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Bio-modulation of Primary Human Tenon's Capsule Fibroblasts Using a Novel Application of Coated Magnesium

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Surgery

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Abstract

Background: Glaucoma is the leading cause of irreversible blindness. The last decade has seen the emergence of minimally invasive glaucoma surgical procedures, with the most recent utilizing implantable devices. The first generation of micro-incisional devices has been made of titanium. Since then other materials have also been introduced. In each case, the presence of a permanent foreign body has been associated with undesirable wound healing responses characterized by fibroblast proliferation and surgical failure. Magnesium alloys have attracted much attention as biodegradable implantable materials due to their excellent biodegradable and biocompatibility properties. The purpose of this research was to evaluate the *in vitro* biocompatibility and anti-proliferative properties of differently coated magnesium alloys in a primary culture of human Tenon's capsule fibroblasts (HTCFs). The goal of the research was to establish a proof of principle for further exploration of this novel material as a potential adjunct to glaucoma surgery.

Materials and Methods: Pure magnesium was cut into discs measuring 14.5 mm in diameter and 1 mm thick. These were coated with Hydroxyapatite (HA), Dicalcium phosphate dehydrate (DCPD) and Dicalcium phosphate dehydrate + Stearic acid (DCPD+SA), respectively. Coated magnesium alloys were immersed in simulated aqueous humor to examine the corrosive properties, the released ions, and the variation of pH. The primary HTCFs were seeded on DCPD, DCPD+SA and HA

disks in 24-well culture and incubated at 37 degrees for 2-7 days.. Glass disks were used as control. The MTT and LDH assays were used to determine cellular metabolic activity and cytotoxicity during the logarithmic phase of HTCFs, respectively. The BrdU assay was used to evaluate cellular proliferation. Western blot was used to assess the expression of alpha-smooth muscle actin (α -SMA).

Results: A total of 453 coated magnesium alloy disks were evaluated. Corrosion was observed in 67 disks (14.8%). The corrosion rate of HA (12.6%) was lower than other two coatings (DCPD 15.9%, DCPD+SA 15.9%, respectively), but the difference was not statistically significant different ($p=0.851$). The immersion test showed that the HA coating had better ability to prevent the coating dissolution and corrosion. The cellular metabolic activity of different coated magnesium alloys gradually declined during the logarithmic phase of HTCFs, and each type of coated magnesium alloy demonstrated significant decreased metabolic activity of HTCFs when compared to the control ($p<0.001$). The cytotoxicity of different coated magnesium alloys slightly increased during the logarithmic phase. The group of DCPD+SA showed higher cytotoxicity than the other coatings, but it was not statistically significant different when compared to the control ($p=0.976$). Significant inhibition of proliferation was observed with the DCPD+SA coating ($p=0.47$). The expression of α -SMA was decreased in the cells when seeded on all of the coated magnesium alloy disks.

Chemical coatings are able to affect the corrosive properties of magnesium. HA was the most resistant to corrosion. No significant difference was found in metabolic activity or necrosis at different times during the logarithmic phase of HTCFs. DCPD+SA demonstrated a stronger ability to reduce metabolic activity while its cytotoxic profile was the same as the titanium and glass controls. In comparison to titanium, coated magnesium alloys attenuated HTCFs proliferation. Coated magnesium alloys reduced the expression of α -SMA. The expression of α -SMA was significantly decreased in cells exposed to the HA coated magnesium

Conclusions: Different chemical coatings on magnesium were able to affect the corrosive properties which, in turn, influenced the morphology and function of human Tenon's capsule fibroblasts. These results support the further study of coated magnesium for its potential modulatory role in Tenon's capsule wound healing.

Keywords:

Glaucoma, glaucoma drainage device, fibrosis, tenon's capsule fibroblasts, biodegradable, magnesium, magnesium alloys, coating

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List of Abbreviations

POAG	Primary open angle glaucoma
PACG	Primary angle closure glaucoma
IOP	Intraocular pressure
MMC	Mitomycin C
5-FU	5-Fluorouracil
ECM	Extracellular matrix
HA	Hydroxyapatite
DCPD	Dicalcium phosphate dehydrate
DCPD+SA	Dicalcium phosphate dehydrate +Stearic acid
HTCFs	Human tenon's capsule fibroblast
SEM	Scanning electron microscope
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
MTT	3- (4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide
LDH	Lactate dehydrogenase
BrdU	5-Bromo-2-deoxyUridine
α -SMA	α -Smooth muscle actin
GAPDH	Anti-glyceraldehyde-3phosphate dehydrogenase
MMPs	Matrix metalloproteinases

DMEM	Dulbecco's Modified Eagle Medium
mRNA	Messenger RNA
ng/mL	Nanograms per milliliter
P/S	Penicillin/Streptomycin
BSS	Balanced salt solution
ANOVA	Analysis of variance
BSA	Bovine serum albumin
RGCs	Retinal ganglion cells
Al	Aluminum
Mg	Magnesium
Ca	Calcium
K	Potassium
Na	Sodium
AH	Aqueous humor
TM	Trabecular meshwork
TRAB	Trabeculectomy
PBS	Phosphate buffered saline
CaP	Calcium phosphate
mmHg	Millimeter of mercury
CO ₂	Carbon dioxide
Mg (OH) ₂	Magnesium hydroxide
PVDF	Polyvinylidene difluoride

BCA

Bicinchoninic acid

Review

A feasibility study of using biodegradable magnesium alloy in glaucoma drainage device

Abstract

Technological advances in glaucoma have challenged the traditional treatment paradigm. Historically, incisional surgery has been used in cases of advanced disease and/or uncontrolled intraocular pressures resistant to medical or laser interventions. Despite these trends, surgical manipulation of the tissues and unpredictability of wound healing continue to result in surgical failure. Magnesium is an essential element for the human body and plays a critically important role for maintaining the functional and structural integrity of several tissues, including the eye. Due to several of its advantageous properties such as non-toxicity, biodegradability, and high biological compatibility, magnesium alloy has attracted great attention as a novel biomaterial. Biodegradable cardiovascular stents made of magnesium alloy have already been introduced into clinical practice. The purpose of this review is to determine if bio-absorbable magnesium alloys can be utilized as a promising candidate for the development of a new generation of glaucoma surgical assistive devices.

Key words: Glaucoma, Tenon's capsule fibroblasts, fibrosis, Glaucoma drainage

devices, Magnesium alloy, Coating, Anti-scarring.

Introduction

Magnesium is an essential trace element for human life, and also is one of the most important regulatory cations involved in several biological processes. Magnesium is the second most common cation in the intracellular fluid. Mg^{2+} plays a crucial role in regulating vascular functions and energy metabolism as well as maintaining water and electrolyte balance^{1,2}. The level of Mg^{2+} in the serum ranges from 0.8 to 1.2mmol/L, with homeostasis being maintained by the kidney and intestine. Hypomagnesemia (serum Mg^{2+} < 0.8mmol/L) has been suggested to be associated with several disorders, such as vascular spasm and arrhythmia. Magnesium deficiency may also lead to cardiovascular, respiratory, and digestive illnesses, as well as abortion, fetal abnormalities and other obstetric diseases³. A large number of studies have shown that dietary intake of magnesium can prevent osteoporosis and femoral neck fracture⁴⁻⁷, and help to manage diabetes and coronary artery disease^{8,9}. Serum Mg^{2+} levels exceeding 1.2mmol/L may cause muscular paralysis, respiratory distress and hypotension¹⁰; however hypermagnesemia is rare due to the efficient excretion of the element in the urine¹¹.

Glaucoma is the second most common cause of blindness and the leading cause of irreversible worldwide blindness¹². Surgery is an important treatment option for glaucoma. Incisional surgery, with and without glaucoma drainage devices, is a major part of the treatment paradigm when medical options cannot be tolerated and/or fail to

reach a pressure that halts progression. Long-term complications are often related to variability in the wound healing process. Devices used adjunctively in surgical glaucoma management represent a foreign body under the conjunctiva, causing fibroblast proliferation. This may be due to the persistent effect of inflammatory mediators and cytokines at the level of the conjunctival-Tenon-episcleral interface resulting in fibrosis and obstruction of the fistula^{13,14}. There continues to be significant interest in adjunctive approaches to minimize scarring in the subconjunctival space, reducing inflammation, and in slowing or halting the excessive fibrotic healing process.

Bleb scarring is a major contributor to increased intraocular pressure (IOP) and surgical failure. In order to improve the success rate of glaucoma surgery, various intraoperative anti-metabolites have been used to inhibit fibroblast proliferation, such as 5-fluorouracil (5-FU) and mitomycinC (MMC). However, these antimetabolic agents interfere indiscriminately with cellular proliferation, and can contribute to a number of postoperative adverse events, including bleb leaks, hypotony, choroidal detachment, endophthalmitis, and keratitis^{15,16}. There continues to be a significant need for a safe, non-toxic, and effective approach to reduce postoperative scarring and adhesions after glaucoma surgery. The relatively recent introduction of bleb-forming micro-invasive glaucoma surgery has heightened this interest.

Magnesium alloy is a biodegradable material which possesses many advantageous

properties such as high strength, light weight, and high biological compatibility. Additionally, magnesium alloy has been shown to be able to reduce irritation and inflammatory reactions in the body¹⁷⁻²⁰. We were thus motivated to determine the feasibility of developing a magnesium-based bio-absorbable device for modulation of wound healing associated with glaucoma surgery. Moreover, the possibility that the slow release of Mg²⁺ by the device could have a local neuroprotective role is an intriguing concept worthy of future investigation.

Application of magnesium alloy in modern medicine

The application of magnesium alloy as a bio-absorbable material in clinical practice can be traced back to the early 1900s. The first use of magnesium was reported by Lambotte *et al.* in 1907, who utilized a plate of pure magnesium with gold-plated steel nails to treat a fracture involving the bones of the lower leg. The initial attempt failed as the pure magnesium plate corroded too rapidly *in vivo*, disintegrating only eight days after surgery and producing a large amount of gas under the skin^{21,22}. In 1938, McBride *et al.* developed magnesium alloy fixtures which successfully treated 20 cases of fracture without any significant adverse effects, and the fixtures were completely absorbed three months after surgery²³. In 1944, Troitskii *et al.* applied cadmium magnesium alloy as an internal fixation device to secure the bones of 34 consecutive fracture patients. They observed that the fixture could maintain the mechanical integrity up to two months, and it was completely absorbed after 10-12 months. Nine cases were unsuccessful, and these failures were attributed to infection.

A hard callous was found around the fracture site with no increase in serum levels of magnesium and no obvious inflammatory reactions to the implant in all 34 patients who were studied²³. Znamenski *et al.* reported similar results in 1945, where magnesium alloy containing 10% aluminum (Al) was used to treat two patients with gunshot wounds. The magnesium implant and nails were completely absorbed after 4-6 weeks²³. These early reports demonstrated that magnesium alloy was a non-toxic biomaterial, and it had the ability to promote bone healing. However, due to its characteristics of hydrogen emission and low corrosion resistance in the electrolytic and aqueous environments of the physiological system, biomaterial research on magnesium alloy was suspended. Thereafter, stainless steel materials were widely applied in bone internal fixation devices. Until recent years, with the use of advanced techniques, more complicated alloy compositions have been introduced. Advancements in corrosion protection technologies have effectively reduced the production of hydrogen gas. It is for these reasons that interest in the medical use of magnesium alloys has once again increased.²³.

Numerous *in vitro* and *in vivo* studies have focused on the use of magnesium alloys in internal fixation devices for fracture repairs. The corrosion resistance of different types of coated magnesium alloys has been studied in cytological and animal experiments to determine the reduction in hydrogen evolution and tissue compatibility^{24,25}. *In vitro* experiments confirmed that there were minimal cytotoxicity and cell apoptosis when fibroblasts and osteoblasts were exposed to various coated

magnesium alloys²⁵. *In vivo* experiments found that Mg^{2+} could enhance bone formation during the degradation process of the implant, and no inflammation was observed^{26,27}. F.Witte *et al.* successfully implanted magnesium alloy stents into the femur of rabbits, and the magnesium implants substantially degraded after three months. Their study also showed that the degradation process of the magnesium alloy stents could promote trabecular bone formation and resorption, without any significant harm to their neighboring tissues. They concluded that even fast-degrading magnesium alloy stents could show favourable biocompatibility thus establishing a more convincing potential role, in musculoskeletal surgery^{28,29}. In addition, F.Witte *et al.* also carried out an *in vitro* experiment to investigate the properties of a metallic matrix composite made of magnesium alloy AZ91 as a matrix with hydroxyapatite (HA) particles as reinforcements. The results revealed that the HA particles could stabilize the corrosion rate of the magnesium alloy, and this biodegradable metallic matrix composite HA was a cyto-compatible biomaterial with adjustable mechanical and corrosive properties³⁰. Okazaki M *et al.* developed a novel material containing magnesium, calcium, and phosphate for use as oral implants. They found that magnesium increased the metabolic rate of osteoblasts³¹. Zreiqat *et al.* found that the protein levels were significantly higher in human bone-derived cells cultured on [Mg]- Al_2O_3 (alumina doped with magnesium ions) compared with those grown on Al_2O_3 alone³². Hunt *et al.* reported that the magnesium-coated Ti-6Al-4v implant could activate bone cell signal transduction and hence improve protein synthesis and accelerate bone formation³³. The mechanical and degradation properties of

magnesium have been used to develop novel cardiovascular stents, so as to maintain the endothelial function of coronary arteries and to reduce the risk of coronary ischemia and occlusion³⁴⁻³⁷. Based on a large number of clinical trials^{38,39}, magnesium coronary stents have been introduced into clinical practice⁴⁰.

The important roles of Mg²⁺ in the eye

Mg²⁺ is important for maintaining the structural and functional integrity of several vital ocular tissues such as the cornea; lens and retina. The concentration of magnesium in aqueous humor is 2.97 ± 0.75 mg/ 100 ml. The magnesium levels in the lens are far higher than those in the anterior chamber and vitreous body. The concentration in the lens periphery is four times greater than the axial regions⁴¹. Mg²⁺ plays an extremely important role in maintaining retinal function. The reason for this is because magnesium acts as a co-factor involved in the catalytic function of more than 350 enzymes in the body and regulates neuro-excitability. Membrane associated ATPase functions that are crucial in regulating the intracellular ionic environment, are also magnesium-dependent. As a result, a reduced level of Mg²⁺ may affect the functions of Na⁺/K⁺ ATPase and calcium dependent ATPase, causing an increase in the intracellular concentrations of calcium and sodium as well as a decrease in the potassium⁴² concentration. Such ionic imbalances induced by magnesium deficiency may contribute to the pathogenesis of many eye disorders, such as cataract, corneal, conjunctival, choroidal and retinal diseases^{43,44}.

Maintenance of corneal structure and function

Mg²⁺ is one of the most important cations in the cornea as it is involved in the metabolism and maintenance of corneal transparency⁴⁵. As early as 1920, Kirkpatrick *et al.* reported the use of magnesium sulfate in the treatment of keratitis, conjunctivitis, and corneal ulceration⁴⁶. In 1985, Bachman and Wilson performed an animal experiment and found that the epithelial surface of the excised rabbit cornea was maintained best with a buffered solution containing Mg²⁺, K⁺, and Ca²⁺⁴⁷. Hogan *et al.* reported that Mg²⁺ loss in the corneal stroma was associated with corneal edema⁴⁸. In 2001, Gong *et al.* investigated the effect of magnesium deficiency on the cornea in rats that were fed a low magnesium diet for 3 weeks. Their findings revealed that magnesium deficiency affected the structural and functional integrity of the cornea⁴⁹.

Keratoconus is defined as a progressive eye disease which causes thinning and fragmentation of membranes, degenerated cells and collagen fibers, swelling of the mitochondria, and biochemical abnormalities in protein synthesis and expansion of central area of the cornea. Thalasselis *et al.* reported that hypomagnesemia was commonly seen in the serum samples of keratoconus patients, and magnesium deficiency could pathologically affect the integrity of the cornea⁵⁰. Prior studies also showed that magnesium deficiency was associated with a reduced number of microvilli and their irregular arrangement. Vesicular degeneration and swelling of the mitochondria were observed in the cytoplasm of epithelial cells, resulting in abnormal apoptosis of these cells^{51,52}. Magnesium deficiency was also associated with rupture

and fragmentation of the Bowman's layer, which may be an early change leading to keratoconus^{53,54}.

Supporting lens metabolism and preventing cataract

Cataract is the most common cause of blindness worldwide. It is characterized by progressive lenticular opacities. It is known that cataractous lenses have an abnormal intracellular ionic environment with lower concentrations of potassium and magnesium and higher concentrations of sodium and calcium relative to the cytosol of most cells. The lens membrane has increased permeability in the presence of a cataract⁵⁵. Studies have explored the relationship between magnesium deficiency and cataract. These studies have shown that the alterations in lenticular redox status and ionic imbalances form the basis of the relationship between magnesium deficiency and cataract. It is believed that Mg^{2+} plays an important role not only in maintaining a low lenticular Ca^{2+} and Na^{2+} concentration but also in preserving the lens redox status, which has been shown to reduce lenticular oxidative stress^{56,57}. Nagai *et al.* reported that the incidence of cataract was significantly lower in Shumiya rats fed 200mg/L magnesium compared to controls. It was noted that the intracellular Ca^{2+} level of the lenses was also significantly lower⁵⁸. Based on these findings, they concluded that an appropriate supplementation of magnesium could delay cataract genesis, probably by inhibiting the increase in Ca^{2+} levels in the lens⁵⁹. After a thorough literature review, Agarwal *et al.* concluded that magnesium supplementation might be of therapeutic value in preventing the onset and progression of cataract⁶⁰.

