline) which is responsible for its conformational stiffness of the OVD, in other words, it determines the formation of the thickness of the hyaluronic acid gel (see figure 3).



**Figure 3.** Hyaluronic acid gel formation when buffer is applied (Price *et al.*, 2007).

The purpose of this study was to evaluate the potential of a buffered hyaluronic acid (BHA), to be utilized as an OVD, to produce skin sensitization in the guinea pig using the technique applied for single chemicals in the guinea pig maximization test. The study consisted of a preliminary test and a main test. During the preliminary test the concentration at which the test substance would be used was determined.

## **MATERIALS**

BHA was supplied by Southern Medical (Pty) Ltd, Temgesic was supplied by the UPBRC, Saline and Vaseline was obtained from Dischem, Pretoria, South Africa. Freud's complete adjuvant (F5881) and Sodium lauryl sulphate (L4509) was obtained from Sigma-Aldrich (Pty) Ltd, Midrand, South Africa. Syringes, needles, gauze, occlusive patches and Opsite transparent wound covering were supplied by the UPBRC.

## **METHODS**

The test was conducted according to the maximization method as described by the OECD guideline no. 406 (1992). Thirty female albino guinea pigs of single strain aged between 21 and 67 days weighing between 300g and 500g were used. None of the animals were used in

previous studies. The animals were kept in isolation, housed in groups of three per cage with a in a room with a 12-hour light/dark cycle at a temperature of 17-24°C and humidity of between 30-60%. The room was equipped with full spectrum fluorescent lights so that accurate observations could be made of the test subjects. The cages were enriched with cardboard tunnels, autoclaved pinecones and wood sticks. The animals were fed irradiated Epol rabbit pellets, *ad libitum*, Vitamin C supplement (250mg), Teff, Lucern, Carrots and spinach. Water was given *ad libitum*. The animals were kept in identical conditions as for the actual test for 5 days before commencing the test. Before commencing the test the fur of the animals were shaved and clipped closely on all treatment sites on the upper back region in an area of about 3x4 cm. The animals were weighed before dosing.

### **Preliminary test**

During the preliminary test three animals were used in order to determine the concentration of hyaluronic acid that will be used in the main test. A range of hyaluronic acid dilutions (0.8%, 1.0%, 1.2%, 1.8% and 2.3%) was topically applied to the upper back region of the guinea pigs and was then closed with an occlusive dressing as follows:

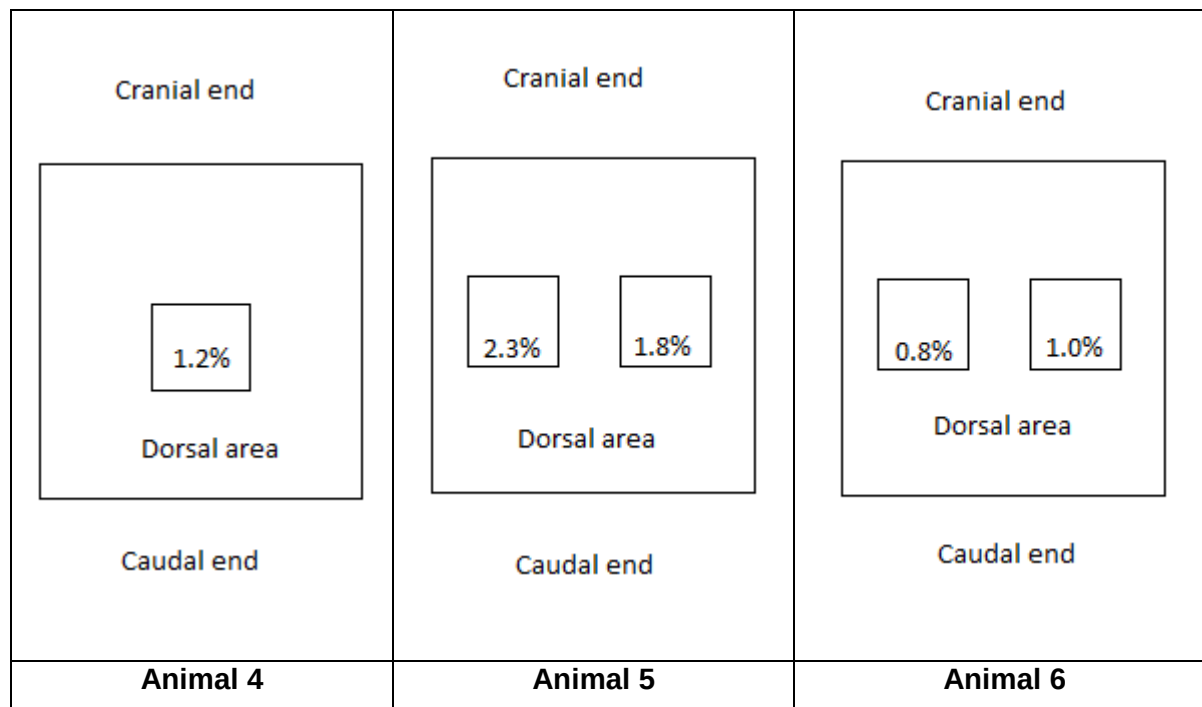


**Figure 4.** Topical application sites of test concentrations of hyaluronic acid.

The occlusive patches were removed after 24 hours and the application sites were assessed for erythema and oedema using the Magnusson and Kligman grading scale, see table 1.

### Induction phase

For the induction phase three pairs of 0.1ml intradermal injections of each of the following, were made into each animal, at the injection sites as seen below:

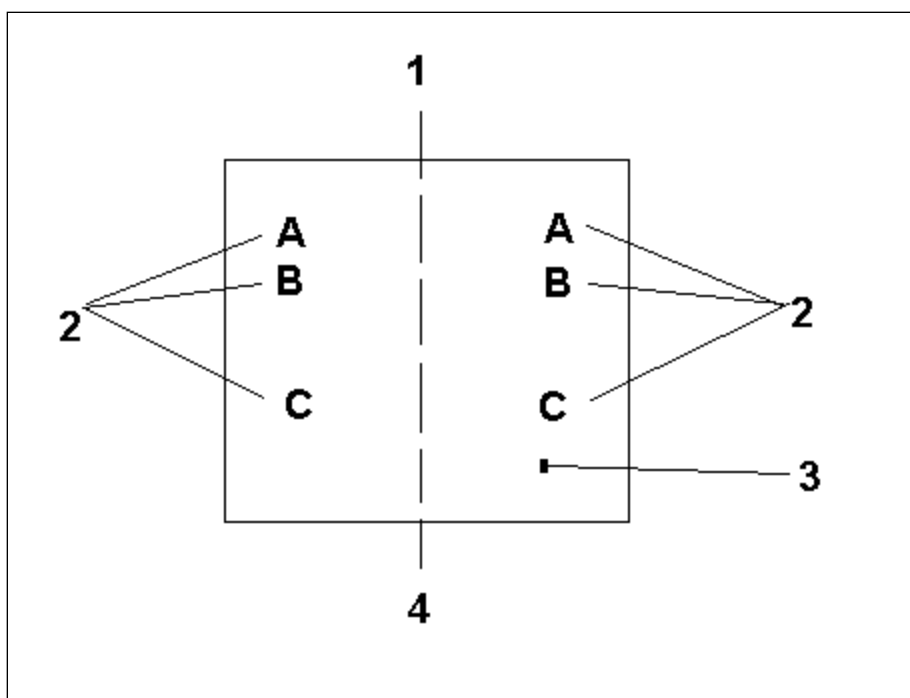


**Figure 5.** Injection sites for guinea pig maximization test. 1):Cranial end, 2): 0.1ml injection sites, 3): Clipped dorsal area, 4): Caudal end. **For the test animals:** Site A): A 50:50 (volume ratio) stable emulsion of FCA mixed with the chosen solvent, saline. Site B): The test sample (undiluted extract); inject the control animals with the solvent alone. Site C): The test sample at concentration used at site B, emulsified in a 50:50 volume ratio stable emulsion of FCA and the solvent (50%). **For the control animals:** Site A): A 50:50 (volume ratio) stable emulsion of FCA mixed with the chosen solvent, saline. Site B): The undiluted vehicle (saline). Site C): A 50% w/v formulation of the vehicle in a 1:1 mixture (v/v) FCA/saline.

Temgesic (0.8ml, every 8 hours) were administered as pain medication to all the animals to control pain. The animals were left to rest for 7 days before the topical induction phase. One day prior to the topical induction phase, the injection sites were clipped free of fur and evaluated. The test area was then painted with 0.5ml of a 10% sodium lauryl sulphate in Vaseline in order to create local irritation. The test substance was then administered by topical application to the upper back area of each animal using a patch of approximately  $2 \times 4 \text{ cm}^2$  (absorbent gauze soaked in the test substance covered by Opsite), so as to cover the intradermal injection sites. The occlusive dressings were removed by the guinea pigs during the application thereof. It was decided that the substance would be applied 4 hourly for the remaining duration of the test in order to create continuous exposure of the chemical to the skin. The test substance was washed off after 48 hours with warm water and a non-irritating soap. At 14 days after completion

of the topical induction phase, all the test and control animals were challenged with the test substance.

### Challenge phase

A day prior to the commencement of the challenge phase the fur was clipped from the back and sides with an electric clipper and excess hair were removed with a razor, care was taken not to damage the skin. The test substance was administered by topical application to sites that were not treated during the induction stage. Throughout the study, 0.2ml of the test substance was applied to lower back region of the animals. The test substance was applied every 4 hours for 24 hours and was then washed off with warm water and a non-irritating soap. Skin sites were evaluated at 24 and 48 hours after removing the test substance of the challenge phase and scored according to the Magnusson and Kligman scale, table 1. Erythema scores of 1 or more were considered a positive response. Scoring was done in full-spectrum lighting in order to visualize the skin reactions. Animals were weighed at the end of the observation period and their weights recorded. The animals were then sacrificed by carbon dioxide inhalation. For this test the animals were distributed as follows: three animals in the preliminary group, 20 animals in the experimental group and 6 animals in the control group.

**Table 1.** The Magnusson and Kligman scale for scoring skin responses in the maximization test.

| Magnusson and Kligman scale |                                |
|-----------------------------|--------------------------------|
| 0                           | No visible change              |
| 1                           | Discrete or patchy erythema    |
| 2                           | Moderate or confluent erythema |
| 3                           | Intense erythema and swelling  |

A sensitization response/incidence is the number of animals showing a response of 1 or more, at 24 and 48 hours divided by the number of test animals.

## Facility and ethical clearance

The test was conducted at the UPBRC research Centre, Onderstepoort, South Africa. All procedures were approved by the animal use and care committee (AUCC), a subcommittee of the Committee for Research Ethics and Integrity of the University of Pretoria.

## RESULTS

### Preliminary test

During the preliminary test no skin reactions were seen with any of the test animals. Table 2 shows the skin score results of the five injection sites.

**Table 2.** Preliminary test results.

| <b>Hyaluronic acid concentration</b> | <b>Score</b> | <b>Comments</b> |
|--------------------------------------|--------------|-----------------|
| 0.8%                                 | 0            | No reaction     |
| 1.0%                                 | 0            | No reaction     |
| 1.2%                                 | 0            | No reaction     |
| 1.8%                                 | 0            | No reaction     |
| 2.3%                                 | 0            | No reaction     |

No skin sensitization reactions were seen in any of the concentrations used for viscosurgical devices.

**Table 3.** Animal weights of the preliminary test.

|                      | <b>Body weight (g)</b> |                 |                      |                          |
|----------------------|------------------------|-----------------|----------------------|--------------------------|
| <b>Animal number</b> | <b>23/02/09</b>        | <b>27/02/09</b> | <b>Weight change</b> | <b>Signs of toxicity</b> |
| 4                    | 416                    | 446             | +30                  | None                     |
| 5                    | 387                    | 407             | +20                  | None                     |
| 6                    | 424                    | 439             | +15                  | None                     |

All the animals gained weight throughout the preliminary test period as can be seen in table 3. No adverse reactions or signs of toxicity were seen in any of the animals at any of the concentrations.

### Challenge phase

The challenge phase skin reactions were evaluated using the Magnusson and Kligman scale, Table 1.

The results of the study were graded at “site B” (test substance) by three blinded observers (RP, QW, TP) at 24 and 48 hours (tables 4 and 5 respectively). Animals 1-20 were the test animals, where animal numbers 24-30 were the control animals.

**Table 4.** Test scores of the challenge phase at 24 hours.

| 24 H Challenge |               |       |    |    |
|----------------|---------------|-------|----|----|
|                | Animal Number | Score |    |    |
|                |               | RP    | QW | TP |
| 1              | 511E          | 0     | 0  | 0  |
| 2              | 513           | 0     | 0  | 1  |
| 3              | 619           | 0     | 0  | 1  |
| 4              | 467C          | -     | -  | -  |
| 5              | 577           | -     | -  | -  |
| 6              | 761A          | -     | -  | -  |
| 7              | 70E           | 0     | 0  | 0  |
| 8              | E10           | 0     | 0  | 0  |
| 9              | D3D           | 0     | 0  | 0  |
| 10             | 117           | 0     | 0  | 0  |
| 11             | C0E           | 0     | 0  | 0  |
| 12             | 440A          | 0     | 0  | 0  |
| 13             | 436           | 0     | 0  | 1  |
| 14             | 82F           | 0     | 0  | 0  |
| 15             | 834           | 0     | 0  | 0  |
| 16             | DIED          | -     | -  | -  |

|    |      |   |   |   |
|----|------|---|---|---|
| 17 | 707D | 0 | 0 | 0 |
| 18 | 753  | 0 | 0 | 0 |
| 19 | 71F  | 0 | 0 | 0 |
| 20 | 618  | 0 | 0 | 0 |
| 21 | 31E  | 0 | 0 | 0 |
| 22 | 67F  | 0 | 0 | 0 |
| 23 | E29  | 0 | 0 | 0 |
| 24 | 22E  | 0 | 0 | 0 |
| 25 | 435  | 0 | 0 | 0 |
| 26 | A38  | 0 | 0 | 0 |
| 27 | 744  | 0 | 0 | 0 |
| 28 | 576  | 0 | 0 | 0 |
| 29 | 653  | 0 | 0 | 0 |
| 30 | 700  | 0 | 0 | 0 |

The photo documentation of the test results for the challenge phase at 24 hours can be seen in table 8 at the end of this chapter.

