

**EFFECT OF RIGID GAS PERMEABLE (RGP)  
CONTACT LENS CARE SOLUTIONS ON  
CULTURED EPITHELIAL CELLS**

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LENS CARE SOLUTIONS ON CULTURED  
EPITHELIAL CELLS**

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### تفويض

نحن الموقعين أدناه، نتعهد بمنح جامعة العلوم والتكنولوجيا الأردنية حرية التصرف في نشر محتوى الرسالة الجامعية، بحيث تعود حقوق الملكية الفكرية لرسالة الماجستير إلى الجامعة وفق القوانين والأنظمة والتعليمات المتعلقة بالملكية الفكرية وبراءة الاختراع.

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## **DEDICATION**

*To whom who have the limitless contribution*

*(My father and my mother)*

*To my uncle Dr. Mohammad Almasri*

*(May Allah have mercy on him)*

*I dedicate my thesis*

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## LIST OF ABBREVIATIONS

| <b><u>Abbreviation</u></b> | <b><u>Description</u></b>                                    |
|----------------------------|--------------------------------------------------------------|
| 7AAD                       | 7-amino-actinomycin D                                        |
| ATCC                       | American Type Culture Collection                             |
| CE                         | Corneal Epithelial                                           |
| Dk                         | Oxygen permeability                                          |
| DMEM                       | Dulbecco's Modified Eagle Medium                             |
| DMSO                       | Dimethyl Sulfoxide                                           |
| DNA                        | Deoxyribonucleic Acid                                        |
| DPBS                       | Dulbecco's Phosphate Buffered Saline                         |
| EDTA                       | Ethylenediaminetetraacetic Acid                              |
| EWC                        | Equilibrium Water Content                                    |
| FITC                       | Fluorescein Isothiocyanate                                   |
| G1 or G2                   | Gap1 or Gap2                                                 |
| HCE                        | Human Corneal Epithelium                                     |
| HPMC                       | Hydroxypropyl Methyl-cellulose                               |
| MCC                        | Minimal Cysticidal Concentration                             |
| MPS                        | Multipurpose Solution                                        |
| MTT                        | 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide |
| NAD                        | Nicotinamide Adenine Dinucleotide                            |
| O.D                        | Optical Density                                              |
| °C                         | Degree Celsius                                               |
| PHMB                       | Polyhexamethylene Biguanide                                  |

| <b><u>Abbreviation</u></b> | <b><u>Description</u></b>        |
|----------------------------|----------------------------------|
| PI                         | Propidium Iodide                 |
| PMMA                       | Polymethylmethacrylate           |
| PolyHEMA                   | Poly-2-hydroxyethyl methacrylate |
| PQ-1                       | Polyquaternium-1                 |
| PVP                        | Poly Vinyl Pyrrolidone           |
| Rb                         | Retinoblastoma                   |
| RGP                        | Rigid Gas Permeable              |
| RPM                        | Revolutions Per Minute           |
| SCL                        | Soft Contact Lenses              |
| SEM                        | Standard Error of the Mean       |
| SV40 LT                    | Simian Virus40 Long T            |

## ABSTRACT

# EFFECT OF RIGID GAS PERMEABLE (RGP) CONTACT LENS CARE SOLUTIONS ON CULTURED EPITHELIAL CELLS

By

**Alshefaa Shokry Almasri**

**Background:** Contact lenses are appliances that clinically used to correct ocular refractive errors as an alternative management option to spectacles correction. There are two main types of contact lenses that are used extensively today. These include soft contact lenses (SCL) and rigid gas permeable (RGP) contact lenses.

Contact lens care solutions are chemical formulas that used by contact lens wearers along with contact lens use in order to clean the lens from debris and dirt, wet the lens surface and disinfect the lens from microbes. Multipurpose solutions (MPSs) which are named due to the different purposes of their use (cleaning, wetting, and disinfection) are considered the main type of contact lens care solutions.

During contact lens wear, contact lenses that have been soaked overnight in MPS are applied to the ocular surface (the most anterior part of the eye). It is suggested that a trace amount of MPS could enter into the corneal cells upon contact lens insertion and through continuous release from the lens.

It has been reported that everyday usage of MPS for long term to clean and disinfect contact lenses may result in toxicity and adverse effect on the corneal epithelial cells. Such possible toxicity has been extensively reported during soft contact lens wear. However, the safety of RGP contact lens solutions is poorly studied.

**Purpose:** In this study, epithelial cell culture was used to investigate and compare possible toxicity of MPSs that used with RGP lenses.

**Methods:** Cultured epithelial cells were treated with different types of RGP MPSs and an appropriate control was used. Cytotoxicity assay such as 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was used to study possible toxic effect caused by MPSs against the cells. Additionally, cell cycle analysis using flowcytometry was used to assess the impact of MPSs on cell cycle profile. Wound healing model was also used to study the effect of different MPSs on cell migration during healing process.

**Results:** The results show a higher toxic effect of polyhexanide 0.0005% and chlorhexidine-based MPS compared to others tested solution. Some toxic effects seem to

be reversible as it is observed after re-growing the MPSs-treated cells in fresh cell culture media.

**Conclusion:** The toxic effect on epithelial cultured cells differs between the three tested RGP MPSs. These differences may be due to different formula of preservatives in MPSs. Polyhexanide 0.0005% and chlorhexidine 0.003% showed the most toxic effect on epithelial cultured cells. RGP MPS that contains polyhexanide 0.0002% showed less toxic effect and Polyhexanide 0.0001%-based MPS showed the least toxic effect against cultured cells.

## Chapter One: Introduction

The cornea is the most anterior part of the eye. It is avascular transparent tissue that mainly works as the light refracting surface in the eye. It also considered as a source of ocular defense that works to protect the whole eye from the microbes and external environment (Trattler, *et al.*, 2010).

It has been extensively reported that corneal health could be affected by external environment such as in contact lens wear. However, there is a reported variability of contact lens interaction with the corneal cells. This may be attributed to the type of contact lenses material, contact lens solutions and biocompatibility of contact lenses and their care solution (Bakkar, 2012).

Contact lenses are classified according to the material that they are made of into three main categories; soft lenses are made of hydrogel or silicone hydrogel, Rigid gas permeable lenses (RGP), hybrid lenses made of RGP focal optical zone, surrounded by soft lens material (Heiting, 2015).

Contact lens is applied to the ocular surface during contact lens wear. Once the lens is removed from the eye, it should be placed in multipurpose care solution (MPS) to remove the deposits attached to the lens surface and to disinfect the lens from microbes (Jones & Senchyna, 2007). It is suggested that a trace amount of MPS could be absorbed by lens material and expose to corneal cells upon contact lens wear and through continuous release from the lens (Gorbet, *et al.*, 2011).

It has been reported that everyday usage of MPS for long term to clean and disinfect contact lenses may result in toxicity and adverse effect on the corneal epithelial cells. Such possible toxicity has been extensively reported during soft contact lens wear. However, the safety of RGP contact lens solutions is poorly studied (Choy, *et al.*, 2013).

In studies that reported the effects of soft lens care solutions on corneal cells, the reported toxicity was attributed mainly to the preservatives or disinfecting agents in the solution.

Rigid gas permeable contact lenses (RGP) lenses are made of materials that relatively do not absorb solutions but still small amount could enter the eye through release from the lens surface or during lens handling via patient's hand. Compared to soft contact lens solutions, RGP lens solutions have higher percentage of disinfecting agents that may suggest higher level of toxicity if exposed to corneal epithelium (Choy, *et al.*, 2013).

Generally, in studies that aim to assess possible toxicity of contact lens solutions, the main model used is an *in vitro* model where the effect of solutions on cultured cells has been studied using different toxicity assays and cell markers. The most common assay is 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay; a colorimetric assay that depends on reduction of MTT formazan by cellular enzymes, so it determines the cytotoxicity based on cellular metabolic dysfunction and decreased viability that caused by studied agents (Morgan, 1998). Wound healing is another method to assess cell migration and proliferation in a deliberately-induced scratch area after exposure to toxic agent. Based in cell cycle analysis, flowcytometry using propidium iodide stain is another method to study the impact of agents on cell cycle stages in order to investigate any apoptotic effect of studied agents on cells.

The aim of our study was to study possible toxicity of different RGP multipurpose solutions on cultured epithelial cells and to compare the cytotoxicity profile of these solutions.

The significance of this work arises from the need to study potential toxicity of RGP MPSs among lifelong lens wearers such as keratoconus and orthokeratology patients. By studying the toxicity profile of RGP MPSs, we sought to provide better understanding of possible solutions toxicity at corneal level which may help in avoidance and management of possible ocular complications related to RGP lens solution.

The main objectives of this project are to:

1. Assess potential toxicity of RGP contact lens care solutions.
2. Compare the cytotoxicity profile of different RGP solutions.
3. Study the effect of RGP solutions on cells proliferation rate.
4. Assess the recovery rate of cells exposed to different RGP solutions using wound healing model.

5. Study the effect of RGP solutions on epithelial cells cycle and to investigate any occurrence of apoptosis in MPS-treated cells.

## Chapter Two: Literature Review

### 2.1 Anatomy and Physiology of the Cornea

The cornea together with the sclera and the limbus create the outer ocular surface of the eyeball (Figure 2.1) (Ostler, 2004). The cornea is the most anterior superficial part of the eye that composed of six layers as illustrated in (Figure 2.2). These include an epithelial layer, Bowman's layer, stroma, Descemet's membrane, a monolayer of endothelial cells (Trattler, *et al.*, 2010) and a recently discovered pre-Descemet's layer named as (Dua's layer) (Dua, *et al.*, 2013). The average diameter of the cornea is 11.5 mm horizontally and 10 mm vertically (Trattler, *et al.*, 2010). The cornea is covered by the pre-corneal tear film anteriorly, and posteriorly is in contact with the aqueous humor inside the eye.