Oxidative stress is another important contributor to the pathogenesis of cataract. It has been shown in many studies that nitric oxide (NO) could be a risk factor in cataract formation⁶¹. NO production via inducible nitric oxide synthase (iNOS) causes an oxidative stress response in the lens⁶². Mg²⁺ has been shown to prevent the increase of NO in the lens which provides protection by inhibiting the nitrosylation of gap junctional proteins and maintaining membrane permeability⁶³. It is also known to block iNOS expression and reduce the oxidative stress response. A cytological experiment found that the expression of iNOS was 6 times higher in the lens epithelial cells cultured in the magnesium-deficient medium compared to those cultured in the magnesium-supplemented medium. In addition, Mg²⁺ was shown to regulate Ca²⁺ ATPase thus modulating the concentration of Ca²⁺ in the lens.

Both magnesium deficiency and excessive NO production have been shown to decrease ATP levels⁵⁸. The reduction in ATP levels affects the membrane-associated Ca²⁺ ATPase and Na⁺/K⁺ ATPase, causing ionic imbalance in the lens. Interestingly, the decreased ATP levels in turn may have inhibitory effects on the expression of iNOS⁶⁴. Therefore, it has been speculated that magnesium deficiency may cause an acceleration of the progression of lens opacification (Figure 1).

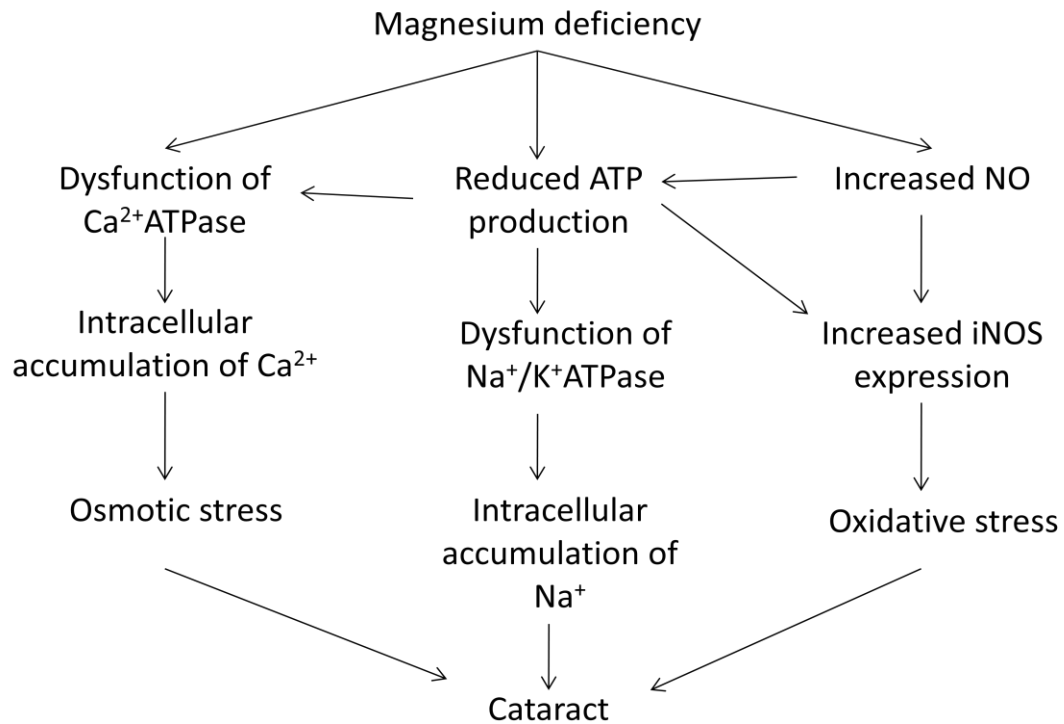


Figure 1. Magnesium deficiency contributes to the pathogenesis of cataract development.

Glaucomatous neuroprotection

Elevated intraocular pressure (IOP) is not the sole risk factor for chronic glaucomatous neuropathy. In fact, the search for putative non-IOP dependent factors continues to be of significant interest. Vasomotor dysfunction has also been suggested to contribute to optic neuropathy, by mediating abnormal hemodynamics and oxidative stress⁶⁵. In an epidemiological study, Bonomi *et al.* reported that reduced diastolic perfusion pressure could be an important risk factor for primary open-angle glaucoma⁶⁶. Leske *et al.* reported an association between cardiovascular disease and glaucoma which suggested a vascular role in glaucomatous progression⁶⁷. It has been well-established that Mg^{2+} can function as a physiological Ca^{2+} channel blocker which,

in turn, prevents ischemia and provides protection to the optic nerve. A large number of studies concerning ocular blood flow and oxidative stress response have confirmed that Mg^{2+} may regulate the current strength and in activation process of Ca^{2+} channels^{68,69}. Even small changes in extracellular Mg^{2+} levels may have significant effects on vascular tone. Mg^{2+} has been shown to have a direct vasodilatory effect⁷⁰⁻⁷² while magnesium deficiency has been shown to increase intracellular Ca^{2+} levels leading to vasoconstriction^{73,74}. Moreover, as Mg^{2+} regulates Na^+/K^+ ATPase, magnesium deficiency is associated with reduced activity of Na^+/K^+ ATPase and increased intracellular Na^+ and Cl^- levels causing cellular swelling and apoptosis of retinal ganglion cells (RGCs)⁷⁵.

Other studies have demonstrated an association between plasma endothelin-1 (ET-1) levels and normal tension glaucoma (NTG). Patients with NTG in the initial stage of visual field loss demonstrated higher plasma ET-1 levels than those with moderate visual field damage⁷⁶. An increase in extracellular Mg^{2+} levels inhibits ET-1, which may induce constriction and vasospasm of the ciliary arteries, with reduced blood supply to the optic nerve. Furthermore, ET-1 may affect the functions of axons and astrocytes, and accelerate the apoptosis of RGCs⁷⁷. It has been suggested that the favorable effects of magnesium on the visual field may be attributed to ET-1 inhibition, suppressing vasoconstriction, improving the ocular blood flow, and preventing glaucomatous neuropathy⁴³.

The ion channel of the N-methyl-D-aspartate (NMDA) receptor is calcium dependent and subject to voltage-dependent regulation by Mg^{2+} . Mg^{2+} can regulate the glutamate-gated ion channel which, in turn, has been shown to prevent excitotoxicity and cell apoptosis⁷⁸⁻⁸¹. In the presence of a magnesium deficiency, the toxic effects of glutamate on RGCs are mediated by the over stimulation of NMDA receptors, leading to glutamate excitotoxicity and loss of RGCs in glaucoma patients. Lambuk *et al.* reported that intravitreal Mg^{2+} could prevent retinal and optic nerve damage induced by NMDA⁸². Moreover, intracellular accumulation of Ca^{2+} associated with magnesium deficiency has been associated with the production of free radicals^{83,84}.

Magnesium deficiency directly increases the expression of iNOS. Lower levels of Mg^{2+} have been associated with vasospasm and retinal ischemia which also enhance the expression of iNOS⁸⁵, and produce large amounts of free radicals⁸⁶. Numerous studies have shown that Mg^{2+} plays a neuroprotective role in inhibiting the elevated iNOS activity of neurons in retinal ischemia^{87,88}.

In a study by Gaspar *et al.*, 10 glaucoma patients including 6 with primary open-angle glaucoma (POAG) and 4 with NTG were administered magnesium 121.5mg, twice a day for one month. Results showed that magnesium significantly increased the ocular blood flow and improved the peripheral circulation, exerting a beneficial effect on the visual field in glaucoma patients with vasospasm⁸⁹. Aydin *et al.* also reported that in 15 NTG patients who received 300 mg oral magnesium citrate for a month, an

improvement in the visual field was observed, but the ocular blood flow remained unchanged. They speculated that mechanisms other than increased ocular blood flow may be responsible for the improvement in the visual field when given oral magnesium therapy⁹⁰. Based on these findings, it was suggested that Mg^{2+} may play an important role in optic neuroprotection in patients with glaucoma (Figure 2).

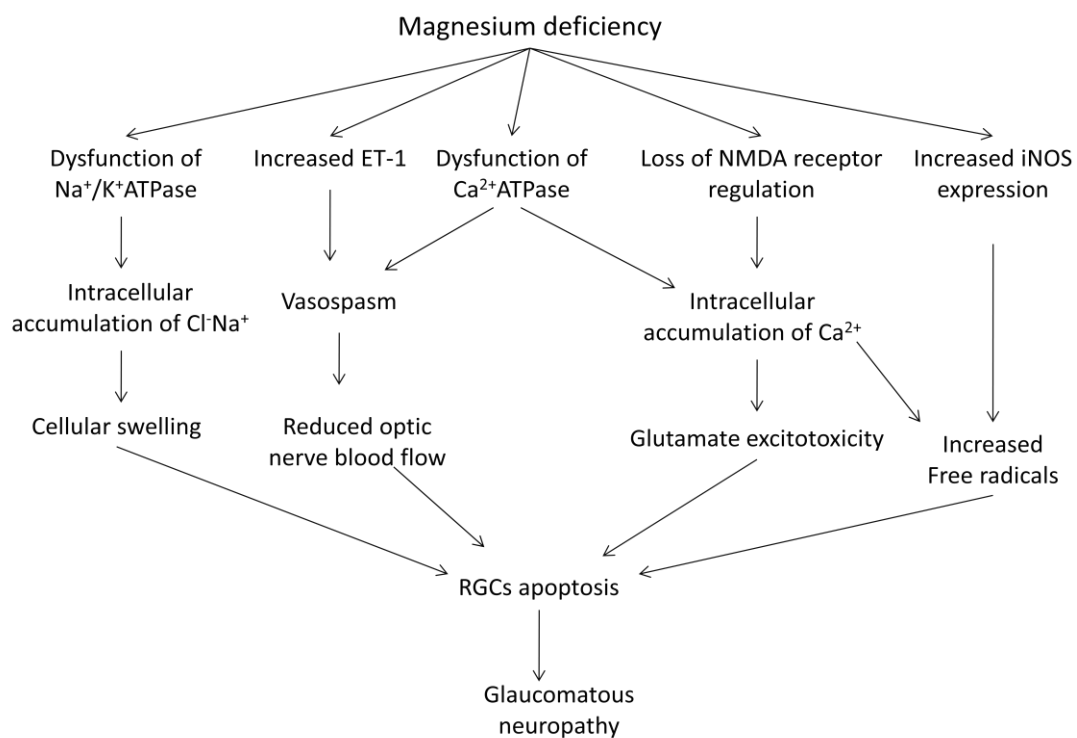


Figure 2. Magnesium deficiency contributes to the pathogenesis of glaucomatous neuropathy.

The history and prospects of using magnesium materials in glaucoma drainage surgery

Due to the unique properties of magnesium and its satisfactory bio-compatibility, the use of the pure materials was considered as an implant for the treatment of glaucoma

in the middle of the 20th century. In 1940, Troncoso utilized pure magnesium implants to increase the aqueous outflow in the treatment of glaucoma⁹¹. Five years later, Boshoff used this technique to treat a patient with neovascular glaucoma who refused enucleation. Twelve hours after operation, the patient's whole anterior chamber was filled with gas and the IOP was significantly increased, resulting in severe pain. The pressure of the gas was relieved by venting with a thin hypodermic needle. The IOP normalized on the sixth postoperative day, but huge bubbles were still found in the anterior chamber and the conjunctiva, which disappeared on the tenth day. Interestingly, before the operation the cornea was opaque at an IOP of 54 mmHg, but it became absolutely clear when the anterior chamber was full of gas, despite the elevated IOP which varied from 49 to 55 mmHg. The postoperative IOP was controlled at 1-4 months after surgery, but elevated to 45mmHg again at the fifth month. Gonioscopy revealed complete circumferential iridocorneal synechiae. Coarse pigment granules were observed in the dependent part of the angle. Eventually, the eye was removed. Pathological examination revealed a closed filter channel and massive fibroblast proliferation around the incision⁹².

These experiences indicated that pure magnesium had significant adverse effects due to the excessive corrosion when in contact with body fluids due to hydrogen gas emission. This limited its potential therapeutic benefits when used as a pure, uncoated material. Since then, advancements in the area of coating technology have allowed the synthesis of novel coated magnesium alloys with optimized compositions

(containing aluminum, zinc, manganese, calcium, praseodymium, or neodymium) allowing control over the corrosive properties. Coated magnesium alloys have shown significant promise as effective biodegradable materials *in vivo*.

During the last 15 years, biodegradable metallic stents have been developed and investigated as alternatives for the currently-used permanent cardiovascular stents⁹³⁻⁹⁵. Traditional cardiovascular stents have significant drawbacks including irritation and damage to the vascular endothelium, leading to stenosis and blockage⁹⁶⁻⁹⁹. In order to solve these problems, absorbable magnesium materials have shown success. *In vitro* studies have demonstrated that magnesium cardiovascular stents can maintain the integrity and function of endothelial cells, as well as reduce inflammation and fibroblast proliferation. In these studies, stents could be fully absorbed within 2-4 months with few complications observed during a two years of follow-up period^{37,39}. Subsequently, a large number of clinical trials were conducted^{37,38}. In 2007, a prospective multi-center trial in patients with coronary heart disease was published in the *Lancet*, which showed that biodegradable magnesium stents achieved an immediate angiographic result similar to the result of traditional metal stents and was safely degraded after 4 months¹⁰⁰.

The introduction of magnesium cardiovascular stents has led to interest in how their use can be expanded into other areas of the body. In particular, their potential utility as an adjunctive device in glaucoma surgery is of high interest due to recent

developments in the area of micro-invasive glaucoma procedures. The excellent biocompatibility and bio-degradability have the potential to allow magnesium alloy devices to effectively decrease postoperative irritation, reduce scar formation, improve the success rate of glaucoma surgery, and minimize the long-term effects of permanent implants which tend to produce local complications related to tissue compression. The liberation of Mg^{2+} cations during the natural degradation process has many potential beneficial effects on the cornea, lens, retina, choroid, and optic nerve.

In conclusion, bio-absorbable coated magnesium alloys may be a very promising candidate for the development of a new generation of glaucoma drainage devices.

Chapter 1

Introduction

1.1 Glaucoma

The glaucoma is a group of diseases that have in common a characteristic optic neuropathy with associated visual field loss for which elevated intraocular pressure (IOP) is one of the primary risk factors.

Most patients with early glaucoma are asymptomatic. The great majority of patients lack pain, ocular inflammation, or halos. Significant peripheral vision can be lost before the patient notices visual disability. If glaucoma is detected early and treated medically or surgically, blindness can be prevented.

1.1.1 Epidemiology of glaucoma

The World Health Organization ranks glaucoma as the second most common cause of blindness, and as the leading cause of irreversible blindness worldwide^{101,102}. Since 2010 there are about 60.5 million patients who suffered from primary open angle glaucoma (POAG) and primary angle-closure glaucoma (PACG) all over the world. The amount will become 79.6 million considerably, and it has been estimated that over 5.9 million people worldwide will be bilaterally blind with open-angle glaucoma by 2020¹⁰³. Two main types of glaucoma can be classified according to etiology: primary angle closure glaucoma and primary open-angle glaucoma. The highest prevalence of open-angle glaucoma occurs in Africans, and the highest prevalence of

angle-closure glaucoma occurs in the Inuit.

1.1.2 Aqueous Humor and Outflow

Elevated IOP is a well-known causative risk factor for both the development and progression of glaucoma. Within the eye is a mechanism for the continuous production and drainage of fluid. This fluid is called aqueous humor (AH). Intraocular pressure is regulated by aqueous humor production in the ciliary body and drainage from the eye occurs by way of the trabecular meshwork (TM) and uveoscleral pathways.

Aqueous humour is secreted into the posterior chamber by the ciliary body, specifically the non-pigmented epithelium of the ciliary body. It flows from the ciliary processes into the posterior chamber, bounded posteriorly by the lens and the zonules of Zinn, and anteriorly by the iris, to escape through the pupil into the anterior chamber, and then to drain out of the eye via the trabecular meshwork. From here, it drains into Schlemm's canal by one of two ways: directly, via aqueous vein to the episcleral vein, or indirectly, through collector channels to the episcleral vein by intrascleral plexus into the veins of the orbit and eventually general blood circulation. Because of some resistance to the flow of aqueous through the trabeculum and Schlemm's canal, pressure is created in the eye.

