Chemical Prevention of Corticosteroid-induced Ocular Hypertension

in vitro and *in vivo*

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Abstract

Topical and systemic use of corticosteroids causes ocular hypertension in general population, leading to glaucomatous damage, in particular the optic nerve and this could result in vision loss. Intraocular pressure (IOP) rise could be due to the resisted aqueous humor outflow in trabecular meshwork (TM) region. Medical intervention includes standard medication and/or argon laser trabeculoplasty. Filtration surgery may also require for lowering the high IOP.

My study explored a novel therapeutic intervention to steroid-induced ocular hypertension using small molecule chemicals. These chemicals are demonstrated to promote cell survival and improve tissue function in cell and animal models of various diseases. In our laboratory, sodium 4-phenylbutyrate (NaPB) and trimethylamine N-oxide (TMAO) have been shown to maintain functional TM cells in myocilin-caused glaucoma and functional lens epithelial cells in crystallin-caused congenital cataract. The objectives of my work were to identify small molecule chemicals that correct steroid-induced IOP rise and investigate the effect of small molecule chemical chaperone on preventing and/or reducing ocular hypertension

caused by corticosteroids. I hypothesized that the chemical treatment can give functional aqueous humor drainage, hence maintaining a constant low IOP level and preventing the onset of steroid-induced glaucoma. This work can throw light on a new design of potential medication that can be used in parallel with steroids, without causing ocular hypertension.

Firstly, the effect of NaPB and other small molecule chemicals on steroid-induced IOP changes was investigated using a rabbit steroid-induced ocular hypertension model. Male New Zealand albino rabbits (7 weeks old) were recruited for topical Maxi-Dex® (0.1% dexamethasone, Dex) followed by small molecule chemical treatment daily. IOP was monitored regularly by an independent researcher. In 150 rabbits used in this study, 75% of them showed IOP rise by >20% at 14 days of steroid treatment. Histochemistry (hematoxylin and eosin, periodic acid-Schiff and masson trichrome staining) and transmission electron microscopy of TM region revealed a reduced cell density and extracellular matrix (ECM) deposition after Dex treatment, which could attribute to IOP rise.

Topical NaPB demonstrated a dose-dependent reduction of Dex-induced IOP rise. The optimal dose frequency was 0.5 mM NaPB (equivalent to 0.01%, weight/volume), 4 times daily ($p=0.025$, paired Student's *t*-test, compared to vehicle control). Similar prevention of IOP rise by NaPB was replicated in eyes with topical Pred Forte® (0.5% prednisolone acetate). Histochemistry and ultrastructural analyses showed that

topical NaPB reduced extracellular matrix (ECM) deposition in the TM region and maintained an open drainage channel. No systemic and ocular side effects and toxicity was observed by cornea surface examination using slit lamp biomicroscopy and corneal endothelial cell count using specular microscopy. Treatment with TMAO did not alter the steroid-induced IOP rise.

Next, I investigated the possible mechanistic action of NaPB on Dex-treated human TM cell line. Using expression microarray and gene ontology term analyses, NaPB might regulate ECM metabolism, inflammatory and defense responses. It reversed the Dex-caused expression changes of insulin growth factor binding protein 4, carbonic anhydrase 12 and interleukin 1B, whereas no effect was found for other selected genes. By MTT cell viability/proliferation assay, terminal deoxynucleotidyl transferase dUTP nick end labeling assay and caspase-3 expression by western blotting, NaPB reduced Dex-caused TM cell apoptosis.

Conclusively, topical NaPB prevented steroid-caused IOP rise. It could preserve healthy TM cells and reverse steroid-caused gene expression changes, leading to reduced resistance to aqueous humor outflow. Its application did not have any side effect or was not toxic to ocular tissues. Hence, NaPB could be a novel therapeutic agent for steroid-induced ocular hypertension.

摘要

人群中，全身及局部使用激素可造成高眼壓，從而導致青光眼性損害，特別是視神經，這可引起視力喪失。眼壓的升高是由於小梁網區域房水外流阻礙。醫療干預包括常規藥物及氫鐳射，病人甚至需濾過手術降低眼壓。

我的研究探索了使用小分子化合物治療激素性高眼壓的新方法。在多種疾病的細胞和動物模型中，這些物質被證實可提高細胞生存及改善組織功能。在我們實驗室的研究顯示，苯基丁酸鈉 (NaPB) 及氧化三甲胺 (TMAO) 分別在 myocilin 引起的青光眼中維持人小梁網細胞功能以及在 crystallin 引起的先天性白內障中維持有功能的晶體上皮細胞。我的工作目標是尋找可糾正激素引起的眼壓升高的小分子化合物以及探究此類物質對激素性高眼壓的預防或降眼壓效應。我假設小分子化合物治療可維持房水排出功能，從而穩定眼壓，預防激素性青光眼。此項研究設計一種可與激素同時使用，並且不引起高眼壓的有潛力的藥物。

首先，我們建立動物模型，探索苯基丁酸鈉及其它小分子物質對激素導致的眼壓變化的作用。雄性新西蘭白兔（7 周大）每天局部滴百分之 0.1 地塞米松及小分子物質眼藥水。由研究員獨立定期監測眼壓。研究使用的 150 只兔子中，百分之 75 的兔子在使用激素 14 天時出現至少基礎值百分之 20 的眼壓升高。組織化學(蘇木素伊紅、希氏高碘酸與麥森三色染色)及透射電鏡顯示小梁網區域細胞密度減少及細胞外基質沉積，這些均促使眼壓升高。

局部苯基丁酸鈉對激素誘導眼壓升高的抑制作用與劑量有關。最佳劑量為0.5 毫摩爾每升(百分之 0.01) 每天 4 次 (p=0.025, 配對 t 檢驗, 與溶質對照相比)。類似的預防作用可在局部使用百分之 0.5 潑尼松龍醋酸鹽的實驗中重複。組織化學及超微結構分析顯示局部苯丁酸鈉可減輕小梁網區域細胞外基質沉積並維持房水流出道打開。裂隙燈眼表檢查與角膜內皮細胞計數確認其無全身及局部毒副作用。氧化三甲胺不影響眼壓水準。

隨後，我探究了苯基丁酸鈉對加入地塞米松的人小梁網細胞可能的作用機制。微陣列表達及基因本質分析顯示苯基丁酸鈉可能調節細胞外基質代謝、感染及防衛應答。它同時逆轉地塞米松導致的胰島素樣生長因數連接蛋白 4、碳水化酶 12 和白細胞介素 1B 的表達變化，然而其他被選基因無明顯變化。噻唑藍細胞增殖能力檢測、原位末端標記法及蛋白質印跡法半胱天冬酶 3 表達檢測顯示，苯基丁酸鈉減少地塞米松導致的人小梁網細胞凋亡。

綜上所述，局部苯基丁酸鈉預防激素誘導的眼壓升高。它能保護健康細胞及逆轉激素導致的基因表達改變，從而減少房水外流阻力。苯基丁酸鈉對眼部組織無毒副作用。因此，苯基丁酸鈉可作為治療激素性高眼壓的新物質。

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Abbreviations

ATF6	Activated transcription factor 6
BiP	Binding immunoglobulin protein
Bp	Base pair
BSA	Bovine serum albumin
β-ME	β-mercaptoethanol
CA12	Carbonic anhydrase 12
CHOP	C/EBP-homologous protein
CRYAA	αA-crystallin
CRYGD	γD-crystallin
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acids
dNTPs	Deoxyribonucleotides
DTT	DL-dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde phosphate dehydrogenase
HSP70	Heat shock protein 70

HSP90a	Heat shock protein 90a
IGFBP4	Insulin-like growth factor binding protein 4
IL1B	Interleukin-1β
IOP	Intraocular pressure
IRE1	Inositol requirement 1
JNK	c-Jun amino terminal kinase
ml	Milliliter (10^{-3} L)
mM	Millimoler (10^{-3} M)
mm	Millimeter (10^{-3} meter)
mmHg	Millimeters of mercury
MW	Molecular weight
MYOC	Myocilin
NaPB	Sodium 4-phenylbutyrate
OHT	Ocular hypertension
PACG	Primary angle closure glaucoma
PBA	Phenylbutyric acid (active ingredient of sodium 4-phenylbutyrate)
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PERK	Double-stranded RNA-activated protein kinase-like ER kinase
POAG	Primary open-angle glaucoma
PMSF	Phenylmethyl sulfonylfluoride

RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
SDS	Sodium dodecylsulfate
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TMAO	Trimethylamine N-oxide
TUDCA	Tauroursodeoxycholic acid
Tx	Triton X-100
µl	Microliter (10^{-6} L)
µM	Micromoler (10^{-6} M)
UPR	Unfolded protein responses

Publications

1. Research papers

1.1 Liu DTL, **Xu L**, Pang CP, Lam DSC, Yam GHF. Disruption of Bevacizumab (Avastin) Activity by Vitreous Matrix Gel. *J Clinic Experiment Ophthalmol* 2011 Mar, 2:140 doi: 10.4172/2155-9570.1000140.

1.2 **Xu L,** Yam GHF, Leung KS, Lau JYN, Lam DSC. Sodium 4-phenylbutyrate as a novel anti-glaucoma drug. *In preparation*

2. Conference papers

2.1 **Xu L,** Ng TK, Pang CP, Yam GHF. Unfolded protein response in mouse stage-specific retinal progenitor cells. The association for research in Vision and Ophthalmology, *Fort Lauderdale, Florida, USA*, May 2-6, 2010.

2.2 **Xu L**, Liu HM, Lam DS, Pang CP, Liu DT, Yam GHF. Disruption of Bevacizumab (Avastin) activities by vitreous *in vitro*. Asia Pacific Academy of Ophthalmology Congress, *Beijing, China*, Sep. 16-20, 2010.

3. Patent Application

US Application Serial Number: 10/MED/367-1 (Provisional)

Authors: Dennis SC LAM, Johnson YN LAU, Gary HF YAM, CP PANG, Christopher KS LEUNG, HY CHEN, Srinivas K RAO, Dorothy SP FAN

Title: "Method and Medication for Prevention and Treatment of Ocular Hypertension and Glaucoma"

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Chapter 1: Introduction

1.1 Glaucoma

1.1.1 Characterization, signs and symptoms

Glaucoma is a chronic progressive eye disorder which leads to permanent damage of the optic nerve including increase in cup-disc ratio, visual field loss leading to defect in visual acuity, and possible progression to complete blindness if untreated. It is the second leading cause of blindness in the world, and more often affects elderly people (Serge et al., 2002).

Glaucoma is often, but not always associated with an elevation of intraocular pressure (IOP), and the optic nerve damage involving the loss of retinal ganglion cells (RGCs) (Osborne et al., 1999). There are many different sub-types of glaucoma, but it can be divided into two major categories: angle closure glaucoma and open-angle glaucoma.

Angle closure glaucoma is more common in the Orientals. Nearly half of the glaucoma patients in China have angle closure glaucoma, in contrast with ~10% in the United States or European countries (Wang et al., 2002). About 1 in 10 angle closure glaucoma patients presents with acute angle closure, which is an ocular emergency characterized by red eye, sudden ocular pain, very high intraocular pressure (usually above 30 mmHg), seeing halos around lights, sudden decrease in vision, nausea and vomiting, and a fixed, mid-dilated pupil (Vadot et al., 1989).

In contrast, open-angle glaucoma is more prevalent in the United States and European countries. It accounts for almost 90% of the glaucoma patients in that region (Friedman et al., 2004). It does not have acute attacks and is often painless. The only signs are progressive visual field loss and optic nerve damage (increased cup-to-disc ratio on fundus examination), which can progress to blindness.

1.1.2 Pathophysiology

It is known that for glaucoma, rise of intraocular pressure is the most important risk factor. In our eyes, aqueous humor, which is produced by ciliary body, flows into the posterior chamber, bounded posteriorly by the lens and anteriorly by the iris (Mozaffarieh et al., 2008). It then flows through the pupil into the anterior chamber, bounded posteriorly by the iris and anteriorly by the cornea. Trabecular meshwork is located around the base of cornea, near the ciliary body. It is spongy and is lined by trabeculocytes. The main function of trabecular meshwork is to drain the aqueous humor via Schlemm's canal from the anterior chamber into scleral venous plexuses and general blood circulation (Soules et al., 2005; Chen et al., 2008; Mozaffarieh et al., 2008). Hence, intraocular pressure is a function of aqueous humor production and its drainage through the trabecular meshwork, and any of the disorders of production or drainage of aqueous humor may cause abnormal IOP. In normal condition, the secretion and the outflow of aqueous humor is a dynamic balance, however, increased production of aqueous humor, and/or its blocked outflow cause a rise in IOP

(Mozaffarieh et al., 2008). The persistent high pressure will be transmitted to the posterior part of the eye damaging the optic nerve and resulting in poor vision.