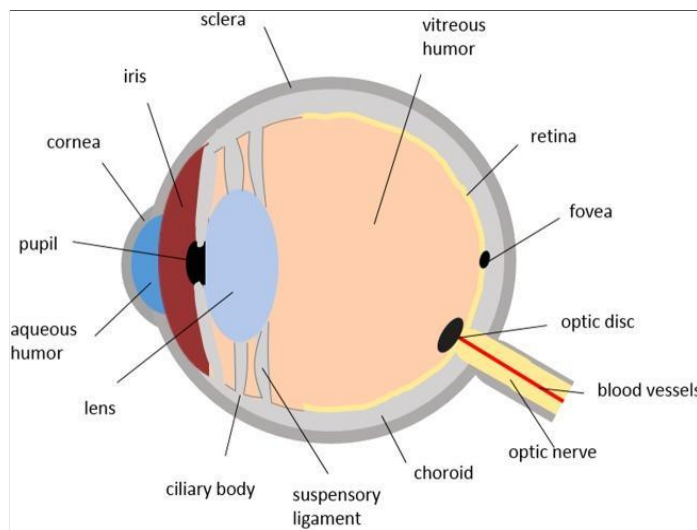


**Figure 2.1:** Anatomy of the human eye (Shafaie, et al., 2016).

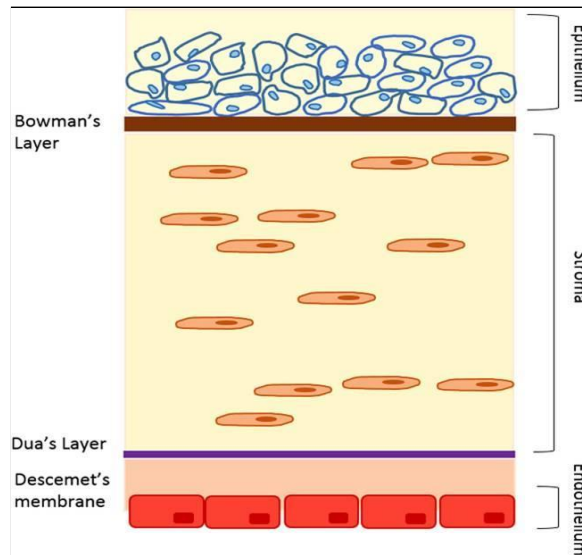


**Figure 2.2:** Histology of the cornea (Shafaie, et al., 2016).

### 2.1.1 The Corneal Epithelium

Corneal epithelial cell layer is about 50-52  $\mu\text{m}$  in thickness and consists of stratified squamous non-keratinized epithelial cells that arranged in 5-7 layers, these layers composed of a layer of basal cells, 2-3 layers of wing cells and 3-5 layers of squamous cells (Trattler, *et al.*, 2010). Similar to other epithelial cells, corneal epithelium works as a diffusion barrier to transmit solutes and water into the cornea and as a defense barrier against microorganisms. Because of its avascular structure, the corneal epithelium works as a transparent surface for refracting light into the eye (Acharya, *et al.*, 2008).

The epithelial cells are adhered strongly to each other and to their basement membrane by intercellular junctional complex such as desmosomes or hemidesmosomes, tight and gap junctions, these intercellular complexes associated with elements such as intracellular cytoskeleton to maintain the epithelial cells' shape and motility (Trattler, *et al.*, 2010).

The layers of epithelial cells are categorized into three groups:

#### 1. Basal epithelial cells

Are columnar shape epithelial cells forming a single layer which adheres to basement membrane (Trattler, *et al.*, 2010). The basement membrane is normally 0.05  $\mu\text{m}$  thick, comprises of laminin and type IV collagen, which secreted by these basal cells (DelMonte & Kim, 2011). The basal cell layer acts as barrier between the superficial epithelium and the stroma. Additionally, the basal layer serves to keep the polarity and give matrix for

structure and migration of epithelial cells as these basal cells are the only corneal cells that undergo mitosis (Trattler, *et al.*, 2010; DelMonte & Kim, 2011).

## 2. Wing cells

The wing cells are considered as an intermediate state of basal cells differentiation, that consists of 1-3 layers of polygonal wing shaped cells. These cells are connected with the basal cells via gap junctions and desmosomes (Trattler, *et al.*, 2010).

## 3. Squamous cells

The cells move from the basal cells layer to wing cells layer and finally differentiated into 3-5 layers of squamous non- keratinized cells (Trattler, *et al.*, 2010).

### **2.1.2 Bowman Layer**

The Bowman layer is located anterior to the stroma. It is acellular, smooth, 15  $\mu\text{m}$  thick layer. Its main function is to maintain the firm shape of cornea (DelMonte & Kim, 2011).

### **2.1.3 The Stroma**

The stroma forms the bulk part of the corneal structure that comprises approximately 80-85% of the corneal thickness. It is composed of parallel bundles of collagen fibers that arranged in fibrils. These fibrils are packaged in lamellae that also arranged in parallel. This network, which is highly organized, contributes to the mechanical strength and the high transparency of the cornea (DelMonte & Kim, 2011).

The main cell type in the stroma is keratocytes that play a crucial role in keeping homeostasis of the stroma, through synthesizing glycosaminoglycans and collagen molecules and creating matrix metalloproteases (DelMonte & Kim, 2011).

### **2.1.4 Descemet Membrane**

It is secreted by the endothelium to form unbanded amorphous region between the stroma and the endothelium. The thickness of Descemet membrane is around 10  $\mu\text{m}$  that increases with age (DelMonte & Kim, 2011).

### **2.1.5 The Endothelium**

The endothelial layer forms the posterior interface of the cornea with the aqueous. It is a monolayer of polygonal cells which have many organelles, such as endoplasmic reticulum, mitochondria and Golgi apparatus, more than other corneal cells. The presence of such organelles indicates the high metabolic importance and secretory role of endothelium cells (Trattler, *et al.*, 2010). The endothelium has an important function in regulation the water content in the stroma, this function is confirmed by layer's folds and microvilli in the apical surface that work to increase the absorption area with the aqueous (Acharya, *et al.*, 2008). The average range of density of corneal endothelial cells in adults is (2500-3000 cells/ mm<sup>2</sup>) and it decreases dramatically by age at rate of 0.6% per year. The corneal endothelial density is loosed shown to decrease in trauma, disease and after open eye surgeries (Trattler, *et al.*, 2010).

## **2.2 Keratoconus**

The most common ectasia corneal disorder is keratoconus which is described as a non-inflammatory thinning, protrusion and irregularity of the corneal surface (Krachmer, *et al.*, 1984) (Figure 2.3). The disease is described as a multi-factorial with unknown etiology (Espandar & Meyer, 2010) and its prevalence ranges from 50-230 per 100,000 (Romero-Jiménez, *et al.*, 2010). The exact etiology of keratoconus is unknown but it is suggested that there is a progressive stromal cornea loss due to an increase in protease enzyme activity or decrease in the activity of protease inhibitors in the stroma (Colin & Velou, 2003). It was shown that deep injury of epithelial cells due to; surgery, trauma or disease could cause the loss of anterior keratocytes of the stroma through apoptosis caused by interleukin-1 which may result in development of keratoconus (Colin & Velou, 2003). Ectopic eye disease, aggressive eye rubbing (McMonnies, 2009) and improper contact lens fitting were also suggested to increase the risk of keratoconus (Macasai, *et al.*, 1990).

At molecular level, many studies have been done to study genetic association in keratoconus. However, the results indicated a genetic heterogeneity of the disease (Espandar & Meyer, 2010).

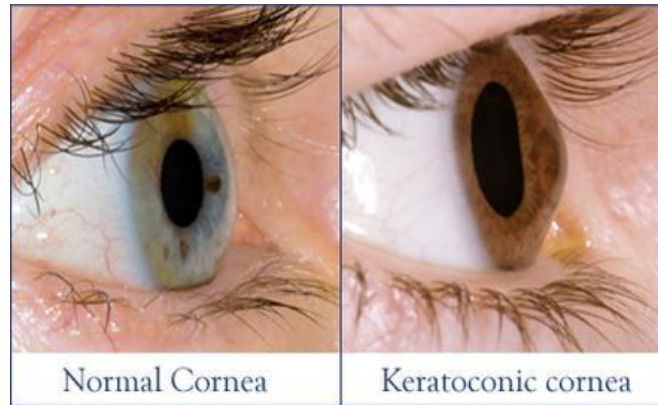


**Figure 2.3:** Normal versus keratoconic cornea  
(retrieved from: <http://www.kcnz.co.nz/what-is-keratoconus.html>).

Keratoconus and other ectatic diseases are associated with corneal irregularities and distortion that result in reduction in visual acuity in affected patients. Hence, management of keratoconus disease has an important role in vision rehabilitation and improvement of quality of life related to visual tasks (Gore, *et al.*, 2013).

Management options for keratoconus include spectacle, soft contact lenses or rigid gas permeable (RGP) lenses. However, RGP lenses could be used in all stages of the disease and proved to give superior optical quality and improved visual functions over spectacles and soft contact lenses (Espandar & Meyer, 2010). Other surgical treatment options are also available to manage keratoconus. These include; collagens cross linking, corneal intrastromal rings and corneal keratoplasty (Gore, *et al.*, 2013).

## 2.3 Contact Lenses and their Care Solutions

### 2.3.1 Types of Contact Lenses

According to the materials that used in contact lenses manufacturing, there are two types of contact lenses; these are soft contact lenses and rigid contact lenses.

Soft contact lenses were firstly introduced in 1960's by Wichterle and Lim who succeed in developing the first type of soft contact lens material known as poly-2-hydroxyethyl methacrylate (polyHEMA) (Jones, 2002). This material mainly made of hydrogel material which are polymeric, water absorbance, hydrophilic materials (Subbaraman, 2009). The polyHEMA was extensively used in contact lens manufacturing, however, the main limitation of polyHEMA lenses is the low equilibrium water content

(EWC) which result in decrease in lens oxygen permeability (Dk). In order to avoid this problem and to increase the EWC of soft lenses, hydrophilic groups have been added to polyHEMA to increase its oxygen transmissibility (Jones, 2002; Subbaraman, 2009). In 1990's silicone hydrogel lenses were developed by silicone-based technology, the silicone content provides much higher Dk value compared to the conventional hydrogel lens material (Gill, 2010).

Rigid contact lenses were first manufactured using polymethylmethacrylate (PMMA) in 1940 (Jones, 2002). These lenses which made of PMMA have a main drawback of low to no oxygen transmissibility into the cornea that accompanied in some cases with corneal hypoxia and corneal neovascularization (Jones, 2002). With the advent of new rigid lens materials, the PMMA lenses wear had been declined (Gill, 2010). Nowadays, new rigid lens material that characterized by high wettability and oxygen transmissibility allow lens wearers to benefit of comfort and better vision during extended period of use and decreased the complications related to low oxygen transmission (Gill, 2010).