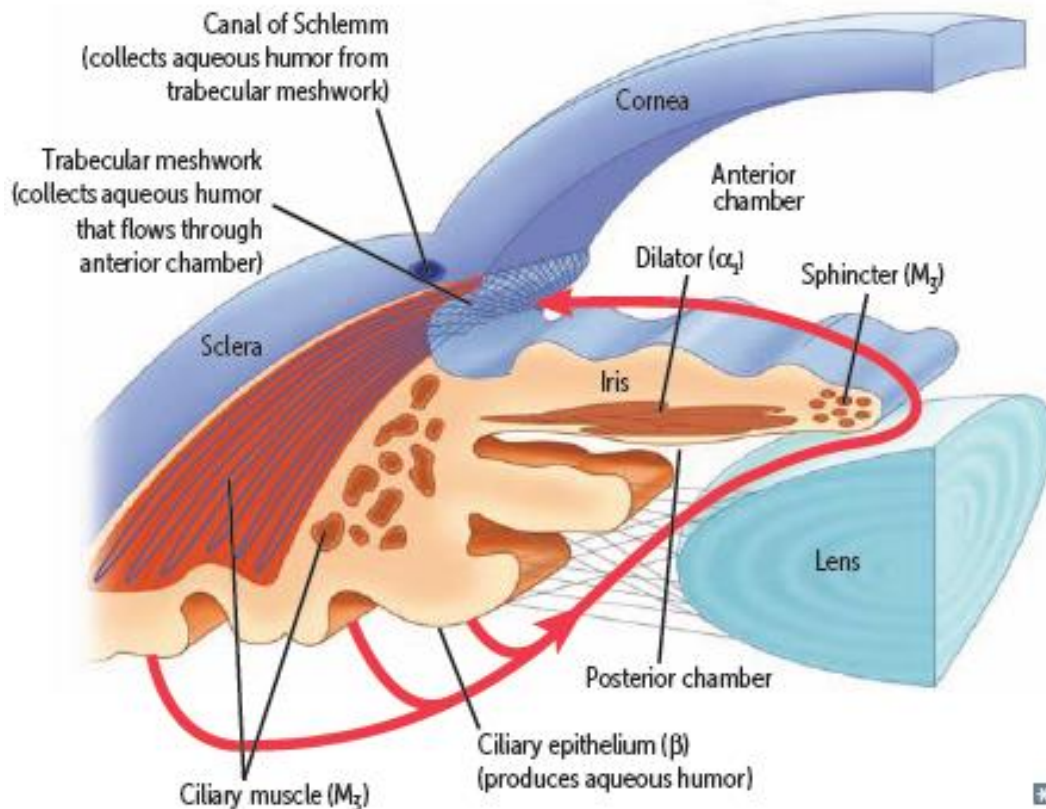


Figure 1.1.2 The trabecular meshwork outflow pathways.

1.1.3 Pathogenesis

Although the pathogenesis of glaucoma is not fully understood, the level of intraocular pressure is related to retinal ganglion cell death. Intraocular pressure can cause mechanical stress and strain on the posterior structures of the eye, notably the lamina cribrosa and adjacent tissues. In addition, elevated intraocular pressure is not the only risk factor for chronic glaucomatous neuropathy. In fact, the search for putative non-IOP dependent factors continues to be of significant interest. Vasomotor dysfunction has also been suggested to contribute to optic neuropathy, by mediating abnormal hemodynamics and oxidative stress⁶⁵.

1.2 Glaucoma treatment options

Slowing disease progression and preservation of quality of life are the main goals for glaucoma treatment. Current management guidelines from the American Academy of Ophthalmology Preferred Practice Patterns recommend lowering the intraocular pressure toward a target level, which is a value or range of values at which the rate of disease progression will be slowed sufficiently to avoid functional impairment from the disease¹⁰⁴. The target intraocular pressure should be achieved with the fewest medications and/or surgeries, and with a minimum of adverse effects. When glaucoma is no longer controlled by maximally tolerated medical therapy or laser trabeculoplasty, incisional *ab* externo filtering surgery has been the traditional next step in the therapeutic management.

1.2.1 Filtration surgery

Filtration surgery still plays a mainstream role in the treatment of glaucoma despite the fact its development dates back to the 19th century. Trabeculectomy (TRAB) is still the most popular surgical intervention in patients who are affected by primary glaucoma, and is the most commonly performed incisional surgical procedure to lower intraocular pressure. The trabeculectomy was introduced almost 50 years ago with very few modifications since that time. Trabeculectomy is generally recommended for patients with glaucoma that continues to progress despite use of medications and/or laser treatments. It consists of excision of a small portion of the trabecular meshwork tissue to provide a drainage route for aqueous humor from within the eye to underneath the conjunctiva where it is absorbed. However, the

trabeculectomy can contribute to a number of adverse effects, such as: scarring, bleeding, infection, malignant glaucoma. Therefore, a new class of glaucoma procedures, termed microinvasive glaucoma surgery, has emerged, which aims to fill the gap between conservative medical management and more invasive surgery.

1.2.2 Glaucoma drainage devices

Although the procedure itself has not changed much in the last 50 years, chemical agents that modulate the fibroblastic response to the surgery have been more recently introduced. These agents, known as 5-fluorouracil and mitomycin C have effects at the cellular level in blunting the wound healing response. In addition, trabeculectomy is not the only choice of *ab externo* glaucoma surgery. These procedures have been augmented, traditionally in more advanced cases, with macroglaucoma drainage devices and implantations. Glaucoma drainage devices are designed to divert aqueous humor from the anterior chamber to an external reservoir, where a fibrous capsule forms about 4-6 weeks after surgery and regulates flow. Lowering IOP can be achieved by increasing aqueous humour drainage through this artificial route. These devices have shown success in controlling intraocular pressure (IOP) in eyes with previously failed trabeculectomy and in eyes with insufficient conjunctiva because of scarring from prior surgical procedures or injuries. They also have demonstrated success in complicated glaucomas, such as uveitic glaucoma, neovascular glaucoma, and pediatric and developmental glaucomas, among others. Therefore, glaucoma drainage devices are being used increasingly in glaucoma

surgery. Currently, the glaucoma drainage devices are available in different materials. These “macro” *ab externo* adjunctive devices provided a basis for the emergence of the first generation of micro-incisional devices. The latter have been made of various materials with one of the first being titanium.

1.3 Fibrosis

1.3.1 Anti-fibrotic agents

In general, filtering procedures such as trabeculectomy or placement of subconjunctival drainage implants, aim at allowing aqueous humor to leave the anterior chamber through a novel transscleral route towards the subconjunctival space. Scarring is a major cause of increased intraocular pressures and surgical failure. In order to prevent bleb scarring and obstruction of the fistula, the inhibition of cellular proliferation and inflammation is both necessary and desirable after surgery for glaucoma. Currently, various substances have been used for the modulation of wound healing in filtering glaucoma surgery. In filtering glaucoma surgery, the most frequently used anti-fibrotic agents are mitomycinC (MMC), and 5-fluorouracil (5-FU). These anti-fibrotic agents improve the success rate for long-term intraocular pressure (IOP) control and reduce the risk of bleb failure^{105,106}. but their use is associated with an increased complication rate including thin-walled blebs¹⁰⁷, hypotony¹⁰⁸, wound leakage¹⁰⁹ and infection¹¹⁰. Until now, no satisfactory methods have been developed to resolve this issue.

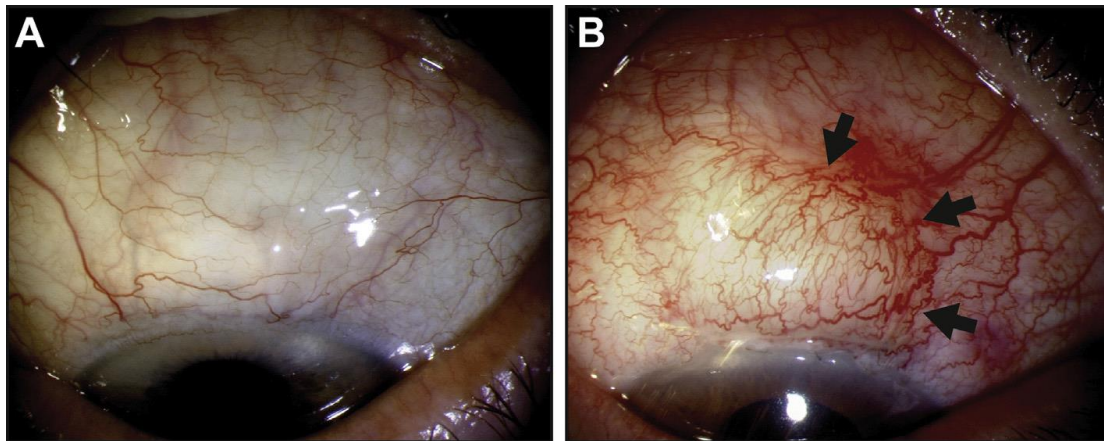


Figure 1.3.1 Clinical presentation of filtering blebs.

(A) Unscarred diffuse filtering bleb 12 months following trabeculectomy. (B) Scarring filtering bleb with beginning encapsulation (arrows) and enhanced vascularity, 6 weeks following trabeculectomy. G.

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1.3.2 Permanent foreign body under tenon's capsule stimulates fibrosis

Currently, permanent and inert metals like Titanium alloy, Polypropylene, and Silicone are the most common materials used in adjunctive glaucoma surgical devices. . However, it has been found that these materials can be associated with long-term complications. One reason is that the presence of the permanent foreign body induces inflammation stimulation under the tenon's capsule. The initial process of inflammatory is characterized by the increasing of monocytes and lymphocytes with the early proliferation of connective tenon's capsule tissue¹¹¹. This is a main risk factor cause fibroblast proliferation¹¹²⁻¹¹⁴ and fibrosis^{115,116}.

Inflammatory responses of organisms against foreign body are a natural defense process, which, in turn, is one of the greatest challenges in the development of

permanent implantable devices^{117,118}. The foreign body may be associated with subconjunctival inflammation due to toxic and allergic responses. The inflammatory phase is characterized by the activation of the innate immune system and the release of inflammatory cytokines. Chronic inflammation is an established risk factor for fibrosis due to the release of mediators such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF- α) which lead to increased tissue levels of TGF- β . Chronic inflammatory reactions associated with the foreign body can accelerate wound scarring, resulting in failure of the glaucoma surgery. It is for these reasons that higher surgical success rates may occur if the inhibitory substances remain in intimate contact with the tissue whose proliferation should be prevented, while supporting the well-being of the tissues whose integrity is necessary to prevent complications and promote healthy function. Ideally, the implantable material would do its job in the acute phases of wound healing and then auto-degrade to limit the potential for chronic responses to a foreign body.

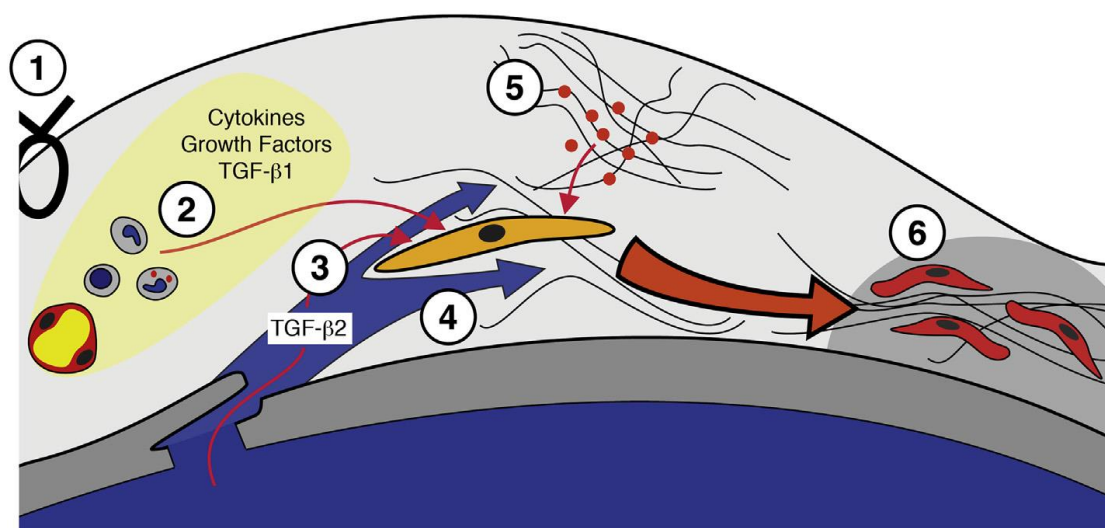


Figure 1.3.2 Conjunctival scarring following filtering glaucoma surgery.

Fibroblasts (orange) proliferate and differentiate into myofibroblasts (red). Drivers of fibrosis: (1)

sutures, (2) blood, vessel-derived cells, cytokines and growth factors, (3) aqueous humor-derived growth factors, (4) shear force stimulation by interstitial fluid flow, (5) signaling molecules released from ECM storage sites, (6) myofibroblast transdifferentiation leading to matrix deposition and tissue compaction. *G. Schlunck et al. / Experimental Eye Research 142 (2016) 76-82*

1.4 Magnesium

1.4.1 The important roles of Mg^{2+}

Magnesium is an essential trace element for human life, and is one of the most important regulatory cations involved in several biological processes. Magnesium is the most common divalent cation in the intracellular fluid, and plays an important role in regulating vascular functions and energy metabolism, as well as maintaining water and electrolyte balance. Mg^{2+} is important for maintaining the structural and functional integrity of several vital ocular tissues such as the cornea; lens and retina¹¹⁹. Magnesium deficiency may contribute to the pathogenesis of many eye disorders, including, cataract, conjunctival diseases, corneal diseases, choroidal diseases, and retinal diseases. Magnesium is a nature's calcium blocker¹²⁰, increase blood flow to tissues through endothelin-1 and endothelial nitric oxide pathways¹²¹. As well as, magnesium has been shown neuroprotective role to glaucoma patients^{122,123} that prevent oxidative stress and apoptosis¹²⁴.

1.4.2 Magnesium alloys

Magnesium alloy has a variety of advantageous characteristics which include:

biodegradable, non-toxic, high strength, light weight, and high biological compatibility¹²⁵. Due to the unique properties and satisfactory biocompatibility the application of magnesium alloy as a bio-absorbable material in clinical practice can be traced back to the early 1900s for treatment of bone fracture patients, which demonstrated it was a non-toxic biomaterial²¹⁻²³. Magnesium alloy also has been considered as a potentially appropriate biomaterial for glaucoma drainage devices. Various studies have found that magnesium alloys are biologically safe and have demonstrated positive biological reactions in ocular tissue¹²⁶. In comparison with polymers, the degradation products of the alloys which includes magnesium ions are needed in the human body for physiological functions, with consumption lying in the range 250–500mg day. About 20g of Mg is always present in the average 70kg human body¹²⁷.

1.4.3 Coating techniques improve corrosive property

Magnesium as a pure metal has poor corrosion resistance, which has been a major obstacle to their application as a human implantable device. If corrosion is rapid and not homogenous, it limits its potential *in vivo* applications. Another issue is the formation of hydrogen gas during corrosion: if evolution of the gas is too rapid it cannot be absorbed and a balloon effect takes place. Fortunately, in recent years, the most attention in the magnesium alloy field has been the study of coatings or surface modification to slow the degradation rates of various Mg alloys. This has caused a renewed interest in the use of magnesium as a surgical adjunctive device. There are a

large number of possible coating technologies for Mg biomaterials, including anodisation, metal–metal coatings, plasma spray, chemical vapour deposition, pulsed laser deposition, ion beam assisted deposition, solution coatings, calcium phosphate (CaP) deposition achieved by various means and the well known methods of electrodeposition and conversion coating. The CaP coatings have garnered the majority of attention due to their intrinsic biocompatibility¹²⁸⁻¹³⁰, due to the formation is similar to the mineral phase of bone, including hydroxyapatite (HA), dicalcium phosphate dehydrate (DCPD) and dicalcium phosphate dehydrate +Stearic acid (DCPD+SA).

Nowadays, the advancement techniques have fabricated a new generation of magnesium alloys, especially the development of coating technique which effectively reduced the production of gas and rapid corrosion in the body. The coated magnesium alloys have been used to develop novel cardiovascular stents resulting in a number of clinic trials³⁷⁻³⁹. It is for these reasons that we were interested in exploring the feasibility of coated magnesium devices as a potential surgical adjunct to glaucoma surgery.

1.5 Hypothesis and Objectives

The preliminary data showed that coated magnesium alloy may reduce metabolic activity and potentially inhibit human tenon' s capsule fibroblasts (HTCFs) cells growth and proliferation. The results indicated that different coated magnesium alloys had different inhibitory ability on HTCFs proliferation, and this effect may be

associated with the capacity of blocking cell cycle.

1.5.1 Hypothesis

Bio-degradable coated magnesium alloys will inhibit cellular proliferation and reduce myofibroblast activity in a primary culture of human Tenon's capsule fibroblasts.

1.5.2 Objectives

The purpose of this study was to evaluate the biocompatibility and anti-proliferative potential of different coated magnesium alloys on the activity of human Tenon's capsule fibroblasts in primary cell culture.

Chapter 2

Primary cell culture and sample preparations

2.1 Materials and methods

2.1.1 Primary HTCFs culture

The tenon's capsule samples were obtained from 32 glaucoma patients without a past history of ocular surgery. The study has been approved by the Research Ethics Committee of St. Joseph's Hospital, Western University, London, Canada. All the patients provided informed consent, and the study was conducted in accordance with the Declaration of Helsinki. The capsule samples were excised during the surgery, and cultured in a 60 mm dish. The culture medium, consisting of Dulbecco's modified Eagle's medium (DMEM, Gbico, USA) supplemented with 10% fetal bovine serum (FBS, USA) and 1% penicillin/streptomycin (Gbico, USA), was changed every 2-3 days and the cells were allowed to reach 80% confluence. Subsequently, the cells were disaggregated with 0.25% trypsin and 0.02% EDTA at 37°C for 5 min. And then, the HTCFs cells were transferred to 25 cm² flasks (BD Falcon, BD Biosciences, Broendby, Denmark), which were placed at 37°C in 5% CO₂ incubator for subculture. The cells were seeded in a 24-well plate when they reached 80% confluence.