**Table 5.** Test scores of the challenge phase at 48 hours.

| 48 H Challenge |               |       |    |    |
|----------------|---------------|-------|----|----|
|                | Animal Number | Score |    |    |
|                |               | RP    | QW | TP |
| 1              | 511E          | 0     | 0  | 0  |
| 2              | 513           | 0     | 0  | 0  |
| 3              | 619           | 0     | 0  | 0  |
| 4              | 467C          | -     | -  | -  |
| 5              | 577           | -     | -  | -  |
| 6              | 761A          | -     | -  | -  |
| 7              | 70E           | 0     | 0  | 0  |
| 8              | E10           | 0     | 0  | 0  |
| 9              | D3D           | 0     | 0  | 0  |
| 10             | 117           | 0     | 0  | 0  |
| 11             | C0E           | 0     | 0  | 0  |

|    |      |   |   |   |
|----|------|---|---|---|
| 12 | 440A | 0 | 0 | 0 |
| 13 | 436  | 0 | 0 | 0 |
| 14 | 82F  | 0 | 0 | 0 |
| 15 | 834  | 0 | 0 | 0 |
| 16 | DIED | - | - | - |
| 17 | 707D | 0 | 0 | 0 |
| 18 | 753  | 0 | 0 | 0 |
| 19 | 71F  | 0 | 0 | 0 |
| 20 | 618  | 0 | 0 | 0 |
| 21 | 31E  | 0 | 0 | 0 |
| 22 | 67F  | 0 | 0 | 0 |
| 23 | E29  | 0 | 0 | 0 |
| 24 | 22E  | 0 | 0 | 0 |
| 25 | 435  | 0 | 0 | 0 |
| 26 | A38  | 0 | 0 | 0 |
| 27 | 744  | 0 | 0 | 0 |
| 28 | 576  | 0 | 0 | 0 |
| 29 | 653  | 0 | 0 | 0 |
| 30 | 700  | 0 | 0 | 0 |

The incidence of the responses of the challenge phase was calculated as follows:

**Table 6.** Incidence of the skin responses of the challenge phase at 24 and 48 hours.

|                                           | TEST       |            | CONTROL    |            |
|-------------------------------------------|------------|------------|------------|------------|
| <b>Amount of animals</b>                  | 19         |            | 7          |            |
| <b>Number of observers</b>                | 3          |            | 3          |            |
|                                           | <b>24h</b> | <b>48h</b> | <b>24h</b> | <b>48h</b> |
| <b>Combined incidence</b>                 | 3          | 0          | 0          | 0          |
| <b>Total scores</b>                       | 57         | 0          | 0          | 0          |
| <b>% Incidence<br/>(Incidence/Scores)</b> | 5.3%       | 0%         | 0%         | 0%         |

A slight skin irritation was seen after the application of Sodium dodecyl sulphate at the application site before the start of the challenge phase. From table 6 it can be deduced there was a 5.3% response to the test article as observed by one observer at 24 hours after the challenge phase. No reactions were seen in the control animals and no adverse skin reactions were seen at 48H after the challenge phase.

Temgesic (0.8ml, every 8 hours) were administered as pain medication to all the animals to control pain that were caused by the lesions as a result of the positive control, FCA. The pain medication was administered the first three days of this test phase.

The study did not require a rechallenge phase. Only one animal (16) died under anesthetic, all the other animals survived the study. All animals gained weight throughout the rest of the study as can be seen in table 7.

**Table 7.** Animal weights for the test period.

| Group | Animal # | Weight change (g) |                    |                   | Signs of toxicity |
|-------|----------|-------------------|--------------------|-------------------|-------------------|
|       |          | Day 0<br>13/02/09 | Day 31<br>24/03/09 | Difference<br>(g) |                   |
| Test  | 1        | 344               | 598                | +254              | None              |
|       | 2        | 323               | 553                | +230              | None              |
|       | 3        | 323               | 543                | +220              | None              |
|       | 4        | 335               | 606                | +271              | None              |
|       | 5        | 300               | 540                | +240              | None              |
|       | 6        | 331               | 598                | +267              | None              |
|       | 7        | 336               | 714                | +378              | None              |
|       | 8        | 339               | 627                | +288              | None              |
|       | 9        | 347               | 612                | +265              | None              |
|       | 10       | 306               | 563                | +257              | None              |
|       | 11       | 310               | 546                | +236              | None              |
|       | 12       | 341               | 561                | +220              | None              |
|       | 13       | 350               | 643                | +293              | None              |
|       | 14       | 355               | 557                | +202              | None              |

|                |    |     |     |      |      |
|----------------|----|-----|-----|------|------|
|                | 15 | 343 | 583 | +240 | None |
|                | 16 | 329 | 0   | 0    | None |
|                | 17 | 322 | 556 | +234 | None |
|                | 18 | 325 | 500 | +175 | None |
|                | 19 | 345 | 622 | +277 | None |
|                | 20 | 316 | 531 | +215 | None |
| <b>Control</b> | 21 | 331 | 660 | +329 | None |
|                | 22 | 347 | 542 | +195 | None |
|                | 23 | 330 | 667 | +337 | None |
|                | 24 | 327 | 529 | +202 | None |
|                | 25 | 298 | 642 | +344 | None |
|                | 26 | 337 | 633 | +296 | None |
|                | 27 | 346 | 581 | +235 | None |
|                | 28 | 330 | 635 | +305 | None |
|                | 29 | 322 | 619 | +297 | None |
|                | 30 | 322 | 562 | +240 | None |

No overt signs of toxicity were evident in any of the animals during the course of the study.

## DISCUSSION

In the cosmetic and medical industry the prediction of the potential of chemicals to produce allergic contact dermatitis are very important in order to protect the users of the end products as well as to protect health workers in their occupation (Maurer, 2007).

During ophthalmic surgeries utilizing OVD's hyaluronic acid used in the solution comes into contact with the most delicate structures of the eye, the corneal endothelial cells. It is therefore of the utmost importance to rule out every possible detrimental effect that the substance might have such as being a sensitizing agent. Because the hyaluronic acid used in the OVD utilized in the study is produced by bacterial cultures every, batch differs and the production methods and sterilization techniques will have a tremendous influence on the purity of the chemical to which the eye is exposed.

The guinea pig maximization test is by far the most sensitive of the sensitization tests in that it includes the intradermal injection of the test substance, thus immediate biocompatibility within the skin, and it uses an adjuvant to increase the sensitization response (Bronaugh *et al.*, 1994). The inclusion of FCA (positive control substance) ensures the enhancement of the immune system in the first phase of the test (Maurer, 2007). FCA non-specifically stimulates the immune system thereby ensuring a higher sensitivity for antigens (Botham *et al.*, 1991; Steiling *et al.*, 2001). This is exploited when re-exposure to the chemical in question is evaluated for an allergic reaction. In a comparative study done by Basketter and Chamberlain in 1995, the authors demonstrated that the Guinea Pig Maximization Test effectively demonstrates the sensitization potential of 25 human contact allergens by comparing human and guinea pig studies (Basketter *et al.*, 1995b). Therefore test results obtained from the maximization test can be considered a dependable source in its indication of the test substance sensitizing potential.

Young adult guinea pigs weighing between 300-500g are used for this test because as their body weight increases with age there is a reduction in their thymus activity resulting in the immune system being less sensitive (Maurer, 2007).

The preliminary test was carried out in order to determine the concentration of the test substance that will not cause systemic toxicity, local necrosis, ulceration or excessive dermal irritation. The choice for the range of hyaluronic acid concentrations for the preliminary test was based on the fact that viscosurgical devices are currently manufactured in concentrations ranging from 0.8% up to a concentration of 2.3%. There were no adverse reactions seen at any of the preliminary test concentrations. The BHA is intended to be used as a cohesive viscosurgical device at a concentration of 1.2%. Therefore this specific concentration was chosen as the test substance concentration for the remainder of the test.

Before the challenge phase BHA was found not to be an irritant as no positive results were seen, therefore the test area was painted with 0.5ml of a 10% sodium lauryl sulphate in Vaseline in order to create local irritation and increase permeation of the test substance. This is also necessary to ensure that weak sensitizing agents which penetrate the skin with more difficulty can now also be incorporated into the skin to reach the Langerhans cells responsible for activating skin cytokines related to the immune reaction (Steiling *et al.*, 2001). In order for a substance to cause allergic contact dermatitis it must penetrate the outer corny layer, the

stratum corneum of the skin (Kimber *et al.*, 2002). The purpose of the SLS in the study is thus to increase the bioavailability of the test substance (Steiling *et al.*, 2001).

Allergic contact dermatitis can only take place after previous exposure to a certain substance (Buehler *et al.*, 1994). The immune system is sensitized by the substance's interaction with the Langerhans cells situated in the suprabasal epidermis areas (Maurer, 2007). Langerhans cells carries surface receptors for antigens and complement molecules and are "active participants in cutaneous delayed hypersensitivity" (Stedman, 2000). The Langerhans cells internalize foreign antigens when they are encountered in the body and then undergo maturation as they migrate to the lymph nodes where they present the antigen to the T-cells of the immune system (Kimber *et al.*, 1998). For an immune reaction to occur several intricate interactions between cells and their immediate environment must take place. Different cytokines are required for each of the steps required in the allergic reaction namely, the migration of the Langerhans cells (immature dendritic cells) to the lymph nodes via the lymphatic system, the maturation of the Langerhans cells, the formation of the mature Langerhans cell/T-helper cell complex and then the migration of the T-cells and their subsequent release of inflammatory mediators (Kimber *et al.*, 2004). The T-helper cells involved in allergic contact dermatitis secrete the cytokines responsible for the initiation and maintenance of IgE antibody responses and those for the stimulation of the immediate type allergic reactions, which include Interleukin (IL)-4, IL-5, IL-9, IL-10 and IL-13 (Kimber *et al.*, 2002). After the effector T-cells released the relevant cytokines intricate cell-matrix interactions occur that results in an inflammatory reaction that presents as an eczematous rash, which is red, inflamed, dry and scaly and is sometimes associated with a discharge depending on the extend of the reaction (Kimber *et al.*, 2002; Krieger *et al.*, 2010).

During the challenge phase an incidence of 5.3% positive reaction was seen by one observer at 24 hours after the challenge phase started. According to the EU for the guinea pig adjuvant assays, a test substance with a score of 30% or more in the challenged group is considered a sensitizing agent (Steiling *et al.*, 2001). It can therefore be concluded that the test substance, BHA in this case to be used as an OVD, is not considered a sensitizing agent. This is consistent with several skin sensitization studies done on hyaluronic acid (Becker *et al.*, 2009). Hyaluronic acid derived from human umbilical cord and from the *Streptococcus aureus* were tested on rabbits and no allergic reactions were seen in the animals (Becker *et al.*, 2009). In one of the studies Hartley guinea pigs were used to compare the sensitization by non-crosslinked, SupArtz, and crosslinked hyaluronic acid, Synvisc. It was concluded at the end of

the study that neither crosslinked or non-crosslinked hyaluronic acid caused a passive allergic response at any concentration tested, as was the findings for non-crosslinked hyaluronic acid in this study (Becker *et al.*, 2009). As Hyaluronic acid is naturally present in the eye and skin as part of the intracellular matrix, the results of this study is consistent and indicative of its non-irritant nature (Tezel *et al.*, 2008).

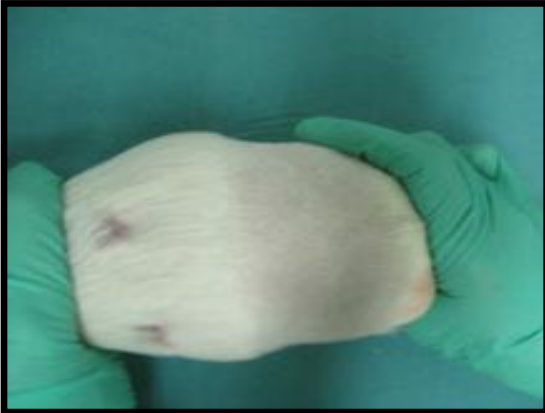
With regard to animal numbers used in the Maximization study, according to Steiling *et al.* a reduction in animal numbers (provided that at least 10 test and 5 control animals are used) will have a 1-2% reduction in the accuracy of the study (Steiling *et al.*, 2001). In this study 16 test animals (excluding the 3 animals used for the preliminary study, and one animal that died) and 10 control animals were used. Because of the small (5.3%) positive reaction to the chemical, a likely 1-2% reduction in accuracy of the study will have no effect on the outcome of the study as a chemical with positive reactions of 30% or more is considered an irritant (Organisation for Economic Cooperation and Development, 1992).