### **2.3.2 Contact Lens Care Solutions**

Contact lens care solutions are chemical formulas that used by contact lens wearers in order to clean contact lenses from debris and dirt and disinfect them from microbes. The main type of contact lens care solutions is multipurpose solutions (MPSs) which is named due to the different purposes of their use that include lubricating the lens (wetting) and prevention of lens material dehydration, removal of deposits from the lens surface and disinfection against microbes (Jones & Senchyna, 2007). Due to its affordability, efficacy and ease of use, contact lens multipurpose solution (MPS) is considered the most popular care regime for soft and rigid contact lenses (Haddad, *et al.*, 2016). However, some level of corneal toxicity is suggested to occur by MPS chemical ingredients (Choy, *et al.*, 2013).

Contact lenses are usually cleaned from debris and microbes through overnight soaking in MPS. However, trace amount of MPS may be absorbed/adsorbed by lens material during overnight storage of the lens in the solution. Upon lens wear this small amount of MPS could be released onto the corneal surface causing cytotoxicity and cell damage (Gorbet, *et al.*, 2011). To minimize possible cytotoxicity in the cornea, MPSs have been manufactured carefully to accomplish a dual purpose; a maximum antimicrobial

activity and least toxicity to the cornea, which is known as biocompatibility of the solution (Choy, *et al.*, 2013).

Contact lens care solution mainly contains preservative, surfactant, buffers, chelating and wetting agent (Bakkar, 2012). However, each solution has special components with specific concentrations (Gromacki & Ward, 2013). The different ingredients of MPS are listed below:

1. Disinfectants: or "preservatives" are antimicrobial agents and are the active agents in MPS. They act to sterilize the lens. Examples of disinfecting agents are polyquaternium-1 and biguanide which are different from each other by structure, concentration and function (Bakkar, 2012).
  - a. Polyquaternium-1: or "polyquad" (PQ1), polycationic quaternary ammonium acts against microorganism through denaturing the protein of microbial cell membrane, 100% of its activity retained over 6 hours, usually 0.001% of PQ-1 is used in most MPSs (Rolando, *et al.*, 2011; Gromacki & Ward, 2013).
  - b. Biguanides also known as polyhexamethylene biguanide (PHMB), polyhexanide and polyaminopropyl biguanide. It is a bactericide derived from chlorhexidine and has adverse potential effect on the bacteria metabolic activity through disrupting its plasma membrane (Gromacki & Ward, 2013). However, 30-60% of biguanide activity has been reported to deplete by 6 hours after lens inserting in the eye (Rolando, *et al.*, 2011).
2. Buffers: they work to maintain the pH of the MPS solution at the range of the pH of eye around 7.4 examples of buffers include; phosphate, bicarbonate, borate, nitrate, and citrate (Gromacki & Ward, 2013).
3. Surfactants: are amphiphilic with hydrophobic tails and hydrophilic heads. So, it can attach and solubilize the debris, proteins and lipids attached to the lens surface, examples of surfactants; polaxamer-407, sodium phosphate, sodium borate, sodium citrate, isopropyl alcohol and polyvinyl alcohol (Gromacki & Ward, 2013).
4. Chelating agents: work to enhance disinfection through prevent proteins that bind to calcium from attaching to the surface of lens, Ethylenediaminetetraacetic acid (EDTA) is the most used one (Gromacki & Ward, 2013).

5. Wetting agents: are moisture attractants that work to wet the lens surface and improve ocular comfort during lens wear. Examples of wetting agents are poloxamine, hydroxypropyl methylcellulose, dexpanthenol, carboxymethylcellulose, hyaluronic acid, hydroxypropylguar, sorbitol and tetronic (Gromacki & Ward, 2013).

MPSs are used in both soft and rigid contact lenses. As the focus of this study was to investigate possible toxicity of RGP lens solution, Table 2.1 summarizes the ingredients of most common RGP MPSs that used in our study.

**Table 2.1:** RGP lens care solutions.

| Product    | Manufacturer  | Active ingredients *                                                                                                                                                                                                                                                     |
|------------|---------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| GPmulti    | AVIZOR        | Preservatives: polyhexanide 0.0002% in a buffered, isotonic and sterile solution. EDTA 0.1%.<br><br>Surfactant: poloxamer 0.25%.<br><br>Wetting agents: Hydroxypropyl methyl-cellulose (HPMC) and Poly vinyl pyrrolidone(PVP).                                           |
| Boston     | Bausch & Lomb | Preservatives: polyamino propyl biguanide 0.0005%, chlorohexidine gluconate 0.003%, edetate disodium 0.05%.<br><br>Wetting agents: Derivatized Polyethylene Glycol, Cellulosic Viscosifier, Cationic Cellulose Derivative Polymer, Polyvinyl Alcohol, polyquaternium 10. |
| Delta plus | SAUFLON       | Preservatives: Polyhexanide 0.0001%<br><br>Surfactant: poloxamer.                                                                                                                                                                                                        |

\* The active ingredients were taken from MPSs' leaflet.

## 2.4 Cell Culture

The growing of specific type of cells under specific conditions is called "cell culture", according to Adamson and his colleagues these conditions include: controlling the temperature, the component of gases that aerating the culture, the component of growth media and the supported layer of the cell (Bakkar, 2012). Cell culture has several types classified according to the methods used to produce the cells, mainly these methods include: suspension, adherent, organotypic, primary and transformed cell culture.

### 2.4.1 Corneal Epithelial Cells Culture

The main purpose of finding proper corneal epithelial (CE) culture is to replace the usage of Draize (rabbit's eye) test (Dart, 2003). The availability of corneal tissue makes corneal epithelium a good source for primary culture to use in investigations in

toxicological studies (Freshney, 2010). CE culture can be sub-cultured up to five times (9-10 population doublings) yields 1 to  $2 \times 10^6$  cells/cornea (Freshney, 2010).

In primary culture, cells reach senescence after a small number of doublings, that made it not practical for investigational used; so, there was an emerged need toward cells immortalization to get cell lines of human corneal epithelium (HCE) with an extended life span and with large supply for experimental purposes (Freshney, 2010).

In immortalization process the primary cells (limited population doublings so limited number of passage) are converted to cell lines which have extended number of passage, this process is achieved by transformation either through virus or plasmid (Walker & Kahn, 1997). Additional advantage of immortalized cell line is the keeping of morphological properties and phenotypic differentiation of primary cultured cells in situ (Walker & Kahn, 1997; Freshney, 2010).

Walker and Kahn enhanced the production of immortalized HCE through transformation of long T Antigen found in hybrid viruses (SV40 LT gene) for ocular investigation purposes. SV40 T Antigen is transforming multi-functional protein which inhibits the function of retinoblastoma (Rb) and p53 genes through binding to their product (Walker & Kahn, 1997; Yamasaki, *et al.*, 2009; Freshney, 2010).

#### **2.4.2 Vero Cell Culture**

Verd reno abbreviated as vero which means green kidney in Esperanto language. Vero cells are kidney epithelial cells derived in 1962 from African green monkey (*Cercopithecus aethiops*) by Yasumura and Kawakita at Chiba University in Japan (Ammerman, *et al.*, 2008).

According to many reports, Vero cell line has many uses in virology study, viruses and viral vaccine production (Ammerman, *et al.*, 2008; Osada, *et al.*, 2014), and to study the propagation of bacteria and parasites (Ammerman, *et al.*, 2008; Lorenzo-Morales, *et al.*, 2015). It has also been used as mammalian cellular model to estimate the impact of toxins, chemicals including ophthalmic solutions (Ammerman, *et al.*, 2008; Osada, *et al.*, 2014).

Throughout this project, the effect of RGP MPSs on epithelial culture was studied using vero cells due to its availability, reproducibility and its nature as epithelial cells that mimic the function of the other epithelial cells including corneal epithelial cells.

## **2.5 Toxicity Assays Used to Study the Effect of Solutions on Cultured Cells**

In 1944 the Draize test has been established for examination of the effect of ocular drug and chemicals on the rabbit eye. Although the Draize test is still used as a standard model for such investigations, researchers in the 1980s have tried to find alternative models to avoid animal sacrifice; they determined two basic models; organotypic models that used whole animal organs and cell culture models that basically used cultured cells as discussed in section 2.4.

In ocular toxicity studies, epithelial cells, or cells from other organs such as cells from connective tissues and keratinocyte cells have been extensively used as *in vitro* models for ocular research (Dholakiya & Barile, 2013). These *in vitro* toxicity models have many advantages over the *in vivo* models. As the former are considered simple, inexpensive and can be manufactured quickly, so the replication data can be obtained and the experiment conditions can be designed and controlled (Wilson, *et al.*, 2015).

Cytotoxicity assays are extensively used for toxicological studies that involved *in vitro* cell model. In such experiments, it is aimed to study the negative effect of substances on the human body at the same time keeping the human far from possible risk (Fotakis & Timbrell, 2006). In ocular cytotoxicity studies, the experiments composed of suitable cell culture, the material need to be tested and an appropriate assay. Examples of these assays are protein assays, enzymatic assays, mitochondrial assays (Wilson, *et al.*, 2015). Enzymatic assays are the most used cytotoxicity assays with the most common examples of these assays are Methyl Tetrazolium (MTT) assay and Lactate Dehydrogenase (LDH) assay (Fotakis & Timbrell, 2006; Wilson, *et al.*, 2015).

In this study, MTT cell proliferation assay, scratch wound healing assay and cell cycle analysis through flowcytometry were used to assess the effect of RGP MPS on cultured cells.

### **2.5.1 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay**

The MTT assay is used as a colorimetric viability assay to assess the proliferation and cytotoxicity effects of chemicals on cells based on the activity of cells' mitochondrial enzymes (Xu, *et al.*, 2015). It was developed in 1983 by Mosmann (Mosmann, 1983).

MTT is a tetrazolium salt which has a positive charge and it is a water-soluble compound. Succinate dehydrogenase enzyme (found in mitochondria) converts tetrazolium ring of MTT by NADH to purple, insoluble and impenetrable material called formazan. The purple formazan accumulates inside the cells as crystals, which is dissolved by organic solvent. The absorbance of these solubilized crystals can then be read at 570 nm to express cell viability (Fotakis & Timbrell, 2006; Xu, *et al.*, 2015).

### **2.5.2 Scratch Wound Healing Assay**

Wound healing process must be maintained in most normal viable tissue. The process involved cell migration then proliferation and differentiation to replace dead/damage cells (Ljubimov & Saghizadeh, 2015).