2.1.2 Assessment of morphology

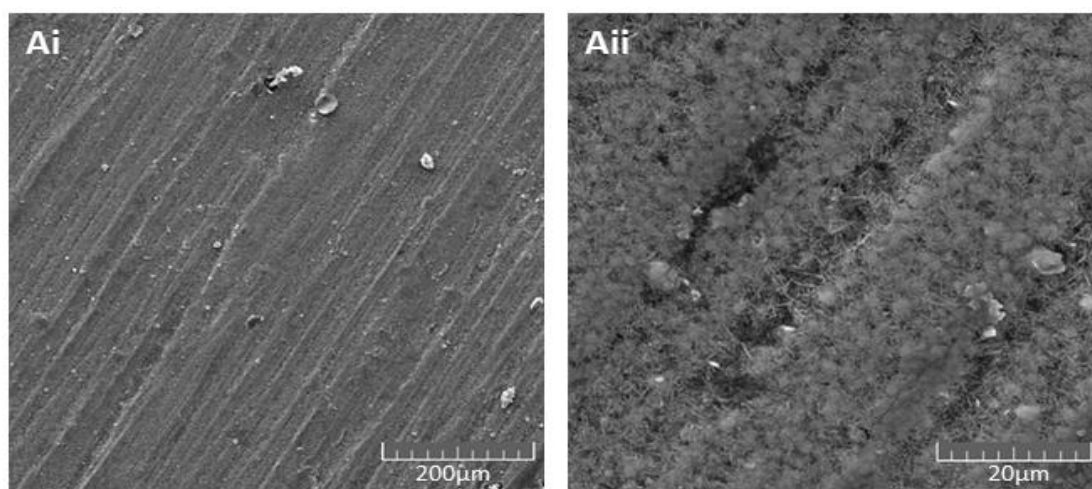
The cells were analyzed 2 days after seeding. Cell counting was performed by fluorescence microscopy (Axio Observer. Z1; Carl Zeiss, Germany) using Hoechst dye (Molecular Probes, Carlsbad, CA, USA) to visualize the nuclei. After 10 minutes

of incubation with the fluorescent dye, the cells were fixed with 4% buffered formaldehyde in phosphate buffered saline (PBS) for 10 minutes, and then permeabilized with 0.1% Triton-X 100 in PBS for 5 minutes. Thereafter, the samples were rinsed with PBS, stained with a 1:100 dilution of monoclonal mouse anti-vimentin and 1:500 monoclonal rabbit anti-keratin, and then left overnight at 9°C. After a PBS rinse, the samples were incubated in a 1:625 dilution of anti-mouse Alexa Fluor 488-conjugated goat anti-mouse IgG for 1 hour at room temperature. For double labeling, the samples were also incubated in a 1:500 dilution of Alexa Fluor 568-conjugated donkey anti-rabbit IgG for 1 hour at room temperature. Finally, the samples were rinsed twice with PBS and kept in PBS at 4°C until the observation time. Image sampling, cellular morphology assessment and cell counting were performed using custom-written routines for the AxioVision rel.4.7 software (Carl Zeiss).

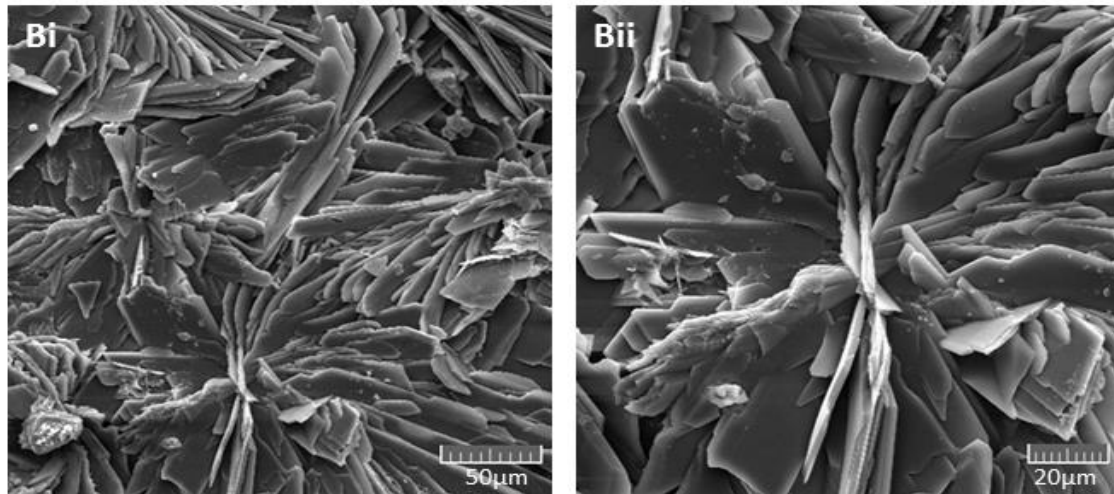
2.1.3 Fabrication of differently coated magnesium alloys

Magnesium alloys with a purity of 99.99% were obtained from the National Engineering Research Centre for Magnesium Alloys, Chongqing University, China. The pure magnesium was cut into disks with 14.5 mm in diameter and 1 mm in thickness after pretreatment at 550°C for 24 hours of annealing. The surface of the disks was polished using 320-800# abrasive paper. All of the disks were ultrasonically cleaned in acetone, air dried and weighed. Thereafter, the magnesium disks were coated with hydroxyapatite (HA, Figure 2.1.3.A), dicalcium phosphate dihydrate

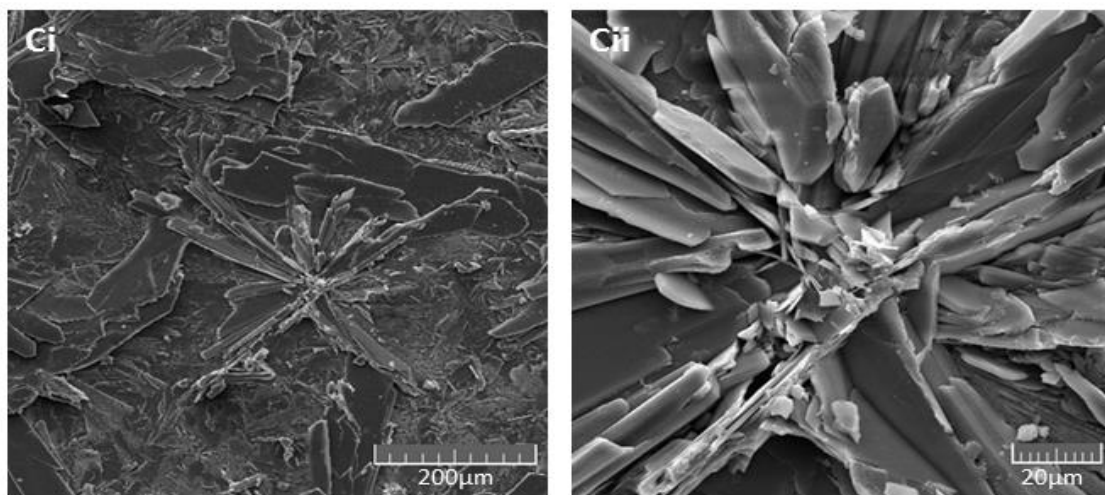
($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, DCPD, Figure 2.1.3.B), and DCPD + stearic acid (DCPD + SA, Figure 2.1.3.C), respectively. The HA coating was composed of 0.25 mol/L ethylenediaminetetracetic acid calcium disodium salt hydrate (Ca-EDTA) and 0.25 mol/L potassium dihydrogenphosphate (KH_2PO_4), and the thickness of coating was 2 μm . The DCPD coating was composed of 3.1% sodium hydrogen phosphate (Na_2HPO_4) and 5.3% calcium nitrate ($\text{Ca}(\text{NO}_3)_2$). The HA and DCPD coatings formed with chemical bond of calcium phosphate and magnesium matrix. The thickness of DCPD coating was 3 μm . The DCPD + SA coating was treated based on the DCPD-coated magnesium disks with the addition of stearic acid. The combination of SA depends on hydrogen bond and physisorption. The thickness of DCPD+SA coating was 4 μm . Finally, all of the coated magnesium disks were ultrasonically cleaned.



(a)



(b)



(c)

Figure 2.1.3 Scanning electron microscope (SEM) images of different magnesium coatings. (A) SEM morphology of HA; (B) SEM morphology of DCPD; (C) SEM morphology of DCPD + SA.

2.1.4 Magnesium sample preparation

The coated magnesium samples were immersed in 70% ethanol for 10 minutes, and rinsed twice with distilled water, and then dried under UV light. The samples were introduced into a separate 24-well plate (BD Falcon, BD Biosciences, Broendby, Denmark), with 5×10^4 primary HTCF cells per well (Invitrogen Countess™). The glass and titanium disks were used as controls (Figure 2.1.4).

During the experiments, if a coated magnesium disk emitted hydrogen gas, the gas would elevate the disk. We defined this phenomenon as corrosion, recorded it and did not use such disks for our experiments.

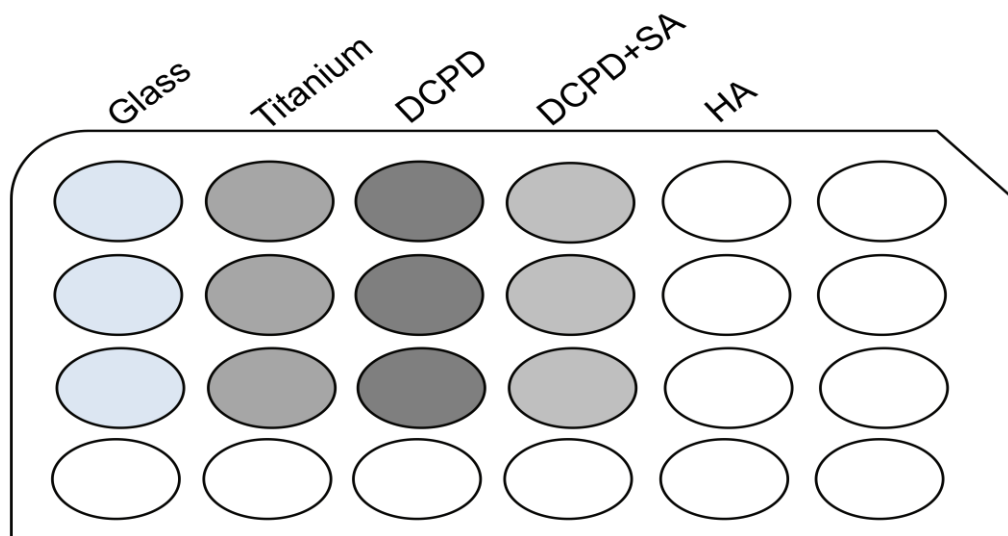


Figure.2.1.4 Different sample groups for experiments.

2.1.5 Statistical analysis

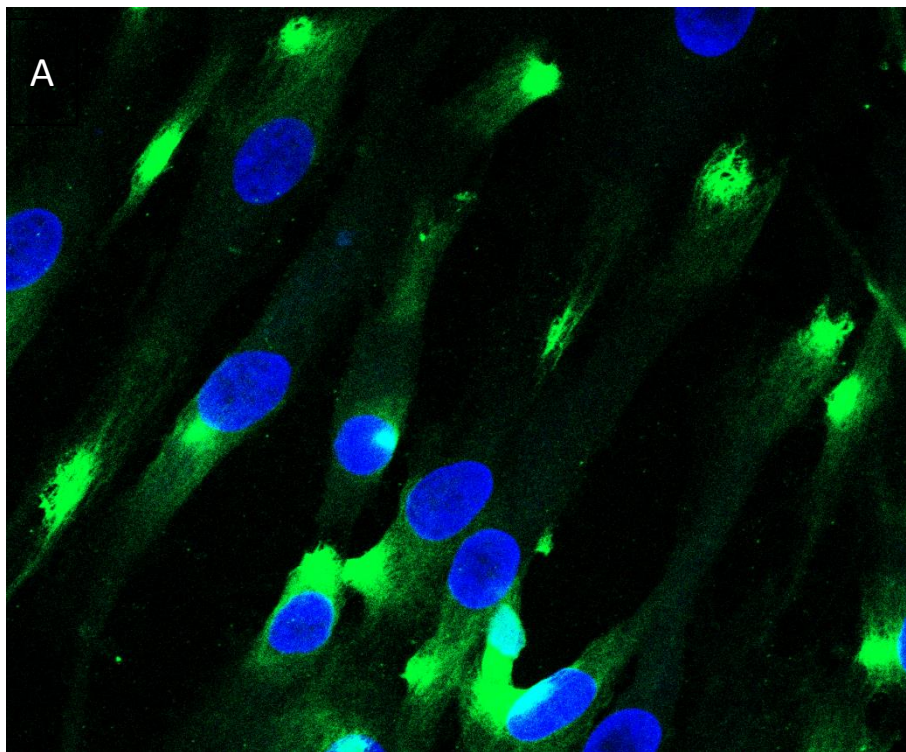
The corrosion rate of differently coated magnesium alloys were compared using chi-square test. The corrosion susceptibility of different coatings from day 2 to day 7 was compared using two-way analysis of variance (ANOVA). Statistical significance was accepted at $P < 0.05$. All analyses were performed using SPSS 24.0 software.

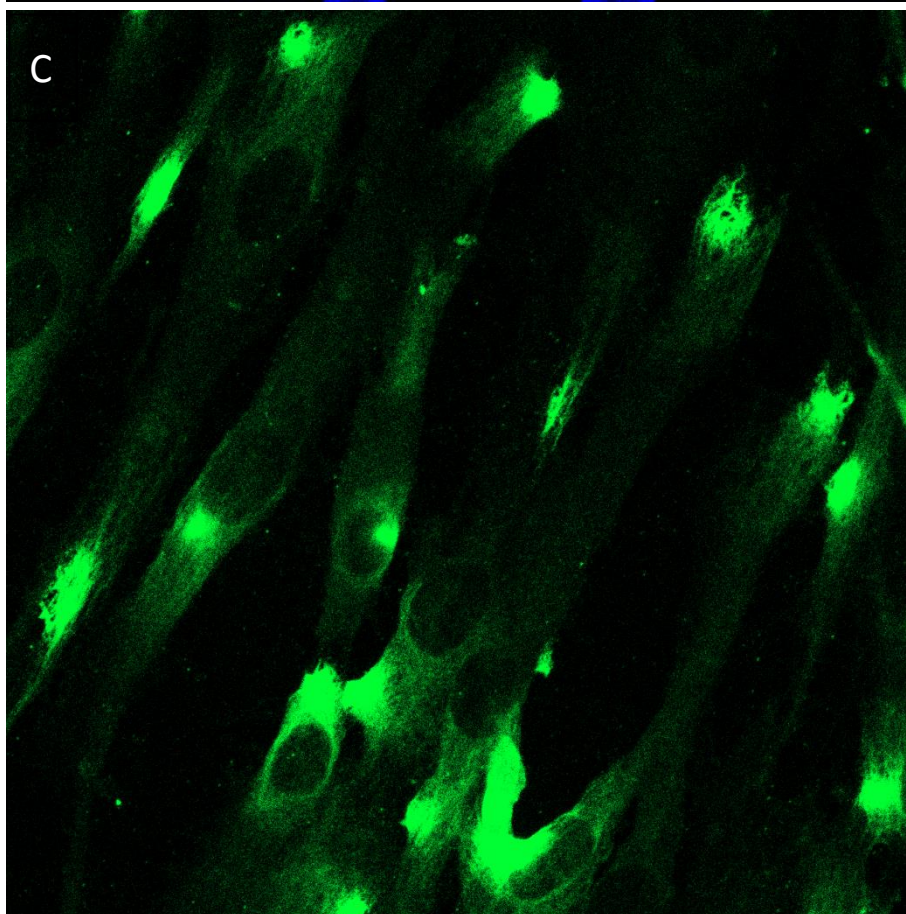
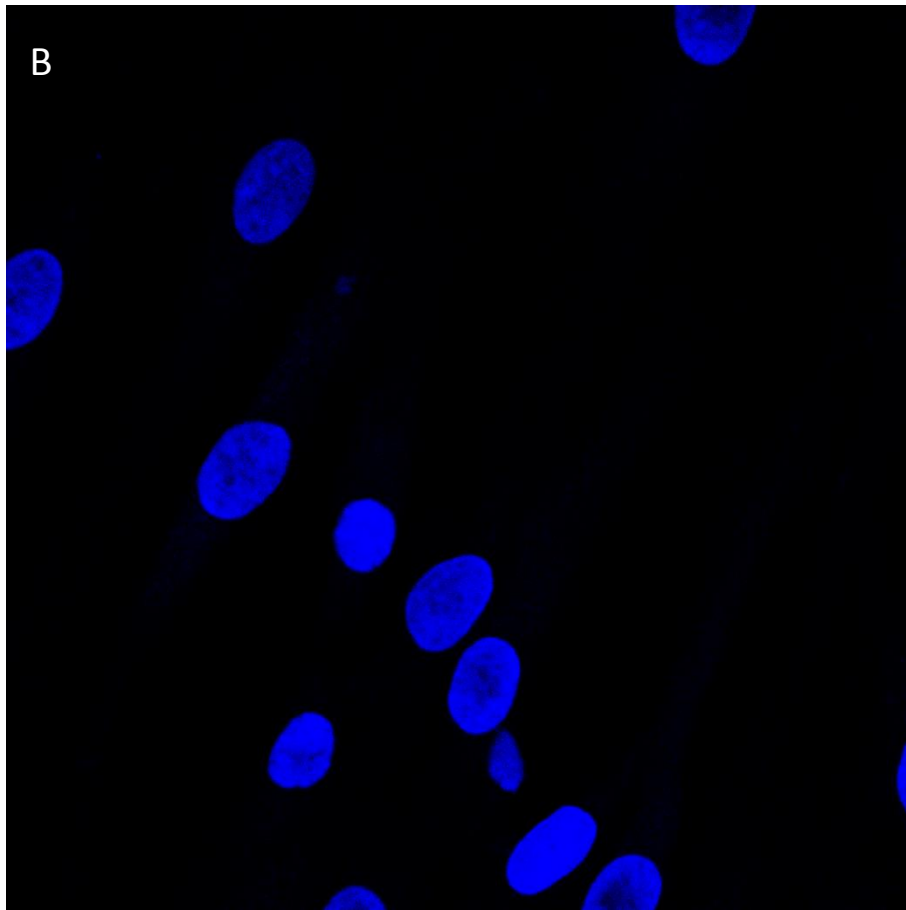
2.2 Results

2.2.1 Cell morphological observation and identification of HTCFs by

immunofluorescence staining.