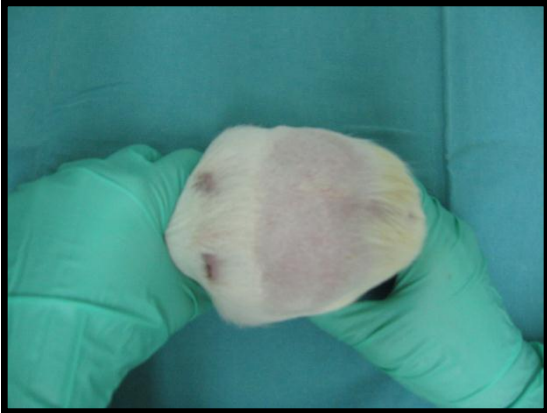
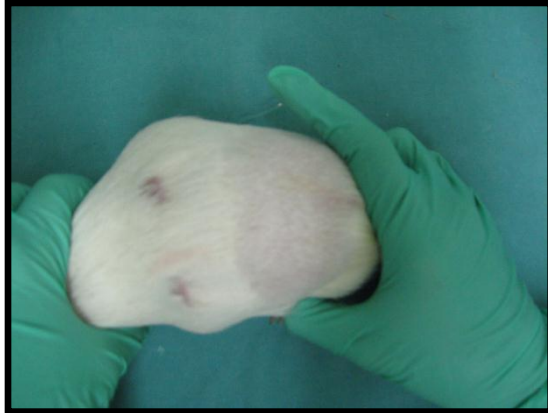
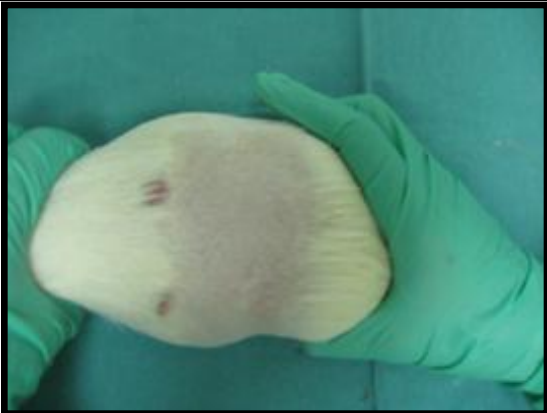
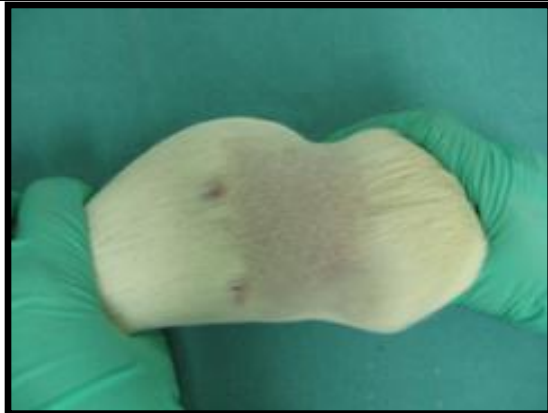
## CONCLUSION

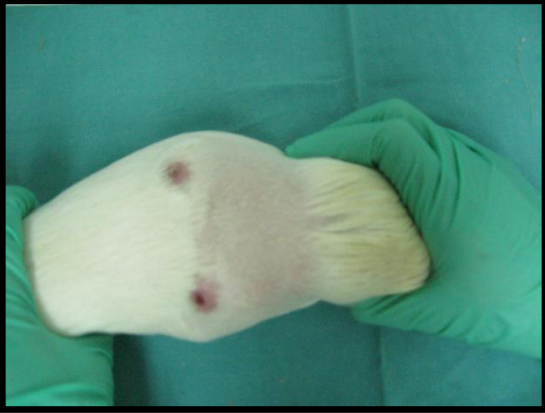
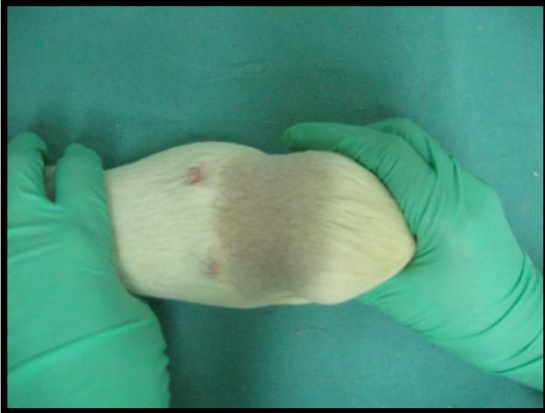
The test article, BHA, was evaluated in this test in order to determine its potential to produce a skin sensitization reaction following intradermal and topical application to albino guinea pigs. As can be seen from the test results, a response of 5.3% was scored by one of the three observers. Substances eliciting responses of more than 30% is considered a sensitizing agent. It can be concluded from the study that the OVD made of Hyaluronic acid, is not considered a skin sensitizer and these results are consistent with previous studies. No reactions were observed in the negative control group.

## GUINEA PIG PHOTO DOCUMENTATION

**Table 8.** Results for the challenge phase at 24 and 48 hours.

| <b>Animal D3D: No positive reaction</b>                                             |                                                                                      |
|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <b>24 hours</b>                                                                     | <b>48 hours</b>                                                                      |
|    |    |
| <b>Animal 511E: No positive reaction</b>                                            |                                                                                      |
| <b>24 hours</b>                                                                     | <b>48 hours</b>                                                                      |
|  |  |

| Animal 513: Positive reaction (TP)                                                  |                                                                                      |
|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| 24 hours                                                                            | 48 hours                                                                             |
|    |    |
| Animal 619: Positive reaction (TP)                                                  |                                                                                      |
| 24 hours                                                                            | 48 hours                                                                             |
|   |   |
| Animal 436: Positive reaction (TP)                                                  |                                                                                      |
| 24 hours                                                                            | 48 hours                                                                             |
|  |  |

| Animal 435: Control                                                                |                                                                                     |
|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| 24 hours                                                                           | 48 hours                                                                            |
|   |   |
| Animal 22E: Control                                                                |                                                                                     |
| 24 hours                                                                           | 48 hours                                                                            |
|  |  |

## CHAPTER 5

# A COMPARATIVE STUDY TO DETERMINE THE POST-OPERATIVE INTRAOCULAR PRESSURE DEVELOPMENT IN TWO VISCOSURGICAL DEVICES

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### INTRODUCTION

Intra ocular pressure (IOP) is the pressure of the intraocular fluid within the eye (Stedman, 2000). IOP pressure is created by the aqueous fluid in the eye that maintains the shape of the eye. One of the main risk factors of glaucoma (a degenerative disease of the optic nerve that can cause blindness if left untreated) is an elevated IOP because of a block in the normal outflow patterns of the aqueous humor (AH) of the eye (Pang *et al.*, 2008).

The AH is produced by the ciliary processes of the eye from where it then moves out of the posterior chamber through the pupil into the anterior chamber (Pang *et al.*, 2008). The aqueous humor is secreted via active and passive secretion (Kanski, 1999). Active secretion is responsible for about 80% of the AH secretion and is secreted by the non-pigmented epithelium of the ciliary body and is enzyme dependant. Enzyme systems, like the  $\text{Na}^+/\text{K}^+$  ATPase pump, secreting  $\text{Na}^+$  ions into the posterior chamber is especially important in the secretion of AH. Active secretion is not dependant on the IOP, whereas passive secretion, responsible for the production of the other 20% of the AH, is dependent on the IOP, the blood pressure and the plasma oncotic pressure. Active secretion is only affected when active metabolism is decreased e.g. as in the case of hypoxia or hypothermia, whereas passive secretion can be influenced by anything that has an effect on the IOP, such as: drugs (e.g. beta-blockers), certain surgical procedures and damage to the ciliary body (Kanski, 1999).

The function of the AH in the eye is to maintain the metabolic homeostasis of all the avascular ocular tissues (like the cornea) and to serve as a non-refractive optical medium for light to travel through the eye to the retina (Pang *et al.*, 2008; Young *et al.*, 2000). The pressure balance in the eye is maintained by the AH formation of the ciliary processes and by the AH outflow via the different pathways: the uveoscleral, trabecular and the episcleral venous pressure outflow pathways, see figure 1 (Pang *et al.*, 2008). The trabecular pathway consists of the trabecular

meshwork (TM) and the canal of Schlemm (Pang et al., 2008). The canal of Schlemm is a circumferential canal lined by endothelium situated next to the angle of the anterior chamber at the root of the iris (Young *et al.*, 2000). The meshwork consists of collagen trabeculae containing the cells of the TM with open spaces between the collagenous threads that gives it a mesh-like appearance (Pang et al., 2008). The TM consists of a uveal part (innermost part of cord-like meshes with large intertrabecular spaces), the corneo-scleral part (middle part with sheet-like meshes and spaces smaller than the uveal part), and an endothelial part forming the link between the corneo-scleral part and the endothelium of the canal of Schlemm (Kanski, 1999). The endothelial part provides for the most resistance against the outflow of the AH. The AH drains non-specifically, as there is no definite communication channel between the meshwork and the canal of Schlemm. It drains through the meshwork into the canal and is then drained with a continuous pressure gradient into the episcleral venous system via minuscule channels (Young *et al.*, 2000). The second outflow pathway, the unconventional pathway, is the uveoscleral pathway in which a percentage of AH leaves the eye through the intracellular spaces of the iris root and ciliary muscle and then drains into the scleral substance, the perivascular and perineural scleral spaces and from there, into the episcleral and orbital tissues (Pang et al., 2008).

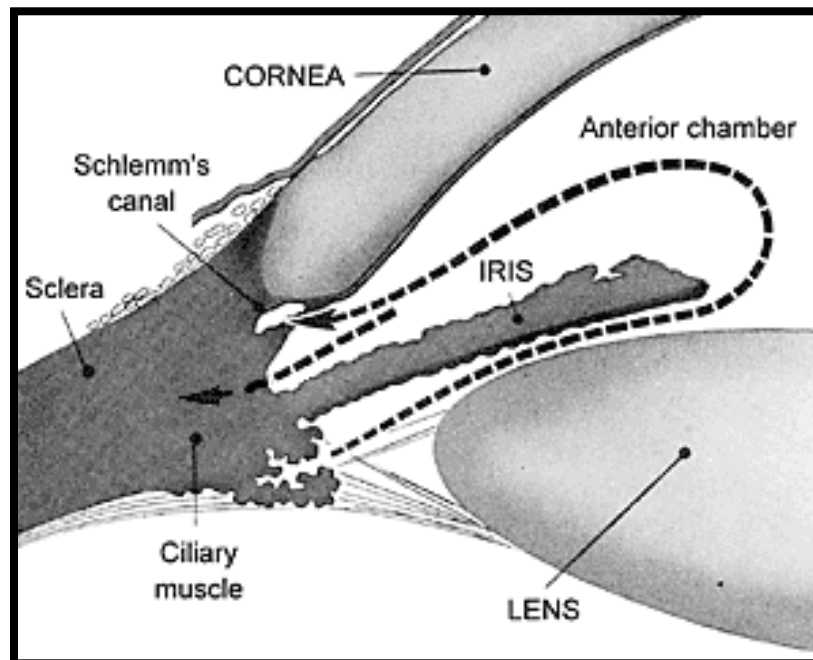


**Figure 1.** The two AH outflow pathways: TM (ends in the canal of Schlemm) and uveoscleral outflow pathway (ends at the iris root) (Medeiros *et al.*, 2002; Pang *et al.*, 2008).

The uveoscleral pathway, unlike the conventional trabecular pathway, is IOP independent while studies have shown that there is almost linear relationship between an increase in IOP and the TM pathway (figure 2).

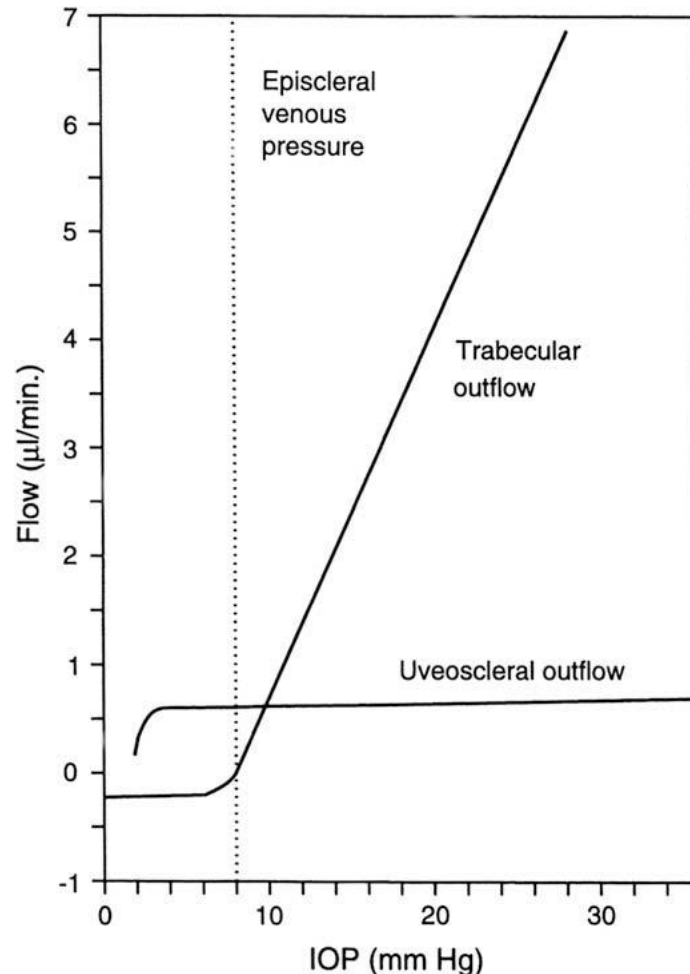


**Figure 2.** The effect of IOP on the conventional trabecular outflow and the unconventional uveoscleral outflow (Alm *et al.*, 2009).

The amount of AC that leaves the eye through the uveoscleral pathway is species specific (40-60% in primates, 3% in cats, 15% in dogs and between 3-8% in rabbits) and age dependent as the spaces between the ciliary muscle reduce with advancing age (Alm *et al.*, 2009).

The resistances for both pathways differ: as stated, for the TM outflow the resistance is situated in the endothelial part of the TM while for the uveoscleral pathway's resistance occurs in the sclera, choroidal vessels and ciliary muscles (Alm *et al.*, 2009). The resistance for the

uveoscleral pathway is minute in relation to the resistance that occurs in the TM but an important function of the TM is herein demonstrated: it provides a pressure gradient that prevents red blood cells from entering the anterior chamber from the venous system should there be a surge in the blood pressure. Also by maintaining the pressure gradient, it contributes to regulate the pressure in the eye necessary for sustaining the rigid structure of the eye (Alm *et al.*, 2009). The TM also acts as a self-cleansing filter (Alm *et al.*, 2009).

The use of ophthalmic viscosurgical devices (OVD) has become a part of ophthalmic surgery since the first OVD, Healon, was introduced into the market in 1979 (Torngren *et al.*, 2000). OVD's are used in ocular surgery as an aid to protect the delicate endothelial cells, to provide a clear anterior chamber with filling pressure in order for the surgeon to manipulate instruments without causing damage to the eye structures (Arshinoff *et al.*, 2005). A transient rise in the IOP postoperatively is a normal consequence as a result of using an OVD during ocular surgery. This is believed to be caused by a blockage by the OVD of the normal TM outflow pattern, and it is therefore advised that all the OVD be removed at the end of surgery (Torngren *et al.*, 2000). Despite several removal techniques that exist a small amount of OVD remains in the eye at the end of surgery causing the expected rise in IOP. As the OVD is cleared by the natural mechanisms of the TM, the IOP is expected to return to normal within 24 hours after surgery (Rainer *et al.*, 2005).