The scratch wound healing assay is an *in vitro*, inexpensive protocol used to measure the migration rate of cells in a deliberately induced scratch area (Liang, *et al.*, 2007). The assay is basically mimics migration behavior in normal cells after tissue injury and the behavior of cancer cells during metastasis (Jonkman, *et al.*, 2014).

The principle of the assay involved making scratch or gap on the surface of confluent monolayer cells by mechanical, chemical or thermal mechanisms (Liang, *et al.*, 2007; Jonkman, *et al.*, 2014). Depending in cell status, the gap found in such confluent monolayer cells leads the cells to migrate to close this space (Jonkman, *et al.*, 2014). For quantification purposes, images of the gap/scratch at the beginning and after a specific period of incubation are captured. Analysis of the length or area of the scratch is then performed to calculate the migration percentage in cells (Liang, *et al.*, 2007).

### **2.5.3 Propidium Iodide Flowcytometry Cell Cycle Analysis**

All cells generate from another cells in a process called cell cycle. During this process, cells go through G1 phase where the cells prepare to DNA synthesis. Then S phase where DNA replication occurs. This is followed by G2 where the cells have two

copies of DNA and prepare for mitosis. Finally, during cell rest or senescence cells enter G<sub>0</sub> phase (Crowley, *et al.*, 2016). Figure 2.4 summarizes these phases.

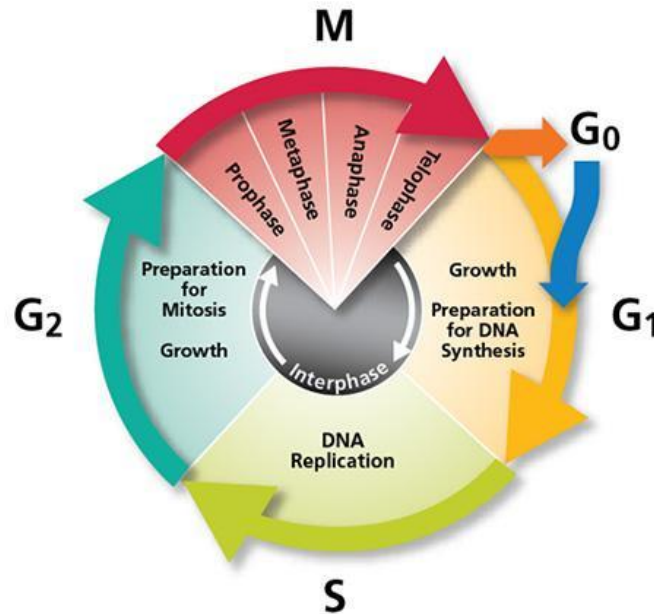


**Figure 2.4:** Cell cycle phases

(retrieved from: <http://www.bdbiosciences.com/anz/research/apoptosis/analysis/index.jsp>)

Propidium iodide (PI) flowcytometry cell cycle analysis is used to determine the status of cell cycle in cell culture. PI is a fluorochrome fluorescence stain, excited at 488nm wavelength and its emission occurs around 600nm. The stain has the ability to bind to the major groove of DNA in stoichiometric manner, which means the intensity of the stain is related to the DNA content in the cells. This fact is used to discriminate different cell cycle phases (G<sub>0</sub>, G<sub>1</sub>, G<sub>2</sub>/M) in cells (Darzynkiewicz, *et al.*, 2001; Darzynkiewicz, 2010; Crowley, *et al.*, 2016). Figure 2. shows histogram representation of normal cell cycle profile.

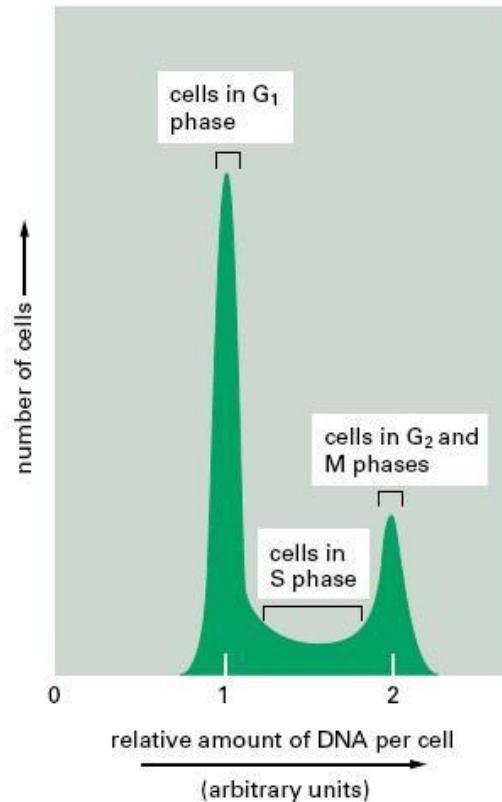


**Figure 2.5:** The results of flowcytometry shown as DNA histogram where the phases of cell cycle appear (retrieved from [slideplayer.com/slide/10880130](http://slideplayer.com/slide/10880130)).

## 2.6 Previous Studies on the Effect of RGP MPSs on Cultured Cells

It has been extensively reported that corneal health could be affected by external environment such as in contact lens wear. However, there is observed variability in studies that reported contact lens interaction with corneal cells. This variability may be attributed to the type of material that used in contact lens manufacturing, contact lens solution and biocompatibility of contact lens and its care solution (Bakkar, 2012).

Many studies have reported the effects of soft lens care solutions on corneal cells. Where toxicity on cultured epithelial cells was attributed mainly to preservatives or disinfecting agents. On the other hand, RGP lenses are made of materials that relatively do not absorb solutions but still small amount could enter the eye through release from the lens surface or during lens handling via patient's hand.

Many studies have investigated the effects of soft contact lenses and care solutions on epithelium to understand the relationship between these effects and risk of epithelium

infection (Robertson & Cavanagh, 2008). Gorbet et al have studied the potential effect of contact lenses materials and MPSs on HCE cells and they found that the phenotypes and the viability of cells are affected by the components of MPS not by the lens itself (Gorbet, *et al.*, 2011).

Using rabbit eye as a model, Begley *et al.* have studied the effect of three RGP care solutions on the Corneas of rabbit by using scanning electron microscope, they noticed that polyaminopropyl biguanide-based lens solution caused significant changes in the cells morphology (Begley, *et al.*, 1991). However, the method that was utilized did not determine what exactly cellular changes.

RGP MPSs apparently have higher percentage of disinfecting agents than soft contact lenses MPSs (Choy, *et al.*, 2013). In a study by Choy *et al.*, the effect of RGP MPS on human cultured epithelial cells was assessed using a mitochondrial activity assay; MTT assay and Annexin V stain, they found RGP MPS that contains chlorhexidine as preservative, has higher toxicity as it resulted in lowest percentage of cell viability and highest number of necrotic cells (Choy, *et al.*, 2013).

## Chapter Three: Materials and Methods

### 3.1 Preparation of Epithelial Cell Culture

All cell culture work was run in a laminar flow hood under appropriate aseptic conditions. Throughout all experiments, Vero cells (ATCC® CCL-81™) were used between cell passage 35 and 46. The cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) without L-Glutamine (EuroClone, Via Figino-Italy). The media were supplemented by adding 50ml of fetal bovine serum (BVS) (Biowest, France) at 10% concentration, and 5ml penicillin-streptomycin (EuroClone, Via Figino-Italy) at 1% concentration (Ammerman, *et al.*, 2008). The final volume of the media was 500 ml.

Vero maintenance had been done periodically, the media were changed twice weekly, and when >90% confluence level achieved (Figure 3.1) the subculture was done according to cell culture laboratory protocol as the following:

1. Media were removed from the flask through aspiration by pipetting.
2. 5ml of Trypsin-EDTA at 0.05% concentration in Dulbecco's phosphate buffered Saline (DPBS) (Capricorn, Germany) was then added to the cells in the flask.
3. The flask was incubated for 5-10 minutes at 37°C, 5% CO<sub>2</sub> incubator until all cells detach from the flasks surface.
4. Then media was added to the detached cells in the flask to neutralize the effect of trypsin.
5. Cells were centrifuge at 1000 revolutions per minute (rpm) for 10 minutes and cell pellet was re-suspended in DMEM.

After suspension of the pellet, cells were counted using hemocytometer following the steps below:

Chamber of the hemocytometer was loaded with cells suspension by pipetting of 10µl volume of the cell suspension. The chamber then covered using a glass cover slip.

Cells were then counted using microscope (10X) in 4 large corner squares of the hemocytometer, each square contains  $10^{-4}$  ml. After that, average of cell counts in these

squares was taken and multiplies by 10000 to get number of cells per ml. Finally, average cell count is multiplied by the volume we have to get the total number of cells. Alternatively, number of cells that is needed for an experiment is calculated to get the volume that contains the number of cells needed. Divided this number by the number of cells/ml, this volume is then transferred to conical tube. Next, conical tube was centrifuged at 1000rpm for 10 minutes. Finally, the cells were re-suspended with required volume of media.

After cell counting, cell suspensions were either split in a ratio of 1 to 5 into 75 cm<sup>2</sup> cell culture flask or seeded into multi-well cell culture plates to be used for subsequent experiments.

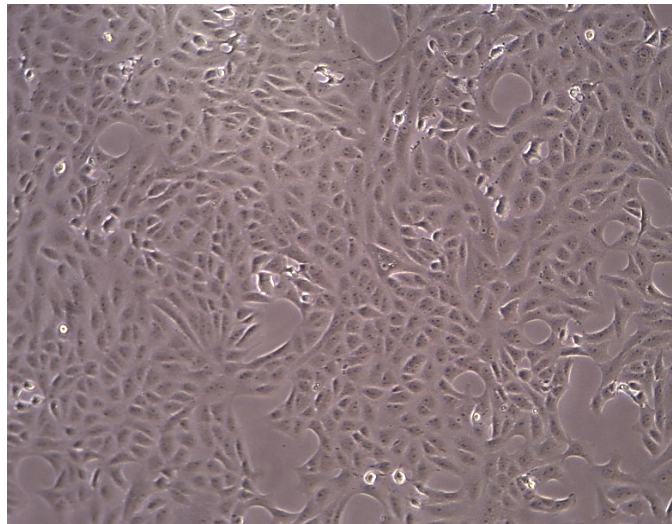


**Figure 3.1:** Cultured Vero cells as seen using inverted microscope (10X)

### **3.2 Cells Treatment with RGP Contact Lens Solutions**

Three types of sterile sealed RGP contact lens solutions were ordered prior to their used in the study. These solutions included GPMulti (AVIZOR, Spain), Boston (Bausch & Lomb, Italy), and Delta (Saulfon, UK). All solutions were checked for their expiry dates and used in the experiments within the expiry date and before the third months of initial opening.