In angle closure glaucoma, the angle of anterior chamber becomes narrow, and this results in the inability of the aqueous humor to flow from the posterior to the anterior chamber of the eye. Thus, the accumulation of aqueous humor in the anterior chamber causes IOP rise. And there is reduced or blocked flow through the trabecular meshwork in open angle glaucoma. Compared with angle closure glaucoma, there is no anatomical restriction to outflow (wide angle) in open angle glaucoma, and the blocked/deterred outflow is from other sources, like TM blockage, TM cell functions alteration, gene expression regulation, extracellular matrix metabolism and cytoskeleton structure changes of TM cells (**Figure 1**).

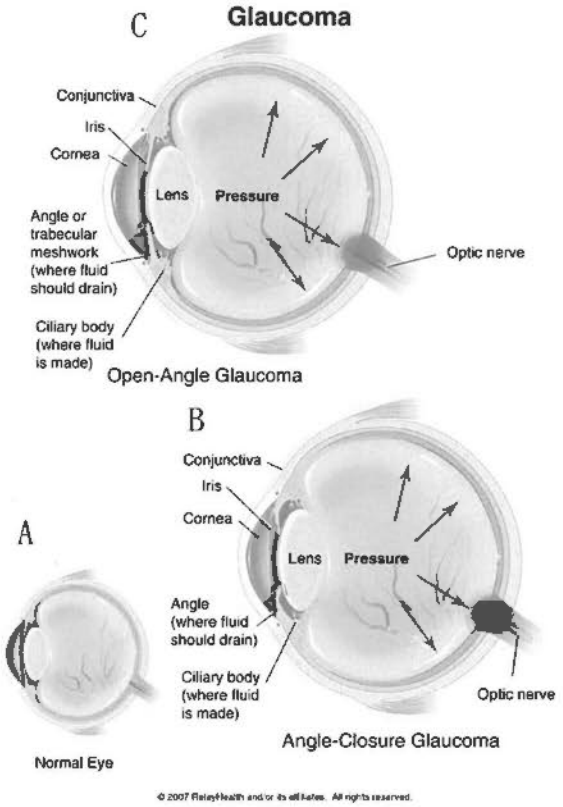

Figure 1. Angle closure glaucoma and open angle glaucoma. (A) Anatomy of normal eye. **(B)** In angle closure glaucoma, the angle of anterior chamber becomes narrow resulting in the inability of aqueous humor to flow from the posterior to the anterior chamber. **(C)** There is no anatomical restriction to outflow (wide angle) in open angle glaucoma.

Picture were modified from www.summitmedicalgroup.com

Nonetheless, the relationship between glaucomatous optic nerve changes with high intraocular pressure is inconsistent (Araie, 2011). Some people may develop optic nerve damage without any elevation of IOP, while some may have high IOP for a long time, even for years and never develop optic nerve damage. It may due to the anatomic structure and development of the eye, including the narrow peripheral anterior chamber, short axis oculi, the abnormal location of lens, and the increased proximity between iris and lens. These anatomic structures in our eyes block the flow of aqueous humor to the anterior chamber, thus pushing the iris and closing the angle (Törnquist, 1958).

As we know, the glaucomatous damage is mainly on the optic nerve and it ultimately causes a loss of retinal ganglion cells (Osborne et al., 1999). The lack of optic nerve blood flow, the excitatory neurotransmitters, retinal ganglion cell/axon degeneration, and aging mechanisms of neuron loss could accelerate the death of RGCs, resulting in damage of the optic nerve (Berry et al., 2006; Morrison, 2006; Knox et al., 2007; Hernández et al., 2008; Levin et al., 2008; Mozaffarieh et al., 2008; Varma et al., 2008).

Recently, immune response was discovered to affect the pathogenesis and optic nerve damage of glaucoma. This immune defence involves lymphocytes, resident and infiltrating innate immune cells, microglia, and macrophages (Schwartz, 2007).

1.1.3 Causes and risk factors

There are several causes and risk factors for glaucoma. Among them, ocular hypertension (above 21 mmHg in human) is the major risk factor for developing glaucoma (Sommer et al., 1991). It can occur in most of the glaucoma patients, but in some populations such as white Americans, only half of the patients with primary open angle glaucoma develop high IOP (Sommer et al., 1991).

Race, sex and age predilection may occur in glaucoma development. Due to shallow anterior chamber depth, East Asian populations are more likely to develop angle closure glaucoma, and women have higher risk than men to develop acute angle-closure glaucoma (Wang et al., 2002). Compared with Caucasians, Inuit also have much higher risk of developing primary angle closure glaucoma (PACG) (Salmon et al., 1993). For Africans, they are more likely to develop primary open angle glaucoma (POAG) (Salmon et al., 1993). People with high myopia and elderly people have thinner corneal thickness and hypermetropia also at higher risk for primary open angle glaucoma (Herndon et al., 2004). Also, primary open angle glaucoma has been found to be associated with mutations in genes at several loci, especially for normal tension glaucoma (NTG) (Stone et al., 1997). For example, myocilin (MYOC) which was named by Kubota et al. based on homology to non-muscle myosin and the identification of the gene product in the connecting cilium of photoreceptor cells in 1997 was a well-known glaucoma-causing gene (Kubota et al., 1997). MYOC was expressed in eye tissues, especially in trabecular meshwork

(Adam et al., 1997; Tamm et al., 1999), and its mutations were found in 2-4% of the patients in primary open-angle glaucoma (Stone et al., 1997).

Congenital eye malformations can also cause the close of angle, resulting in high IOP and glaucomatous optic neuropathy. For example, failure of the normal atrophy of the hyaloid canal and the tunica of lens, which usually combine with other anomalies (Hunt et al., 2005).

Some systemic diseases may also be the risk factors of glaucoma development, and hypertension may be among one of them. In hypertension patients, fluctuations of blood pressure are associated with the damage of optic nerve head and visual field deterioration (Bonomi et al., 2000). In normal tension glaucoma, nocturnal hypotension may play a significant role (Choi et al., 2006; Choi et al., 2007).

Other factors that can cause glaucoma, known as "secondary glaucoma," include long-term use of steroids (topical and systemic), severely restrict blood flow or vitreous haemorrhage, such as proliferative diabetic retinopathy and severe central retinal vein occlusion, ocular trauma, and inflammation such as uveitis (Merayo-Lloves et al., 1999; Foster et al., 2000).

1.1.4 Diagnosis and classification

Screening for glaucoma is usually performed as a part of standard eye examination, including intraocular pressure measurement using tonometry, slit lamp biomicroscopy and gonioscopic to examination of the anterior chamber angle of the eye, and optic nerve to look for any visible damage, such as the pallor of discus opticus, increase in the cup-to-disc ratio, and retinal nerve fiber layer defect. Visual field test should also be performed for the diagnosis of glaucoma to see if there is progressive loss of visual field. The retinal nerve fiber layer can be examined with imaging techniques such as optical coherence tomography (OCT), scanning laser polarimetry (GDx), and/or scanning laser ophthalmoscopy also known as Heidelberg Retina Tomography (HRT3) (Thomas et al., 2006). As mentioned previously in the risk factors of glaucoma, primary open angle glaucoma has been found to be associated with mutations in genes at several loci, especially for normal tension glaucoma (NTG) (Stone et al., 1997). Women usually have narrower anterior chamber angle than men, they are more likely to get primary angle closure glaucoma (Vajaranant et al., 2010). Also, primary open angle glaucoma and primary angle closure glaucoma have the prevalence of races (Salmon et al., 1993; Wang et al., 2002). Keeping all these factors in mind, more attention should be given to sex, race, refraction, family history, and history of drug use.

Glaucoma can be classified into several different types, but mainly, it includes primary glaucoma and secondary glaucoma.

1.1.4.1 Primary glaucoma

1.1.4.1.1 Primary angle closure glaucoma

Primary angle closure glaucoma is caused by contact between the iris and trabecular meshwork, which narrows the angle of anterior chamber, leading to an anatomical obstruction of aqueous humor outflow. The function of trabecular meshwork can gradually be damaged by this contact until it fails or resists draining the aqueous humor from the anterior chamber, which then leads to a rise of IOP. In some of the cases, for example, inflammation, scarring, prolonged contact between iris and TM causes synechiae, which cause a permanent obstruction of aqueous humor outflow. In these cases, IOP may rapidly elevate, causing severe ocular pain and redness. The visual acuity may suddenly decrease, and halos may be seen around bright lights, sometimes in accompany with headache and vomiting. It is called the acute angle closure crises and is an ocular emergency (Vadot et al., 1989).

Diagnosis can be made according to the physical signs and symptoms: mid-dilated pupil, unresponsive to light, corneal edema, visual loss, ocular pain and red eye. However, prior to severe visual loss, most of the cases are asymptomatic and can only be identified by ophthalmic examinations, intraocular pressure measurement, retinal nerve fibre thickness and visual field tests. Once after the symptoms (ocular pain and redness) are controlled, iridotomy performed by laser or surgery is the preferred treatment to prevent the contact between the iris and trabecular meshwork, which can

widen the anterior chamber angle, leading to a better aqueous humor outflow and reduced intraocular pressure. Most of the patients with angle closure glaucoma may need Pilocarpine eye drop to reduce the pupil size or iris incisional surgery.

1.1.4.1.2 Primary open angle glaucoma

Primary open angle glaucoma is an optic neuropathy, usually associated with increased IOP, and it leads to progressive loss of retinal ganglion cells and optic nerve. It results in damaged visual field and tunnel vision. Although not all patients with primary open angle glaucoma develop high IOP, control of IOP has been shown to stop progression even in the cases do not have high IOP (Detry-Morel, 2008).

The elevation of IOP is caused by increased production of aqueous humor and/or the blockage of TM region where the aqueous humor drains out. Because the drainage pathway is blocked, high pressure builds up in the eye and causes optic nerve damage, leading to gradual vision loss. Primary open angle glaucoma usually affects peripheral vision first, but if it is not treated, the entire vision is lost.

Since the patients of open angle glaucoma may not develop high IOP, the most important diagnosis is made by the examination of the optic nerve, especially the cup-to-disc ratio (Charlesworth et al., 2010). The patients require IOP lowering therapy such as prostaglandin agonists (eg. Xalatan, opens uveoscleral passageways), β-blockers (eg. Timolol), and carbonic anhydrase inhibitors (which decrease

bicarbonate formation from ciliary processes), thus reducing the formation of aqueous humor (Nomura et al., 2000; Feldman, 2004; Mincione et al., 2008). When the IOP can not be controlled by drug treatment or laser therapy, many of the patients require filtration surgery.

1.1.4.1.3 Others

Apart from PACG and POAG, the two main types of primary glaucoma, there are also variants of primary glaucoma such as pigmentary glaucoma, congenital glaucoma, which are associated with hereditary of familial diseases. For these types of glaucoma, IOP control and surgery are the main treatments.

Congenital glaucoma is a type of glaucoma characterized by the improper development of trabecular meshwork, which is present at birth and often affects both eyes (Tawara et al., 1981). It occurs more often in boys than girls, and it is one of the leading causes of blindness in children (Franks et al., 1989). Because the development of trabecular meshwork is improper, there is functional defect of the channels that normally drain the aqueous humor from inside the eye. Thus, the continually produced aqueous humor cannot be drained and high IOP develops. In young children or infant, there are three major symptoms of congenital glaucoma, including epiphora, photophobia, and blepharospasm (Seidman et al., 1986). Usually, these children have high IOP, and hazy cornea, and abnormality of iris and angle. Deep anterior chamber hampers the flow of aqueous humor from the eye and ultimately the accumulation of

over-produced aqueous humor causing high IOP. Diagnosis is made by IOP measurement, gonioscopy examination, and ophthalmoscopy under general anesthesia. Surgeries, most commonly goniotomy and trabeculotomy are the main treatments for congenital glaucoma. Before surgery, medications can be used to lower the IOP, and clear the cornea.