The IOP is measured with several instruments of which the handheld TonoVET is one. Measuring the IOP with a TonoVet utilizes the rebound tonometry principle which is based on measuring the rebound motion of an object impacting the cornea (Kniestedt, 2008). The TonoVET contains a probe made of stainless steel covered with a plastic cap that hits the cornea while the microprocessor records the deceleration speed of the probe after it impacted the cornea (Kniestedt, 2008). The higher the IOP the quicker the deceleration speed of the probe will be and this value can be processed and converted into an IOP reading by the TonoVET (Kniestedt, 2008).

A rise in IOP, if left untreated or lasts for prolonged periods, can cause a loss in vision in patients. The preclinical monitoring of the effect of an OVD on the IOP is therefore of the utmost importance before it is released into the market. To regulate this aspect the international standard for OVD's (ISO 15798:2000) stipulates an animal model for the evaluation of the IOP

rise that will be caused by new viscosurgical devices still in the preclinical phases (Torngren *et al.*, 2000).

For this reason the following study was conducted to test and compare the postoperative rise in IOP of two hyaluronic acid OVD's. Biolon (Bio-technology General, Israel), currently in the market worldwide as a cohesive OVD was compared with a buffered hyaluronic acid (BHA), a OVD still in the development phase, also a cohesive OVD.

## MATERIALS

BHA and Biolon (Bio-technology General, Israel) was supplied by Southern Medical (Pty) Ltd. The anesthetics, Ketamine (100mg/ml) and Xylazine (20mg/ml), were supplied by the UPBRC.

## METHODS

The test was conducted according to the protocol as set out in the ISO 15798 for ophthalmic viscosurgical devices, Annex B. Six healthy albino rabbits from a single strain aged between 21 and 67 days all weighing more than 2kg were used for the study. The animals were housed in groups of three and were kept in isolation in a room with a 12-hour light/dark cycle at a room temperature of between 17-24°C and a relative humidity of 30-60%. The cages were enriched with autoclaved pinecones and straw. Animal rations consisted of irradiated Epol rabbit pellets *ad libitum*, Teff, Lucern, Carrots, Spinach, Lettuce and apples. Water was boiled and allowed to cool down and was given *ad libitum*. The animals were allowed to acclimatize for 5 days before commencing the test. Animals were healthy and both eyes were inspected with an indirect ophthalmoscope and hand slit lamp 24 hours before the start of the test. On the day of the test the eyes were inspected with a slit-lamp and ophthalmoscope and the IOP was measured before commencing the test. The eyes were rinsed/wiped with 0.05% Hibitane in water just prior to the paracentesis. Under general anesthesia (0.35ml/kg Ketamine/0.25ml/kg Xylazine), 0.2 ml of AH was removed from the anterior chamber of the eye by paracentesis and was replaced with 0.2ml of the test substance, BHA. The contra lateral eye received 0.2ml of Biolon. Care was taken not to damage any of the eye structures. The intraocular pressure was evaluated with a TONOVET Tonometer (Tiolat, Tiolat Oy, Finland) at the following evaluation times: 1h  $\pm$  15min, 2h  $\pm$  30min, 4h  $\pm$  1h, 8h  $\pm$  2h, 12h  $\pm$  2h, 24h  $\pm$  4h, 1 week  $\pm$  2d and 1 month  $\pm$  7d by a masked observer.

## Facility and ethical clearance

The test was conducted at the UPBRC research Centre, Onderstepoort, South Africa. All procedures were approved by the animal use and care committee (AUCC), a subcommittee of the Committee for Research Ethics and Integrity of the University of Pretoria.

## STATISTICS

Quantitative data was statistically analyzed using the average mean, standard deviation and p-value at a confidence interval of 95%.

## RESULTS

Before the study all the eyes were found to be normal, without any abnormalities or damage. All observers were masked. All animals gained weight throughout the study. Table 1 and 2 illustrates the IOP of Biolon and BHA at the different time intervals of the study.

| Time           | 1h ± 15 min  |        | 2h ± 30min   |        | 4h ± 1h      |        | 8h ± 2h    |        |
|----------------|--------------|--------|--------------|--------|--------------|--------|------------|--------|
| Animal #       | R            | L      | R            | L      | R            | L      | R          | L      |
| 11             | 18           | 22     | 18           | 34     | 55           | 34     | 10         | 11     |
| 12             | 7            | 8      | 4            | 6      | 3            | 14     | 10         | 7      |
| 13             | 20           | 4      | 35           | 5      | 20           | 18     | 12         | 10     |
| 14             | 14           | 28     | 16           | 43     | 20           | 44     | 7          | 20     |
| 15             | 15           | 16     | 27           | 24     | 58           | 15     | 11         | 20     |
| 16             | 20           | 23     | 32           | 12     | 17           | 16     | 5          | 15     |
| <b>Average</b> | 15.667       | 16.833 | 22           | 20.667 | 28.833       | 23.5   | 9.167      | 13.833 |
| <b>SD</b>      | 4.97         | 9.839  | 11.726       | 16.832 | 24.139       | 13.342 | 1.871      | 6.025  |
| <b>P-VALUE</b> | <b>0.801</b> |        | <b>0.877</b> |        | <b>0.646</b> |        | <b>0.1</b> |        |

**Table 1.** IOP values for Biolon (R) and BHA (L) at 1h ± 15min, 2h ± 30min, 4h ± 1h and 8h ± 2h postoperatively.

| Time           | 12h ± 2H     |        | 24h ± 4h     |       | 1 Week± 2d   |        | 1 Month ± 7d |       |
|----------------|--------------|--------|--------------|-------|--------------|--------|--------------|-------|
| Animal #       | R            | L      | R            | L     | R            | L      | R            | L     |
| 11             | 12           | 9      | 8            | 12    | 9            | 8      | 15           | 16    |
| 12             | 4            | 13     | 21           | 10    | 10           | 9      | 18           | 18    |
| 13             | 9            | 10     | 9            | 12    | 13           | 9      | 16           | 15    |
| 14             | 4            | 16     | 7            | 7     | 13           | 10     | 18           | 14    |
| 15             | 11           | 12     | 11           | 8     | 15           | 16     | 18           | 20    |
| 16             | 5            | 23     | 4            | 7     | 13           | 12     | 18           | 16    |
| <b>Average</b> | 7.5          | 13.833 | 10           | 9.333 | 12.167       | 10.667 | 17.167       | 16.5  |
| <b>SD</b>      | 3.808        | 2.739  | 5.675        | 2.28  | 2.449        | 3.209  | 1.414        | 2.408 |
| <b>P-VALUE</b> | <b>0.008</b> |        | <b>0.795</b> |       | <b>0.348</b> |        | <b>0.571</b> |       |

**Table 2.** IOP values for Biolon (R) and BHA (L) at 12h ± 2h, 24h ± 4h, 1 week ± 2d and 1 month ± 7d postoperatively.

An intraocular pressure spike was seen in all animals for both BHA and Biolon post-operatively. The peak IOP rise was at 4H ± 1h after the surgery for both Biolon (28.83 ± 22.4 mm Hg) and BHA (23.5 ± 12.5 mm Hg). There was a significant difference seen at 12h ± 2h between the two OVD's, with Biolon having a IOP of 7.5 ± 3.808 mmHg and BHA a pressure of 13.833 ± 2.739 mmHg. No other significant differences were seen at any of the other times between the two OVD's with a confidence level of 95% ( $p > 0.05$ ). For both OVD's the IOP returned to normal within 24 hours postoperatively.

The following figures shows the comparative IOP pattern for Biolon and BHA per test animal:

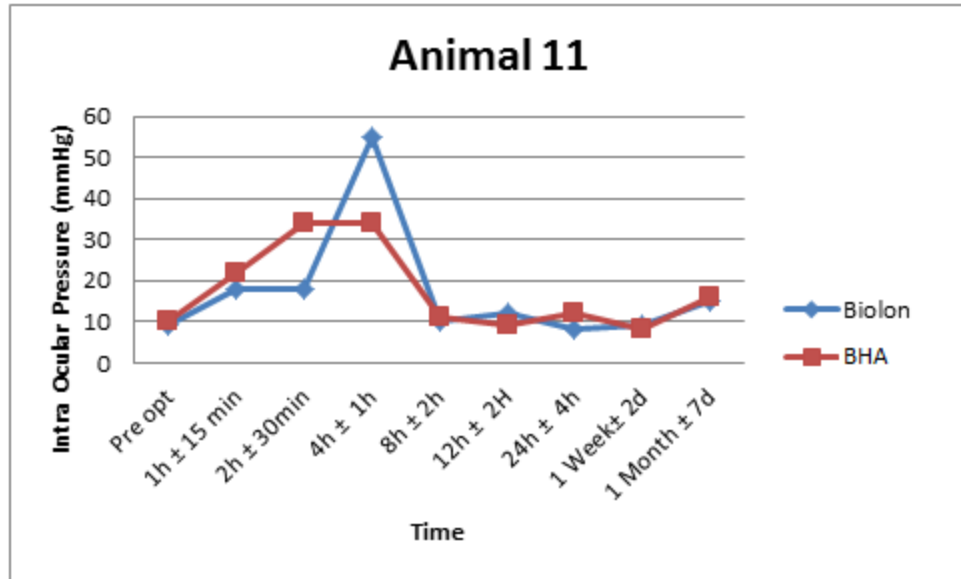


**Figure 3.** IOP pattern for Biolon (peak IOP 4h ± 1h) and BHA (peak IOP 2h up to 4h ± 1h).

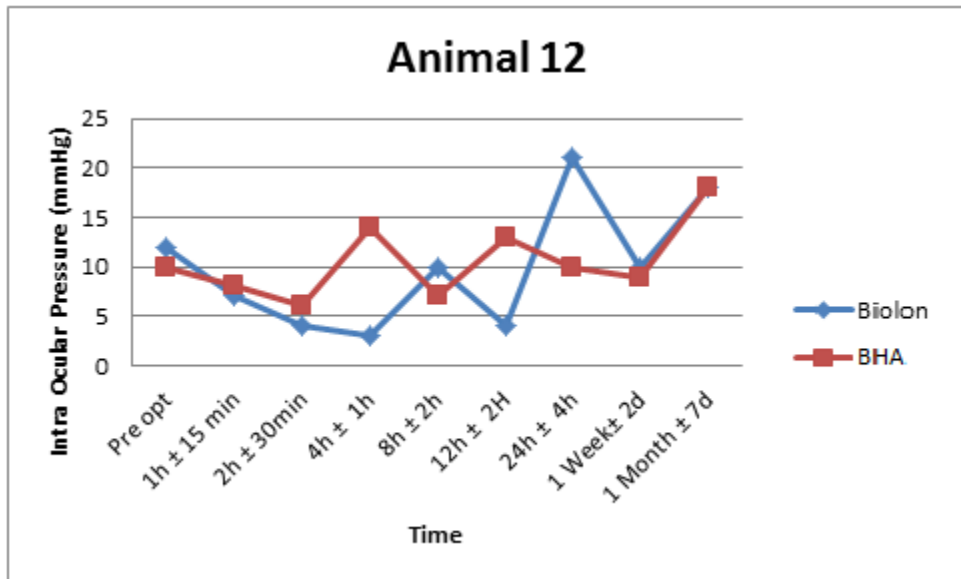


**Figure 4.** IOP pattern for Biolon (peak IOP 24h ± 4h ) and BHA (peak IOP 1 month ± 7d).

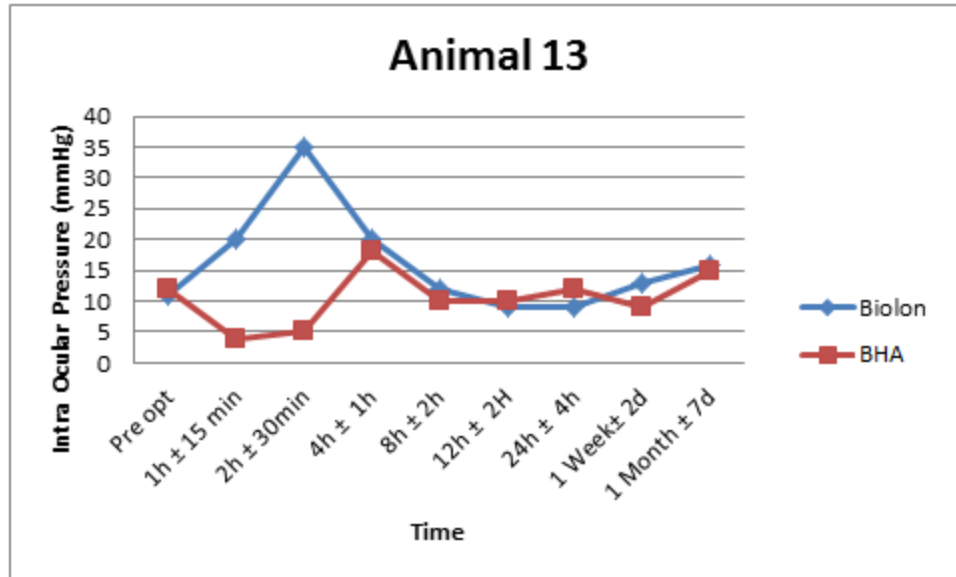


**Figure 5.** IOP pattern for Biolon (peak IOP 2h ± 30 min) and BHA (peak IOP 4h ± 1h).

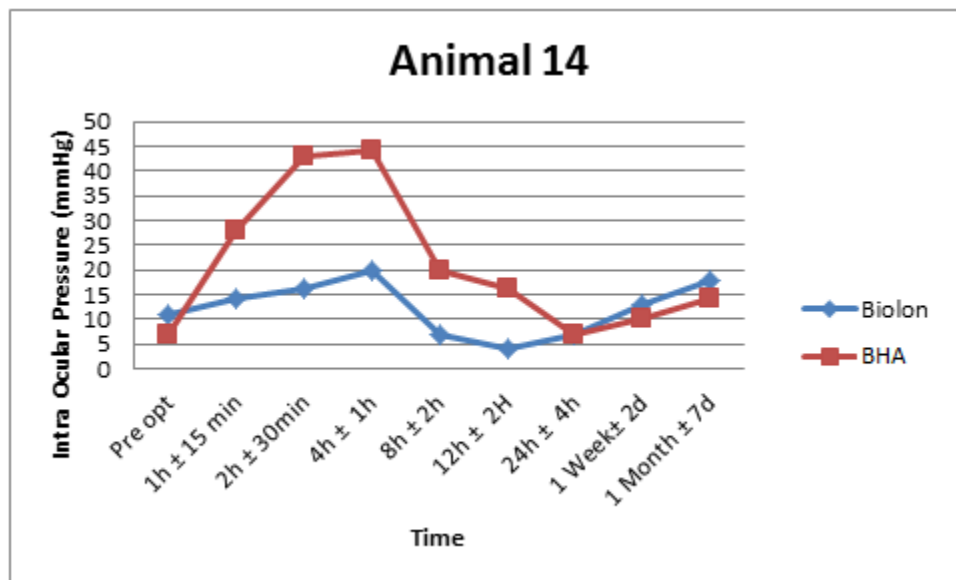


**Figure 6.** IOP pattern for Biolon (peak IOP 4h ± 1h) and BHA (peak IOP 4h ± 1h).

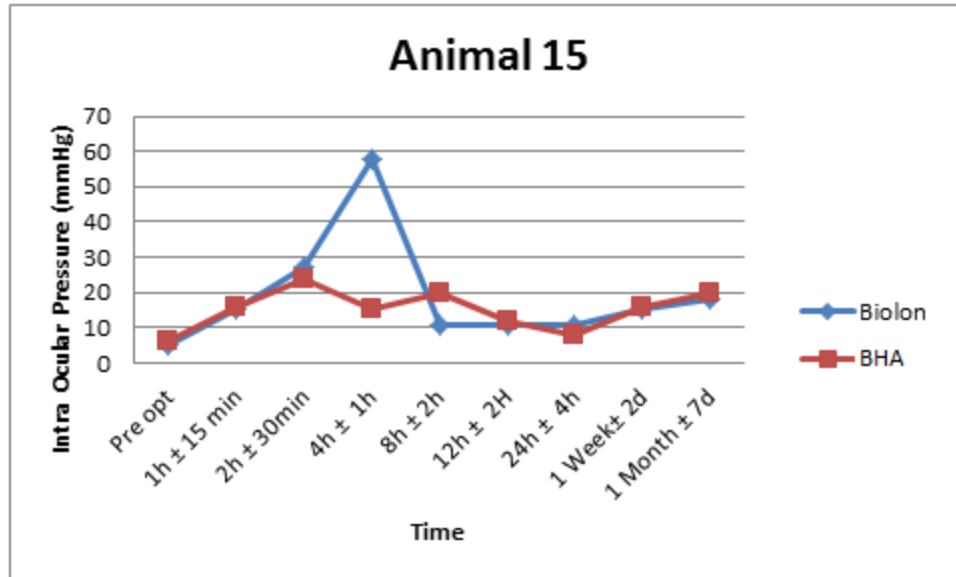


**Figure 7.** IOP pattern for Biolon (peak IOP 4h ± 1h) and BHA (peak IOP 2h ± 30 min).

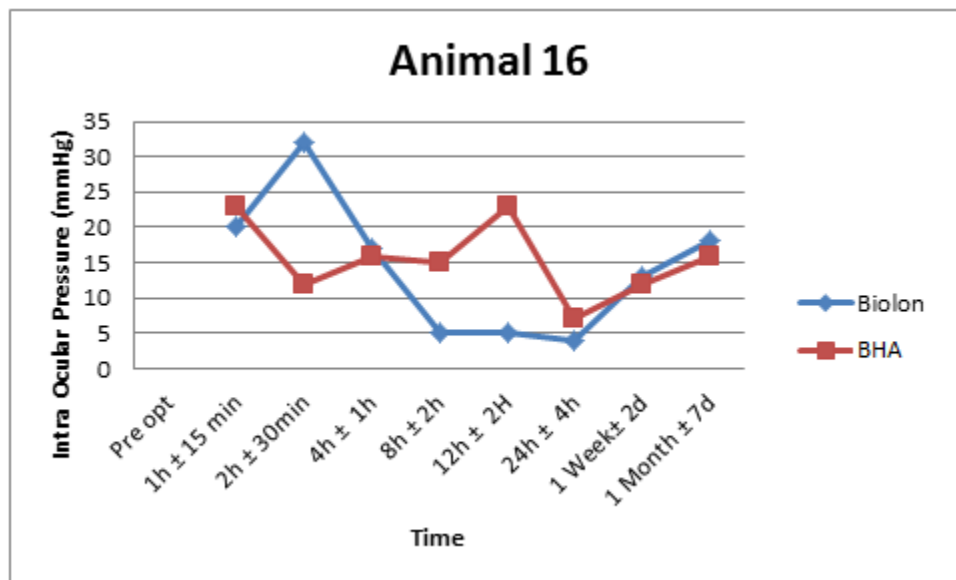


**Figure 8.** IOP pattern for Biolon (peak IOP 2h ± 30 min) and BHA (peak IOP 12h ± 2h).

Mean IOP patterns for both OVD's can be seen in figures 9 and 10 as well as a comparative graph between the two OVD's for the different time intervals in figure 11.

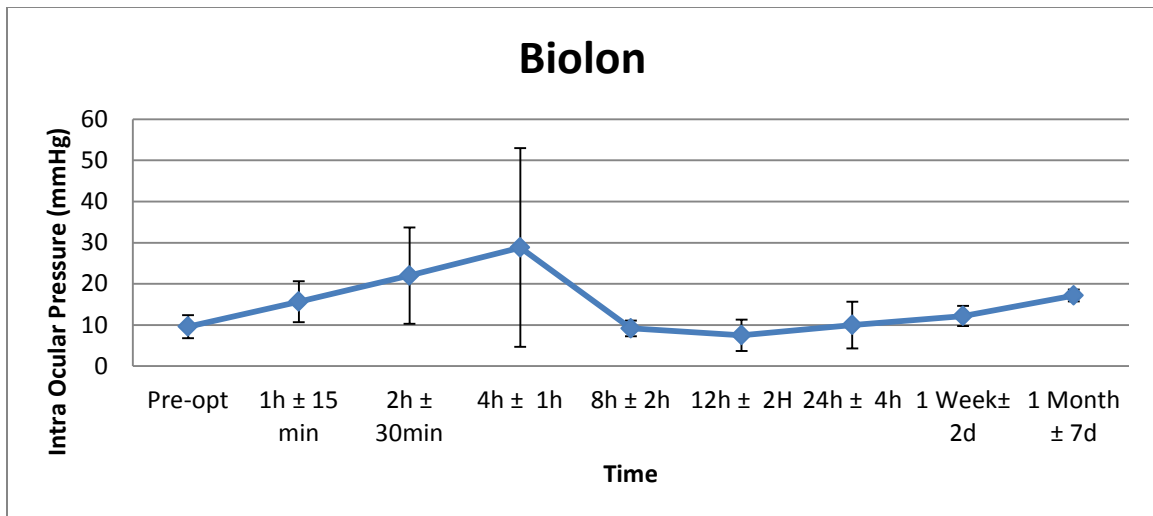


**Figure 9.** Biolon IOP changes after intraocular surgery at different time intervals.

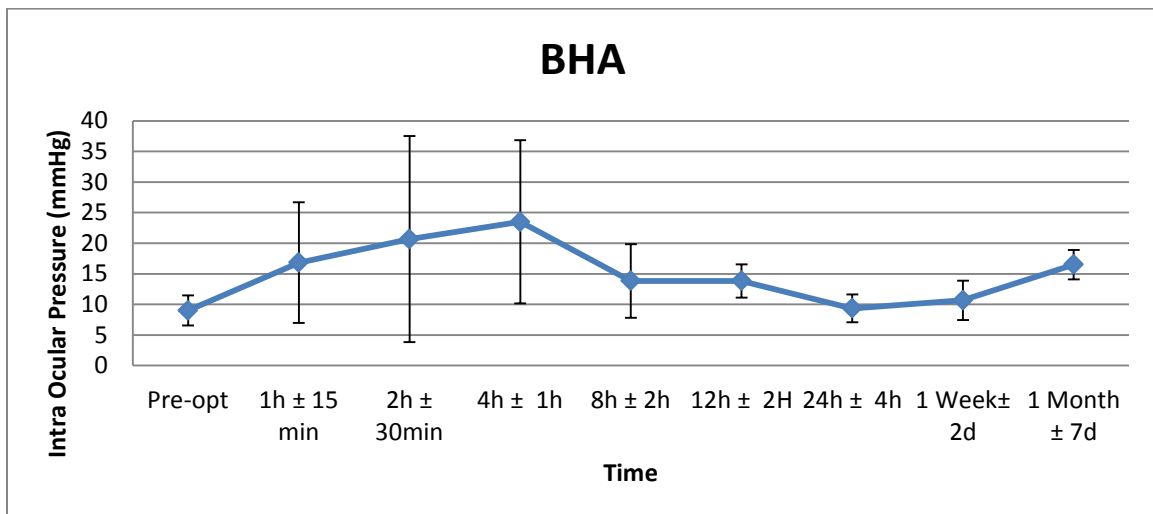


**Figure 10.** BHA IOP changes after intraocular surgery at different time intervals.

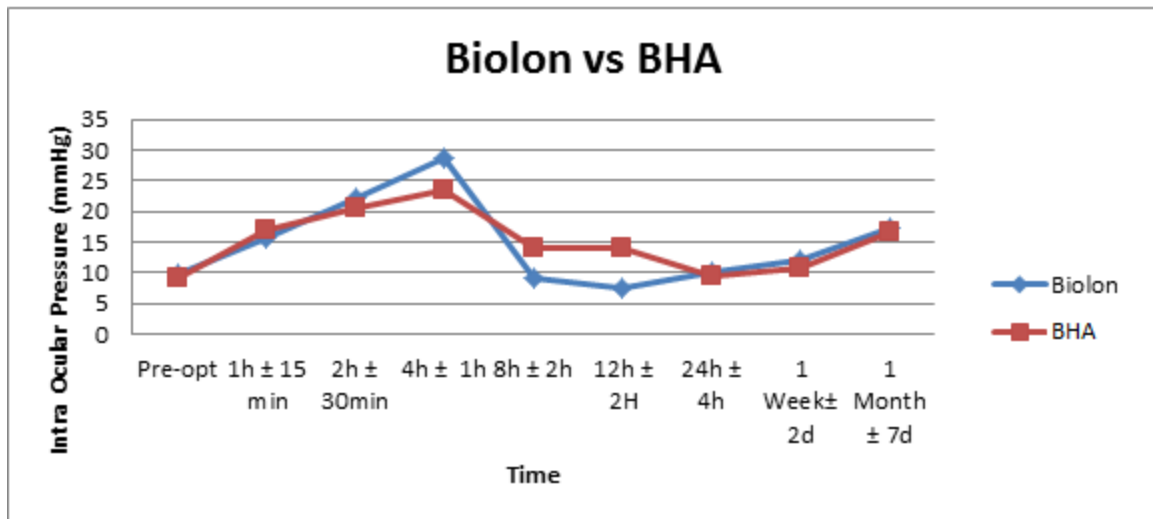


**Figure 11.** Biolon and BHA IOP changes after intraocular surgery at different time intervals.

## DISCUSSION

A sustained rise in the IOP is one of the leading factors causing the potentially blinding disease glaucoma. It is a well-known fact OVD's causes a transient rise in IOP when used during ophthalmic procedures (Glasser *et al.*, 1986; Liebmann *et al.*, 1990). This comparative study was done to evaluate the rise in IOP of two OVD's (BHA and Biolon) both consisting of hyaluronic acid.

In this study the test (BHA) and control (Biolon) OVD caused a rise in the IOP postoperatively. The peak IOP rise for both OVD's was at 4h ± 1h and both OVD's exhibited the same IOP pattern throughout the different time intervals with IOP's returning to normal after 24H. These results correlate with a study by Liebmann *et al* done on human eyes with 1% hyaluronic acid in which the postoperative rise in IOP was also shown to peak at 4-6 hours postoperatively (Liebmann., 1990). These findings differs however from the study done by Glasser *et al.* in which the peak IOP rise was found to be at 2 hours after the surgery in cat eyes for Healon and Amvisc, also 1% hyaluronic acid containing OVD's. The IOP returned to normal, as was found in this study, after 24 hours. Several human IOP studies were done by Rainer *et al.* in which they found the peak rise in IOP for Healon to be 6 and 8 hours postoperatively (Rainer *et al.*, 2007). In these studies the peak IOP rise for Viscoat (chondroitin sulphate 4%-sodium hyaluronate 3%) was found to be at 1 hour and for Ocucoat (Hydroxypropylmethylcellulose 2%) at 2 hours (Rainer *et al.*, 2005 and Rainer *et al.*, 2007). They also confirmed that the composition of an

OVD has an influence on the peak IOP as Viscoat had a much higher spike in the IOP than Ocucoat (Rainer *et al.*, 2007). From the present study it can be concluded that for a hyaluronic acid OVD with a molecular weight of approximately 3 million Dalton the intraocular pressure spike is expected between  $4h \pm 2h$  postoperatively.

The main reason for the rise in IOP after the ocular surgery is believed to be the blockage of the TM by the OVD that then limits the normal outflow of the aqueous fluid through the TM (Glasser *et al.*, 1986; Tornngren *et al.*, 2000). In a study done by Berson *et al.* it was shown that there is a 65% decrease in the outflow through the TM after the installation of a 1% hyaluronic acid OVD (Berson *et al.*, 1983). Other factors influencing the IOP are pre-existing morbidity, the ease of OVD removal, chemical composition and postoperative inflammation (Glasser *et al.*, 1986; Rainer *et al.*, 2007; Tornngren *et al.*, 2000). The molecular weight of the OVD's used in this study was both approximately 3 million Dalton which explains the similarity in the IOP rise and peak at  $4H \pm 1h$  with no significant difference between the two values, as the molecular weight is directly related to the peak IOP after surgery (Tornngren *et al.*, 2000).