Three different dilutions were prepared from each MPS to get 10% (v/v), 25% (v/v) and 50% (v/v) concentration of the tested MPSs. All solutions were diluted in growth medium.

Vero epithelial cells were seeded in multi-well plates in specific density and treated with different dilutions of the solutions. For MTT assay experiments, cells were seeded in a density of 20000 cells/well in 96-well plate. For flowcytometry experiments cells were seeded in a density of 400000 cells/well in a 6-well plate and for wound healing model cells were seeded in a density of 400000 cells per well in 12 or 24-well plates. In all experiments control cells were treated with growth media.

### 3.3 Measuring Cell Viability Using MTT Assay

MTT cell proliferation assay Kit (Cat.No.21180, iNtRON Biotechnology, Korea) was used throughout the experiments to measure cells viability after exposure to different dilutions of MPSs. Prior to treatment with MTT, cells were seeded in 96-well plate at a density of 20000 cells/well, 100µl of cell culture were added per well. After 24 hours incubation of the cells, the cells were treated by three MPSs (GP multi, Boston, Delta) at three different dilutions for each solution (10%, 25%, 50%). For each treatment six well replicates were used and each experiment was repeated four times. The effect of MPSs on Vero cells were monitored after 4 hours and 20 hours of exposure to different MPSs.

After cell exposure to different MPSs, MTT assay was run following iNtRON protocol as the following:

1. A volume of 10µl MTT solution was added per well in the 96-well plate.
2. Cells then incubated for four hours.
3. Media in the well were removed and plate is checked under the microscope for the formation of formazan crystals.
4. Formazan crystals were then dissolved by adding 100µl DMSO per well.
5. Resultant absorbance was read by spectrophotometer (BioTek®, USA) measured at 490 nm.

The viability percentage was computed according to the following equation:

$$Viability \% = (O.D_{Sample} - O.D_{Blank}) / (O.D_{Control} - O.D_{Blank}) * 100$$

The mean viability of the control cells was standardized to 100%. Results were calculated as a ratio of percentage reduction of cell viability relative to that of control cells, and were expressed as mean  $\pm$  standard error of the mean (SEM).

### 3.4 Measuring Cell Viability after Exposure to MPSs and Following Re-Culturing in Growth Media

In this experiment, the aim was to study the viability of Vero cells after recovery from initial exposure to MPSs. Cells were initially seeded in 96 well plates at a density of 20000 cells/well and a volume of 100µl/well.

After overnight growth, cells were treated with different dilutions of MPSs. Control cells were exposed to growth medium.

For one plate cells viability were measured using MTT assay as described in section 3.3 and after 20 hours exposure to MPSs.

In the second plate and in order to study the effect of treatments washout, the media/treatments were aspirated and replaced with fresh media. Cells were then incubated for further 24 hours. The MTT assay was then run following the protocol as described in section 3.3.

The viability percentage of the first and the second plate (washout) was calculated using viability based absorbance formula in section 3.3. Viability of the two conditions (treatment Vs washout) of the treatment was compared. Figure 3.2 summarizes the wash out/ re-culturing experiments.

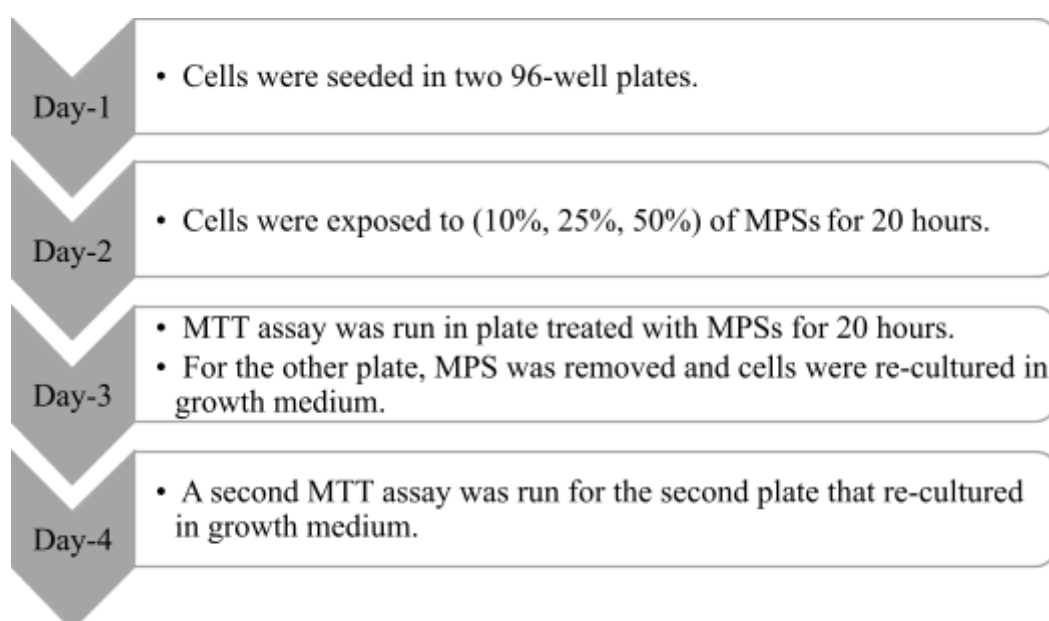


**Figure 3.2:** Summary of re-culturing experiments.

### 3.5 Wound Healing Assay

The first step that occurs when healing happens is migration of the cells to fill in the wound place. Based on that, the wound healing assay aims to measure the ability of treated cells to migrate through a scratch to close it (Liang, *et al.*, 2007).

Throughout this experiment, cells were seeded in 12-well plate at a density of 400000 cells/well. Cells were then incubated for 72 hours until 100% confluence level was confirmed by microscopy. Next, cells were treated with three concentrations of each tested MPSs (10%, 25% and 50%). Control cells were exposed to cell culture media.

Cells were incubated for 20 hours. After that, 200µl pipette tip was used to scratch the surface of cells. Immediately after the scratch, images of the wells were captured by microscope and accordingly named baseline image, as shown in Figure 3.3.



**Figure 3.3:** A scratch on cultured cells induced by 200µl pipette tip (baseline image).

Following that, the treatments were changed carefully and replaced by fresh cell culture media. Following 24 hours of incubation, a second image of the wells was captured and named final image. Baseline and final images were analyzed By ImageJ software (1.51j8, USA) through measuring the length of scratch at baseline and final images. The migration percentage was then calculated using the following equation:

$$\text{Cell Migration\%} = (\text{Baseline scratch length} - \text{Final scratch length}) / \text{Baseline scratch length} * 100.$$

Data were represented by a plot the concentrations of different MPSs versus the percentage of cell migration.

### **3.6 Assessment of Cell Cycle Profile after Exposure to MPSs Using Flowcytometry**

The aim of this experiment was to study the cell cycle profiles in cells treated with different MPSs through using propidium iodide (PI) flowcytometry kit for cell cycle analysis (ab-139418, abcam, UK).

Prior to the experiment, Vero cells were seeded in 6-well plates at a density of 400000 cells/well. Plates were then incubated for 72 hours before cells were treated with a concentration of 25% (v/v) of different types of MPSs. Plates were then incubated for four hours.

Following the four hours incubation with different treatments, the cells were harvested from the wells by aspiration of the media and washing the cells by 1ml of phosphate buffered saline (PBS). Both media and PBS were collected in 15ml tube. Then, 1ml of Trypsin-EDTA at 0.05% concentration in DPBS was added to each well and incubated until all cells detached. The trypsin was then neutralized with media and collected in same tubes, then, tubes were centrifuged at 1500 rpm for 5 minutes. Cells were then washed by PBS and centrifuged again. After that, the formed cells pellet was fixed in 66% Ethanol (400µl PBS+ 800µl Ethanol) in ice. Cells were then stored in tubes overnight at 4°C.

Next day, the PI flow cytometry assay was run as the following: the tubes of cells that stored overnight at 4°C were centrifuged at 1500 rpm for 5 minutes, and then were washed by PBS, and the centrifuge was repeated. 1X of PI+Rnase was added to the cells and cells were incubated in dark at 37°C for 20-30 minutes. Samples were then kept in ice prior to their imaging by flowcytometry machine (Becton Dickinson FACSCalibur™, USA). Flowcytometry data acquisition and analysis were performed by Cell Quest Pro program (BD Bioscience, Canada).

### **3.7 Statistical Analysis**

Statistical analysis of the data was performed using Statistical Package for the Social Sciences (SPSS) program (version 19, 2010, USA). Paired t-test was used to compare results in the same group. Analysis of variance (ANOVA) and Post hoc test were used to compare the results between different solutions at different concentrations. Data were

presented as mean $\pm$  standard error of the mean. Significance has been considered when P value is less than 0.05.

## Chapter Four: Results

### 4.1 Cell Viability Measured by MTT Assay

Treatment of cultured epithelial cells with three concentrations of three MPSs for 4 hours yields a pattern of response as illustrated in Figure (4.1), where cell viability in Boston<sup>®</sup> < GPmulti<sup>®</sup> < Delta<sup>®</sup> for all used concentrations of the solutions. Both GPmulti and Boston showed a significant reduction in cell viability at all concentrations ( $p=0.00$ ). Delta solution only showed slight reduction in cell viability only at 50% concentration of the solution. However, the effect of 50% Delta solution on cell viability was not statistically significant ( $p=0.975$ ).