1.1.4.2 Secondary glaucoma

1.1.4.2.1 Secondary angle closure glaucoma

Secondary angle closure glaucoma is usually due to coexisting conditions, including inflammation, blood vessel disorders, and intraocular surgery (Merayo-Lloves et al., 1999; Foster et al., 2000; Knox et al., 2007). In conditions with inflammation such as uveitis, scarring pulls the iris into the angle, resulting in angle closure and a rise of IOP.

For ocular neovascular diseases, for example, proliferative diabetic retinopathy (PDR) and central retinal vein occlusion (CRVO), or other ischemic disorders of the retina or ciliary body, abnormal vessel growth, neovascular membrane contraction and vitreous hemorrhage obstructing the angle of anterior chamber, cause IOP elevation. In some cases, this may appear quickly after cataract surgery or vitrectomy. It is called as neovascular glaucoma, which is uncommon, and very difficult to treat. Patients with neovascular glaucoma lose their vision rapidly.

Abnormal location of the lens can also induce glaucoma, especially the rupture of lens capsule, subluxation of lens, and mature cataract. In these situations, increased proximity between iris and lens blocks the flow of aqueous humor to the anterior chamber, thus pushing the iris, closing the angle, leading to high IOP.

Diagnosis is made by detecting the related ocular diseases or the history of ocular surgery. The main methods of treating secondary angle closure glaucoma are to treat the primary diseases related to the elevation of IOP and lower the IOP. Almost all the patients have secondary angle closure glaucoma require filtration surgery or drainage implants.

1.1.4.2.2 Secondary open angle glaucoma

Pigmentary glaucoma is a type of inherited open-angle glaucoma, which develops more frequently in males than in females, and mostly developed in the twenties and thirties (Loyo-Berríos et al., 2007). Myopic patients are more affected because of the anatomy of the eyes. Myopic eyes have a concave-shaped iris, which creates an unusually wide angle. This causes the pigment layer of the eye to rub on the lens. This causes the iris pigment to shed into the aqueous humor and onto trabecular meshwork (Loyo-Berríos et al., 2007). Because trabecular meshwork is a spongy region, it is likely to be blocked by pigment and results in the rise of IOP. Diagnosis is made by IOP measurement and also family history. Miotic therapy such as pilocarpine eye drops is the first choice for the treatment of this disorder, and laser iridotomy is

presently being investigated (Veselovský et al., 1998).

The injury of the eye could cause a lot of complications such as corneal epithelial defect, cataract and glaucoma. Post-traumatic glaucoma is one of the complications (Strohl et al., 1999). After injury or trauma, hemorrhage of the eye tissues, for example, vitreous hemorrhage, obstructing the trabecular meshwork, angle of anterior chamber, cause the resistance of aqueous humor IOP elevation. It could also appear after intraocular surgeries such as cataract surgery or vitrectomy.

Among secondary open angle glaucoma, steroid-induced ocular hypertension/glaucoma is the most commonly seen. Topical and systemic use of corticosteroids may cause an elevation of intraocular pressure leading to secondary open angle glaucoma. Mainly, the patients using topical, oral or high dose of inhaled steroids are at higher risk of steroid-induced ocular hypertension, and about 30% of patients on topical steroid therapy can be affected (Sapir-Pichhadze et al., 2003). In spite of IOP elevation, there are patients who need long term using of corticosteroids, for example, patients with severe uveitis. Hence, these patients have much more risk to get steroid induced glaucoma.

Whether a person will have elevation in IOP depends on his/her genetics. 25% of the general population will develop steroid-induced elevations in IOP (above 6 mmHg) after 4 weeks of topical steroid 4 times daily. They are labeled as steroid-responders.

5% of the population is "super-responders" developing greater than 10 to 15 mmHg IOP rise with topical steroid use within 2 weeks (Tripathi et al., 1999). There is no racial and sexual predilection for steroid responsiveness in human and it can occur at any age. It has been reported that although chamber angle components are fully present at birth, the maturation of angle cellular and extracellular components occurs 1 to 8 years after birth (Remé et al., 1981).

Because of the structural and functional immaturity of trabecular meshwork, children have been reported to have more drastic increase of IOP after using steroids (Ohji et al., 1991; Ng et al., 2000; Fan et al., 2001; Fan et al., 2003). Chronic glaucoma patients usually have the obstruction of aqueous humor outflow or the over-production of aqueous humor. Steroid application aggravates the imbalance between the production and outflow of aqueous humor, thus resulting in high IOP.

Fluctuation of IOP is mostly due to the outflow resistance of aqueous humor from the anterior chamber through the trabecular meshwork, which is regulated by trabecular meshwork cells (Tian et al., 2000). Upon steroid application *in vivo* and *in vitro*, increased production and release of glycosaminoglycans and other proteins, along with cytoskeletal changes, like actin cross-linking, cause cellular stress and morphological changes in trabecular meshwork cells and ultimately lead to cell death (Wilson et al., 1993; Clark et al., 1994; Clark et al., 1996; Clark et al., 2005; Wang et al., 2008). As a consequence, the disrupted trabecular meshwork tissue cells have

ineffective phagocytic ability and reduced pinocytosis of aqueous humor, causing obstruction to humor flow and increase of IOP (Shirato et al., 1989; Matsumoto et al., 1997; Zhang et al., 2007). This persistent high pressure was transmitted to the posterior part of the eye and damaged the optic nerve, resulting in poor vision.

Diagnosis is made by the signs of IOP rise and history of using steroids. Subjects with steroid-induced ocular hypertension usually respond poorly to argon laser trabeculoplasty, because laser cannot change the structure of drainage pathway (Zhao et al., 2005). These patients may also be resistant to standard medical management such as IOP lowering eye drops (Rhee et al., 2001). Patients with sustained high IOP after mediation may require filtration surgery to alleviate the block of aqueous humor outflow, thus lowering the IOP.

1.1.5 Management

The primary goals of glaucoma management are to avoid glaucomatous optic nerve damage and to preserve the visual field. This would delay the disease progression, hence improve the quality of life for patients and minimize the effect of severe glaucoma occurrence. Appropriate diagnosis, judicious treatment selection which is suitable for individual patient is important for glaucoma. Since the increase of intraocular pressure is one of the major risk factors in glaucoma, lowering the IOP level by different methods (eg. medications, surgery, etc.) is still the mainstay of treatment. In recent years, neuroprotective therapy aims at slowing down the

progression of glaucoma (Hernández et al., 2008).

In addition, herbal medicines such as *Ginkgo biloba* have been tested as neuroprotective agents on the rabbit model of steroid-induced ocular hypertension (Jia et al., 2008; Qin et al., 2010), and erythropoietin on trial (Rhee et al., 2001; Tsai et al., 2007). The major methods of treating glaucoma are medications and surgery.

1. Medication

The stabilization of intraocular pressure is very important for every glaucoma patient. Intraocular pressure can usually be controlled through the application of the eye drops.

There are several kinds of medications for glaucoma treatment. They are meant to treat different types and stages of glaucoma. Broadly speaking, two major ways of controlling IOP are either via increasing the outflow of aqueous humor or decreasing aqueous humor production. Many medications focus on either one of them and some can achieve both.

To reduce the secretion of aqueous humor, topical β-adrenergic receptor antagonists like timolol, levobunolol (Betagan®, USP), and betaxolol are commonly used. Through the vasoconstriction of ciliary body blood vessels, less-selective sympathomimetics such as epinephrine have the effect of decreasing aqueous humor

production (Feldman, 2004). Carbonic anhydrase inhibitors like dorzolamide (Trusopt®, Merck & Co.), brinzolamide (Azopt®, Alcon), acetazolamide (Diamox®, Merck & Co.) have this effect by inhibiting carbonic anhydrase in the ciliary body, and they are used in lots of glaucoma patients (Mincione et al., 2008).

For the pharmaceuticals enhancing the outflow of aqueous humor, miotic agents (parasympathomimetics) like pilocarpine is one of the most commonly used eye drops. By contracting the ciliary muscle and tightening the trabecular meshwork, this kind of anti-glaucoma drug allows more outflow of the aqueous humor (Veselovský et al., 1998).

Prostaglandin analogs like latanoprost (Xalatan®, Pfizer), bimatoprost (Lumigan®, Allergan) and travoprost (Travatan®, Alcon) also increase aqueous humor outflow (Nomura et al., 2000).

Alpha2-adrenergic agonists such as brimonidine (Alphagan®, Allergan) and apraclonidine are also commonly used drugs, because they do not only lower the aqueous humor production, but also can enhance the trabecular outflow drainage capability (Brubaker, 2003).

Every drug including the anti-glaucoma medications may have their local and systemic side effects. For example, long term use of beta-adrenergic receptor antagonists may slow down the heart rate, and even result in atrioventricular block. Thus, it should not be used on asthma patients (Taira et al., 2008). Prostaglandin analogs may cause conjunctival hyperemia, elongation, and iris darkening, and they have abortive potential, which is the most dangerous (Cracknell et al., 2009). Alpha2-adrenergic agonists have become more popular, but they also have side effects like allergy in eyes, eye jumpiness, fast or atypical heartbeat and elevated blood pressure (Camras et al., 1999). So, patients with hypertension, diabetes, hyperthyroidism, or cardiopathy should use α2-adrenergic agonists with utmost care.

2. Surgery

For glaucoma treatment, there are two main types of surgery: laser surgery and conventional surgery, which includes iridectomy and filtration surgery (Sharaawy et al., 2011). The aim of conventional surgery is to create a new opening in the trabecular meshwork region to provide a channel for steady aqueous humor from the eye and thus prevent or reduces the rise of intraocular pressure.

Laser trabeculoplasty (LTP)

Laser beam is applied to burn out some areas of the trabecular meshwork tissue near the base of the iris (Francis et al., 2011). This can increase the efficiency of fluid outflow. LTP is used in the treatment of various open angle glaucoma. Argon laser

trabeculoplasty (ALT) and selective laser trabeculoplasty (SLT) are the two types which uses argon laser and Nd:YAG laser, respectively. As the power of Nd:YAG laser is much lowered than argon laser and Nd:YAG laser can selectively target on the melanocytes, SLT usually causes lesser thermal damage in the trabecular meshwork than ALT (Zhao et al., 2005). Hence, SLT surgery can be repeated for three to four times, whereas ALT can only be performed once.

Iridotomy

Iridotomy is commonly used in patients with suspected angle-closure glaucoma (Ng et al., 2008). Making puncture-like openings in trabecular meshwork can be performed by laser or standard surgical instruments. Iridotomy can lower the risk of developing acute angle closure attack.

Iridectomy

Iridectomy involves the removal of a portion of iris tissue. It is different from iridotomy which removes trabecular meshwork tissue. However, the aim and application of iridectomy is similar as that of iridotomy.

Filtration surgery: penetrating versus non-penetrating

Among the IOP management in glaucoma patients, filtration surgery is the main mode of surgical treatment (Sharaawy et al., 2011). An anterior sclerotomy or sclerostomy is performed to create a new opening in trabecular meshwork region, such that the

aqueous humor can seep from the anterior chamber into a bleb under the conjunctiva. It is divided into penetrating and non-penetrating types, depending on the extent of surgery into the anterior chamber or not.

Penetrating filtration surgery is further divided into partial thickness and full thickness filtering procedures. Trabeculectomy is one of the most commonly performed partial filtering procedures (Schwenn et al., 1998). A scleral flap is made over the sclerostomy site, and part of the trabecular meshwork is removed. Sclerectomy, trephination, and sclerostomy are the full thickness procedures to remove the trabecular meshwork, so as to prevent the block of aqueous humor outflow (Mermoud, 2000).

Non-penetrating filtration surgery does not involve entry into the anterior chamber of the eye. There are two major types: bleb-forming and viscocanalostomy. Non-penetrating trabeculectomy (NPT) is the most common type of non-penetrating filtering surgery. It involves making of a scleral flap which is large and very deep, so that the Schlemm's canal can be exposed (Lachkar et al., 2002). The inner wall of Schlemm's canal is then stripped off. Viscocanalostomy is also a commonly used non-penetrating filtering surgery. It involves opening of the Schlemm's canal which is then cannulated for the injection of viscoelastic substance. This can dilate the Schlemm's canal and the channels for the collection of aqueous humor.