The cells migrated out from the tissue after 7-10 days of adherent culture. Microscopically, the cells exhibited a fusiform appearance with clear outline, and the cellular plasma was abundant, bright, and uniform-sized. The cells were arranged in fasciculus or swirling patterns. No differences were observed between the recovered cells and those before cryopreservation in terms of morphology and growth characteristics. The results revealed that the cells were positive for the expression of vimentin, and the specific fluorescence could be observed within the cytoplasm (Figure 2.2.1). The cells were negative for the expression of keratin. This supported their identity as fibroblasts.





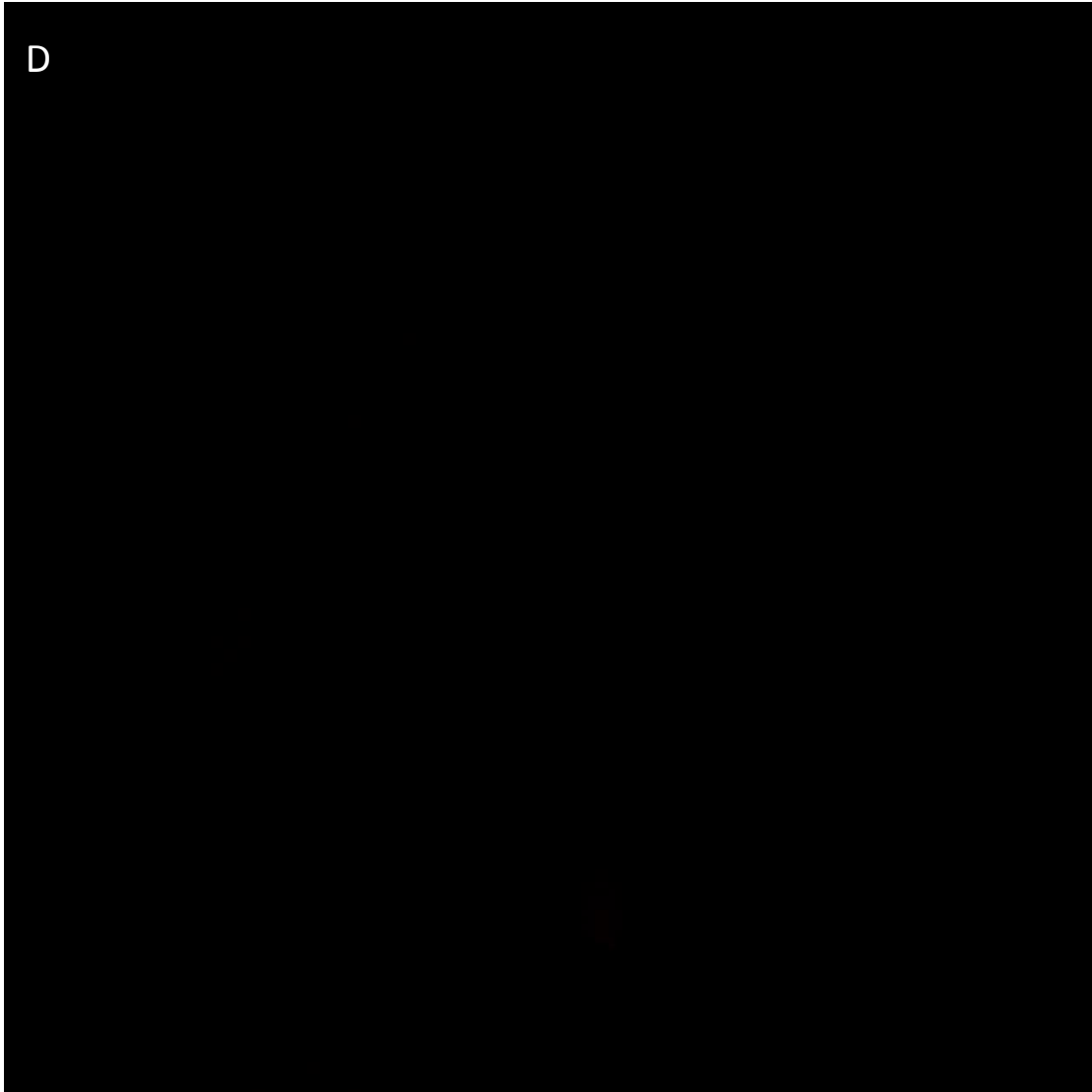


Figure 2.2.1 Immunostaining and characterization of primary human Tenon's capsule

cultures. Cells were stained for nuclei (blue), vimentin (green), and keratin (red) under 40x magnification. Image A shows a complete image while B, C, and D show isolated images for nuclei, vimentin, and keratin stains respectively. Images displayed are representative of all images of samples taken.

2.2.2 Corrosion of different coated magnesium alloys

A total of 453 coated magnesium alloy disks were used in our experiments: 399 for assessment of metabolic activity, cytotoxicity, and cellular proliferation rates, and 54

for western blot analysis (Table 2.2.2). The phenomenon of corrosion was observed in 67 disks, accounting for 14.8%. The coating of DCPD and DCPD + SA had the same corrosion proportion (both 15.9%), while the HA coating had the lowest corrosion proportion (12.6%). The difference, however, was not statistically significant (chi-square test, $\chi^2 = 0.876$, $P = 0.645$).

Table 2.2.2 Corrosion rate of different coated magnesium alloys (events).

	DCPD	DCPD+SA	HA	Total
Corrosion	24	24	19	67
No corrosion	127	127	132	386
Total	151	151	151	453
Proportion	15.9%	15.9%	12.6%	14.8%

The difference was not statistically significant (chi-square test, $\chi^2 = 0.876$, $P = 0.645$).

2.2.3 The corrosion susceptibility of different coatings from day 2 to day 7

A total of 324 samples were used for assessment of metabolic activity and cytotoxicity from day 2 to day 7. As shown in Table 2.2.3, corrosion of the HA coating was more frequently found during the last three days (8/10), while corrosion of the DCPD coating was more commonly seen during the first three days (6/9). The two-way ANOVA analysis revealed that the corrosion susceptibility of different coatings was not statistically different ($P_{\text{coating}}=0.729$, $P_{\text{day}}=0.903$).

Table 2.2.3 The corrosion susceptibility of different coatings on different days (events/disks).

	DCPD	DCPD+SA	HA
Day 2	2/18	1/18	0/18
Day 3	3/18	1/18	1/18
Day 4	1/18	1/18	1/18
Day 5	0/18	2/18	3/18
Day 6	1/18	1/18	3/18
Day7	2/18	1/18	2/18

The difference was not statistically significant (two-way ANOVA, $P_{\text{Coating}}=0.729$, $P_{\text{Day}}=0.903$).

2.3 Discussion

Coatings increased the corrosion resistance of magnesium alloys

The total corrosion rate was 14.8%, and the HA coating showed a relatively more stable property compared with DCPD and DCPD + SA.

Mg alloys have unique properties, providing the mechanical benefits of a metal combined with the degradable and biological advantages of biomaterials¹³¹. However, in spite of significant recent research, corrosion is a major challenge to successful implementation of Mg-based materials in the body. In addition, the wear resistance of Mg and Mg alloys is not very high. Therefore, a broad range of coating systems have been developed to overcome these weaknesses for numerous applications^{132,133}.

Recently, calcium phosphate-based coatings have attracted special interests for biomedical application as bone substitutes and orthopaedic materials¹³⁴, including DCPD, DCPD + SA, and HA. Particularly, the formation of a HA layer is similar to the mineral phase of bone¹³⁵⁻¹³⁷, and more smoothly than other two coatings. In our experiments, the HA coating was more stable than DCPD or DCPD + SA. Interestingly, in the MTT and LDH assays, where disks were immersed in the culture medium for 7 days, most of the corrosion events of the HA-coated magnesium alloys occurred during the last three days, accounting for 80%. Therefore, we conclude that the HA coating has better corrosion resistance at the beginning of the cellular logarithmic phase.

Chapter 3

Corrosive properties of coated magnesium alloys in simulated aqueous humor

3.1 Background

Due to their excellent bio-compatibility, magnesium alloys are being considered as promising implant materials. However, the poor corrosion resistance becomes a major obstacle to their widespread applications. Currently, the focus of bio-magnesium studies is on coatings or surface modification to slow the degradation rates of Mg alloys. If coated magnesium alloys are going to be used a biomaterial candidate for glaucoma drainage device, not only gradual degradation is needed to control the overall corrosion process, but also pH maintenance and control of ions released into the anterior chamber are also required to maintain a non-toxic concentration of each ions. These conditions very rapidly alter as a result of the corrosion process, with rapid changes in pH levels and metal ion concentrations, as well as presence of soluble corrosion products and hydrogen gas evolution. Thus, we need a complicated solution that considers the corrosion resistance, metal ion concentrations, pH and mechanical performance of coated magnesium alloy implants when immersed in the aqueous humor. It is essential to fully understand the long term corrosion engineering and potential biological interactions. Thus we invited our collaborators (National Engineering Research Center for Magnesium Alloys, Chongqing University, China) using balanced salt solution to test the variation of pH, sample's weight, and ion concentrations.

3.2 Materials and methods

3.2.1 Corrosion resistance test

The corrosion resistance, ion release, sample weight change, and pH change were investigated by immersion of coated and uncoated magnesium alloy samples in balanced salt solution (BSS, Alcon, USA). The BSS components are shown in Table 3.2.1.

Four prepared samples were placed on a specially made PVC plastic stent and soaked in a 150ml containing balanced salt solution (BSS), and each sample was weighed before test. Each sample set 4 groups, each group of a specimen in a ground glass stoppered flask. And then remove flasks to a 37°C 5% CO₂ incubator(Shanghai Shengke 101) .

3.2.1.1 The SEM morphologies

The SEM morphologies were observed at 5 and 9d. The coated sample was covered with specific conductive liquid to allow it to conduct electricity. The pure magnesium sample can be directly placed in the electron microscope.

3.2.1.2 The pH test

The pHs of three groups in each sample were measured at 1, 2, 5, 9, and 15d. The electrode was rinsed by BSS before test. The instrument of pH test was Shanghai Lida phs-3c.

3.2.1.3 Variation of weight

3 discs of each sample were taken out respectively at 2, 5, 9, 15 and 21d. The discs were rinsed by deionized water and 100% ethanol, and then slowly dried the discs with cold wind. After weighed on an electronic scale, the discs continued to immerse in BSS.

3.2.1.4 Variation of ion concentrations

2ml different soaking solutions were extracted at 1, 5, and 9d, respectively, diluted two times, detection of magnesium, calcium, phosphorus content in solution, and compared with BSS solution. The result of these three ions content was removed the content of ions in the blank solution. The instrument used is inductively coupled plasma emission spectrometry (ICP-OES)

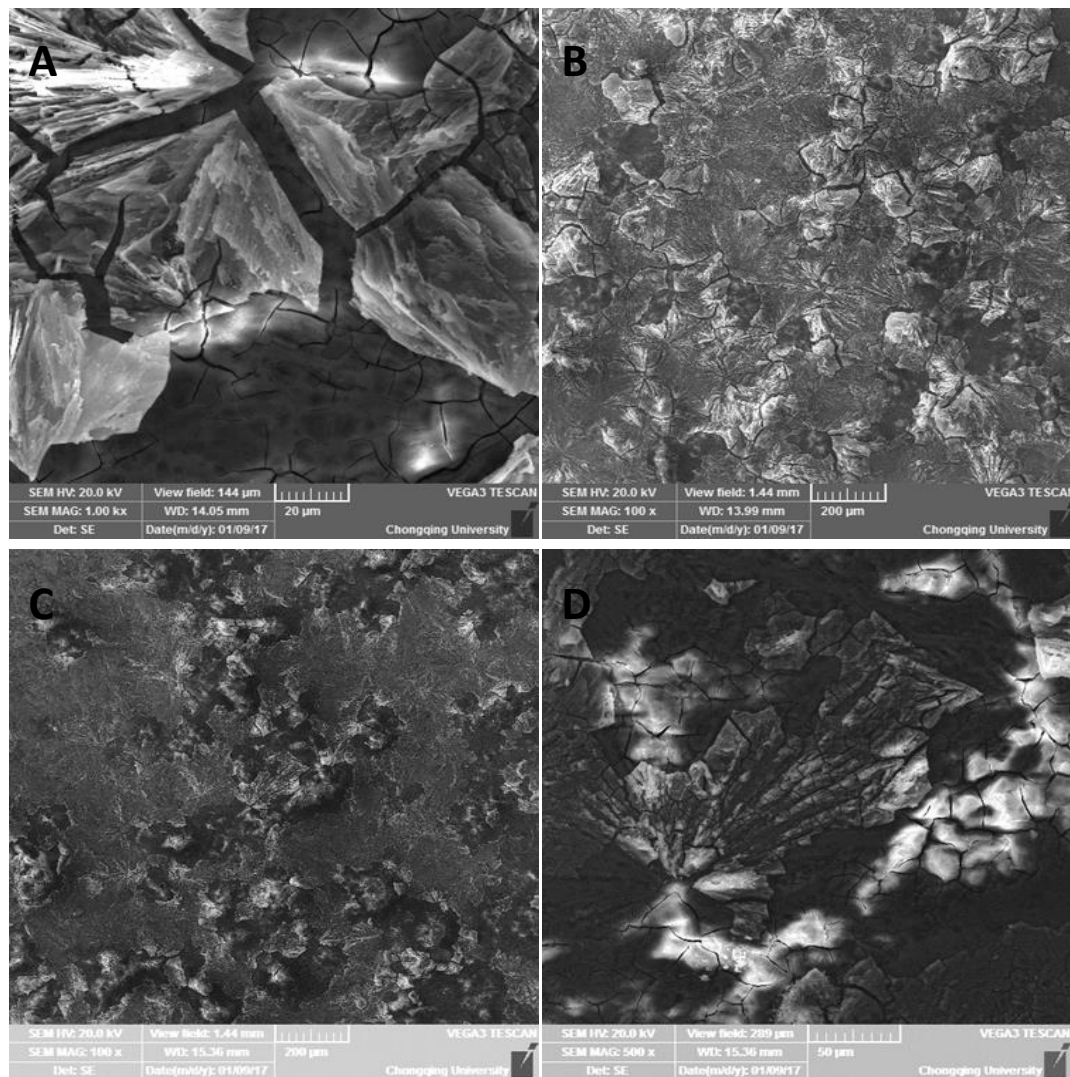
Table 3.2.1 Components of aqueous humor and simulated aqueous humor.

	Aqueous humor	Balanced salt solution (Alcon)
Na ⁺ (mmol/L)	163	156
K ⁺ (mmol/L)	2.3-3.9	10.1
Ca ²⁺ (mmol/L)	1.8	3.3
Mg ²⁺ (mmol/L)	1.1	1.5
Cl ⁻ (mmol/L)	132	129
pH	7.38	7.4
Osmotic pressure (mmHg)	304	305

3.3 Results

3.3.1 Microstructure of different coatings

The SEM morphologies for different coatings are shown in Figure 3.3.1. After immersion for 9 days, the coating of DCPD and DCPD + SA was dissolved and flaked, with cracked surface. The HA coating was exfoliated from the matrix on day 5, but without any obvious changes on the surface of non-exfoliated areas.



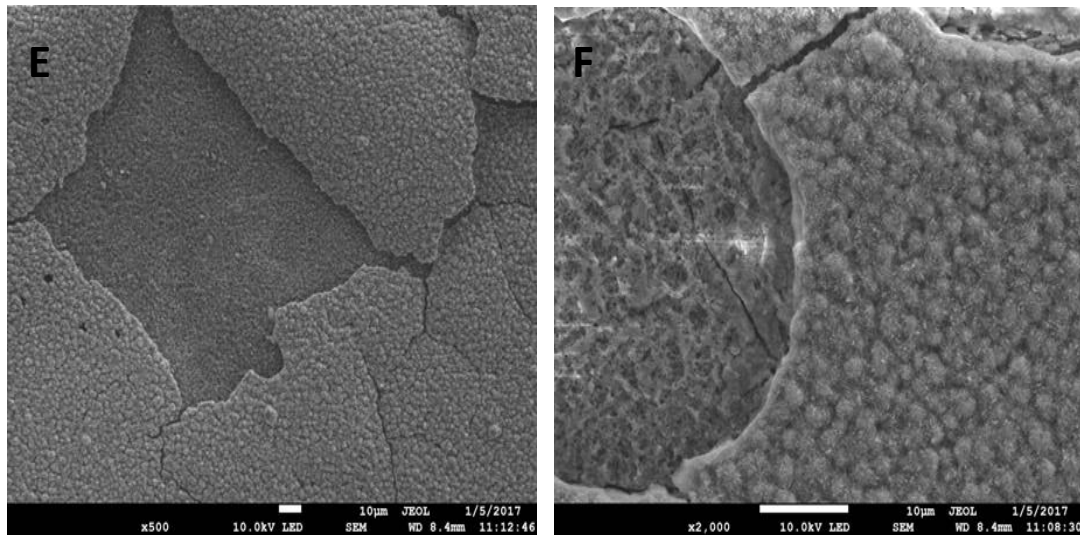


Figure 3.3.1: SEM images of coated magnesium samples after immersion under different magnifications. Image A and B shows the DCPD coated sample at 100x and 1000x magnification. Image C and D shows the DCPD+SA coated sample at 100x and 500x magnification. Image E and F shows the HA coated sample at 500x and 2000x magnification. **Graphs from National Engineering Research Center for Magnesium Alloys, Chongqing University, China**

3.3.2 pH variations of BSS

As shown in Figure 3.3.2, the pH levels of all samples rapidly increased during the first two days. The pure magnesium group had higher pH levels compared with other groups, but after day 5, the pH levels of the pure magnesium group slightly declined. The pH levels of all coating groups gradually increased after day 2. The pH levels of the HA coating group was constantly higher than those of the DCPD and DCPD + SA groups.