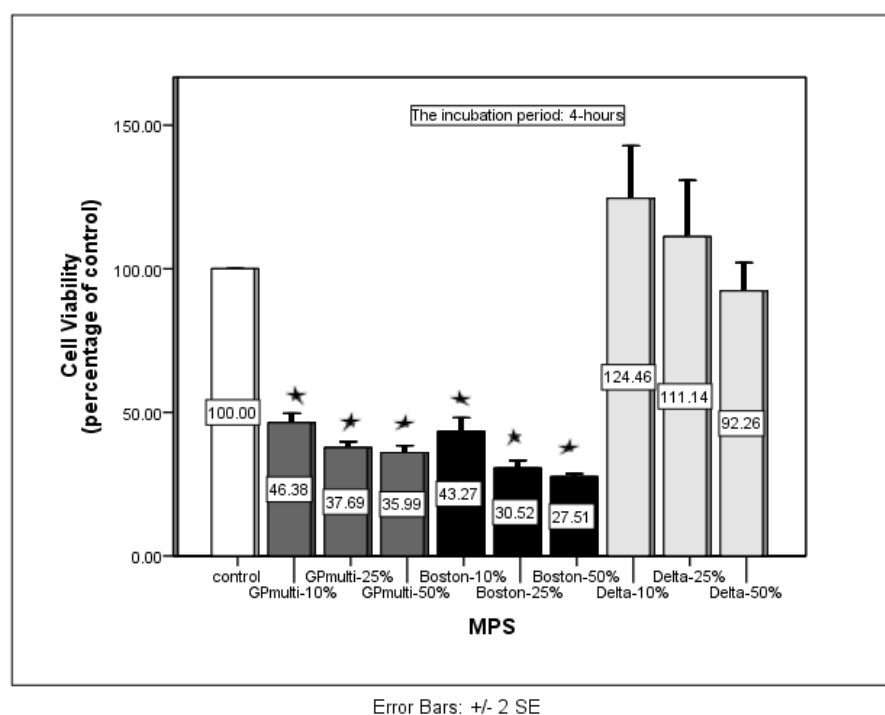


**Figure 4.1:** Percentage of cells viability after 4 hours treatment of cells with three concentrations (10%, 25% and 50%) of three MPSs. Number of wells per treatment= 6.

- Indicates significantly decreased in cell viability comparing to control.

In different experiments, cells were also treated with different concentrations of the MPSs for 20 hours. Similar to the 4 hours exposure, cell viability percentage were also decreased at all concentrations compared to the control cells as it is shown in Figure 4.2, and the reduction in cell viability was significant at all used concentrations ( $p=0.00$ ).

Similar to the 4 hours treatment, the 20 hours treatment also showed similar effect on cell viability. Where cell viability profile was as the following: Boston® < GPmulti® < Delta® for all used concentrations ( $p=0.00$ ). However, the prolonged effect of 20 hours exposure showed much lower level of cells viability in all solutions and at all concentrations compared to 4 hours treatment ( $p=0.00$ ), that may suggest time-dependent toxicity effect of MPSs.

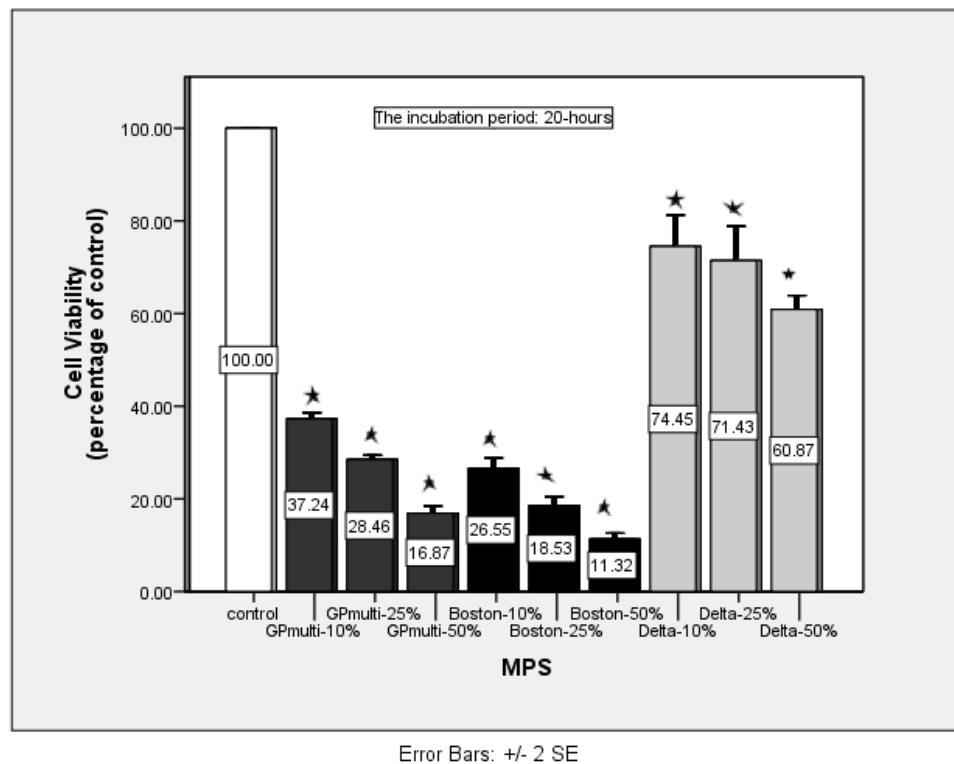


**Figure 4.2:** Percentage of cells viability after 20 hours treatment of cells with three concentrations (10%, 25% and 50%) of three MPSs. Number of wells per treatment= 6.

\* indicates significantly decreased in cell viability comparing to control.

The data also showed difference in cell viability between the three different concentrations for the same solution with higher concentration of the same solution results

in lower cell viability, this significance in 20 hours treatment. That may suggest a concentration-dependent toxicity of MPSs.

## 4.2 Cell Viability after Exposure to MPSs and Following 24 Hours of Re-culturing in Growth Media

After 20 hours treatment, the cells were re-cultured in fresh media for 24 hours to test recovery rate of these cells in term of cell viability measured by MTT assay. The data showed significant increase in cell viability among all MPSs at all concentrations after re-culturing in fresh media ( $p=0.00$ ) as it shown in Figure 4.3. The data also showed the highest cells viability after recovery as the following Boston<GPmulti<Delta.

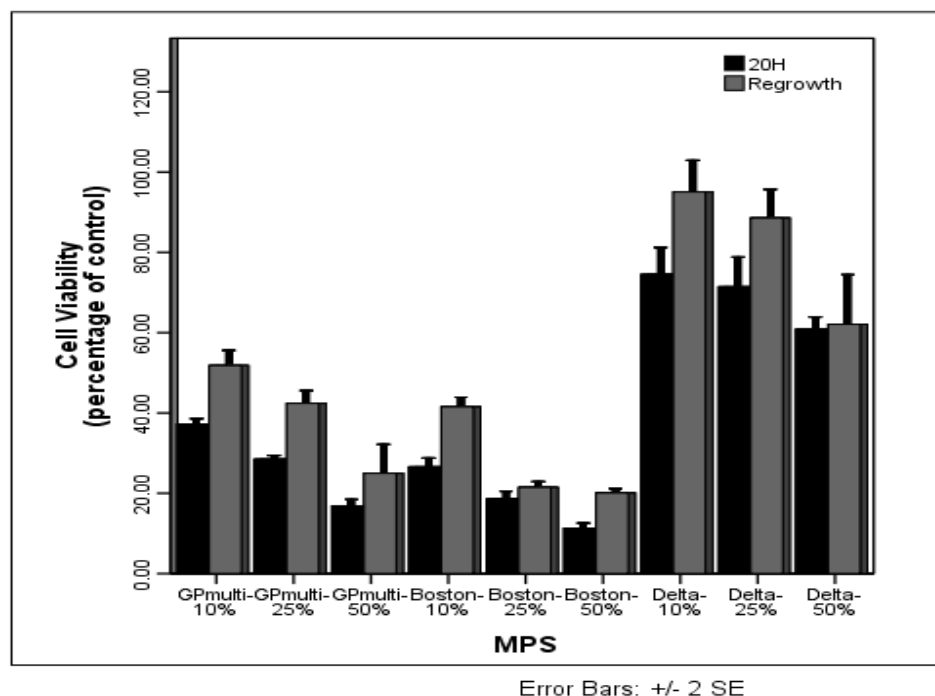


**Figure 4.3:** Comparison between percentage of cell viability after 20 hours of exposure to MPSs and following 24 hours re-culturing in growth medium.

### 4.3 Cells Migration Percentages Using Wound Model

In general, the data showed that the wound healing process is better in treated samples than in negative control, as it shown in Figure (4.4). The data also showed insignificant change in migration percentages with different tested MPSs at 10% and 25% concentrations. For higher concentrations, no data were obtained due to complete cells dislodge from the treatment well surface and impossibility to make a scratch. Moreover, the migration percentages did not show significant difference between different concentrations of the same MPS ( $p=0.299$ ), that may suggest the effect of MPSs on cell migration and wound healing after damage is independent of MPSs concentration.

Figure 4.5 shows photographs of cell cultures after a scratch induced and following 24 hours of growth in fresh media.

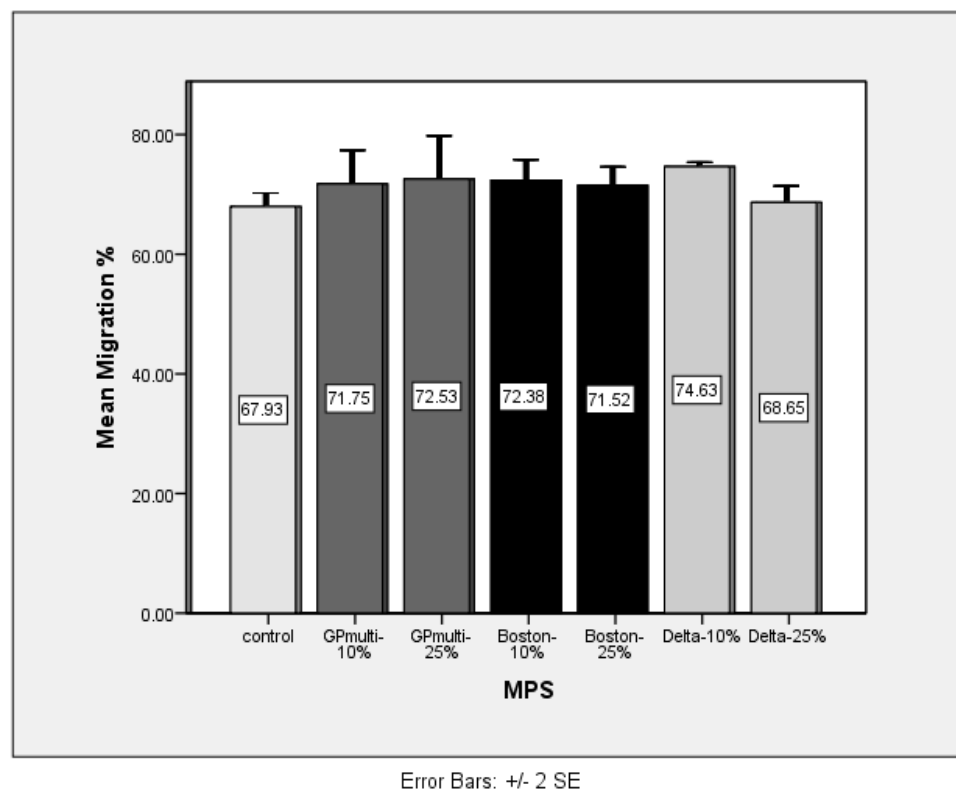


**Figure 4.4:** The mean migration percentages of control and cells treated with different MPSs following 24 hours of re-culturing in fresh media.