Other surgeries

Goniotomy and trabeculotomy are similar techniques of microsurgical dissection that mechanically disrupt the trabecular meshwork. Goniotomy is performed by both laser and surgical instruments. Among them, surgical goniotomy is relatively more common, and it involves cutting the fibers of trabecular meshwork to allow a smooth aqueous humor outflow. In patients with congenital glaucoma, trabeculotomy is usually preferred than an endoscopic goniotomy (Kulkarni et al., 2010). This is because the patient's cornea is usually too hazy for a clear observation of the anterior chamber angle. Hence, a trabeculotomy which can go towards the angle from the exterior surface of the eye will be more suitable to be performed and the corneal transparency will be less crucial.

The placement of tube or glaucoma valves in glaucoma surgery is performed in tube-shunt surgery or drainage implant surgery (Sarkisian, 2009). Glaucoma drainage implant surgery is usually performed on patients who do not respond to maximal medical therapy or experienced previous failure of filtering surgery (eg. trabeculectomy). During the surgery, the flow tube is inserted into the anterior chamber and the plate is implanted under the conjunctiva to facilitate the outflow of aqueous into a bleb (Nguyen, 2009).

Cyclodialysis, cyclogoniotomy, and ciliarotomy are rarely used surgeries on ciliary zone. Their aims are to separate the ciliary body from the sclera.

Canaloplasty

Canaloplasty is a non-penetrating procedure utilizing microcatheter technology (Godfrey et al., 2009). It involves making an incision similar to viscocanalostomy, and placement of a microcatheter to maintain the drainage and collector channels circumnavigating the canal around the iris. A sterile, gel-like viscoelastic material is then injected. Thus, IOP may be lowered by canalization.

Ciliary destructive surgery

Aqueous humor is produced by cells in ciliary body. A ciliary destructive surgery is aimed to destroy those humor-producing cells in order to reduce the flow of aqueous humor, hence a decrease in the intraocular pressure (Ferry et al., 1995). Cyclocryotherapy, cyclophotocoagulation, cyclodiathermy, and cycloelectrolysis, are the ciliary destructive surgeries. These surgeries are performed using different methods, such as freezing probe, laser, heat generated from a high frequency alternating electric, and chemical action.

For the medications, they may induce adverse effects not only topically, but also systemically, and some of the side effects are risky to the patients. For surgeries, every surgery may have the risk of failure and infection. The prevention of glaucoma could be the best means.

1.2 Corticosteroids

1.2.1 Structure of corticosteroids

Corticosteroids are a class of steroid hormones synthesized from cholesterol within the adrenal cortex (Arlt et al., 2005). They include both the glucocorticoids (GC), like cortisol controlling carbohydrate, fat and protein metabolism and, mineralocorticoids such as aldosterone. They are involved in a wide range of physiological systems, such as stress response, immune response and regulation of inflammation, carbohydrate and blood metabolism.

Steroids are usually composed of a core skeleton of gonane, in which 17 carbon atoms are bonded together in the form of three cyclohexane rings (Elliot et al., 1987). This forms the skeleton of phenanthrene and one cyclopentane ring (four fused rings). Usually methyl groups are present at the carbon C-10 and C-13, and an alkyl side chain at carbon C-17. There are various types of steroids due to different number of methyl groups, the structure of side chains and the functional groups attached to the rings.

In general, corticosteroids are grouped into four classes, according to the conformation of chemical structure. Class A (short-to-medium-acting glucocorticoids) includes hydrocortisone, cortisone acetate, tixocortol pivalate, and prednisolone. Among them, prednisolone ($C_{21}H_{28}O_5$, 1,4-pregnadiene-17a,21-diol-3,11,20-trione; 17,21-dihydroxypregna-1,4-diene-3,11,20-trione) is one of the commonly used

corticosteroids. Class B contains triamcinolone acetonide (TA), fluocinonide, and halcinonide. Among them, TA ($C_{24}H_{31}FO_6$, 9a-fluoro-11b,16a,17a,21-tetrahydroxy-1,4-pregnadiene-3,20-dione 16,17-acetonide; 9a-fluoro-16a-hydroxyprednisolone 16a,17a-acetonide) is the most commonly used corticosteroid for eye diseases. Class C has betamethasone, dexamethasone, and fluocortolone. Among them, dexamethasone ($C_{22}H_{29}FO_5$, 9alpha-fluoro-11beta,17alpha,21-trihydroxy-16alpha-methylpregn-1,4-diene-3,20-dione) is the most common corticosteroid for clinical use. Class D includes hydrocortisone-17-butyrate, betamethasone valerate, prednicarbate, clobetasone-17-butyrate, fluocortolone caproate, fluocortolone pivalate and fluprednidene acetate. Among them, hydrocortisone-17-butyrate ($C_{25}H_{36}O_6$, 11b,17,21-trihydroxypregn-4-ene-3,20-dione 17-butyrate) is the most common and is often used in allergic testing (**Figure 2**). If a person is allergic towards one kind of steroid, he/she is highly intolerant to other kinds of steroids of the same class (Coopman et al, 1989).

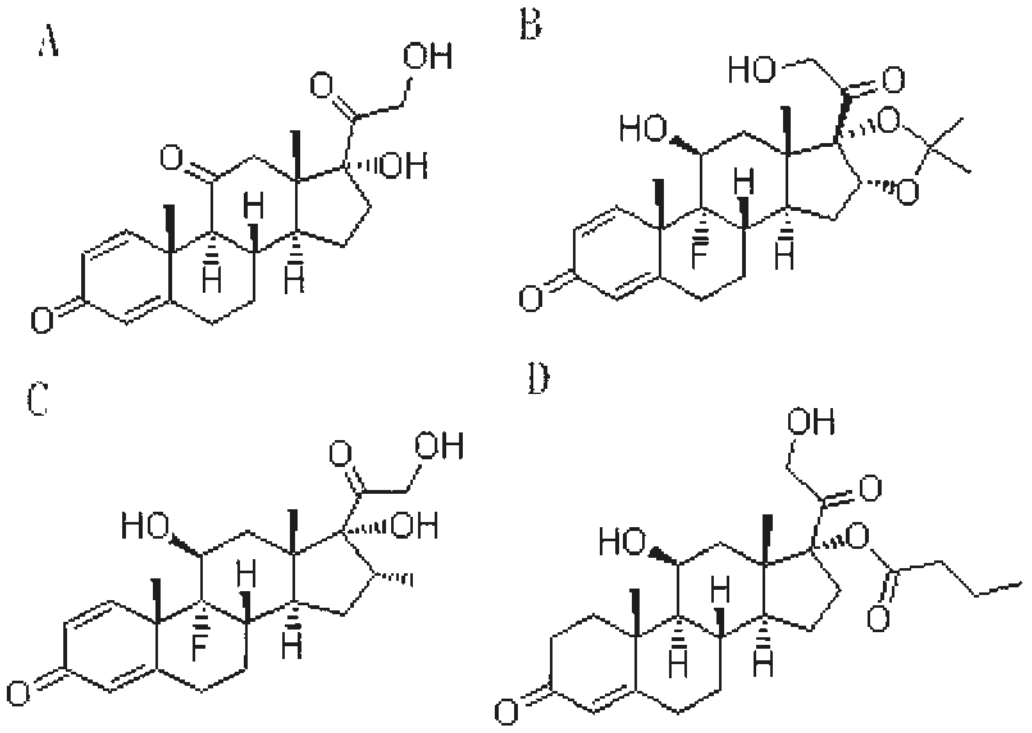

Figure 2. Chemical structure of commonly used corticosteroids. (A) Prednisolone ($C_{21}H_{28}O_5$,1,4-pregnadiene-17a,21-diol-3,11,20-trione;17,21-dihydroxypregna-1,4-diene-3,11,20-trione), **(B)** triamcinolone acetonide (TA) ($C_{24}H_{31}FO_6$, 9a-Fluoro-11b,16a,17a,21-tetrahydroxy-1,4-pregnadiene-3,20-dione 16,17-acetonide; 9a-Fluoro-16a-hydroxyprednisolone 16a,17a-acetonide), **(C)** dexamethasone ($C_{22}H_{29}FO_5$,9alpha-Fluoro-11beta,17alpha,21-trihydroxy-16alpha-methylpregn-1,4-diene-3,20-dione) and **(D)** hydrocortisone-17-butyrate ($C_{25}H_{36}O_6$, 11b,17,21-Trihydroxypregn-4-ene-3,20-dione 17-butyrate).

Pictures were modified from www.chemblink.com

1.2.2 Treatment of corticosteroids on systemic diseases

Corticosteroids are very useful in treating various medical conditions, ranging from skin problems to tumors. Their application can reduce itching, edema, redness, and suppress allergic reactions (Ito et al., 2006). In general, they are widely used for treating dermatitis, severe allergies, and asthma (Horvath et al., 2006). They are prescribed to patients who have undergone organ transplantation to reduce tissue rejection or to patients with autoimmune diseases, such as systemic lupus erythematosus, or diseases related with immune response, like inflammatory bowel disease (ulcerative colitis and Crohn's disease), and sarcoidosis (Cronin, 2010; Ford et al., 2011; Taylor et al., 2011). In patients with deficiency of endogenous corticosteroid production, for example, Addison's disease or other types of adrenal insufficiency, the symptoms can be alleviated by replacement therapy of recombinant corticosteroids (Lennemäs et al., 2008).

Corticosteroids are also used in patients with cancers, and to reduce inflammation like joint pain or inflammation (arthritis), temporal arteritis, and hepatitis (Hoes et al., 2010; Inaba et al., 2010; Ahmad et al., 2011).

Medicinal corticosteroids exist in many formulations, depending on their applications for different diseases (Reid et al., 2005; Horvath et al., 2006). As mentioned, corticosteroids were widely used to treat systemic diseases. For example, intravenous or intramuscular injection of steroid used in systemic lupus erythematosus,

and oral steroid is used in inflammatory bowel disease (Ford et al., 2011; Taylor et al., 2011). Apart from the formulations for injection, topical steroids are commonly used because of the convenience. Inhalant corticosteroids are used in patients with asthma attacks, whereas corticosteroid ointment, cream and gel are for patients with skin problems. Corticosteroid eye drops are used in patients with inflammatory or allergic eye diseases like uveitis and vernal conjunctivitis, or reduce inflammatory reaction after surgery (Bielory et al., 2010; Lobo et al., 2010).

1.2.3 *Effect of corticosteroids*

1.2.3.1 Systemic effects

Corticosteroids have been shown to play an important role in many biological processes and reactions in our body (Munck et al., 1986). These include metabolism of proteins, fats, and carbohydrates, homeostasis of the physiological levels of salt and water, regulation of blood pressure and sugar level and nervous system activity. With these widespread effects on the biological activities, corticosteroids have been commonly used to treat many medical conditions. Beside the medical benefits, corticosteroids are known for their side effects (Coombes et al., 2010; Nair, 2011).

Generally, side effects seldom appear when corticosteroids are used for a short time. However, with long-term use of corticosteroids, the body's ability to fight off bacterial or fungal infections may be lowered and the infections become more difficult to treat. In children with chickenpox or measles, corticosteroids may make

the disease more serious. It may also cover up the symptoms of infection, such as tuberculosis. Because of the immunosuppressive effects of corticosteroids, wound healing or ulcer formation may be impaired. So, patients with serious infections, especially fungal infections or serious trauma, should be cautious of using corticosteroids.

Cushing's syndrome is the most typical systemic undesired effect of corticosteroids (Smets et al., 2010). The most common symptom of Cushing's syndrome is central obesity, including the signs of a round face often referred to as a "moon face", and fat deposition on the back of the neck referred to as "buffalo hump". Other symptoms include hyperhidrosis, telangiectasia, thinning and weakness of the skin, other mucous membranes and connective tissues, proximal muscle weakness, and hirsutism. Corticosteroids also affect the regulation of blood pressure and the balance of water and salt (Guyton et al., 1986; Zennaro et al., 2009).

For patients on long-term corticosteroids, they can be at high risk of developing hypertension, electrolyte imbalance, such as hypokalemia and hypernatremia, and problems of metabolism, like peripheral edema, and metabolic alkalosis, especially in old age. Patients with high blood pressure and diabetes should consult their physicians for the use of corticosteroids. In young people, it may affect the function of the adrenal glands, resulting in a poor production and secretion of natural steroids (Bruni et al., 2009; Priftis et al., 2009).