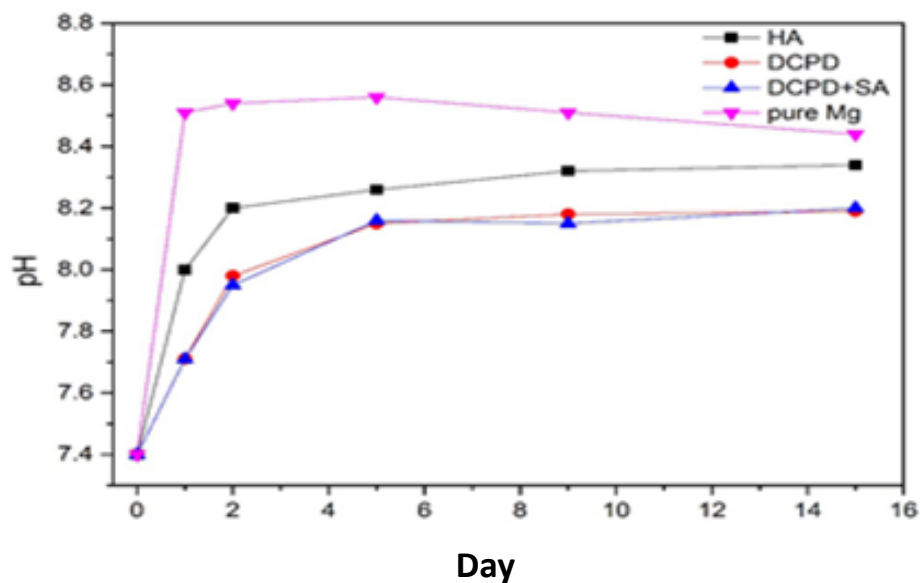


Figure 3.3.2 Variation of BSS pH of coated and uncoated samples over time.

The pHs of three groups in each sample were measured at 1, 2, 5, 9, and 15 days. The electrode was rinsed by BSS before each test. **Graphs from National Engineering Research Center for Magnesium Alloys, Chongqing University, China**

3.3.3 Sample weight changes in BSS

The changes of sample weight during the immersion test are shown in Figure 3.3.3.

The sample weight was slightly reduced in the pure magnesium group and the HA coating group during the first 5 days, but gradually increased after day 5. The sample weight of the DCPD and DCPD + SA groups was dramatically reduced during the first 2 days, but gradually increased after day 2 in the DCPC + SA group. In the DCPC group, weight loss became slower from day 2 to day 5, but the sample weight began to increase after day 5.

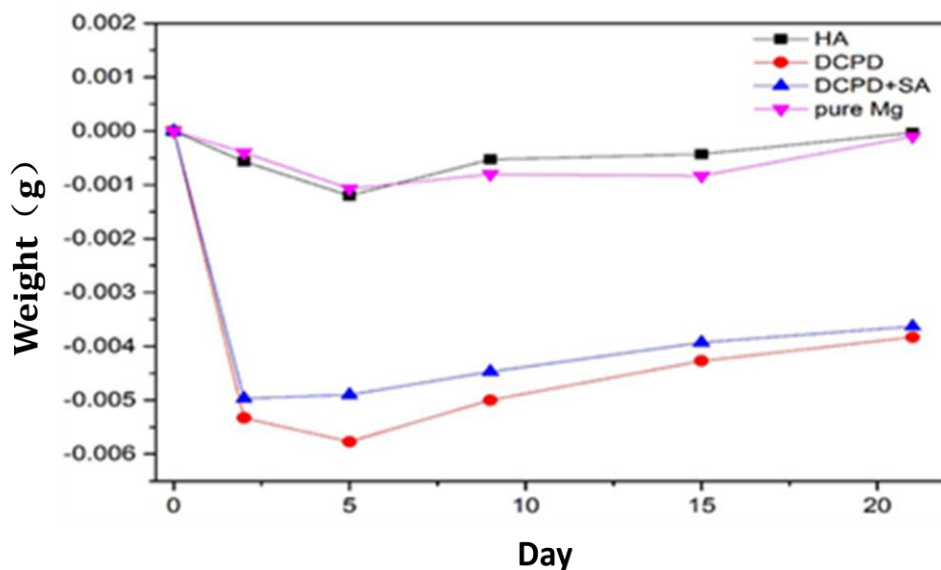


Figure 3.3.3 Weight changes of coated and uncoated samples over time.

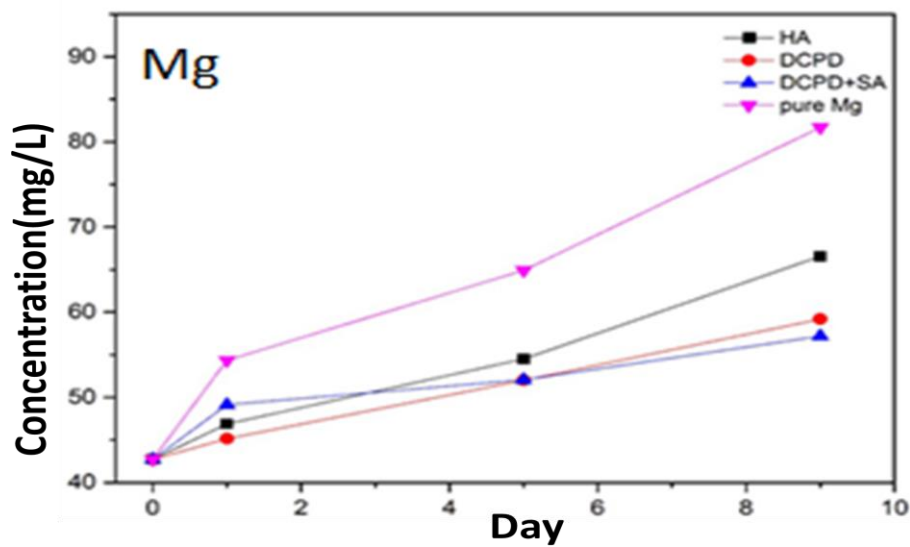
Discs were taken out at 2, 5, 9, 15 and 21 days. The discs were rinsed by deionized water, 100% ethanol, air-dried and then weighed on an electronic scale. **Graphs from National Engineering Research Center for Magnesium Alloys, Chongqing University, China**

3.3.4 Changes of ion concentrations during immersion test.

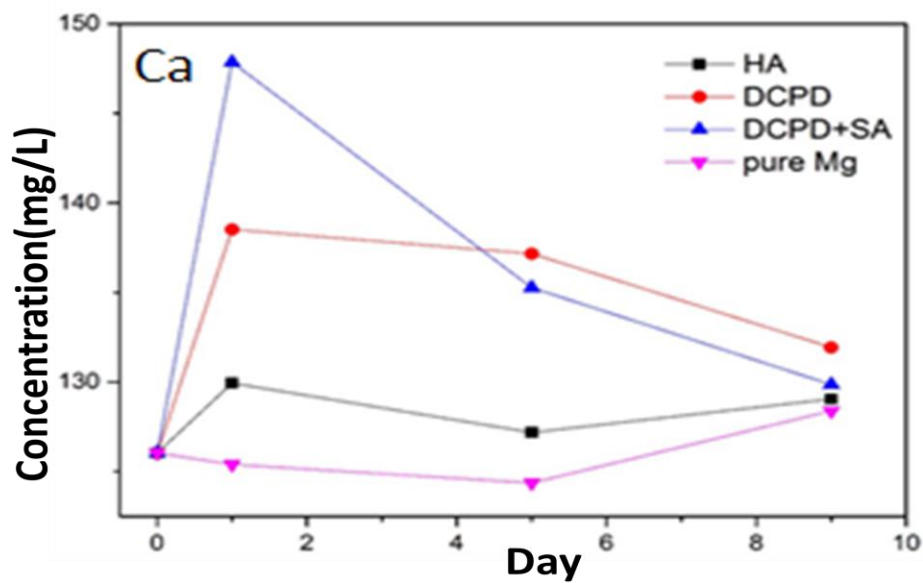
As shown in Figure 3.3.4.a, the magnesium concentration of all groups gradually increased from day 1 to day 9. The magnesium concentration in the pure magnesium group was obviously higher than that of other groups.

The calcium concentration of the DCPD and DCPD + SA groups was much higher than that of the HA group (Figure 3.3.4.b). The calcium concentration of all the coating groups rapidly increased on day 1, and then declined afterwards. The calcium concentration of the DCPD + SA group rocketed at the beginning, but rapidly dropped after day 1 and became lower than that of the DCPD group after day 5.

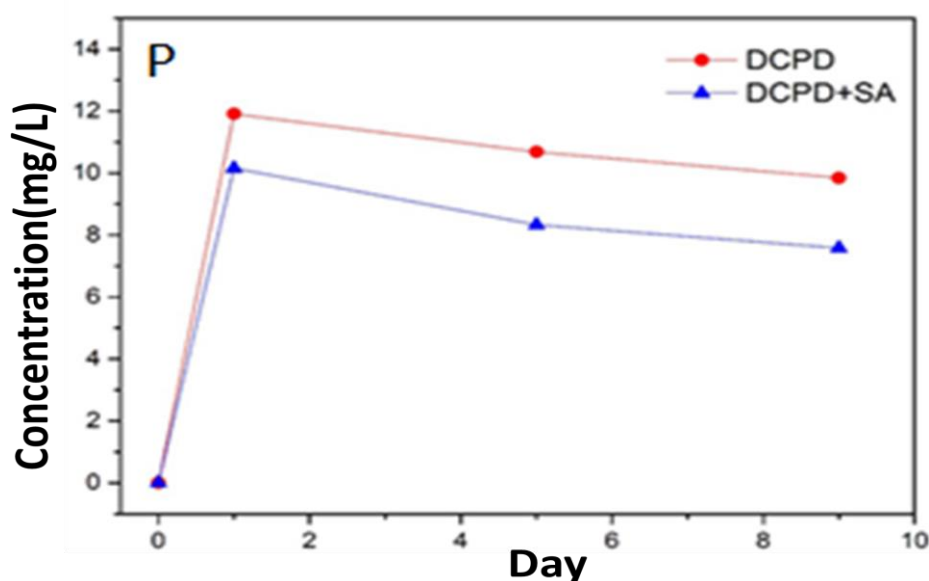
As for the changes of the phosphorus concentration (Figure 3.3.4.c), the DCPD and DCPD + SA groups showed a similar trend with that of calcium, i.e. rapid increase on day 1, and then gradual reduction afterwards. The DCPD group had a higher phosphorus concentration than that of the DCPD + SA group. Interestingly, the phosphorus concentration was not detectable in the HA group.



(a)



(b)



(c)

Figure 3.3.4 Ion concentration changes during immersion test.

(a), (b) and (c) illustrate the changes of magnesium, calcium and phosphorus concentrations during immersion test, respectively. 2ml different soaking solutions were extracted at 1, 5, and 9d, respectively, diluted two times, detection of magnesium, calcium, phosphorus content in solution.

Graphs from National Engineering Research Center for Magnesium Alloys, Chongqing University, China

3.4 Discussion

3.4.1 Effect of coating on corrosion process

BSS is an intraocular irrigating solution commonly used in ophthalmic surgeries to perfuse and support the anterior chamber to prevent its collapse during operation. Its properties, including pH value, ion concentrations and osmotic pressure, are generally similar to those of human aqueous humor. Thus, BSS was applied in the immersion test as simulated aqueous humor.

The pH level of BSS and aqueous humor is 7.4, which may corrode magnesium alloy, while the presence of chloride (Cl⁻) ions would aggravate the corrosion leading to dissolution of magnesium^{138,139}. The dissolution of magnesium consumes the hydrogen (H⁺) ions in the solution, resulting in a continuous rise in the pH of the solution¹⁴⁰. During the immersion test, the pure magnesium group had higher pH levels than other groups, indicating that coating could prevent dissolution of the matrix and protect against corrosion, thus delaying changes in the pH level.

The samples is stable in the dry air is long-term and stable. In vitro experiment, by immersion in BSS for 21 days, the DCPD and DCPD+SA coated samples lost about 1% weight loss, HA and pure magnesium did not test to weightlessness. Weight loss of samples is caused by dissolution of coating and matrix. The surfaces of coatings have some small pores that causing the solution permeate into the matrix. Although the corrosion of coating and matrix almost occur at same time, but in the early stage, corrosion medium is slow and difficult to penetrate the coating. According to the pure magnesium disks almost did not lose any weight during the test, and thus it could be concluded that dissolution of the coating was the major contributor to weight loss. Similarly, the weight of the HA disks remained almost the same throughout the test, indicating that the coating of HA had better ability to protect against dissolution. Compared with the DCPD and DCPD + SA coated samples, the HA coated samples had lower weight loss rate because of the protective effect on the matrix. Additionally, the oxide layer of the original metal surface grows inwards and outwards at the same

time, and thus the weight change of samples consists of two parts: dissolution causing weight loss and deposition of magnesium hydroxide ($\text{Mg}(\text{OH})_2$) causing weight gain¹⁴¹. During the later stage of this test, magnesium hydroxide deposition might exceed dissolution, leading to increases in the sample weight. This explains why the weight of each group gradually increased after rapid decline.

According to these results, our collaborators estimated that the pure magnesium in BSS solution can maintain the stability for more than 1 month and the coated magnesium alloys can maintain the stable state for longer periods. But vitro experiments had big difference compare to vivo experiments, because there is no bioactivity in vitro experiment. Therefore, the purpose of immersion corrosion test is screening for a promising coating for future research.

3.4.2 Ion release of different samples during immersion test.

Compared with the DCPD and DCPD + SA coated samples, the HA coated samples released fewer ions contained in the coating. This finding demonstrated that the coating of HA could prevent dissolution and corrosion of magnesium alloys in an effective and stable manner. Interestingly, DCPD is a precursor for the synthesis of HA^{141,142}; it can be dissolved in physiological environment and release calcium and phosphorus which form of HA with precipitation¹⁴³. This is why more calcium and phosphorus ions were released in the DCPD and DCPD + SA groups. However, the dissolution rate of the DCPD and DCPD + SA coating was higher than inducing the

formation of HA crystals.

3.4.3 SEM morphologies of different coatings after immersion test

According to the SEM photographs, the coating of DCPD and DCPD + SA was dissolved and flaked, with cracked surface observed on day 9. Although the HA coating was exfoliated from the matrix, no obvious changes were noted on the surface of non-exfoliated areas. Therefore, we speculate that the main problem of the HA coating is an increased risk of detachment from the matrix. If it can be improved to attach more closely with the matrix, we believe that this type of coating could effectively reduce the corrosion rate of magnesium alloys.

Chapter 4

The effect of different coatings on metabolic activity and necrosis of HTCFs

4.1 Background

Various kinds of metal-based tissue implants have been developed since the 20th century. But they still have many defects that remain to be addressed. For instance, nickel-titanium alloy implants cause potential harm to the human body¹⁴⁴, because of the release of Ni⁺. The titanium has been widely used as a biocompatible surgical device. Despite the wide belief that it is inert, allergic reactions and stress shielding effects have been reported after implantation¹⁴⁵.

Magnesium is the second most common cation in the intracellular fluid, also the fourth most common metallic ion in the body, mainly located in the cytoplasm. It participates in a series of metabolic processes in the body. In order for magnesium alloys to be developed as a tissue implantable material in the future, we not only need to solve the key adverse effect of corrosion in the human body, but also consider the cytotoxicity of coating and its effects on cellular metabolism. Therefore, it is very important to investigate the biocompatibility of differently coated magnesium alloys.

In our previous experiments, differently coated magnesium alloy disks were used to treat cells for only 24 hours, but as a potential biomaterial for future glaucoma drainage devices, coated magnesium alloys would exist in the conjunctiva for a

relatively longer period of time, and thus it would be necessary to investigate the long-term effect of differently coated magnesium alloys on HTCFs.