At  $12H \pm 2h$  after surgery there was a significant difference between the IOP of the two OVD's with BHA ( $13.833 \pm 2.739$ mm Hg) being slightly higher than Biolon ( $7.5 \pm 3.808$ mm Hg). The possible reason for the discrepancy between the two OVD's might be found in their composition. The concentration of BHA (1.2%) is slightly higher than that of Biolon (1.0%). The intraocular pressure of all the test eyes (BHA) are higher at  $12h \pm 2h$  than that of the control eyes (Biolon) except for one, indicating the higher concentration of BHA might have an effect on the breakdown of the OVD at this time. Another possible reason could be the manufacturing of the OVD's. The TM serves as a sieve to remove all particles from the AH while it moves with the pressure gradient. An analysis of the particle sizes and chain lengths of the two OVD's might be helpful to support this notion. As Englert *et al.* suggests, there may be a correlation between the IOP and the molecular size of an OVD (Englert *et al.*, 2000).

Knowing the exact time of the IOP spike for a specific OVD is of clinical importance as most patients undergoing cataract surgery are discharged almost directly after surgery. Most IOP spikes occur between 4-7 hours post operatively, as was seen in this study, while patients are not being monitored (Rainer *et al.*, 2007). Patients with existing glaucoma or compromised optic discs should especially be made aware of the symptoms of a severe increase in the IOP. A rise in IOP (especially above 30mmHg or higher in humans) can cause pain, corneal epithelial

oedema, nausea and vomiting and even damage to the optical disc (Rainer *et al*, 2001; Englert *et al*). These patients might be at risk for retinal artery occlusion and anterior ischemic optic neuropathy (Rainer *et al.*, 2007). Surgeons are advised to monitor patients, especially in the first few hours after surgery, for a rise in the IOP that might be detrimental to their eyes.

Because OVD's are normally removed at the end of surgery, this type of test is not directly applicable to the clinical setting because the OVD remains in the eye and can thus rather be used as an aid to compare OVD's while they are still in development as part of the preclinical studies. In a study done by Glasser *et al.* in cats' eyes the intraocular pressure after washing the OVD out from the eye was considerably lower and shorter than in the eyes where it was not washed out (Glasser *et al.*, 1986). The animal model differs from the human eye in that the animal has a thinner cornea than the human and the animal eye does not have a Bowman's membrane. The thinner cornea has an effect on the measurements of IOP and therefore this test is mainly used to compare substances with each other especially when measured with a TonoVet (Lim *et al.*, 2005). Rabbits are chosen for these studies because of their large eye structure that are easy to evaluate, also because their eyes' response to ocular surgery with regard to inflammatory reactions and rise in IOP is the same as in man's and thus provides a good *in vivo* model (Gwon, 2008). The results obtained is thus a good indication of when to expect the postoperative rise in the IOP, but clinical tests on humans are required to determine the actual IOP values that will occur.

## CONCLUSION

The test article, BHA, was assessed to determine its postoperative IOP pattern when compared to a control substance Biolon in the New Zealand white rabbit. The peak IOP rise was at  $4h \pm 1h$  postoperatively for both OVD's. There was a significant difference found at  $12h \pm 2h$  after the surgery with possible reasons being the slightly higher concentration of the test substance. No other differences were found between the two OVD's.

## CHAPTER 6

# DETERMINING THE EYE IRRITATION POTENTIAL OF HYALURONIC ACID USING THE DRAIZE EYE TEST

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## INTRODUCTION

Before any medical product is released into the market they are subjected to stringent safety evaluations to ensure the safety of the end user, the patient. One of these safety evaluations is the eye irritation test. Eye irritation is defined as "the production of changes in the eye following the application of test substance to the anterior surface of the eye, which are fully reversible within 21 days of application" (Alttox.org. 2008; <http://alttox.org/ttrc/toxicity-tests/eye-irritation/>). Eye corrosion is defined as "the production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application" (Alttox.org. 2008; <http://alttox.org/ttrc/toxicity-tests/eye-irritation/>).

The Organization for Economic Cooperation and Development (OECD) is an organization concerning itself with defining and setting up of protocols for different kinds of toxicity tests (Wilhelmus, 2001). Several methods exist for testing eye toxicity of which Guidance document number 405 is specifically assigned for the Acute Eye Irritation/Corrosion methods.

## THE MACROSCOPIC STRUCTURE OF THE EYE

The eye is the organ of sight where light is converted by highly specialized cells into nerve impulses which are then interpreted by the brain in order for vision to take place, see figure 1. The eye is made up of three layers: the outer cornea-scleral layer, the middle uveal layer and the inner retinal layer (Young *et al.*, 2000). As the outer cornea-scleral layer and middle uveal layer are involved in ocular irritation testing, these two layers will now be described in further detail.

The outer corneo-scleral layer of the eye serves as the supportive layer of the eye and is made up of a tough, fibroelastic capsule which is divided into the posterior sclera making up five-sixth of the capsule and the anterior one-sixth called the cornea. The cornea is responsible for

refracting the light rays and concentrating it on the retina thereby enabling vision. The limbus is the junction between the sclera and cornea and contains the limbal stem cells (Young *et al.*, 2000). The anterior part of the sclera (the “exposed” part) is covered by the conjunctiva which serves as an outermost epithelial layer covering the sclera and inner surface of the eye lids (Young *et al.*, 2000).

The middle uveal layer consists of three parts: the choroid, ciliary body and the iris. The choroid is pigmented and is present between the retina and the sclera in the posterior five-sixth of the eye and it supplies the retina with nutrients. The choroid joins anteriorly with the ciliary body that is attached to the lens of the eye via suspensory ligaments called the zonules. The ciliary body contains smooth muscle fibres that control the shape of the lens enabling focus of the image. The iris is the diaphragm that incompletely extends in front of the lens from the ciliary body and it divides the anterior compartment of the eye into an anterior and posterior chamber. The iris is the pigmented part of the eye and is responsible for regulating the amount of light that reaches the retina through the pupil (Young *et al.*, 2000).

Both the anterior and posterior chambers are filled with a non-refractive fluid called the aqueous humor that is secreted by the ciliary body responsible for maintaining the IOP in the eye (Young *et al.*, 2000). The fluid serves as a source of nutrients to the cornea and lens (no vascular supply) and is secreted in the posterior chamber circulated through the pupil and drains through a channel called the canal of Schlemm as it was discussed in further detail in chapter 5.

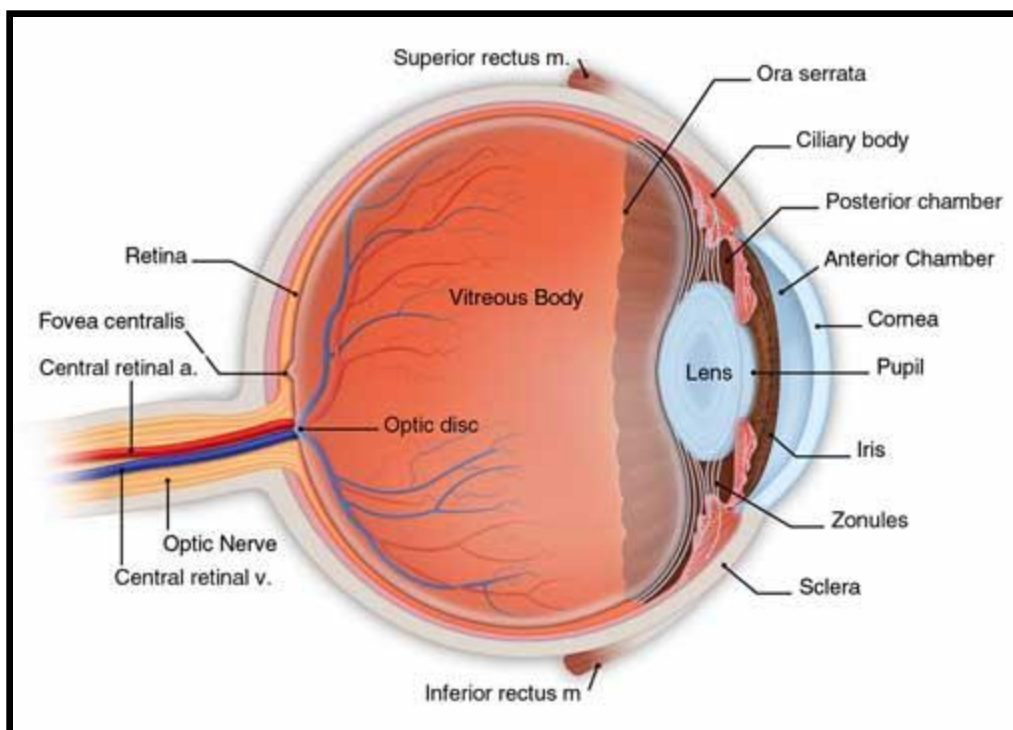


**Figure 1.** The internal structures of the eye (Retina Consultants of Western New York, <http://www.wnyretina.com/eye.html>).

## THE MICROSCOPIC STRUCTURE OF THE EYE

The cornea is the part of the eye mainly responsible for focussing the light image on the retina and any damage therefore to the cornea would result in a loss of vision. The cornea consists of 5 layers. The outer layer consists of stratified non-keratinised squamous epithelium of about 5 cell layers thick that is underlined by a thin specialized basal membrane called the Bowman's membrane (Young *et al.*, 2000). The cells are attached via tight junctions allowing access of molecules into the stroma (Altox.org. 2008; <http://alttox.org/ttrc/toxicity-tests/eye-irritation/>). The next layer, the corneal stroma, which forms the largest part of the cornea, consists of highly organized dens collagenous lamellae. Stromal cells consist of fibroblasts (sometimes called keratocytes) and at times, lymphocytes (Altox.org. 2008; <http://alttox.org/ttrc/toxicity-tests/eye-irritation/>; Young *et al.*, 2000). Descemments' membrane, the next layer of the cornea serves as a supporting layer consisting of elastic fibres that supports the final crucial layer of corneal endothelial cells. The flattened endothelial cells are responsible for the homeostasis of fluids into and from the corneal stroma, any detriment to these cells results in a dysfunctional barrier function of the cells and the cornea becomes cloudy accompanied by a loss in vision (Young *et*

*al.*, 2000). The cornea is innervated by nerves extending through the stroma reaching the tight junctions between the cells on the surface layer (Alttox.org. 2008; <http://alttox.org/ttrc/toxicity-tests/eye-irritation/>). Because of its rich innervation any damage to the tight junctions will cause an immediate pain sensation (Alttox.org. 2008; <http://alttox.org/ttrc/toxicity-tests/eye-irritation/>).

The conjunctiva that covers the external surface of the eye and lines the inner surface of the eyelids consists of cells containing goblets cells that are responsible for the lubrication of the surface of the eye and for the ease of movement of the eyelids.

The iris contains many melanocytes scattered in its loose, vascular tissue and is therefore visible as the “colour” part of the eye. The anterior and posterior surfaces of the iris differs, with the anterior surface being irregular consisting of an inconsistent layer of fibroblasts and melanocytes while the posterior surface is smooth and lines the surface of the ciliary body. The posterior layer contains two types of myoepithelial cells which is responsible for the movement of the iris. The first type is the dilator papillae muscle which is innervated by the sympathetic nervous system and the second, the constrictor papillae muscle innervated by the parasympathetic nervous system (Young *et al.*, 2000).

In the event of chemicals coming into contact with the eye surface, the cornea and conjunctiva are the first structures to be exposed to its potentially harmful effects. Depending on the chemicals’ composition it will penetrate and damage the corneal layers of the eye and may cause irreversible damage, resulting in a loss of vision. When new cosmetic products (like mascara) are designed, these products are tested on various ways, of which the Draize eye test is one way, to ensure the safety of the product before it is released into the market for consumer usage.

## **HISTORY OF EYE IRRITATION TESTS**

During World War I “mustard gas” (*bis*-(2-chloroethyl)sulphide) was used as a chemical warfare mechanism by the German allies (Wilhelmus, 2001). This yellow chemical substance was released as a deadly vapour during the war having devastating effects when coming in contact with the skin or eyes. The chemical denatures enzymes and crosslinks DNA strands resulting in the epithelial sloughing of the conjunctiva of the eye and causing epidermal blisters that sometimes resulted in ischemic ulceration of the skin.

After these horrible incidents several research centres were set up to study the effects of chemical warfare on animals. In 1939, Ida Mann, one of the few female ophthalmologists in Great Britain at the time, managed to describe the effects of mustard gas qualitatively. In the 1940's Jonas S Friedenwald was the first to use a numeric scale to grade the severity of the effects of poisonous chemicals in the eye. Conjunctival redness, for example, could now be scored from 0 up to 2 and the scores correlated with the dose of different chemical substances, different chemicals could also now be compared with regards to its toxicity. As time went by, the method was improved with the addition of extra measures namely recovery time and residual corneal scarring. The final step was for John Draize to modify the Friedenwald findings from a way of assessing toxic components into a useful tool for ensuring the safety chemical substances to be used for topical means (Wilhelmus, 2001).

In 1957 Draize received a medal from the America Society of Cosmetic Chemists for his research on cosmetic product safety. His methods for testing irritation on animals became well known and to this day are referred to as the Draize tests (Draize eye test specifically when testing irritation in the eye). Draize concluded: "Assessment of data, a consideration of species differences, and the allowance for a margin of safety, should make possible an estimation, in the case of a given compound, of the amounts, concentration, and frequency of application that may be tolerated by man." (Wilhelmus, 2001).

## **THE DRAIZE EYE TEST**

The Draize eye test is considered the golden standard for determining the ocular irritation potential of a substance. Many variations on this method exist and are recognized as suitable alternatives to the method, but most of these methods are not "stand alone" methods and have to be verified with sometimes several other tests.

### **Animal models and numbers**

Because of the familiarity with their anatomy and physiology and them being readily available, rabbits are the animals of choice when it comes to eye irritation tests (Wilhelmus, 2001). Their eyes are much larger than that of the rodent and guinea pig and because of their ease of handling it makes them even a more attractive model for eye tests.

Several differences exist between the human and rabbit eye and the Draize eye test performed on rabbits is considered an overestimation of the irritant effect than of that it would have in the

human eye (Alttox.org. 2008; <http://alttox.org/ttrc/toxicity-tests/eye-irritation/>; Wilhelmus, 2001). Differences between the structure of human and rabbit eyes are the presence of a nictitating membrane and the absence of a Bowman's layer in the rabbit, humans have a smaller corneal surface area and a thinner cornea (Wilhelmus, 2001).