Bone problems are also a common side effect after prolonged use of corticosteroids (de Nijs, 2008). Beside the slow growth in children and teenagers, corticosteroids can cause osteoporosis in old people, especially in old women.

It can also trigger problems in subjects with other medical conditions. For example, overuse of corticosteroids during pregnancy or breastfeeding period may cause growth problems of the fetus (Michael et al., 2008).

Other side effects of corticosteroids involve indigestion, nervousness, restlessness, and sleep problems. It may induce peptic ulcer, or epilepsy (Ng et al., 1991; Rosen et al., 1994). With overuse of topical corticosteroids, some topical side effects may also occur. Nasal spray forms may irritate the nose and may cause dry throat, headache, nausea, and unpleasant taste (Magyar et al., 2000). Use of ointments, gels, or creams of corticosteroids may cause skin atrophy and allergies (Schoepe et al., 2006).

Normally, these side effects will disappear when the patients stop the use of corticosteroids.

1.2.3.2 Ocular effects

In our eye, corticosteroids are effective for suppression of inflammation, allergy, and immune response (Edelman, 2010). Steroid eye drops with medications, such as

dexamethasone, fluorometholone, and prednisolone, are prescribed to patients after surgery to reduce the inflammation (Waseem et al., 2009). Intravitreal injection of corticosteroids is also widely used for the treatment of choroidal neovascularization, proliferative diabetic retinopathy (macular edema), retinal vein occlusion, and uveitis (Gopal et al., 2007; Kiernan et al., 2009; Taylor et al., 2010). Although steroids are effective in the treatment of eye diseases, they may carry the risk of side effects.

Mild and temporary side effects, which include irritation, redness, and blurred vision, commonly occur with the use of steroid eye drops. Although they are not serious, they may persist and get worse upon long-term use of steroids. Some patients may experience allergic reaction to the eye drops as well. Too much corticosteroid can cause impaired wound healing ability after operation or injury (Güngor et al., 2005).

Improper use of corticosteroids may cause permanent damage in the eye, including the onset of cataract, central serous retinopathy, and, the most important, ocular hypertension (Nootheti et al., 2006). Although not all subjects taking corticosteroids will develop high intraocular pressure (IOP), patients may remain undiagnosed with elevated IOP, and may have glaucomatous optic nerve damage. In most cases, after the patients stop using steroids, the IOP can return to the baseline level. However, in some instances, IOP can remain at high level and patients require medical intervention.

1.2.3.3 Cellular effects

1.2.3.3.1 Reported changes of steroids in retinal ganglion cells

Steroid may cause some changes in cultured cells, mainly the induction of cell stress and death. For example, dexamethasone was reported to increase the production of hydrogen peroxide in a variety of cell types like vascular endothelial cells, and this can induce oxidative stress, leading to cell growth arrest and death (Iuchi et al., 2003). Dexamethasone also induces apoptosis in some types of cells, including hippocampal cells (Sekita-Krzak et al., 2002). Triamcinolone acetonide (TA) has been shown to suppress the viability and growth of RGC5 cells, and the suppressing effect was dose-dependent (Szurman et al., 2007). Although intravitreal TA injections are used for treating many retinal diseases, its toxicity on retina cannot be overlooked. TA deposits may be harmful to the retina especially in eyes that have received vitrectomy with inner limiting membrane (ILM) peeling as TA in these cases may come in contact with retinal cells directly. Because of the disturbance of vitreoretinal interface after ILM peeling, retinal ganglion cells may be more sensitive to the cytotoxic effects.

1.2.3.3.2 Reported changes of steroids in trabecular meshwork cells

1.2.3.3.2.1 Cell changes

The morphology of cultured trabecular meshwork (TM) cells is altered by steroid treatment. Treating TM cells with steroid for a period of time causes an increase in TM cell size, which is associated with steroid-induced TM cytoskeletal changes, and

increase of nuclear size caused by increased DNA content (Tripathi et al., 1989; Wilson et al., 1993; Clark et al., 1994; Clark et al., 1996). A significant increase of fusion vesicles was found and they were arranged in linear rows beneath the cell membrane (McCartney et al., 2006). Dexamethasone also induced ultrastructural changes, including the induction of proliferation and activation of the endoplasmic reticulum (ER) and Golgi apparatus, and also the deposition of extracellular matrix materials (Wilson et al., 1993). Clark and his team observed that dexamethasone caused the cross-linkage of actin fibres, resulting in the formation of networks within the cultured human trabecular meshwork cells, and the network structure was reversible after the cessation of corticosteroid treatment (Clark et al., 1994; Clark et al., 2005). In addition, it caused the microtubules tangles in cultured TM cells, especially at the cell periphery (Clark et al., 1996). Thus, these cells with dense actin networks became less pliable and more likely to inhibit aqueous humor outflow, leading to IOP rise.

Steroid also alters the function of TM cells. Normal TM cells have the capability of phagocytozing cellular debris and pigments present in the aqueous humor and this function has been reported in cultured TM cells and trabeculectomized samples, as well as in organ culture of anterior segment (Shirato et al., 1989; Matsumoto et al., 1997). This phagocytosis activity was found to be involved in the turnover of extracellular matrix materials. However, upon the treatment with dexamethasone for several weeks, cultured TM cells had reduced phagocytic activity, leading to

deposition of materials in intercellular region (Matsumoto et al., 1997; Zhang et al., 2007). It was also demonstrated that glaucomatous TM cells had a decreased phagocytic capability and were more responsive to dexamethasone treatment compared to normal TM cells (Zhang et al., 2007).

In addition to inhibiting phagocytosis, TM cell migration, proliferation, and retraction could also be inhibited by dexamethasone exposure (Clark et al., 1994; O'Brien et al., 1996; Matsumoto et al., 1997). Dexamethasone also affected the arachidonate metabolism produced in normal TM cells, which was similar to that seen in perfusion cultured human eyes (Shaw et al., 1993). But the role of arachidonic acid metabolism in the regulation of IOP and/or TM cell function is yet to be investigated.

Steroid also induced structural changes in the TM region. There is report showing loss of glycogen in the cytoplasm of TM cells, increase of basement membrane-like materials, termed as plaques, and enlarged cisternae of rough ER in the TM cells in steroid-treated bovine eyes. The plaques consisted of densely packed fine fibrils connected to the sheaths of subendothelial elastic fibers. They were cross-linked or contact with increased basement membrane-like materials (Tektas et al., 2010).

1.2.3.3.2.2 Gene expression changes

1.2.3.3.2.2.1 Microarray studies (Table 1)

In 2002, Ishibashi and colleagues showed 30 out of 2400 human genes analyzed in cultured human TM cells were up-regulated for more than two folds after treatment with dexamethasone (100 nM) for 7 days (Ishibashi et al., 2002). Five of the most up-regulated genes were myocilin (MYOC), decorin, insulin-like growth factor binding protein 2 (IGFBP2), ferritin L chain and fibulin-1C, and they were confirmed by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Concurrently, 34 genes were significantly down-regulated. Lo and his group reported multiple genes differentially up-regulated in dexamethasone (100 nM, treated for 8 to 10 days)-treated human TM cells including myocilin (TIGR/MYOC), α1-antichymotrypsin (a serine protease inhibitor), pigment epithelium-derived factor (PEDF), cornea-derived transcript 6, and prostaglandin D2 synthase (Lo et al., 2003). Fifteen of the 249 differentially regulated genes with four or more fold changes were mapped to glaucoma-linked loci.

For long-term dexamethasone treatment (for 21 days) on human TM cells, among 1260 genes analyzed using U133A GeneChip, 23 genes were up-regulated and 18 were down-regulated by more than three folds (Rozsa et al., 2006). Confirmed by quantitative PCR, genes with significant changes were *SLP1, SAA2, ANGPTL7, MYOC, SAA1, SERPINA3* and *ZBTB16*.

Our laboratory also reported gene expression profile in steroid-treated human TM cells by using microarray and RT-PCR analyses. After treatment with Dex at 100 nM for 10 days, genes including *GAS1, CDH4, MT1L, CST3, ATF4, ASNS/TS11, CHOP, HSPA5* were expressed at levels more than 1000 times than at normal condition, and *TSC22, LDHA, IGFBP2, TAGLN, SCG2, WARS* more than 100 times (Leung et al., 2003). Several of them were reported to function in anti-inflammatory response and humor outflow resistance. In TM cells treated with either DEX or TA, *MYOC* and *GAS1* were up-regulated (Fan et al., 2008). In contrast, *SENP1, ZNF343* and *SOX30* were down-regulated.

In these 5 Microarray studies, MYOC which is in POAG loci was all upregulated when HTM cells were treated with steroid.

Table 1. Microarray studies of gene expression on Dex-treated HTM cells.

Study	Treatment of Dex (dose and day)	Analysis/gene number	Up-regulated genes	Down-regulated genes
Fan et al., 2008 *IOVS*	100 nM, 7 d	Standford Functional Genomics Factory, UtraGAPS /22904	MYOC, GAS1, SERPINA3, SAA1, SAA2	SENP1, IGFBP3, TCF7L2, IL1B, ZNF343, SOX30
Rozsa et al., 2006 *Mol Vis*	100 nM, 21 d	U133A GeneChip /1260	23 genes (SAA1, SAA2, MYOC, SERPINA3, SLP1, ANGPTL7, ZBTB16)	18 genes (KCNJ15, NDP, GRP, IL6, etc.)
Leung et al., 2003 *Mol Vis*	100 nM, 10 d	MicroMax Human cDNA System I/2400	MYOC, GAS1, MT1L, CDH4, CST3, ATF4, CHOP, ASNS/TS11, HSPA5, etc.	
Lo et al., 2003 *IOVS*	100 nM, 8-10 d	Affymetrix U95Av2 Chip, n=6/12627	SERPINA3, MYOC, PEDF, APOD	
Ishibashi et al., 2002 *IOVS*	100 nM, 7 d	Laser detection system (ChipReader) /2400	30 genes (MYOC, decorin, IGFBP2, ferritin L chain, fibulin-1C, etc.)	34 genes (Pig7, P78 protein, etc.)

1.2.3.3.2.2.2 Candidate genes affected by steroids and relationship to TM aqueous outflow regulation

Steroid-induced ocular hypertension is mainly due to the increased outflow resistance of aqueous humor. *In vitro* exposure of Dex/steroid to TM cells was reported to affect the expression of genes and proteins that may be involved in resistance to aqueous humor outflow and/or other glaucomatous modifications (Leung et al., 2003; Zhang et al., 2003; Tane et al., 2007).

Dexamethasone has been reported to reduce the expression of endothelin-1 receptor B (ET-1rB) in cultured HTM cells (Zhang et al., 2003). Under normal conditions, ET-1rB binding with endothelin-1 drives nitric oxide release and subsequent vasodilation. Upon dexamethasone treatment, synthesis of ET-1rB was reduced resulting in a drop of nitric oxide levels (Hirata et al., 1993). Thus, only endothelin-1 receptor A remains available for the binding with endothelin-1, which starts a vasoconstriction signaling pathway. This may reduce aqueous humor outflow in the TM region leading to IOP rise.

Dexamethasone also affects the expression of extracellular matrix (ECM)-associated genes, which are involved in the regulation of the outflow system (Steely et al., 1992; Tane et al., 2007). As reported, untreated HTM cells were subjected to pressure rise up to 50 mmHg and they showed up-regulation of matrix-metalloproteinase (MMP)-3. Upon dexamethasone treatment, MMP3 was

down-regulated (Ehrich et al., 2005). MMP-3 cleaves the extracellular matrix proteins. Its suppression by dexamethasone thus results in extracellular matrix protein deposition and may block the aqueous humor outflow.

In addition, the expression level of beta isoform of glucocorticoid receptor (GRβ) was reported to regulate the cellular responsiveness to dexamethasone in terms of phagocytic activity in TM cells (Zhang et al., 2005). The lower expression of GRβ in glaucomatous TM cells may contribute to a steroid-induced decrease in phagocytosis, thus resulting in an exacerbation of aqueous humor outflow resistance.

For myocilin (MYOC), it was reported as a delayed secondary glucocorticoid responsive elements (GRE)-mediated gene, as the application of 100 nM dexamethasone (Dex) to cultured HTM cells resulted in a delayed (8-16 hours) induction of myocilin (Shepard et al., 2001).