4.2 Materials and methods

4.2.1 MTT assay

The primary HTCFs were seeded on DCPD, DCPD + SA, and HA disks in a 24-well culture plate, 2×10^4 cells per well from Day 2 to Day 7. Glass disks were used as controls. The assay was performed according to the manufacturer's instructions. MTT (Sigma, USA) was added at the final concentration of about 450 $\mu\text{g/ml}$. MTT concentrations in the stock: 5 mg/mL for 1 mL cell culture medium, and 0.5 mL was added to each well. Cells were placed in a 37°C 5% CO_2 incubator. After 3 hours, the supernatant was aspirated from culture wells. The precipitate in each well was resuspended by adding 0.5 mL dimethyl sulfoxide (DMSO, Sigma, USA) to solubilize formazan crystals. Subsequently, the solution was completely mixed, and 100 μL was removed from each well to 3 wells of a 96-well plate. Absorbances at 575 nm were determined using a microplate reader (Molecular Devices, Sunnyvale, CA). Control cells, which represented 100% metabolic activity, were used to normalize absorbances. At least 6 samples per group were measured in duplicate.

4.2.2 Lactate dehydrogenase cytotoxicity(LDH) assay

The supernatant of different groups were collected from Day 2 to Day 7. Glass was used as control. The collected supernatant was analysed using a LDH cytotoxicity kit (Roche, USA), and the assay was performed according to the manufacturer's

instructions. The collected supernatant was centrifuged at 1000 x 10 min. Three 1.5 mL tubes were labeled as 1, 2 and 3. Next, 7.5 uL LDH (Sigma, USA) solution was diluted with 992.5 uL PBS solution in tube 1, and then 100 uL solution was extracted from tube 1 to tube 2 which contained 900 uL PBS solution. Finally, 100 uL solution was extracted from tube 2 to tube 3 which also contained 900 uL PBS solution. To the final row standard lanes, 200 uL of the diluted LDH solution (0.5 U/mL) was added to 2 wells of the 96-well plate. Using a multipipette with PBS, 100 uL PBS was placed into the other wells of the standard lanes. The standards were serially diluted so that each well had a final volume of 100 uL, and then each standard was mixed well. And no LDH was contained in the first row. The amount of the total reaction solution was determined and 100 uL reaction solutions was added to each well and allowed to incubate for 10-15 min at room temperature. Optical densities of the solutions were read at dual wavelength of 490/655 nm. Control cells, which represented 100% cytotoxicity, were used to normalize absorbances. At least 6 samples per group were measured in duplicate.

4.2.3 Statistical analysis

The MTT and LDH assay results were analyzed using two-way ANOVA test. Statistical significance was accepted at $P < 0.05$. All analyses were performed using SPSS 24.0 software.

4.3 Results

4.3.1 Time and cellular metabolic activity curves

The MTT assay was used to determine cellular metabolic activity. In the presence of viable cells, MTT was enzymatically reduced to the purple dye formazan. The multiple curves represented the variation of metabolic activities of HTCFCs which were treated by different disks from day 1 to day 7 (Figure 4.3.1). The p value of different treatments was statistically significant ($p_{\text{treatment}} < 0.001$). The metabolic activity of the DCPD + SA group, which gradually declined from day 2 to day 7, was significantly lower compared with other groups. The HTCFCs seeded on the titanium plates had the highest viability. The HA group showed a higher metabolic activity during the first two days, but the viability gradually decreased and became lower than that of the DCPD group since day 4. The P value of time was not statistically significant ($p_{\text{time}} = 0.195$), indicating that time had no obvious influence on the metabolic activity of HTCFCs.

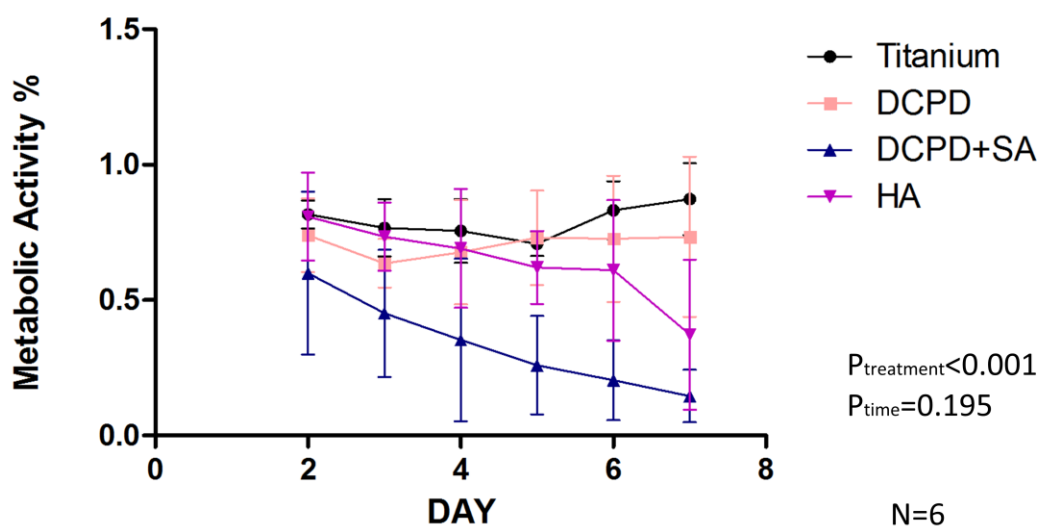


Figure 4.3.1 Cellular metabolic activity of different samples.

MTT results from HTCFCs treated with different coated magnesium alloys. The multiple curves represent the variation of metabolic activities of HTCFCs which were treated by different disks from day

1 to day 7. MTT was added at a final concentration of 450 ug/ml, and 0.5 mL was added to each well. Cells were placed in a 37°C 5% CO₂ incubator. After 3 hours, the supernatant was aspirated from culture wells. The precipitate in each well was resuspended by adding 0.5 mL dimethyl sulfoxide (DMSO, Sigma, USA) to solubilize formazan crystals. Data was analyzed using two-way ANOVA test. The source of statistical significance was determined by applying Tukey test (SPSS 24). *** $p_{\text{treatment}} < 0.001$, $p_{\text{time}} = 0.195$; N = 6.

4.3.2 Time and cytotoxicity curves

The LDH assay was utilized to examine the cytotoxicity of coatings or necrosis of HTCFs seeded on different disks. Liberated nicotinamide adenine dinucleotide, formed from LDH's conversion of lactate into pyruvate, subsequently reduced a tetrazolium dye into formazan, which could be detected using a spectrophotometer. The P value of treatments was statistically significant ($p_{\text{treatment}} < 0.001$). The HA group had the lowest cytotoxicity, which was statistically different compared with other groups. The cytotoxicity of the DCPD + SA group was higher than that of the HA and DCPD groups, but similar to that of the titanium ($p = 0.468$) and glass ($p = 0.976$) groups. The p value of time was not statistically significant ($p_{\text{time}} = 0.260$).

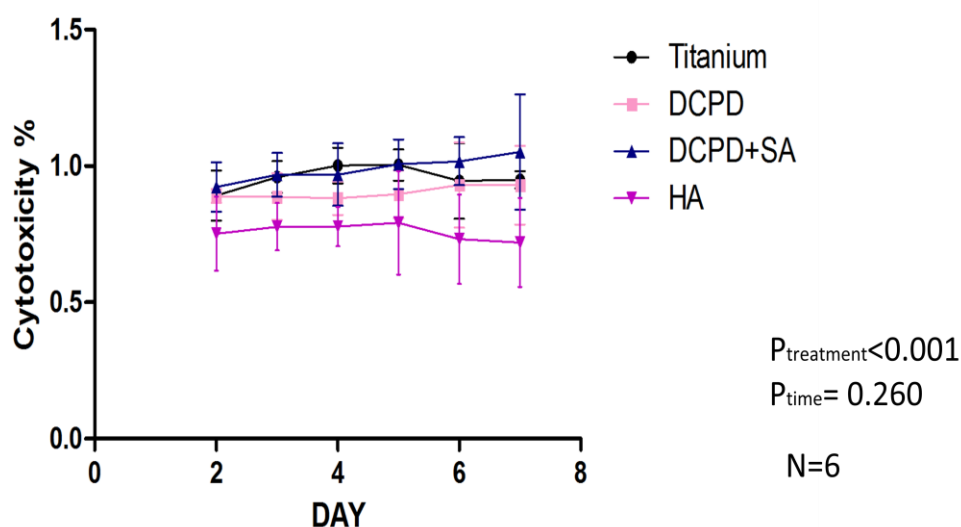


Figure 4.3.2 Time and cytotoxicity curves of different samples.

The supernatant of different groups were collected from Day 2 to Day 7. Glass was used as control. The collected supernatant was analysed using a LDH cytotoxicity kit (Roche, USA), and the assay was performed according to the manufacturer's instructions. Data was analyzed using two-way ANOVA test. The source of statistical significance was determined by applying Tukey test (SPSS 24).

*** $p_{\text{treatment}} < 0.001$, $p_{\text{time}} = 0.260$; N = 6.

4.4 Discussion

4.4.1 The influence of time

A prior study reported that the logarithmic phase of HTCFCs was from 48 hours to 7 days¹⁴⁶. Therefore, in order to examine the relationship between the HTCFCs' metabolic activity and time, and to investigate the variation of cytotoxicity during this period, we performed the MTT and LDH assays of treated HTCFCs from day 2 to day 7. The results revealed that no statistically significant difference was observed in metabolic activity or necrosis at different time points during the logarithmic phase ($p=0.195$, $p=0.260$, respectively). Based on these findings, the factor of time was not a major contributor to reduced metabolic activity and increased cytotoxicity in this in vitro experiment. Thus, those two curves could basically describe the influence of different treatments on HTCFCs during the logarithmic phase. These results were used to as a basis for the design of future experiments to assess cellular function, such as BrdU, Western blotting and RT-PCR.

4.4.2 Biocompatibility of coated magnesium alloys

The purpose of this study was to evaluate the biocompatibility of coated magnesium alloys which might be promising materials for novel drainage devices. Lorenz A *et al.*¹⁴⁷ reported that cell death on untreated pure magnesium samples occurred within 1

day, whereas surface passivation could enable survival of a number of cells on Mg. However, cell densities were found to be reduced on Mg samples even with prior passivation treatments, compared with glass substrates used as a reference. Cell death was thought to be related to the ongoing corrosion of Mg in cell culture medium, leading to a pH increase. A recent study indicated that cell culture tests could not provide enough volume to compensate for the high concentration of Mg^{2+} ions or the alkaline pH shift in solution, and hence might not be appropriate for testing resorbable materials¹⁴⁸. Although coatings could reduce Mg^{2+} release from pure magnesium matrix and prevent pH increase, the coatings might influence the viability of cells and their cytotoxicity might lead to necrosis of HTCFs.

In the MTT and LDH assays, the p value of different treatments was statistically significant ($p < 0.001$). The DCPD + SA group had the lowest metabolic activity but higher cytotoxicity than the other two coatings. The coatings of both HA and DCPD had lower cytotoxicity than titanium and glass.

HA is attributed to the compositions of bioactive and bioresorbable ceramics or substances close to it in composition which forms HA crystals by the reaction with the organism at the implant-biomedium interface¹⁴⁹. Synthetic HA is a complete chemical and crystallochemical analog of bone mineral. This chemical similarity with bone accounts for their osteoconductive potential and excellent biocompatibility^{135,136}.

In fact, HA, as a biological material, has been used in ophthalmological clinical

practice for a long time, such as HA orbital implants. Because of its excellent biocompatibility, HA orbital implant exposure rarely causes rejection after implantation

In our experiments, the HA coating also showed excellent biocompatibility which had higher metabolic activity and the lowest cytotoxicity compared with other coatings. Therefore, we believe that HA is a very promising type of coating for glaucoma drainage devices.

Chapter 5

Anti-proliferative potential of coated magnesium alloys on HTCFs

5.1 Background

Nowadays, glaucoma drainage implants have become increasingly useful in surgical management of glaucoma, especially for refractory glaucoma¹⁵⁰, including neovascular glaucoma, secondary glaucoma and remnant glaucoma. Glaucoma drainage devices are designed to divert aqueous humor from the anterior chamber to an external reservoir¹⁵¹. However, postoperative scarring is a major risk factor affecting surgical success rate. Moreover, glaucoma drainage device encapsulation may result in loss of the external reservoir function, leading to IOP increase and surgical failure.

It has been shown that inflammatory reaction is a main risk factor leading to postoperative scarring¹⁵². Foreign bodies inevitably induce rejection and activate some specific proteins which will trigger a localized inflammatory response and provide a short-term structural support. The inflammatory phase is characterized by the activation of the innate immune system and the release of inflammatory cytokines. At present, the commonly used materials for glaucoma drainage devices are titanium and silicone which are not biodegradable materials. Although they have good biological compatibility, their presence as permanent foreign body under the conjunctiva, stimulates fibroblast proliferation and glaucoma drainage device

encapsulation¹⁵³.

Magnesium alloy possesses many advantageous properties such as non-toxicity, absorbable and high biological compatibility. However, it has been reported that the corrosion of magnesium could increase pH levels and lead to cell death¹⁴⁷. In this research we took advantage of the ability to modulate these corrosive properties by the employing the use of coating materials. It was our goal to determine if we could use the more controlled corrosive properties to modulate the behavior of fibroblastic cells.

5.2 Materials and methods

5.2.1 BrdU assay

The proliferation of HTCFCs was monitored by 5-bromo-2-deoxyuridine (BrdU)-incorporation into cellular DNA using the Cell Proliferation BrdU assay kit (Merck KGaA, Darmstadt, Germany) as recommended by the manufacturer. Cell suspension in medium containing 10% FBS was placed on a 24-well culture plate (BD Falcon, BD Biosciences, Broendby, Denmark), 0.5 mL per well at a concentration of 0.5×10^4 cells/mL and was incubated for 48 hours. Cellular DNA was labeled for 24 hours using BrdU labeling reagent. After fixation, the cells were incubated with anti-BrdU antibody for 60 min at room temperature. Then the cells were washed, peroxidase goat antimouse IgG conjugate was added and the cells were incubated for 30 min. After addition of substrate solution, the plate was read using a spectrophotometer microplate reader with dual wavelength set at 450/595 nm. The

glass group was used as control. At least 6 samples per group were measured in duplicate.

5.2.2 Protein extraction and Western blotting analysis

Western blotting was performed on HTCFs samples derived from glaucoma patients. After treatment, the cells were rinsed with ice-cold PBS twice, and the total cell proteins were extracted using RIPA lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing phosphatase inhibitors and protease inhibitors. Protein concentrations were determined by micro BCA protein assay (Thermo Scientific, CA). Following the manufacturer's instructions, 10% SDS-polyacrylamide gel and 5% stacking gel were prepared and placed inside the electrophorator containing running buffer. And then, 6 μ L marker (Invitrogen, Thermo Fisher Scientific, CA) was loaded on the left lane, followed by 10 mg of each sample. The gel was run at 120 V for stacking gel and at 100 V for 10% SDS-polyacrylamide gel. Subsequently, the gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen, Thermo Fisher Scientific, CA) using a Bio-Rad gel-blotting apparatus (Invitrogen, Thermo Fisher Scientific, CA). Membranes were blocked in 5% milk in Tris-Buffered Saline Tween (TBST; 10 mM Tris HCl, 150 mM NaCl, 0.1% Tween 20) for 1 hour, and probed with antibodies against α -smooth muscle actin (α -SMA, Abcam, USA) at a dilution of 1:1000, then incubated with primary antibody overnight at 4°C and with a peroxidase-conjugated secondary antibody for 1 hour at room temperature. After these incubations, membranes were washed in TBST for 30

minutes. Western blotting detection kit (WesternBright Quantum, USA) was used to visualize for enhanced chemiluminescence (Chemi genius², Syngene, USA) and exposure. To ensure equal protein loading, nitrocellulose membranes were stripped with stripping buffer (Restore, Thermo Scientific, CA) and reprobred with anti-glyceraldehyde-3phosphate dehydrogenase (GAPDH) antibody at a dilution of 1:2000 (Santa Cruz, Biotechnology, USA). At least 6 samples per group were measured in duplicate.

5.3 Results

5.3.1 Cell proliferation study

The BrdU cell proliferation assay was used to determine the proliferation of HTCFs. The HTCFs seeded on the titanium disks showed a greater proliferative ability; the cell proliferation rate of the titanium group was significantly higher than that of the glass group ($p=0.049$). The proliferation rate of the DCPD + SA group was significantly lower compared with the glass group ($p=0.047$); that of the DCPD group was almost the same as the glass group; and the HA group had a slightly higher proliferation rate than that of the DCPD group. In addition, all types of coated magnesium alloys showed a lower proliferation rate compared with the titanium group.