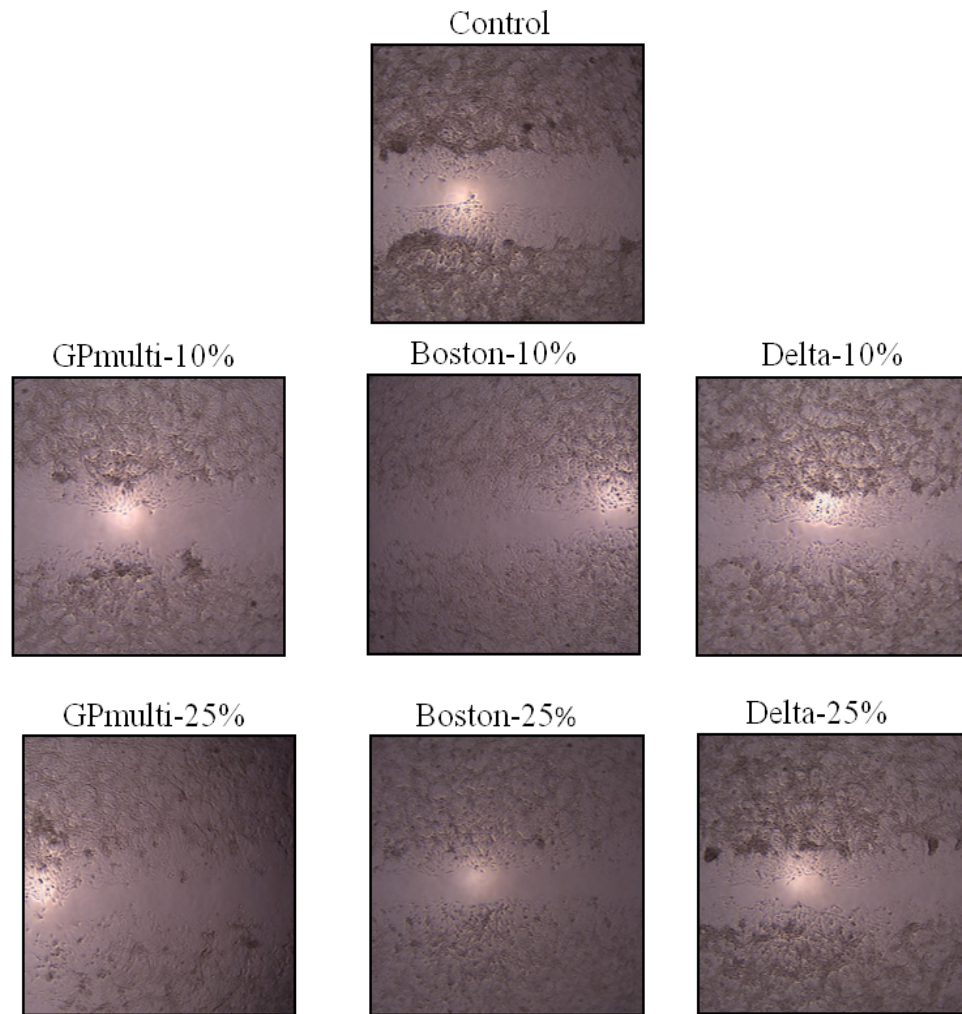


**Figure 4.5:** Response of control and MPS-treated cells to the scratch after 24 hours of growth in DMEM. Images captured by inverted microscope at 10X magnification.

#### 4.4 Cell Cycle Profile Assessment Using Propidium Iodide Flowcytometry

The aim of this experiment was to assess cell cycle phases obtained through propidium iodide flowcytometry. The data showed that in all control and 25% MPSs treated cultured cells, the percentage of G0 was  $\leq 0.2\%$ . The number of cells in G0 was as the following Boston>Delta >GPmulti. However, only cells treated with Boston solution showed a significance increase in cells in G0 phase compared to control cells ( $p=0.000$ ).

The data also showed numerical differences in cells number in all cell cycle phases (G1, S, G2M) between different treatments, however, these changes were not statistically significance.

The mean percentages of cells and standard error of mean in all cell cycle phases (G0, G1, S, G2M) are shown in Table 4.1. A graphical distribution of cell cycle phases in control and treated cells is shown in Figure 4.6.

**Table 4.1:** The mean percentages and standard error of mean (SEM) of cells in (G0, G1, S, G2M) obtained from control and 25% MPSs treated cells.

| Cell cycle phase | G0       |      | G1       |      | S        |      | G2M      |      |
|------------------|----------|------|----------|------|----------|------|----------|------|
|                  | Mea<br>n | SEM  | Mea<br>n | SEM  | Mea<br>n | SEM  | Mea<br>n | SEM  |
| Control          | 0.03     | 0.02 | 62.13    | 0.67 | 12.42    | 1.29 | 26.77    | 0.66 |
| GPmulti-25%      | 0.02     | 0.01 | 67.15    | 3.31 | 10.45    | 1.46 | 22.38    | 2.62 |
| Boston-25%       | 0.19     | 0.00 | 62.20    | 2.35 | 9.25     | 0.44 | 28.35    | 2.79 |
| Delta-25%        | 0.06     | 0.02 | 62.95    | 0.84 | 12.05    | 0.56 | 24.94    | 1.23 |

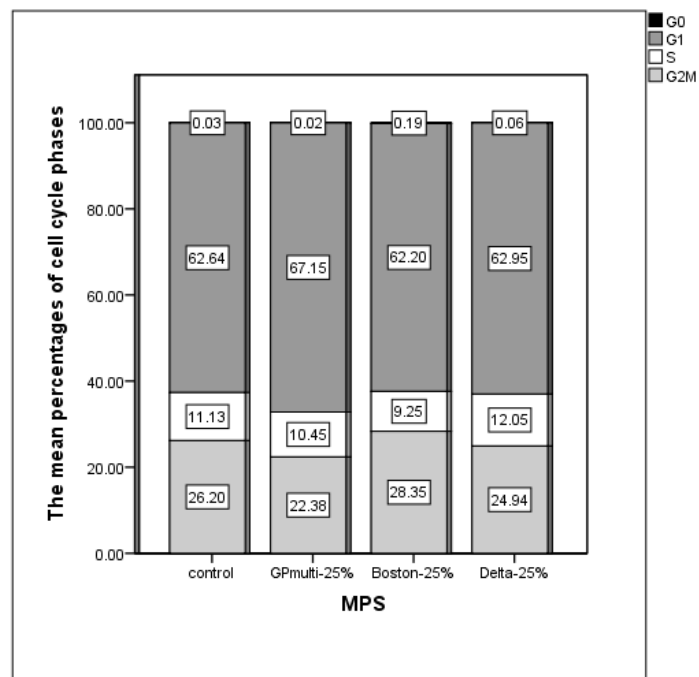


**Figure 4.6:** Distribution of cell cycle phases (G0, G1, S, G2M) in control and 25% MPSs treated cell cultures.

## Chapter Five: Discussion and Conclusions

### 5.1 Discussion

In this project, it was aimed to study the effects of different RGP MPSs on cultured epithelial cells to investigate any possible toxicity that may result from ingredients in the solutions. Three RGP MPSs were used; GPmulti, Boston, Delta. The Delta is the only one that used in Jordan. The methods included culturing of an epithelial cell line that has been used extensively in toxicology studies to test the impact of different agents on epithelial cells. Cultured epithelial cells were exposed to three brands of RGP MPSs that are available in the market and are used for cleaning and disinfection of RGP lenses against dirt and microbes. Effect of solutions on the cells was examined using MTT viability assay, wound healing scratch model assay and cell cycle analysis by PI-assisted flowcytometry.

The monolayer of epithelial cells that were treated with different MPSs did not significant effect on cell migration process. This may suggest low to none potential influence of RGP MPSs on wound healing mechanisms. This finding agrees with a study by Hamil et al, who found non-significant difference in epithelial cell healing in rabbit eye between control (treated with saline) and chlorhexidine (<1%) (Merani, *et al.*, 2016).

Interestingly, our results showed significant reduction in cell viability revealed by MTT assay after 4 hours exposure to both Boston and GPmulti MPSs only, but not for the Delta solution. However, increasing exposure time to 20 hours resulted in further reduction in cell viability for all tested solutions; GPmulti, Boston and Delta. This pattern may suggest a time-dependent toxicity effect.

It has been showed that increasing the time of exposure to chemical is inversely related to cell viability and testing the toxicity at different time points resulted in significant increase in measured toxicity level. In this regard, Lee et al. studied the Minimal Cysticidal Concentration (MCC) of both polyhexanide and chlorhexidine at three

periods of incubation (8, 24, 48 hours) on corneal keratinocytes using MTT assay. They showed that the survival rate of the cells - after 24 hours incubation in fresh media- has an

inverse relationship to the exposure time; with an increased toxic effect when exposure time increased. According to this study, it was suggested that for a 5µg/ml (0.0005%) polyhexanide solution it requires approximately 8 hours to disinfect the lens from microbes. A further reduction in polyhexanide concentration to ≤2µg/ml (0.0002%) needs more than 48 hours to disinfect the lens and a Chlorhexidine at 30µg/ml (0.003%) needs less than 8 hours to disinfect the lens (Lee, *et al.*, 2007).

In the current study, the reduction in cell viability revealed by MTT assay was also observed when increasing the concentration of contact lens solutions used to treat the cells from 10% v/v to 50% v/v as the following cell viability in Boston<GPmulti< Delta, which may suggest a concentration-dependent toxicity response. Our results of the concentration-dependent toxicity response are in agreement with a report by Müller *et al.*, who reported that when cultured L929 cells were exposed to different concentration of polyhexanide, cell viability was also decreased dramatically when increasing the concentration of polyhexanide (Müller, *et al.*, 2013).