Considering the animal numbers per test the desired outcome of the study needs to be taken into account. Three to six animals are the norm for this type of test.

### **Exposure and formulation considerations**

One eye is exposed to the chemical in question while the other serves as a control. The mucus membrane of the eye would be damaged by a substance having a pH of lower than 2 or exceeding 11.5 and these substances are therefore automatically omitted from these studies (Wilhelmus, 2001).

Volume wise, 0.1ml of test substance was chosen by Draize and Friedenwald as this is the conventional amount used for both intraocular and topical applications although it has been proven that an amount of 0.01ml is a more accurate representation of a real life situation. This volume is inserted onto the eye into the lower conjunctival sac (Wilhelmus, 2001).

The ocular response is then quantified in terms of its severity by a subjective observer and evaluations are made at 1, 24, 48 and 72 hours following exposure. Observations are made with a slit lamp where the mucosal layer and epithelial effects are monitored and recorded. The following parameters are evaluated during the test: the opacification of the cornea, the presence of iritis, conjunctival redness, edema and discharge. Each reaction is given a score and a maximum of 110 can be reached with the opacification of the cornea, being the most exposed and the most important structure, carries 73% of the weight (Wilhelmus, 2001).

For a chemical to be considered an irritant at least four out of six rabbits should have a positive response, retesting is required if two or three animals shows a positive response, and the test can be considered negative if one or no animal shows a positive response. The time of onset and duration of the irritation reaction is followed during the test and is a good indication of the grade/severity of the irritant potential of the test chemical. The reversibility of the irritation reaction is therefore used as an indicator to distinguish between a substance being an eye irritant ("constellation of reversible structural and inflammatory changes") or an eye corrosive

("irreversible structural and inflammatory changes and eye corrosion"). (Alttox.org. 2008; <http://alttox.org/ttrc/toxicity-tests/eye-irritation/>; Wilhelmus, 2001).

## NON-ANIMAL ALTERNATIVE METHODS

The need to eliminate the use of animals is of great concern to anti-animal advocates for preclinical testing (Bordeau *et al.*, 1990). Therefore several large cosmetic companies (such as Revlon) have donated millions of dollars to academic and private institutions to find alternative means of testing chemicals irritation potential (Wilhelmus, 2001).

Several methods already exist and can be grouped into organotypic methods (utilizing live tissue), chorioallantoic membrane-based tests, tests utilizing cell function as a mean of evaluating toxicity, cytotoxicity and others (Wilhelmus, 2001; Bordeau *et al.*, 1990). Newer methods include the usage of macromolecular matrixes to test the effects of chemicals on it. As stated, the Draize eye test remains the golden standard as each one of the alternative tests can only evaluate a single parameter at a time. Some are of opinion that this is not enough to determine the irritation grade of a chemical before it is approved for human use and have combined many of these alternative tests into a "battery of tests" for evaluating irritation potential (Bordeau *et al.*, 1990).

Some of the alternative tests to the Draize eye test include the Bovine Corneal Opacity and Permeability (BCOP) assay, the isolated chicken eye (ICE) assay, the EpiOcular assay, the HCE-T transepithelial permeability (HCE-T TEP) assay, the MDCK Fluorescein leakage (FL) assay and the Hen's egg Test- Chorioallantoic Membrane (HET-CAM) assay.

The BCOP assay uses enucleated cow eyes, which would be discarded by the slaughter house, to test chemicals on. The chemicals are introduced onto the cornea and the corneal transparency and permeability is then assessed. In the same way a second alternative test to the Draize eye test, the ICE test, utilizes enucleated chicken eyes and assesses corneal opacity, corneal swelling and fluorescein retention (Alttox.org. 2008; <http://alttox.org/ttrc/toxicity-tests/eye-irritation/>).

Examples of cell culture assays as alternative means includes the EpiOcular assay (normal, human derived epidermal keratinocytes cultured to represent the layered cornea), the HCE-T TEP assay (uses HCE-T cell line to determine the fluorescein transepithelial permeability of a test substance) and the MDCK Fluorescein Leakage (FL) assay (measures the effect of a test

material on a monolayer MDCK cells by evaluating the amount of fluorescein that penetrates the monolayer because that is related to the amount of tight junction damage that took place because of the chemical) (Alttox.org. 2008; <http://alttox.org/ttrc/toxicity-tests/eye-irritation/>).

Another widespread alternative method, the HET-CAM test, is based on the principle of and describes the level of capillary permeability (Wilhelmus, 2001). Of these methods the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) endorsement states that "In 2007, BCOP and ICE were recommended as screening tests for identifying corrosives and severe irritants, with certain limitations; HET-CAM is not recommended for regulatory hazard classification purposes until further developed and evaluated." (Alttox.org. 2008; <http://alttox.org/ttrc/toxicity-tests/eye-irritation/>).

## **EYE IRRITATION TESTS AS PART OF PRECLINICAL STUDIES FOR OPHTHALMIC VISCOSURGICAL DEVICES**

Ophthalmic viscosurgical devices (OVD) come into contact with all the most important structures of the eye when used in ocular surgery in procedures such intra-ocular lens insertion, intra and extra capsular lens extraction, glaucoma surgery and corneal graft surgery. As part of its preclinical evaluation, an irritation test to ensure its safety is required.

The purpose of this study was to evaluate the irritation potential of a glycosaminoglycan OVD, consisting of buffered hyaluronic acid (BHA), on the eyes of New Zealand white rabbits. Being the golden standard of the irritation tests, the trusted Draize eye test was chosen for this study.

## **MATERIALS**

Both viscosurgical devices, BHA (test) and Biolon (control) was supplied by Southern Medical (Pty) Ltd.

## **METHODS**

The test was conducted according to the Draize eye test protocol as set out in the ISO 10933-10:2002(E). Six healthy albino rabbits from a single strain and approximately three years of age were used for the study. Animals were healthy and the eyes were inspected before commencing the study. The animals were housed in groups of three and were kept in isolation in a room with

a 12-hour light/dark cycle at a room temperature of between 17-24°C and a relative humidity of 30-60%. The cages were enriched with autoclaved pinecones and straw. Animal rations consisted of irradiated Epol rabbit pellets *ad libitum*, Teff, Lucern, Carrots, Spinach, Lettuce and apples. Water was boiled and allowed to cool down and was given *ad libitum*. The animals were allowed to acclimatize for 5 days. One day (24 hours) before commencing the test both eyes were visually examined with an indirect ophthalmoscope and a hand slit lamp to ensure no abnormalities were present. A volume of 0.1ml of the test substance was then instilled into the lower conjunctival sac of the right eye by everting the eye lid. The eye lid was kept closed for approximately one second and was then released to allow the test substance to spread onto the surface of the eye. The contra lateral eye served as the control eye for each rabbit. The eyes were then evaluated at 1h, 24h, 48h and 72h after instillation according to the following table for scoring eyes in the Draize eye test:

**Table 1.** Draize eye test system for grading ocular lesions:

| Reaction                                                                       | Numerical grading |
|--------------------------------------------------------------------------------|-------------------|
| <b>1. Cornea</b>                                                               |                   |
| <b>A. Degree of opacity (most dense area)</b>                                  |                   |
| No opacity                                                                     | 0                 |
| Scattered or diffuse areas, details of iris clearly visible                    | 1 <sup>a</sup>    |
| Early discernible translucent areas, details of iris slightly obscured         | 2 <sup>a</sup>    |
| Opalescent areas, no details of iris visible, size of pupil barely discernible | 3 <sup>a</sup>    |
| Opaque, iris visible                                                           | 4 <sup>a</sup>    |
| <b>B. Area of cornea involved</b>                                              |                   |
| One-quarter (or less), not zero                                                | 0                 |
| Greater than one-quarter, but less than half                                   | 1                 |
| Greater than half, but less than three quarters                                | 2                 |
| Greater than three quarters, up to whole area                                  | 3                 |
| <b>Score equals A x B x 5 Total maximum = 80</b>                               |                   |

|                                                                                                                                                                        |                |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| <b>2. Iris</b>                                                                                                                                                         |                |
| <b>A. Values</b>                                                                                                                                                       |                |
| Normal                                                                                                                                                                 | 0              |
| Folds above normal, congestion swelling, circumcorneal injection (any or all in combination of these), iris is still reacting to light (sluggish reaction is positive) | 1 <sup>a</sup> |
| No reaction to light, hemorrhage, gross destruction (any or all of these)                                                                                              | 2 <sup>a</sup> |
| <b>Score equals A x 5 Total possible maximum = 10</b>                                                                                                                  |                |
| <b>3. Conjunctivae</b>                                                                                                                                                 |                |
| <b>A. Redness (refers to palpebral and bulbar conjunctiva excluding cornea and iris)</b>                                                                               |                |
| Vessels normal                                                                                                                                                         | 0              |
| Vessels definitely injected above normal                                                                                                                               | 1              |
| More diffuse, deeper crimson red, individual vessels no easily discernable                                                                                             | 2 <sup>a</sup> |
| Diffuse beefy red                                                                                                                                                      | 3 <sup>a</sup> |
| <b>B. Chemosis</b>                                                                                                                                                     |                |
| No swelling                                                                                                                                                            | 0              |
| Any swelling above normal (including nictitating membrane)                                                                                                             | 1              |
| Obvious swelling with partial eversion of lids                                                                                                                         | 2 <sup>a</sup> |
| Swelling with lids about half-closed                                                                                                                                   | 3 <sup>a</sup> |
| Swelling with lids about half-closed to completely closed                                                                                                              | 4 <sup>a</sup> |
| <b>C. Discharge</b>                                                                                                                                                    |                |
| No discharge                                                                                                                                                           | 0              |
| Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)                                                          | 1              |

|                                                                                   |   |
|-----------------------------------------------------------------------------------|---|
| Discharge with moistening of the lids and hairs just adjacent to lids             | 2 |
| Discharge with moistening of lids and hairs, and considerable area around the eye | 3 |
| <b>Score equals (A + B + C) x 2 Total maximum = 20</b>                            |   |
| <sup>a</sup> Positive result                                                      |   |

The animal rooms were maintained as a limit-access facility.

### Facility and ethical clearance

The test was conducted at the UPBRC research Centre, Onderstepoort, South Africa. All procedures were approved by the animal use and care committee (AUCC), a subcommittee of the Committee for Research Ethics and Integrity of the University of Pretoria.

## RESULTS

The following table displays the results of the irritation test scored at 1h, 24h, 48h and 72 hours after exposure to hyaluronic acid.

**Table 2.** Results for eye irritation test using White New Zealand rabbits for BHA (test, T) and Biolon (control, C).

| EYE IRRITATION TEST                        |                          |            |          |            |          |            |          |            |          |
|--------------------------------------------|--------------------------|------------|----------|------------|----------|------------|----------|------------|----------|
| <b>ANIMAL NUMBER</b>                       | 69                       |            |          |            |          |            |          |            |          |
| <b>Reaction</b>                            | <b>Numerical grading</b> |            |          |            |          |            |          |            |          |
| <b>1. Cornea</b>                           | <b>#</b>                 | <b>1 H</b> |          | <b>24H</b> |          | <b>48H</b> |          | <b>72H</b> |          |
| <b>T=TEST C=CONTROL</b>                    |                          | <b>T</b>   | <b>C</b> | <b>T</b>   | <b>C</b> | <b>T</b>   | <b>C</b> | <b>T</b>   | <b>C</b> |
| <b>Degree of opacity (most dense area)</b> |                          |            |          |            |          |            |          |            |          |
| No opacity                                 | 0                        | X          | X        | X          | X        | X          | X        | X          | X        |
| <b>2. Iris</b>                             |                          |            |          |            |          |            |          |            |          |
| Normal                                     | 0                        | X          | X        | X          | X        | X          | X        | X          | X        |

|                                                                                       |                          |          |          |          |          |          |          |          |          |
|---------------------------------------------------------------------------------------|--------------------------|----------|----------|----------|----------|----------|----------|----------|----------|
| <b>3. Conjunctivae</b>                                                                |                          |          |          |          |          |          |          |          |          |
| <b>Redness (refers to palpebral and bulbar conjunctiva excluding cornea and iris)</b> |                          |          |          |          |          |          |          |          |          |
| Vessels normal                                                                        | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>Chemosis</b>                                                                       |                          |          |          |          |          |          |          |          |          |
| No swelling                                                                           | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>Discharge</b>                                                                      |                          |          |          |          |          |          |          |          |          |
| No discharge                                                                          | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>ANIMAL NUMBER</b>                                                                  | 84                       |          |          |          |          |          |          |          |          |
| <b>Reaction</b>                                                                       | <b>Numerical grading</b> |          |          |          |          |          |          |          |          |
|                                                                                       |                          |          |          |          |          |          |          |          |          |
| <b>1. Cornea</b>                                                                      | #                        | 1 H      |          | 24H      |          | 48H      |          | 72H      |          |
| <b>T=TEST C=CONTROL</b>                                                               |                          | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> |
| <b>Degree of opacity (most dense area)</b>                                            |                          |          |          |          |          |          |          |          |          |
| No opacity                                                                            | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>2. Iris</b>                                                                        |                          |          |          |          |          |          |          |          |          |
| Normal                                                                                | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>3. Conjunctivae</b>                                                                |                          |          |          |          |          |          |          |          |          |
| <b>Redness (refers to palpebral and bulbar conjunctiva excluding cornea and iris)</b> |                          |          |          |          |          |          |          |          |          |
| Vessels normal                                                                        | 0                        | X        | X        | X        | X        |          | X        | X        | X        |
| Vessels definitely injected above normal                                              | 1                        |          |          |          |          | X        |          |          |          |
| <b>Chemosis</b>                                                                       |                          |          |          |          |          |          |          |          |          |
| No swelling                                                                           | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>Discharge</b>                                                                      |                          |          |          |          |          |          |          |          |          |
| No discharge                                                                          | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>ANIMAL NUMBER</b>                                                                  | 80                       |          |          |          |          |          |          |          |          |