1.3 Cell stress

1.3.1 Characterization

Cell stress response belongs to a defensive reaction when cells are faced with deleterious stimulations or in environment related to drug toxication, chemical toxicity, viral and bacterial infections. The main pathways of cell stress response include the initiation of cell signaling pathways, which regulate the activity of target proteins, synthesis of stress-related proteins, and/or induction of apoptosis when the

stress response cannot solve the damaging problems.

1.3.2 Pathophysiology and impact of stress on diseases

Stress can make changes in our body, including the changes of metabolism and function. The pathophysiology and diseases related with stress affect almost all systems in our body.

In central nervous system, stress results in upregulated norepinephrine level which causes anxiety, nervousness, and anger, and the activation of hypothalamic-pituitary-adrenal axis which cause depression, anorexia, and suicidal tendency (Nestler et al., 1999; Aguilera, 2011). In cardiovascular system, stress activates sympatheticoadreno-medullary system, which increases the contractility of heart, heart rate, and the speed of blood flow, and contract peripheral small vessels, resulting in hypertension or blood flow rearrangement (Stalcup et al., 1982; Oliveira-Sales et al., 2009). In the digestive system, stress also causes the activation of sympatheticoadreno-medullary system, causing the contraction of vessels in the stomach and intestine, and ulcer formation (stress ulcer) (Choung et al., 2008). In immune system, acute stress upregulates the phagocyte, complements, and expression of c-reactive protein (Downing et al., 2000; Schroecksnadel et al., 2006; Chou et al., 2008; Essler et al., 2009)

Long-term stress promotes the secretion of glucocorticoid and catecholamine, which suppresses the immune response, causing autoimmune diseases or immunodeficiency (Dhabhar, 2009). In circulatory system, acute stress causes the increase in the number of leukocytes and enhances the functions of platelets (Doré, 1998; Montrucchio et al., 2000).

But chronic stress often results in anemia (Yamashita et al., 2007). In genital-urinary system, the activation of sympatheticoadreno-medullary system and renin-angiotensin-aldosterone system contracts the renal vessels, thus reduces glomerular filtration rate, and leads to increased secretion of anti-diuretic hormone leading to oliguria (Rohatgi et al., 2010). Stress also reduces the secretion of gonadotropin-releasing hormone (GnRH), leading to growth problems (Hayes et al., 1998).

In our eyes, there are also several diseases associated with stress, including glaucoma, cataract, diabetic retinopathy, and age-related macular degeneration. In POAG, oxidative stress plays an important role. In the trabecular meshwork tissue as well as in the posterior pole, glaucoma patients show an increased oxidative stress (Erb et al., 2011). Oxidative damage is also important in age-related cataract formation. It is suggested that exposure to increased levels of molecular oxygen accelerates the age-related opacification of the lens nucleus, resulting in nuclear cataract (Beebe et al., 2010). It was also reported that ER stress and oxidative stress is

associated with diabetic retinopathy. ER stress leads to the death of the vascular cells in the retina, and oxidative stress that lead to structural and functional changes and accelerated loss of capillary cells in the retinal microvasculature (Madsen-Bouterse et al., 2008; Oshitari et al., 2008). Also, age-related macular degeneration (AMD) is related with the imbalance between the levels of reactive oxygen species (ROS) and antioxidants (Khandhadia et al., 2010). This causes damage and apoptosis of the retinal pigment epithelial cells, leading to neovascularization and degeneration of macula.

1.3.3 Types of cell stress

1.3.3.1 Oxidative stress

Oxidative stress is a kind of stress which represents an imbalance between the production and manifestation of reactive oxygen species (ROS), including free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that produces free radicals or are activated by them and the ability of biological system to detoxify the reactive intermediates or to repair the stress-related damage (Wei et al., 2001). The production of peroxides and free radicals can disturb the normal redox state of tissues, thus causing toxic effects and damage of cell components, including polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA.

ROS are mainly produced by aerobic respiration *in vivo*, although they are also produced by peroxisomal beta-oxidation of fatty acids, microsomal cytochrome P450 metabolism of xenobiotic compounds, stimulation of phagocytosis by pathogens or lipopolysaccharides, arginine metabolism, and tissue specific enzymes (Buonocore et al., 2010). Normally, through the action of superoxide dismutase (SOD), catalase, or glutathione (GSH) peroxidase, ROS are cleared from the cell (**Figure 3**) (Paravicini et al., 2004; Izzotti et al., 2006).

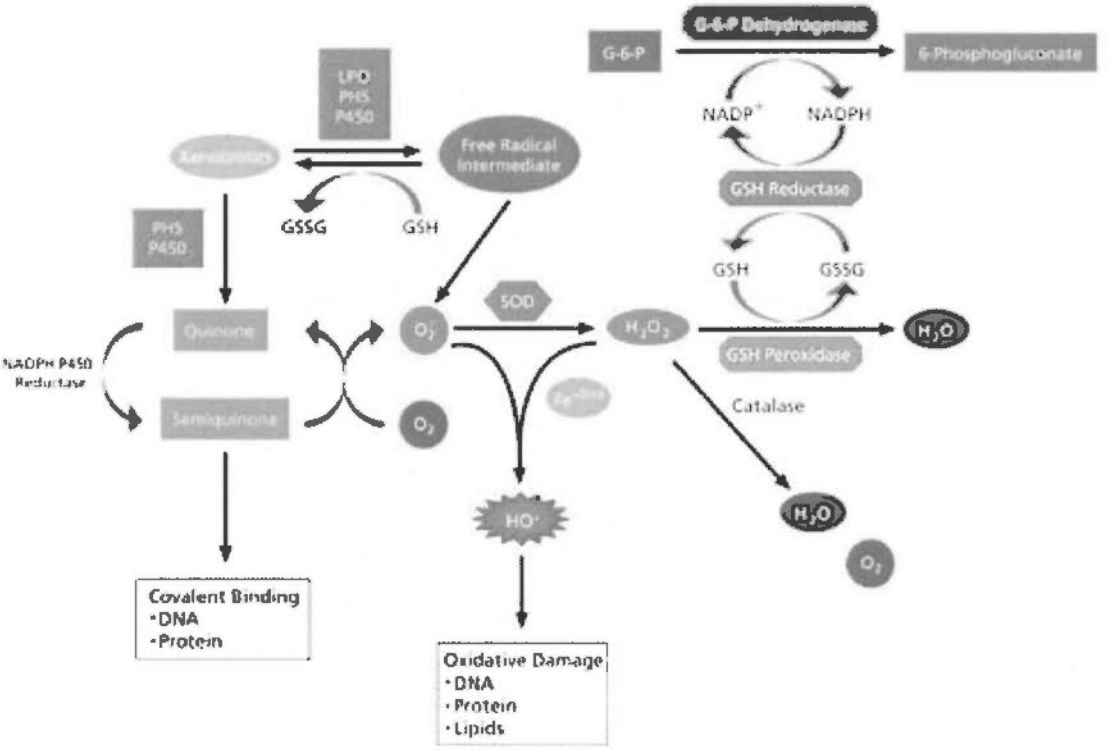

Figure 3. Overview of oxidative stress and ROS activation. Under the condition of oxidative stress, ROS are mainly produced by aerobic respiration *in vivo*. They are also produced by peroxisomal beta-oxidation of fatty acids, microsomal cytochrome P450 metabolism of xenobiotic compounds, stimulation of phagocytosis by pathogens or lipopolysaccharides, arginine metabolism, and tissue specific enzymes (Buonocore et al., 2010). Normally, through the action of superoxide dismutase (SOD), catalase, or glutathione (GSH) peroxidase, ROS are cleared from the cell (Paravicini et al., 2004; Izzotti et al., 2006).

Picture was modified from www.sigmaaldrich.com

Too much ROS can induce cell damage including the damage of mitochondria DNA, apoptosis in the condition of oxidative stress (Birch-Machin, 2006; Matés et al., 2008). For corticosteroids, they are metabolized to reactive quinones and hydroquinones, which can directly damage DNA (Ambrosone, 2000). Thus the use of corticosteroid may induce oxidative stress in the cells.

In the cells, oxidative stress results in an increase in oxidant generation, a decrease in antioxidant protection, or a failure to repair oxidative damage, which is involved in the pathogenesis of many diseases including atherosclerosis, Parkinson's disease, heart failure, myocardial infarction, Alzheimer's disease, schizophrenia, and fragile X syndrome (Hashimoto et al., 2003; Le Strat et al., 2009; Chen et al., 2010). For example, Alzheimer's disease and Parkinson's disease, two of the most common diseases in central nervous system affecting the elderly people. The oxidative stress and mitochondrial dysfunction associated with these two diseases may lead to increased membrane permeability and cytochrome C release, which promotes neurodegeneration (Hashimoto et al., 2003).

In our eye, glaucoma is related to oxidative stress. The pathogenesis of primary open angle glaucoma involves a set of complex phenomena reciprocally interacting such as free radicals, oxidative DNA damage, etc. These result in clinical events such as vascular damage, IOP rise and optic neuropathy (**Figure 4**) (Izzotti et al., 2006). As early as in 1983, there was a study on the role of the redox system in glaucoma, Kahn

et al. demonstrated increased aqueous humor outflow resistance in the presence of high levels of H_2O_2 (Kahn et al., 1983). The effect of H_2O_2 on the adhesion of TM cells to extracellular-matrix proteins results in cytoskeleton changes which caused insufficient adhesion of TM cells and cell loss, thus affect the integrity of TM (Zhou et al., 1999).

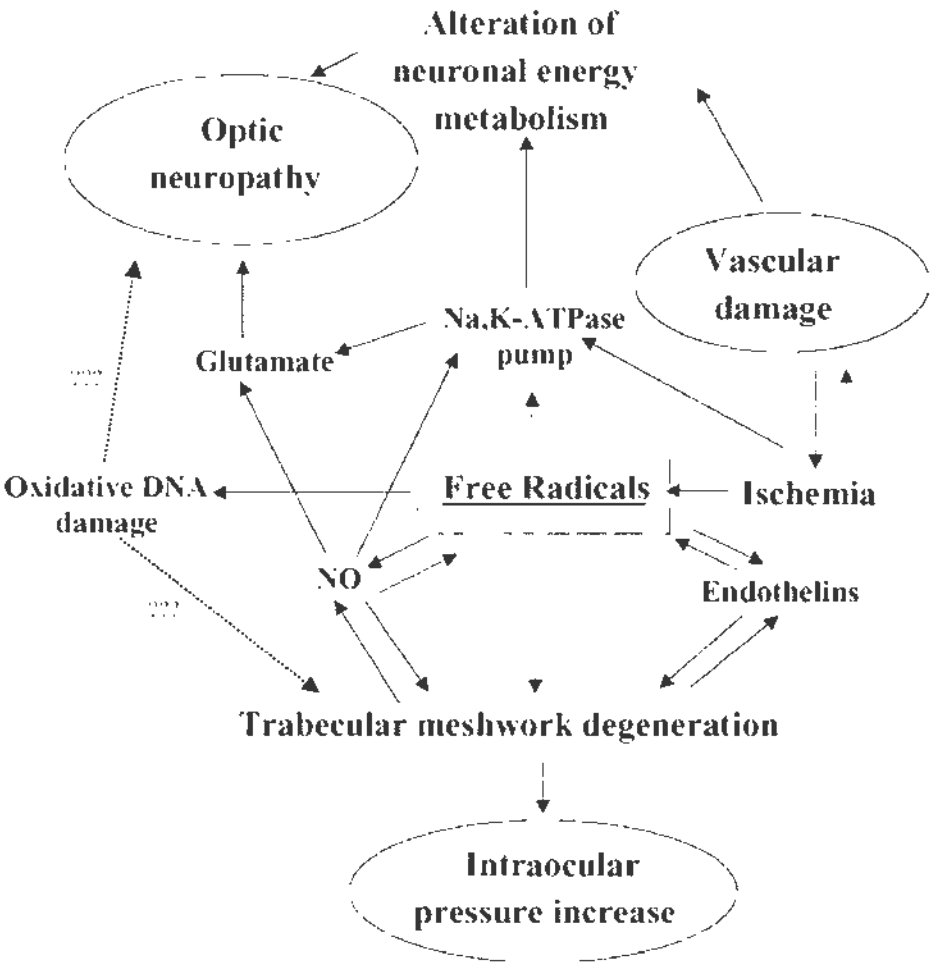

Figure 4. Oxidative stress-related pathogenesis of primary open angle glaucoma. The pathogenesis of primary open angle glaucoma involves a set of complex phenomena reciprocally interacting such as free radicals, oxidative DNA damage, etc. These resulted in clinical events such as vascular damage, IOP rise and optic neuropathy (Izzotti et al., 2006).