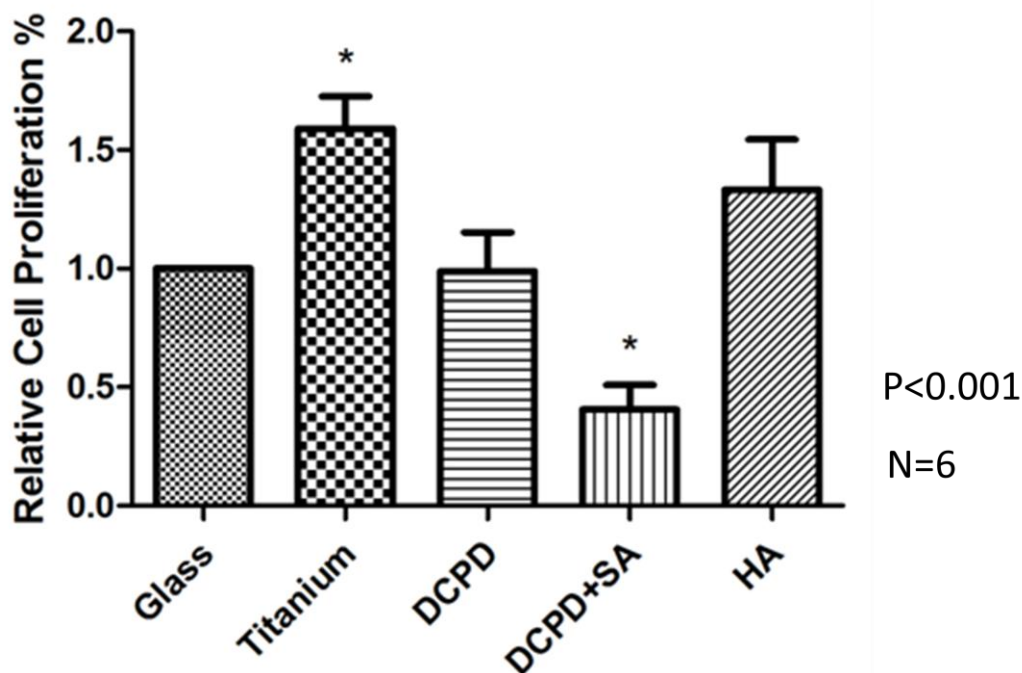


Figure 5.3.1 Relative proliferation rate of HTCFCs.

The proliferation of HTCFCs was monitored by 5-bromo-2-deoxyuridine (BrdU)-incorporation into cellular DNA using the Cell Proliferation BrdU assay kit as recommended by the manufacturer. Data was analyzed using one-way ANOVA test (SPSS 24). * $p < 0.001$ compared with glass; $N = 6$.

5.3.2 Western blotting analysis

The protein of α -SMA is a marker of myofibroblasts which cause fibroblast proliferation and wound scarring¹⁵⁴. Normalized to GAPDH expression, the glass group had the highest α -SMA expression. The α -SMA expression level of the titanium group was slightly lower than that of the glass group. All types of coated magnesium alloys had lower α -SMA expression levels than that of the titanium group or the glass group. The DCPD group and the DCPD + SA group had similar α -SMA expressions. The HA group had the lowest α -SMA expression level, which was significantly lower than that of the glass group ($p=0.037$) and the titanium group (0.001).

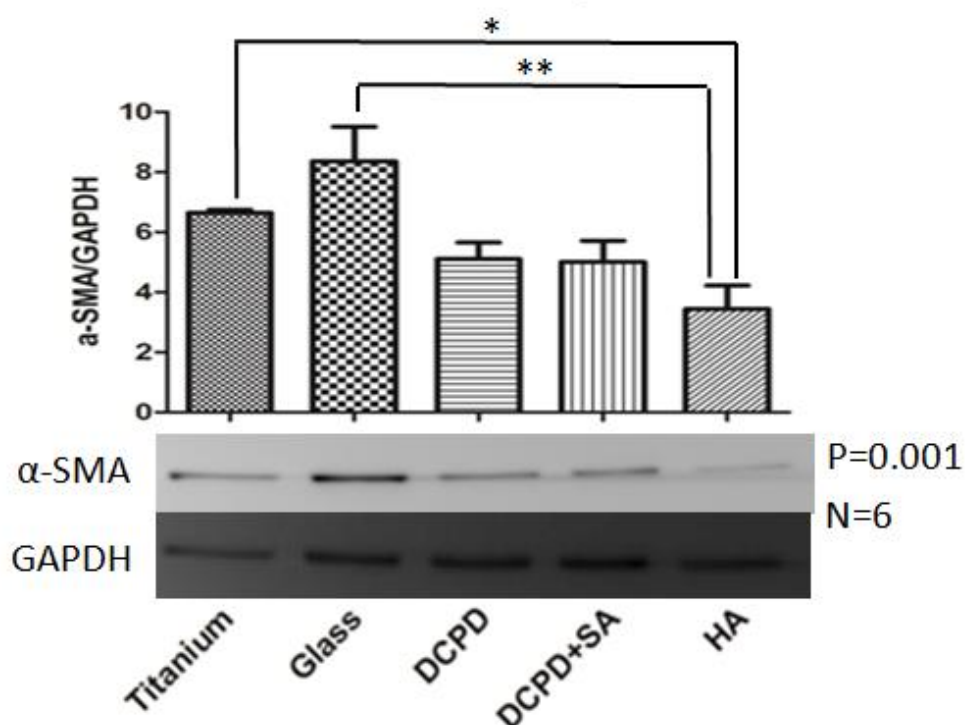


Figure 5.3.2 α -SMA protein expression normalized to GAPDH expression.

Western blotting was performed on HTCFs samples derived from glaucoma patients. The total cell proteins were extracted using RIPA lysis buffer containing phosphatase inhibitors and protease inhibitors. Protein concentrations were determined by micro BCA protein assay (Thermo Scientific, CA). The test performed following the manufacturer's instruction. Data was analyzed using one-way ANOVA test (SPSS 24). ** $p=0.001$; N=6.

5.4 Discussion

5.4.1 Coated magnesium alloys attenuate HTCFs proliferation

According to the BrdU cell proliferation assay, coated magnesium alloys were associated with lower proliferation rates of HTCFs as compared with titanium. In the BrdU assay, the same number of cells was loaded to each well at the beginning, but due to the lower cytotoxicity and better metabolic activity of cells seeded on the titanium disks, more cells grew and proliferated on the titanium disks after 48 hours. The DCPD + SA coating had higher cytotoxicity and lower metabolic activity than other coatings, and thereby a lower proliferation rate was observed in this group. Even

if in the LDH assay the DCPD + SA group showed almost the same necrosis of HTCFs with that of the titanium and glass groups, we still speculated that this type of coating might cause apoptosis of HTCFs. Unfortunately, we have tried many assays to assess apoptosis in our experiments, such as Elisa and caspase-3, but due to the reagent reaction with coated magnesium alloy disks, the effect of each sample on the apoptosis of HTCFs remained unclear, and further studies are needed to address this issue.

5.4.2 HA coating inhibits α -SMA expression

HA is chemically similar to bone, and has excellent biocompatibility^{135,136}. Actually, HA as a biological material has been used to fabricate orbital implants for a long time. And HA orbital implants have been widely used in clinical practice with excellent performance, and no obvious adverse effects have been reported¹⁵⁵. As the data of α -SMA expression had been normalized to that of GAPDH, we believed that the results of western blotting were more reliable and convincing. The expression of α -SMA proteins is a marker of myofibroblasts, which reflects the number and proliferation of myofibroblasts¹⁵⁶. It also is an important marker to estimate scar formation or surgical prognosis. In our experiment, the α -SMA expression levels decreased in all the coated magnesium alloy groups. Furthermore, in comparison with glass and titanium, the HA coating significantly reduced the expression of α -SMA. These findings indicate that HA coated magnesium alloy which has better corrosion resistance, lower cytotoxicity and excellent biocompatibility could inhibit α -SMA

protein expression and prevent scarring. Therefore, HA coating may be considered a very promising biodegradable material for the next generation glaucoma drainage devices.

Chapter 6

Conclusion

6.1 Summary of results

As described in Chapter Two, human tenon's capsule tissue samples were obtained from 32 glaucoma patients. Primary cell culture and subculture were performed to acquire adequate number of HTCFs for the following experiments. Immunofluorescence staining performed for vimentin and keratin verified the purity and identity of the HTCFcultures. Coated magnesium alloy and titanium samples were fabricated and cut into disks with 14.5 mm in diameter and 1 mm in thickness, specific for 24 well culture plate. A total of 453 coated magnesium alloy disks were used in our experiments. The phenomenon of corrosion was observed in 67 disks, accounting for 14.8%. The coatings of DCPD and DCPD + SA had the same corrosion rate (both 15.9%), while the HA coating had the lowest corrosion rate (12.6%). These differences were not statistically significant as determined by the chi-square test. Most of the corrosion events of the HA coated samples were found during the last three days (8/10), whereas the DCPD coated samples were more prone to corrosion during the first four days (6/9). Based on two-way ANOVA analysis, however, the differences were not statistically significant.

In Chapter Three, long-term changes in sample weight, pH levels, and ion concentrations, were determined by immersion of coated magnesium alloys in BSS solution which had similar components of human aqueous humor. The SEM

morphologies revealed that after immersion for 9 days, the DCPD and DCPD + SA coatings were dissolved and flaked, with cracked surface. The HA coating was exfoliated from the matrix after 5 days, but without any obvious changes on the surface of non-exfoliated areas. This finding indicated that the main problem of the HA coating is an increased risk of detachment from the matrix. This coating showed the greatest promise to effectively reduce the corrosion rate of magnesium alloys if further development can be done to improve the attachment to the matrix. The pH levels rapidly increased in all groups during the first two days. The pure magnesium group had higher pH levels than other groups. The dissolution of magnesium consumes the H^+ in the solution, resulting in a continuous rise in the pH of the solution. Thus, this result revealed that coatings could prevent pH increase because of inhibited dissolution and corrosion of the matrix. The DCPD and DCPD + SA groups lost much more weight than the HA and pure magnesium groups, indicating that the coating dissolution was the major reason leading to sample weight loss during the immersion test. Additionally, the magnesium concentration in the pure magnesium group was obviously higher than that of the other three coated groups, which demonstrated that coatings could reduce the release of magnesium ions from the pure magnesium matrix. Compared with the DCPD and DCPD + SA coatings, the HA coating released fewer calcium ions and no phosphorus ions were detected, indicating that the HA coating was associated with a significantly lower risk of dissolution and corrosion.

In Chapter Four, we examined the biocompatibility of differently coated magnesium alloys. We used MTT assay to evaluate the metabolic activity of HTCFCs and LDH assay to determine the cytotoxicity of coated magnesium alloys; the glass and titanium disks were used as controls. Meanwhile, we utilized these *in vitro* experiments to investigate the variation of metabolic activity and necrosis of HTCFCs during the logarithmic phase. The results of MTT assay showed that the P value of treatment was statistically significant. The metabolic activity of HTCFCs in the DCPD + SA group was the lowest, while the HTCFCs seeded on the titanium disks displayed the highest viability. The results of LDH assay also showed that the P value of treatment was statistically significant. The HA group had the lowest cytotoxicity, while the DCPD + SA group had higher cytotoxicity than other coatings, but comparable to the titanium and glass groups in terms of necrosis. Notably, both of the two assays indicated that the P value of time was not statistically significant, indicating that time had no obvious influence on the metabolic activity and necrosis of HTCFCs from day 2 to day 7.

In Chapter Five, we attempted to utilize some of the corrosive properties of coated magnesium alloys to evaluate their potential anti-scarring ability. The BrdU assay was used to assess cellular proliferation, and the cells seeded on the titanium disks showed a greater proliferative ability. Compared with the glass group, the proliferation rate of the DCPD + SA group was significantly lower, and all types of coated magnesium alloys showed lower proliferation rates. These results demonstrated that coated

magnesium alloys have a better potential to inhibit cellular proliferation than titanium. In western blotting analysis, we chose α -SMA proteins to examine the growth of myofibroblasts on different disks, because the expression of α -SMA is a marker of myofibroblasts. Normalized to GAPDH expression, the α -SMA expression was inhibited by coated magnesium alloys. The result of ANOVA showed that the p value was statistically significant difference. Compared with the glass and titanium groups, the expression level of α -SMA was significantly reduced in cells seeded on the HA disks.

6.2 Limitations of the study

Our investigation consisted of an *in vitro* cell culture model designed to investigate the biocompatibility and anti-proliferative potential of differently coated magnesium alloys as novel drainage device materials for glaucoma surgeries. As mentioned in Chapters 2 and 3, the coated magnesium alloys tested still likely have unacceptable corrosion rate as implant materials. In our experiments, the average corrosion percentage was 14.8%, and even the most stable type - HA coating - still had a corrosion proportion of 12.6%. Although the incidence of corrosion is much lower than that of pure magnesium, it would have to be improved before this material could be considered for *in vivo* surgical implantation. Actually, the coating decreased corrosion rate of magnesium, but cannot completely prevent the occurrence of corrosion, because the coating will gradually lose their integrity and degraded. In fact, we do not expect that the coating could completely prevent the magnesium corrosion,

because we expect this is a kind of biodegradable material, if the coating completely prevent corrosion, the material becomes unbiodegradable. Based upon these experiments, the HA coating showed the greatest promise, especially if the material could be modified to be more closely adhere to the magnesium alloy. In the immersion test, we selected BSS as simulated aqueous humor to investigate the longer-term properties of coated magnesium alloys. It would have been informative to also assess hydrogen emission, which would likely have an influence on the tissue properties at the surgical site. Further studies are needed to examine the volume of hydrogen emission based on the size of glaucoma drainage device.

This is an *in vitro* study, and, as such, it is not representative of an *in vivo* biological environment. True biocompatibility profiles in animal studies would be required as a next step towards the development of these materials as a surgical device in the human body. Although this is a historical challenge, it is also well accepted that these types of experiments are necessary to develop the foundation for future *in vivo* experimentation, the latter that require significant resources. In Chapter 4, we found that magnesium alloys could reduce the metabolic activity of HTCFs and are less likely to cause necrosis of these cells. Although attempted by ELISA, it was not possible to assess apoptosis by this technique due to the effects of the materials on the assay. Future experiments that employ alternative methods for determining apoptosis should be considered. The proliferation rate of the DCPD + SA group was significantly lower than other groups, but its cytotoxicity was almost the same as

that of glass and titanium in the LDH assay. Therefore, we infer that the DCPD + SA coating may accelerate cell apoptosis. More studies are required to further investigate the apoptosis of HTCFs seeded on coated magnesium alloy disks.

In Chapter 5, we performed the BrdU assay and detected the expression of α -SMA proteins to examine the anti-proliferative potential of differently coated magnesium alloys. In future studies, it would be useful to assess the expression of collagen-1 to further explore the potential of coated magnesium alloys in inhibiting protein expressions. RT-PCR may also be performed to determine whether coated magnesium alloys could suppress mRNA synthesis.

6.3 Future directions

In this section, future directions are presented to address the identified limitations and expand upon the findings of the current study. Continued study of the corrosion resistance of magnesium alloys would be useful, especially if correlated to the effect on the apoptosis of HTCFs, and their anti-proliferative potentials. Prototypes of coated magnesium alloy-based glaucoma drainage device should be designed. Based upon these designs, the size of the devices would permit determination of the daily volume of hydrogen release, which experiments to determine the safe and acceptable ranges..

Upon completion of the *in vitro* phase of these experiments, future animal studies

would be designed. Implantation of the coated magnesium alloy-based glaucoma drainage device into the eyes of New Zealand rabbits would allow determination of the effects on the tissue and organ. Parameters such as scar formation and encapsulation, as well as the postoperative adverse events related to the corrosion would be useful to obtain.

Anti-scarring will continue to be a focus of our future studies, and the release of different ions from coatings will be assessed in a comprehensive fashion. For example, matrix metalloproteinases proteins are associated with scar formation and regulated by calcium ions ¹⁵⁷. Based on the findings of the immersion test, it was found that calcium ions were released from the coatings when they came in contact with body fluids. Therefore, we speculate that the increased extracellular calcium concentration may influence the expression of matrix metalloproteinases and scar formation, which needs to be validated in further studies.

6.4 Concluding remarks

Magnesium alloy is a novel biodegradable material which possesses many advantageous properties which may render it useful as a surgical adjunctive device. These properties include: non-toxicity, high strength, light weight, and high biological compatibility. Complications related to its corrosive properties caused it to lose interest as a potential implantable device in the middle of the 20th century.

More recent advancements in the area of coating technology have allowed the synthesis of novel coated magnesium alloys, which have shown significant promise as effective biodegradable materials. In this thesis the corrosive response of a selected group of coated magnesium alloys was reported. Although the corrosion rates were still relatively high, the ability of the coatings to differentially modulate these rates, and reduce them in comparison to pure magnesium, was demonstrated.

Scarring caused by fibroblast proliferation is a major risk factor leading to glaucoma surgery failure. In the process of degradation of coated magnesium alloys, different ions are released, changing the pH of extracellular environment and thereby affecting the proliferation of cells. In this study, cellular proliferation when treated by differently coated magnesium alloys was examined, and the potential anti-scarring properties were also investigated. The results showed that, when compared with titanium, coated magnesium alloys could effectively inhibit the proliferation of HTCFs and reduce the expression of myofibroblast specific antibodies.

6.5 Conclusions of full text

1. Coatings are able to effect the corrosive properties of magnesium.
2. HA was most resistant to corrosion.
3. No significant difference was found in metabolic activity or necrosis at different times during the logarithmic phase of HTCFs.
4. DCPD+SA demonstrated a stronger ability to reduce metabolic activity while its

cytotoxic profile was the same as titanium and glass.

5. In comparison to titanium, coated magnesium alloys attenuated HTCFs proliferation.

6. Coated magnesium alloys reduced the expression of α -SMA.

7. The expression of α -SMA was significantly decreased in cells exposed to the HA coated magnesium.

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Oct 2007-Apr 2008, Major in glaucoma

May 2008-Oct 2008, Major in lens and cataract

Nov 2008-Feb 2009, Major in strabismus and pediatric

Mar 2009-Sept 2009, Major in retina and vitreous

Oct 2009-Apr 2010, Major in orbit, eyelids and lacrimal system

May 2010-sept 2010, Major in external and corneal

Sept 2010-may 2012, Major in glaucoma

Specialist:

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Publications:

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2. Xiang-ji Li , Qiang Sun. Common problems and related countermeasures on medical students during ophthalmological internship. Medical information. 2010.23 (5): 1183-1185.
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