| Reaction                                                                              | Numerical grading |          |          |          |          |          |          |          |          |
|---------------------------------------------------------------------------------------|-------------------|----------|----------|----------|----------|----------|----------|----------|----------|
| <b>1. Cornea</b>                                                                      | #                 | 1 H      |          | 24H      |          | 48H      |          | 72H      |          |
| <b>T=TEST C=CONTROL</b>                                                               |                   | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> |
| <b>Degree of opacity (most dense area)</b>                                            |                   |          |          |          |          |          |          |          |          |
| No opacity                                                                            | 0                 | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>2. Iris</b>                                                                        |                   |          |          |          |          |          |          |          |          |
| Normal                                                                                | 0                 | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>3. Conjunctivae</b>                                                                |                   |          |          |          |          |          |          |          |          |
| <b>Redness (refers to palpebral and bulbar conjunctiva excluding cornea and iris)</b> |                   |          |          |          |          |          |          |          |          |
| Vessels normal                                                                        | 0                 | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>Chemosis</b>                                                                       |                   |          |          |          |          |          |          |          |          |
| No swelling                                                                           | 0                 | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>Discharge</b>                                                                      |                   |          |          |          |          |          |          |          |          |
| No discharge                                                                          | 0                 | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>ANIMAL NUMBER</b>                                                                  | 97                |          |          |          |          |          |          |          |          |
| Reaction                                                                              | Numerical grading |          |          |          |          |          |          |          |          |
| <b>1. Cornea</b>                                                                      | #                 | 1 H      |          | 24H      |          | 48H      |          | 72H      |          |
| <b>T=TEST C=CONTROL</b>                                                               |                   | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> |
| <b>Degree of opacity (most dense area)</b>                                            |                   |          |          |          |          |          |          |          |          |
| No opacity                                                                            | 0                 | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>2. Iris</b>                                                                        |                   |          |          |          |          |          |          |          |          |
| Normal                                                                                | 0                 | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>3. Conjunctivae</b>                                                                |                   |          |          |          |          |          |          |          |          |
| <b>Redness (refers to palpebral and bulbar conjunctiva excluding cornea and iris)</b> |                   |          |          |          |          |          |          |          |          |
| Vessels normal                                                                        | 0                 | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>Chemosis</b>                                                                       |                   |          |          |          |          |          |          |          |          |
| No swelling                                                                           | 0                 | X        |          | X        | X        | X        | X        | X        | X        |

|                                                                                       |                          |          |          |          |          |          |          |          |          |
|---------------------------------------------------------------------------------------|--------------------------|----------|----------|----------|----------|----------|----------|----------|----------|
| Any swelling above normal (including nictitating membrane)                            | 1                        |          | X        |          |          |          |          |          |          |
| <b>Discharge</b>                                                                      |                          |          |          |          |          |          |          |          |          |
| No discharge                                                                          | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>ANIMAL NUMBER</b>                                                                  | 94                       |          |          |          |          |          |          |          |          |
| <b>Reaction</b>                                                                       | <b>Numerical grading</b> |          |          |          |          |          |          |          |          |
| <b>1. Cornea</b>                                                                      | #                        | 1 H      |          | 24H      |          | 48H      |          | 72H      |          |
| <b>T=TEST C=CONTROL</b>                                                               |                          | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> |
| <b>Degree of opacity (most dense area)</b>                                            |                          |          |          |          |          |          |          |          |          |
| No opacity                                                                            | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>2. Iris</b>                                                                        |                          |          |          |          |          |          |          |          |          |
| Normal                                                                                | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>3. Conjunctivae</b>                                                                |                          |          |          |          |          |          |          |          |          |
| <b>Redness (refers to palpebral and bulbar conjunctiva excluding cornea and iris)</b> |                          |          |          |          |          |          |          |          |          |
| Vessels normal                                                                        | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>Chemosis</b>                                                                       |                          |          |          |          |          |          |          |          |          |
| No swelling                                                                           | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>Discharge</b>                                                                      |                          |          |          |          |          |          |          |          |          |
| No discharge                                                                          | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>ANIMAL NUMBER</b>                                                                  | 93                       |          |          |          |          |          |          |          |          |
| <b>Reaction</b>                                                                       | <b>Numerical grading</b> |          |          |          |          |          |          |          |          |
| <b>1. Cornea</b>                                                                      | #                        | 1 H      |          | 24H      |          | 48H      |          | 72H      |          |
| <b>T=TEST C=CONTROL</b>                                                               |                          | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> | <b>T</b> | <b>C</b> |
| <b>Degree of opacity (most dense area)</b>                                            |                          |          |          |          |          |          |          |          |          |
| No opacity                                                                            | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>2. Iris</b>                                                                        |                          |          |          |          |          |          |          |          |          |
| Normal                                                                                | 0                        | X        | X        | X        | X        | X        | X        | X        | X        |
| <b>3. Conjunctivae</b>                                                                |                          |          |          |          |          |          |          |          |          |
| <b>Redness (refers to palpebral and</b>                                               |                          |          |          |          |          |          |          |          |          |

|                                                            |   |   |   |   |   |   |   |   |   |
|------------------------------------------------------------|---|---|---|---|---|---|---|---|---|
| <b>bulbar conjunctiva excluding cornea and iris)</b>       |   |   |   |   |   |   |   |   |   |
| Vessels normal                                             | 0 | X | X | X | X |   | X | X | X |
| Vessels definitely injected above normal                   | 1 |   |   |   |   | X |   |   |   |
| <b>Chemosis</b>                                            |   |   |   |   |   |   |   |   |   |
| No swelling                                                | 0 |   | X | X | X |   | X | X | X |
| Any swelling above normal (including nictitating membrane) | 1 | X |   |   |   | X |   |   |   |
| <b>Discharge</b>                                           |   |   |   |   |   |   |   |   |   |
| No discharge                                               | 0 | X | X | X | X | X | X | X | X |

From the results the following with regards to the test substance (BHA) was observed: in animals 84 and 93 the vessels of the conjunctiva was injected above normal at 48 hours after exposure to the chemical. Animal 93 also showed chemosis – swelling above normal at 1 and 48 hours. For the control substance (Biolon): In one animal (97) chemosis - swelling above normal was observed at 1 hour. No eyes showed a positive response and all eyes were found to be normal and without damage at the end of the study.

According to the Draize scoring scale the results of the animals per time period are as follows:

**Table 3.** Average eye injury for two OVD's containing hyaluronic acid.

| Test substance | Average eye injury* |          |          |          |
|----------------|---------------------|----------|----------|----------|
|                | 1 hour              | 24 hours | 48 hours | 72 hours |
| BHA            | 0.333               | 0        | 1        | 0        |
| BIOLON         | 0.333               | 0        | 0        | 0        |

\*Calculated as the maximum total score for each eye

All scores obtained for any of the grading times were between 0-5 according to the Draize grading scale with a classification of: non-irritant.

## DISCUSSION

All new chemicals to be introduced into the market are to be tested and evaluated for their potential to create irritation, especially when the chemicals are to be utilized in delicate structures such as the eye.

The purpose of this study was to evaluate the potential of the ophthalmic viscosurgical device, consisting of hyaluronic acid, to produce eye irritation in the New Zealand white rabbit.

According to the scoring system developed by Draize *et al* in 1944, both the control and test substances were scored as: nonirritant (Draize *et al.*, 1944). This result for hyaluronic acid is consistent with previous studies (Becker *et al.*, 2009; Summary of Safety and Effectiveness Data for BioLon™, 1998, [http://www.accessdata.fda.gov/cdrh\\_docs/pdf/p960011.pdf](http://www.accessdata.fda.gov/cdrh_docs/pdf/p960011.pdf)).

The Draize eye test evaluates cornea, conjunctivae, iris and for any irritation or abnormalities created by a test substance. The cornea is evaluated for the degree of opacity and the area of the cornea involved. In this study no adverse effects were seen in the corneal structure. The next structure that is evaluated is the iris, and it is generally accepted that the iris will only be affected if the substance that is being tested is a severe irritant because for the iris to show any damage the test substance must penetrate through the cornea and into the anterior chamber of the eye. There were two observations of the test substance BHA just above normal regarding the conjunctivae at 48 hours (two eyes), but was not considered a positive reaction. Conjunctivitis is usually characterized by dilatation of the superficial conjunctival blood vessels, and depending on the severity of the inflammation hyperemia and edema of the conjunctiva with or without discharge may follow (Leibowitz, 2000). Chemosis, edema of the bulbar conjunctiva, forming swelling around the cornea (Stedman, 2000), was seen in the control (one eye) and the test (one eye at 1 hour and 48 hours). None of the observed reactions were rated as a positive reaction in any of the eyes and the slight inflammation cleared after 48 hours in all the eyes. The results of this study are consistent with the widely accepted fact that hyaluronic acid is not considered an irritant (Debbasch *et al.*, 2002; Durrani *et al.*, 1995; Wysenbeek *et al.*, 1988). In fact hyaluronic acid is considered to protect the cells of the eye when used in several applications such as that for drug delivery on the surface of the eye (Nepp *et al.*, 2001).

Because of its chemical structure, hyaluronic acid absorbs water and binds to the surface epithelial cells of the eye when administered as a medium to deliver drugs to the surface of the

eye such as artificial teardrops in the treatment of dry eye syndrome (Nepp *et al.*, 2001; Wysenbeek *et al.*, 1988). The hyaluronic acid serves as a viscous substance that prevents the tears from removing the drug from the ocular surface and thereby prolongs its contact with the surface of the eye. This application has also been utilized in the delivery of antibiotics to the surface of the eye (Price *et al.*, 2007). Hyaluronic acid is also believed to release the incorporated water molecules from its structure thereby acting as a wetting medium also alleviating the symptoms of dry eye (Debbasch *et al.*, 2002; Durrani *et al.*, 1995; Wysenbeek *et al.*, 1998). It has been shown to promote epithelial barrier function when incorporated into pharmacological compositions that is applied to the eye surface in that improves the integrity of the corneal epithelial cells, it also promotes corneal wound healing by increasing the migration of cells to the site of injury (Gomes *et al.*, 2004; Yokoi *et al.*, 1997).

The test product is not expected to be used repeatedly in the clinical setting, i.e. repeated exposures to BHA are not likely in short time frame and the test was therefore not conducted more than once, also no adverse reactions were seen in the study.

This test was designed to test products likely to come in contact with the topical surface of the eye such as cosmetic products and all ophthalmological related substances (Wilhelmus, 2001). In the test the observations are subjective, therefore the observers in this study were masked to prevent any bias between the two products.

Some limitations of the Draize eye test include the differences in human and animal eyes, the scoring of the data is subjective and ordered categorical data are obtained i.e. the results cannot be compared to each other as the scores are a representation of a multitude of components for each eye structure (Wilhelmus, 2001). Therefore an accurate statistical analysis cannot be done on a Draize eye test as the data is not related per category in any way as they represent an impartial measurement of a biological mechanism. Also, no evaluation of the molecular changes is taken into consideration as a standard in this test.

Animal models strive to model real life situations in order to test the irritation potential of chemicals, a task that cannot always completely be modelled by an equivalent cell culture study. In 1933 a woman went to tint her eye lashes and eye brows, and after application of “Lash-Lure”, mascara containing paraphenylenediamine (a coal-tar derivative), she went blind because of the toxic effects of this product not being tested (Wilhelmus, 2001). This

unforeseeable event could have been prevented with a simple Draize eye test like this one. Also legislation extensively provides for the extreme care of animals during the test (Wilhelmus, 2001).

## **CONCLUSION**

The Draize eye test was used to determine the irritant potential of two OVD's composed of hyaluronic acid, BHA and Biolon. BHA, a potential new OVD, was found not to be an eye irritant as no positive reactions were seen and this finding correlates with previous studies done on hyaluronic acid.

## CHAPTER 7

### CONCLUSION

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Ophthalmic viscosurgical devices (OVD's) are well known surgical aids used in all types of ophthalmic surgeries throughout the world. Different compositions of OVD's exist and because their composition determines their function, ophthalmic surgeons are provided with a wide variety of choice when having to perform different types of complicated surgeries.

The ideal OVD would create and maintain space, sufficiently protect the corneal endothelial cells and provide the ease of handling of the ocular tissues during surgery. These surgical aids can be used in surgeries in both the anterior and posterior compartments and have also been used to coat surgical instruments and artificial lenses during procedures.

According to ISO 15798, all OVD's is required to be tested in order to evaluate their biological safety before it can be released for further clinical tests on humans and finally into the market. The aim of this study was to determine the safety of a potential OVD, a buffered hyaluronic acid created as a cohesive OVD, by means of *in vitro* and *in vivo* test systems. During the evaluation several parameters were tested which included the cytotoxic potential, the sensitization potential, the IOP pattern and peak rise after surgery and finally the eye irritation potential.

The first of these evaluations entailed the exposure of the test article to human dermal fibroblasts in order to determine its cytotoxic potential at different concentrations. Colorimetric and morphological evaluations showed that the test substance did not have a detrimental effect on fibroblast cell cultures and is therefore not regarded as cytotoxic.

In the guinea pig maximization test the test article was evaluated for its potential to produce a skin sensitization reaction following the intradermal and topical application to the skin of Hartley albino guinea pigs. A response of 5.3% was scored by one of the three observers. Substances eliciting responses of more than 30% is considered a sensitizing agent. It was concluded from this study that the test OVD made of Hyaluronic acid, is not considered a skin sensitizer.

A transient rise in the intraocular pressure (IOP) postoperatively is assumed with the use of an OVD but must not be detrimental to the eye structures. Therefore the IOP pattern of the test substance was assessed. The IOP pattern correlated with that of a commercially available OVD, Biolon, and the peak IOP rise was seen at  $4\text{h} \pm 1\text{h}$  after surgery for both OVD's. A significant difference was found between the two substances at  $12\text{h} \pm 2\text{h}$  with a possible reason being the difference in concentration between the test and control substance. The study concluded that a similar postoperative IOP pattern was seen between the new and currently existing OVD on the market and that all IOP returned to normal 24 hours after surgery without any adverse events occurring.

The Draize eye test was used to determine the irritant potential of the OVD composed of hyaluronic acid. The test substance was found not to be an eye irritant as no positive reactions were seen and this finding correlates with previous studies done on hyaluronic acid.

The buffered hyaluronic acid OVD tested in this study was found to correlate well with a currently existing OVD on the market as well as did not have any detrimental effects on the *in vitro* cell system and proved safe in the *in vivo* studies on New Zealand white rabbits and Hartley guinea pigs.

## CHAPTER 8

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