Picture was modified from Izzotti et al., 2006

1.3.3.2 Endoplasmic reticulum (ER) stress

In eukaryotic cells, the ER is an important organelle for protein and lipid biosynthesis, protein folding and maturation. In the ER, proteins of the plasma membrane, Golgi apparatus and lysosomes, and secretory proteins fold to their proper tertiary and quaternary structure (Stevens et al., 1999). There are very stringent quality control systems in the ER to ensure that only those proteins which are correctly folded and fully functional can leave the ER and transported to the proper destination. If not, the misfolded proteins are usually degraded.

In cells, the proper ER function is essential for most cellular activities and cell survival, because the ER is a major organelle of signal transduction and responds to changes of homeostasis. The stimuli and conditions that interfere with the ER homeostasis can cause the accumulation of unfolded or misfolded proteins inside the ER. These conditions are collectively called the ER stress (Marciniak et al., 2006).

With the onset of ER stress, ER transmembrane proteins such as X-box binding protein-1 (XBP-1), IRE-1 (inositol-requiring and ER to nucleus signaling), ATF6 (activated transcription factor 6) and PERK (ER-resident PKR-like eIF2α kinase) sensor the stress and then activate highly specific signaling pathways called "unfolded protein response" (UPR) to restore normal ER functions (**Figure 5**) (Schröder et al., 2005).

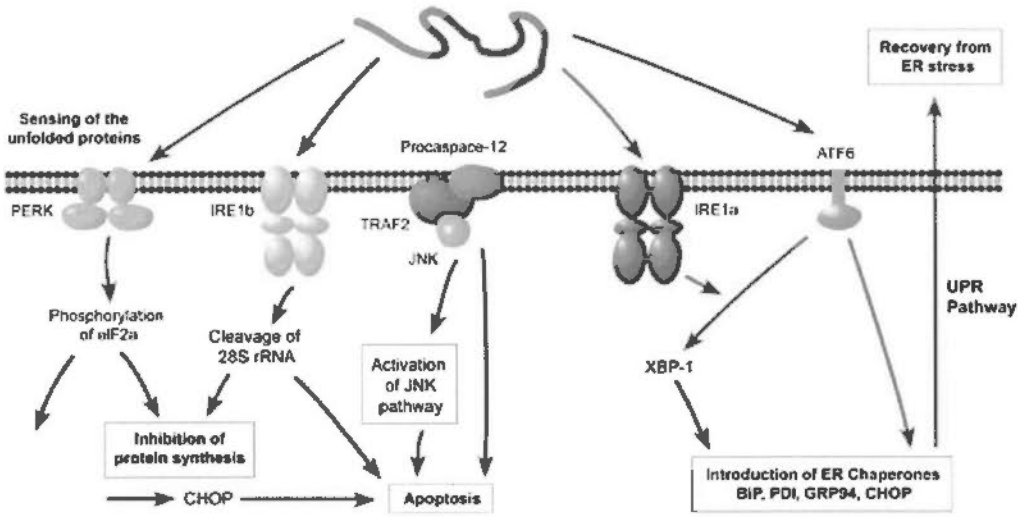

Figure 5. Overview of the ER stress and unfolded protein response (UPR) signaling. In cells, the stimuli and conditions that interfere with ER homeostasis cause an accumulation of unfolded or misfolded proteins inside the ER. These conditions are collectively called as the ER stress (Marciniak et al., 2006). With the onset of ER stress, ER transmembrane proteins such as XBP-1, IRE-1, ATF6 and PERK sensor the stress and then activate highly specific signaling pathways called "unfolded protein response" (UPR) to restore normal ER functions (Schröder et al., 2005).

Picture from www.imgenex.com

UPR relays the ER stress to cytosol and cell nucleus to relieve the imbalance in the synthesis, folding, modification, translocation and degradation of proteins. Some physiological and pathological conditions, for example, nutrient deprivation, elevated protein synthesis, infection, disturbances in Ca^{2+} fluxes and redox regulation have been discovered to cause ER dysfunction and thus activate UPR (Schröder et al., 2005).

UPR is shown to induce BiP/GRP78 expression, which is a common response to the accumulation of mutant unfolded viral haemaglutinin proteins or depletion of glucose (Hendershot, 2004). This phenomenon suggests that the interaction between unfolded proteins and BiP is essential in promoting the cell response under ER stress. However, some unfolded proteins may not interact with BiP and they cannot lead to the induction of BiP (Ng et al., 1992). UPR also upregulates the expression of other chaperone factors such as GRP94 and GRP170 which assist protein folding, calnexin which assists glycoprotein folding and protein disulphide isomerases (PDIs) which assist oxidation/reduction of cysteine (Wu et al., 2006).

UPR is an evolutional mechanism of restoring cellular homeostasis, which is less complex in lower eukaryotes and more complex, diverse and flexible in the mammalian cells. In humans, UPR signals through three ER-transmembrane proteins IRE-1 (inositol-requiring and ER to nucleus signaling), ATF6 (activated transcription factor 6) and PERK (ER-resident PKR-like eIF2α kinase) which represent the three

lines of the signaling pathway (Haze et al., 1999; Jousse et al., 1999; Sha et al., 2009).

UPR plays an important role in embryonic development, maturation of secretory cell types such as plasma cells that secrete antibodies, osteoblasts that secrete collagen, and pancreatic β-cells that secrete insulin. Especially the early stage of embryonic development is related to ER stress and requires chaperone activity for viability. During embryonic development, BiP is induced at 3.5 days, so BiP knockout can cause the embryos' implantation problems, growth problems, and intense apoptosis (Luo et al., 2006).

Since the maturation of secretory cell types such as plasma cells that secrete antibodies, osteoblasts that secrete collagen, and pancreatic β-cells that secrete insulin are associated with ER stress, ER stress plays a significant role in immune and metabolic diseases in humans. X-box binding protein-1 (XBP-1) upregulation is a part of the UPR. Hence the plasma cells carrying IRE-1 mutations that cannot process XBP-1 fail to efficiently secrete antibodies (Iwakoshi et al., 2003). Analyses of homozygous and heterozygous knockout mouse cells in culture, and humans suffering from an infantile type-1 diabetes which is called Walcott Rallison syndrome, reveal that activation of c-Jun N-terminal kinase (JNK) by IRE-1 and subsequent phosphorylation at ser307 of insulin receptor substrate-1 (IRS-1) interferes with the function of β-cells and leads to type-1 and type 2 diabetes/obesity-linked insulin resistance (Marciniak et al., 2006). Anti-inflammatory response, tumor progression,

and hypoxic resistance are also associated with ER stress (Koumenis, 2006; Rzymski et al., 2009). It was reported that, a likely cause of macrophage death was the accumulation of free cholesterol (FC) leading to ER stress-induced apoptosis, and the activation of IRE-1α is the key step in this action (Li et al., 2008). As dexamethasone/steroid had anti-inflammatory effect, the regulation of steroid action could be involved in ER stress response.

When ER stress persists beyond the limits of adaptation, it activates the apoptotic pathway of the UPR. Cell death leads to loss of cell/tissue function and may be the primary reason for the formation and manifestation of several diseases. Although the exact mechanism(s) of ER stress-induced apoptosis is still unclear, the apoptosis is signaled through not only the mitochondria-dependent pathway (intrinsic pathway), but also the activation of proapoptotic downstream kinases (extrinsic apoptotic pathway, mitochondria-independent pathway) (Marciniak et al., 2006). In the intrinsic apoptotic pathway, MDM4 stably localizes at the mitochondria where upon lethal stress conditions was found to promote the mitochondrial localization of p53 phosphorylated at Ser46 (p53Ser46(P)) and facilitate its binding to Bcl-2 (Mancini et al., 2009). Thus, Bcl-2 protein family regulates the ER-Ca^{2+} release and communicates the ER stress signal to the mitochondria, leading to the dysfunction of mitochondria, cytochrome C release and apoptosis (Weston et al., 2010). Long term ER stress can also induce the UPR signaling to switch from pro-survival to apoptotic pathway, like the induction of proapoptotic transcriptional factor GADD153/CHOP

through the PERK-eIF2α pathway, and activation of proapoptotic kinases ASK1 (apoptosis signal regulating kinase) and JNK (c-Jun-N-terminal kinase) through the IRE-1 pathway (Marciniak et al., 2004). This activates mitochondria-dependent caspases, among which procaspase-12 is an ER-associated proximal effector of apoptosis, and the activated caspase-12 cleaves and activates the pro-caspase-9, which in turn activates caspase-3, resulting in apoptosis (Mandic et al., 2003).

In ophthalmologic research, the ER stress has been implicated in many eye diseases, which are usually caused by an accumulation of unfolded or misfolded proteins. The well known ocular diseases related with the ER stress are cataract and retinitis pigmentosa (Rebello et al., 2004; Shinohara et al., 2006; Ohgane et al., 2010). In these two diseases, mutation in carbonic anhydrase IV cause retinitis pigmentosa, and the ER stress was caused by mutant αA-crystallin, and γD-crystallin in cataract. These misfolded proteins could be corrected by chemical chaperones such as TMAO and sodium 4-phenylbutyrate (NaPB) (Bonapace et al., 2004b; Gong et al., 2009; Gong et al., 2010). Mutant MYOC caused POAG is also an ER stress-related disease (Joe et al., 2003), and this mutant MYOC could also be corrected by NaPB and/or TMAO (Yam et al., 2007; Jia et al., 2009).

1.3.3.3 Heat shock response

Heat shock response is the cell response to heat stress, which is the effect of subjecting cells to a higher temperature than normal.

As a part of the internal repair of cells, the heat shock response includes the transcriptional up-regulation of genes encoding heat shock proteins (HSPs) which also called heat stress-proteins (Guisbert et al., 2008; Vabulas et al., 2010). By the activation of several cascade pathways, these proteins respond to heat, cold and hypoxia. Under normal conditions, HSPs are also expressed in cells (Vabulas et al., 2010). Some HSPs have the chaperone effect which ensures the proteins in the cells are in the right shape, right place at the right time by helping correct new or misfolded peptides, which is essential for their function (Guisbert et al., 2008; Vabulas et al., 2010). Similar to most of the chaperones, HSPs also involve in protein trafficking and degradation of misfolded proteins inside the cells (Vabulas et al., 2010). Heat shock proteins are also thought to play a role in the presentation of patches of proteins (or peptides) on the cell surface to help the recognization of diseased cells by the immune system (Tsan et al., 2009).

There are two main effects of heat shock response, induction of HSPs and stress-related apoptosis. Depending on the temperature and duration of heat stress, the response either results in thermotolerance or causes the genetic program of cell death, due to the activation of caspases (Samali et al., 1999).

Short term heat stress leads to thermotolerance which is associated with the induction and accumulation of HSPs. Heat-shock proteins are usually named

according to their molecular weights. For example, the most commonly studied HSPs, including HSP60, HSP70 and HSP90 are referred to heat shock proteins of 60, 70 and 90 kilodaltons (kDa) in size, respectively (Li et al., 2001).

The increase of the heat shock proteins is a key part of the heat shock response which is transcriptionally regulated and is induced primarily by heat shock factor (HSF) (Wu, 1995). Cells are protected from death with the induced expression of HSPs, and those failed to produce HSPs die via apoptosis (Sreedhar et al., 1999). High levels of heat shock protein release can also be induced by a variety of environmental stress conditions, for example, infection, inflammation, toxins (ethanol, arsenic, trace metals and ultraviolet light), starvation, hypoxia, and the loss of water. Although the mechanism by which heat shock or other stress conditions that bring the heat shock factor into action has not been fully determined, some damaged or abnormal proteins are suggested to activate HSPs.

Some heat shock proteins also act as chaperones for other proteins. They are essential in protein-protein interactions, and this assists in proper protein folding and prevents abnormal protein aggregation. Moreover, they participate in metabolic processes ranging from protein synthesis to degradation. By helping to stabilize misfolded or unfolded proteins, HSPs also play an important role in protein transport across cell membranes (Freeman et al., 1996; Ehrnsperger et al., 1997).