In another experiment that aimed to test if the reduction effect on cell viability is due to reversible or it is irreversible cell damage or due to cell growth inhibition, cells were exposed for RGP MPSs for 20 hours before wash out of the solution and re-growth in fresh culture media. Cell viability after the wash-out was measured by MTT assay and the viability results were compared with cells that exposed to MPS for 20 hours (without washout). The results showed a significant recovery in the cells after 20 hours re-culturing which may indicate that some inhibition caused by exposure to MPSs is a reversible process. However, after the re-culturing step, treated cells still did not show normal viability compared to control cells which may suggest some levels of cell inhibition and cell damage that could be further examined by cell cycle profile in the culture. Analysis of cell cycle profile is generally useful in calculating the number of damaged cells through the process of apoptosis. (Elmore, 2007)

In this work, cell cycle profile was studied in both control and treated cells using flowcytometry-assisted by propidium iodide stain. It was shown that the only solution that showed a significant increase in number of apoptotic cells was Boston as it expressed by

its highest apoptotic incidence which is nine and three times higher than GPMulti and Delta solutions, respectively.

The findings of cell cycle analysis support results obtained from the cell viability experiments; that showed lowest viability in Boston solution too. To further expand on that, it is important to note that all the three MPSs used in this project have polyhexanide as preservative but in different concentrations as listed in Table 2.1. Boston solution, however, has the highest concentration of polyhexanide as preservative which is 5 times higher than Delta, 2.5 times higher than GPMulti. Furthermore, Boston solution also contains chlorhexidine gluconate at a concentration of 0.003% as additional preservatives in its ingredients.

Our findings of higher toxicity of chlorhexidine and polyhexanide-based contact lens solutions are in agreement with finding published by Choy et al who investigated the toxicity of different MPSs against cultured epithelial cells using MTT assay and Annexin V-FITC/7AAD staining by flowcytometry (cells in early apoptosis stained with Annexin V-FITC, cells at late apoptosis stained with Annexin V-FITC and 7AAD, cells in early necrosis stained with 7-AAD). Their result showed that cells treated with 100% MPSs that contain polyhexanide at (0.0001% and 0.0005%) had significantly increased level of early necrosis after 10 minutes of exposure. They also showed that MPS with higher concentration of a combination of polyhexanide and chlorhexidine significantly increased the number of cells at late stage of apoptosis and early stage of necrosis; and had the least cell viability *in vitro*. They suggested that such solution of a combination of polyhexanide and chlorhexidine may have a great potential to develop toxic effect against corneal epithelial cells *in vivo* (Choy, *et al.*, 2013).

Chlorhexidine is a water insoluble strong base that found as dicationic compound at physiological pH. It becomes soluble when it binds to gluconate as in MPS solution. Chlorhexidine is active against wide range of microorganisms and it works through disrupting the cell membrane through binding to proteins that contained phosphate in the cell membrane of microbes that lead to leakage the component of cytoplasm (Calogiuri, *et al.*, 2013).

Polyhexanide is cationic, polymeric, water soluble preservative that has the same mechanisms of action as chlorhexidine but its molecular weight is larger than chlorhexidine, so this may explain its lower toxicity effect on epithelial cells compared to chlorhexidine (Davies, *et al.*, 2009; Müller, *et al.*, 2013).

As the most common mechanism of action of polyhexanide and chlorhexidine preservatives is disrupting the microbial cell wall, there is always a possibility that these preservatives can also affect tear film proteins and corneal epithelial cells. These purpose to manufacturers of contact lens solutions to keep a balance between the efficacy (the desired antimicrobial effect of solutions) and safety (to be safe against tear film proteins and corneal cells) (Davies, *et al.*, 2009; Calogiuri, *et al.*, 2013).

The efficacy and possible toxic influence of chlorhexidine and polyhexanide have been studied extensively in eye and the cornea, chondrocytes, skin and surgical wounds (Merani, *et al.*, 2016; Röhner, *et al.*, 2011; Hübner & Kramer, 2010; Hansmann, *et al.*, 2005). Many studies reported that polyhexanide has more antimicrobial action than chlorhexidine (Gilbert & Moore, 2005; Lee, *et al.*, 2007).

To sum up, throughout this work it was shown that MPS contains both polyhexanide 0.0005% and chlorhexidine gluconate 0.003% (commercially known as Boston) has the most toxic effect on epithelial cells, growth inhibition and apoptosis. This is in an agreement with previous studies that showed a combination of polyhexanide at 0.0005% and chlorhexidine at 0.003% in solution is generally considered having higher cellular toxicity compared to other solutions with lower concentrations or only one type of preservatives (Lin, *et al.*, 2016). The results obtained from our study also showed that MPS which contains just polyhexanide 0.0001% (commercially known as Delta) has generally lower toxic effect on epithelial cells and the MPS that has polyhexanide of higher concentration 0.0002% (GPmulti) has higher toxic effect. This is in agreement with Hübner & Kramer who reported that polyhexanide at low concentration has no toxic effect and even though it has positive influence against human skin keratinocytes, when compared to chlorhexidine at low concentration (Hübner & Kramer, 2010).

Finally, the effect of RGP MPSs on cell viability and their potential cell damage have an important clinical application in RGP lens wearers. Clinically, RGP lenses are

mainly prescribed for patients who already have compromised corneas as in cases of keratoconus, post keratoplasty and other corneal irregularities. Accordingly, caution must be taken to completely dry these lenses from solution before their application to the eye to avoid corneal cells toxicity and further cell damage. However, complete lens dryness could be difficult due to expected entry of traces amount of solution to the eye through adsorbance onto lens material or by patient's hands during the lens insertion.

Another issue to consider here is poor patients' compliance toward contact lens care solution use. In an unpublished work by Bakkar et al. it was observed that RGP patients showed low level of compliance with contact lens care solution, where many patients reported to use the RGP MPS solution to lubricate their eyes and in some cases patients reported filling their Scleral RGP lenses as a tear reservoir instead of using recommended saline. Such poor compliance behaviors of using RGP MPS are expected to results in potential adverse effect on the cornea as it is suggested from the obtained *in vitro* results of the current study.

## **5.2 Conclusion**

- The potential toxic effect of three common RGP MPSs against cultured epithelial cells was studied in this project.
- The most toxic effect found was associated to chlorhexidine gluconate at 0.003% and polyhexanide at 0.0005%- based solution known commercially as Boston.
- From wound healing scratch model assay and MTT assay, a percentage of toxic effect of MPS on cells may be reversible once cells retrieved and solutions removed and replaced by fresh media.
- MPS that has the lowest concentration of polyhexanide (commercially known as Delta) showed the least toxic effect against cultured epithelial cells.
- GPmulti solution which is polyhexanide based solution with a concentration between the other two tested MPSs has growth inhibition and moderate toxic effect on cells.

## **5.3 Limitations and Recommendations**

- A future work is recommended to take account of larger number of samples and more types/brands of RGP MPSs.

- This study was performed using Vero cell culture which is considered a relevant epithelial cell line. However, a future work is suggested using corneal epithelial cells to study the effect of RGP MPSs on corneal cells and tear film protein.
- More specific toxic assays could be done to study the exact mechanism of cellular toxicity caused by MPSs at cellular levels.
- The effect of RGP MPS could be studied in compromised corneal models such as keratoconus from donor corneas or *in vitro* induced disease.

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# تأثير محاليل العناية بالعدسات اللاصقة الصلبة على الخلايا الطلائية

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## الملخص

**مقدمة:** تستخدم العدسات اللاصقة كبديل عن النظارات لتصحيح النظر، يوجد نوعين من هذه العدسات تستخدم إلى يومنا هذا: العدسات اللاصقة اللينة والعدسات اللاصقة الصلبة. محاليل العناية بالعدسات اللاصقة عبارة عن مجموعة من المركبات الكيميائية التي تؤدي دوراً فعالاً في تنظيف العدسات وترطيبها، بالإضافة إلى تعقيمها من الميكروبات. المحاليل المتعددة الأغراض (MPS) تعد الأشهر والأسهل والأكثر استعمالاً من بين محاليل العناية بالعدسات اللاصقة، توضع العدسات في المحلول لمدة لا تقل عن عشرة ساعات، من ثم توضع في العين، هذا يرتبط باحتمالية دخول كميات قليلة من المحلول إلى العين. الاستخدام اليومي لهذه المحاليل لمدة طويلة لتنظيف وتعقيم العدسة، ربما يؤدي إلى تأثير عكسي وسمي على الخلايا القرنية الطلائية. هناك الكثير من الأبحاث حول هذا التأثير بالنسبة للعدسات اللينة، أما بالنسبة للعدسات اللاصقة الصلبة فهناك القليل من الدراسات.

**الهدف:** في هذا المشروع تمت دراسة تأثير ثلاثة محاليل العناية بالعدسات الصلبة على الخلايا الطلائية والمقارنة بينها.

**الطريقة:** تم إضافة محاليل العناية بالعدسات الصلبة على الخلايا الطلائية. استُخدم اختبار MTT لدراسة تأثيرها على نمو الخلايا. ولمعرفة إذا كان هناك موت مبرمج للخلايا تم تحليل دورة الخلية باستخدام صبغة بروبيديم أيوديد (PI) وتحليلها بواسطة جهاز (Flowcytometry). لقياس تأثير المحاليل على التئام الجروح، استُخدم اختبار التئام الجروح لدراسة نسبة هجرة الخلايا خلال عملية التئام الجروح.

**النتائج:** تظهر النتائج أن المحلول المحتوي على بوليبيكسانيد 0.0005% وكلوروكسيدين 0.003% كموا حافظة لديه التأثير السمي الأعلى على الخلايا الطلائية، بعض هذا التأثير السمي قد يزول في حالة تم إعادة نمو الخلايا المعالجة في وسط جديد.

**الاستنتاج:** تأثير محاليل العدسات اللاصقة الصلبة على الخلايا الطلائية مختلف تبعاً للمواد الحافظة التي يحتويها، فالمحلول الذي يحتوي على كلاً من بوليبيكسانيد 0.0005% وكلوروكسيدين 0.003% لديه التأثير السمي الأعلى، المحلول الذي يحتوي على بوليبيكسانيد 0.0002% لديه تأثير سمي أقل، بينما المحلول الذي يحتوي على بوليبيكسانيد 0.0001% لديه التأثير السمي الأقل.