In severe heat stress conditions, the apoptosis gene network is activated, causing organelle disruption, condensation of chromatin, degradation of DNA, releasing cytochrome c and apoptogenic factor Apaf-1 from mitochondria, and the activation of caspase 3 (Samali et al., 1999). Apoptosis is associated with activation of caspases, which are constantly produced as proenzymes, in the cytoplasm. When activated, they cleave their specific substrates, which are mostly structural and regulatory proteins (Cohen, 1997; Humke et al., 1998; Van de Craen et al., 1998; Kang et al., 2000; Nakagawa et al., 2000). The released cytochrome c interacts with Apaf-1 and with procaspase 9 to form a complex called apoptosome. This activates procaspase 9, to turn on the subsequent activation of caspases 3, 6 and 7 resulting in apoptosis (Srinivasula et al., 1998; Zou et al., 1999).

HSPs are suggested to suppress apoptosis, activation of caspases and also play a role in generating thermotolerance. HSP70 prevents the release of cytochrome c from the mitochondria and binds with Apaf-1 to prevent apoptosome formation and activation of caspase 9, thus inhibits the processing of caspase 3 (Beere et al., 2000; Li et al., 2000; Mosser et al., 2000; Saleh et al., 2000). Molecular chaperones HSP27 and HSP90 suppress the release of Apaf-1, complexation with cytochrome c, and caspase 9 activation (Bruey et al., 2000; Pandey et al., 2000). HSP10 and HSP60 express in mitochondria and bind with procaspases 3 and 6, which still retain its capacity of proteolysis. This inhibits the activation of caspase 3 and 6 (Xanthoudakis et al., 1999). Heat stress increases the production of ceramide, which activates the

c-Jun N-terminal kinase (JNK), then activates MEKK-1, SEK1 (Mosser et al., 1997; Kondo et al., 2000). Finally, JNK leads to c-Jun phosphorylation, which promotes the expression of the c-jun and c-fos mRNAs to induce apoptosis (Kondo et al., 2000). HSP70 may activate JNK phosphatase to suppress the activation of JNK and kinase. This prevents apoptosis and provides the cells with thermotolerance (Gabai et al., 1997; Mosser et al., 1997; Gabai et al., 2000). In contrast, ceramide destroys HSP70 mRNA, and suppresses the anti-apoptotic activity (Kondo et al., 2000). In severe heat stress, the JNK activity increases rapidly and phosphorylates the regulatory domain of heat shock factor 1 (HSF1) and consequently inhibits HSP70 transcription, eventually leading to apoptosis (**Figure 6**) (Dai et al., 2000).

Heat shock response can cause disorders in many cells. In human, there are also diseases associated with heat shock response, and the abnormal expression level of heat shock proteins. They include atherosclerosis, autoimmune disorders, cancer, and Alzheimer's disease (Rajaiah et al., 2009; Lu et al., 2010; Richardson et al., 2011; Salminen et al., 2011).

Among eye diseases, cataract and glaucoma are related to heat shock response. It was reported that cold cataract induction in cultured mammalian lenses could be enhanced by heat shock and this effect could be prevented by inhibiting heat shock protein production (Banh et al., 2006). Also, heat shock proteins were reported to be related with glaucomatous neurodegeneration. They acted as critical modulators of

both the homeostatic/cytoprotective and pathogenic/neurodegenerative aspects of the immune system in retinal ganglion cells or glial cells (Tezel et al., 2004).

Glucocorticoid sensitivity in glaucoma has been attributed to the expression changes of the two glucocorticoid receptors, GRα and GRβ. For GRα, it binds to HSP90 and its interaction with Dex was blocked, thus activate GRE-mediated gene expressions (Tago et al., 2004). In addition, HSP90 is an essential molecular chaperone for nuclear transport of GRβ (Zhang et al., 2006). As mentioned, myocilin (MYOC) which is in glaucoma loci was reported as a delayed secondary glucocorticoid responsive elements (GRE)-mediated gene (Shepard et al., 2001). Since HSPs are chaperones which regulate incorrectly folded proteins, chaperones (endogenous or exogenous) may have an effect on Dex-induced ocular hypertension.

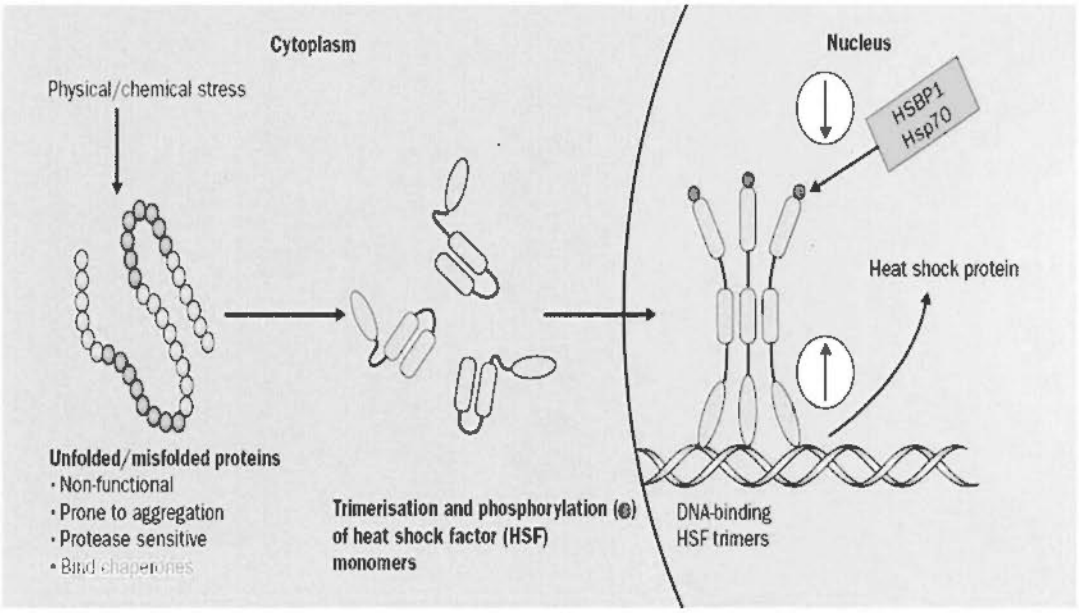

Figure 6. Regulation of heat shock protein expression. Physical or chemical stress induces unfolded/misfolded protein production. In the cytoplasm, HSF monomers form trimers, are phosphorylated, and translocated into the nucleus. HSF trimers bind to heat shock protein gene promoter regions (HSE), and induced HSP gene transcription. Hsp70 gene transcription was down-regulated by interaction of Hsp70 or HSBP1 with the HSF trimers.

Picture was modified from combio.abo.fi

1.4 Chemical intervention of cell stress

1.4.1 Principal

There are four major categories of cellular stress: oxidative stress, osmotic stress, endoplasmic reticulum (ER) or biosynthetic stress, and heat shock-induced stress. Inside the eukaryotic cells, oxidative stress generates oxidation products. Without any induction of anti-oxidative power, the cells are always faced with the problem to repair the oxidative damage. Osmotic stress, on the other hand, causes an intracellular influx of calcium ions and hence volume changes of the cells. The ER stress is usually related to deregulation of protein biosynthesis, folding, modification, translocation and degradation. During and after heat induction, the cells activate heat shock response, which includes the transcriptional up-regulation of genes encoding heat shock proteins and stress proteins (Guisbert et al., 2008; Vabulas et al., 2010). The damage by all such stresses can be alleviated when stress management is initiated at early stages. However, if they are unattended or become severe, the cells will enter into a suicidal cell death pathway. Hence, the principal of stress management is to alleviate the cells from stress, and improve the cell survival.

1.4.2 Stress-associated cell changes

Stress-associated cell changes include structural and functional changes. At the beginning of stress induction, the cells responses include gene expression changes, and cytoskeletal changes. Many of these responses are aimed to protect cells against the stress and help then recover soon after the stress has passed. However, if the stress

sustains and the stress response cannot recover the stress-induced biochemical changes, the cellular death signaling pathways will be activated.

The type and action of cell stress response depend on the type and level of the stress. For example, the production of reactive oxygen species increases in oxidative stress, through the action of SOD, catalase, and/or glutathione peroxidase (Paravicini et al., 2004; Izzotti et al., 2006). ROS are cleared from the cells by antioxidants. During the occurrence of osmotic stress, molecular sensors and signal transduction networks convey the osmolarity information from the surrounding to the cell and this activates the cellular responses to tackle with the osmotic problem. The protective responses of the ER stress and heat shock are UPR and heat shock response, respectively. Both UPR and heat shock responses enhance the protein chaperoning activity, which assists the protein folding. This can alleviate the stress and promote the cell survival.

In general, stress response is a pro-survival strategy carried out by the cells to act as a protective mechanism against stress. If these responses are unsuccessful to deal with the problem, the cell death program will be activated to eliminate the damaged cells from the tissue. The cell death pathways include apoptosis, necrosis, pyroptosis, and autophagy. Among all the forms of cell death, apoptosis is the best characterized and its highly regulated features make it an attractive target for therapeutic intervention (Fulda et al., 2010). Apoptosis is conserved among species throughout

the evolution and plays a physiological role in both embryonic development and aging (Samali et al., 1996; Bree et al., 2002; Lockshin et al., 2007). There are different stress stimuli that induce apoptosis, for example, oxidative stress, ER stress, irradiation and chemotherapeutic agents (anti-cancer therapy). Caspases, a family of cysteine proteases, act as common death effectors for the onset and progression of apoptosis (Degterev et al., 2003). Caspases are synthesized as inactive proenzymes. Upon activation, active caspases cleave various substrates in the cytoplasm or nucleus, leading to morphological and biochemical changes of apoptotic cell death including DNA fragmentation, loss of cell shape, and nuclear shrinking (Degterev et al., 2003; Lockshin et al., 2007; Samali, 2007; Ashkenazi, 2008).

1.4.3 Chaperones

1.4.3.1 Definitions and classification

Molecular chaperones are cellular proteins that assist the non-covalent bond formation and cleavage in folding process (Ellis, 2006). This affects the assembly or disassembly of macromolecular structures. When these structures are properly folded without any hydrophobic patches exposed on the protein surface and can perform their native biological functions, they will not be interacted with chaperones.

Preventing both newly formed polypeptide chains and assembled units from aggregating into non-functional structures is one of the important functions of chaperones. Many chaperones are indeed heat shock proteins. In the condition of

stress, many proteins are denatured, increasing the tendency to aggregate. Chaperones then come in to bind to the denatured proteins and reduce the chance of aggregation or even prevent them from forming aggregates.

1.4.3.2 Small molecule chemical chaperones

1.4.3.2.1 Definition

Chemical chaperones are a group of exogenous small molecules with chaperoning activity. They are reported to successfully correct various misfolded proteins and the associated cellular defects or protein folding abnormalities (Burrows et al., 2000; Tveten et al., 2007; Yam et al., 2007; Caciotti et al., 2009; Janovick et al., 2009). They have been shown to reverse the misfolding or mislocalization of mutant plasma membrane, cytoplasmic, lysosomal and secretory proteins (Morello et al., 2000; Rujoi et al., 2010).

More common representatives of chemical chaperone molecules are glycerol, polyols, dimethylsulfoxide (DMSO), deuterated water (D_2O), tauroursodeoxycholic acid (TUDCA), trimethylamine N-oxide (TMAO) and sodium 4-phenylbutyrate (NaPB).

1.4.3.2.2 Reported use of chemical chaperones on cell rescue

Because of the correction and stabilization effect on misfolded/unfolded proteins/peptides, chemical chaperones can alleviate cell stress, thus protecting the

cells from apoptosis.

As early as in 1997, it was reported that sodium 4-phenylbutyrate (NaPB) enhanced the expression of mutant CFTR on the surface of primary nasal polyp epithelia obtained from cystic fibrosis patients with homozygous ΔF508 mutation (Rubenstein et al., 1997). Also treatment with 100 mM TMAO improved AQP2 expression and function on CHO-K1 cell surface (Rubenstein et al., 1997; Tamarappoo et al., 1998).

There were more reports of using chemical chaperones to rescue the affected cells. NaPB and glycerol significantly induced the secretion of alpha1 anti-trypsin z-variant in skin fibroblasts of patients diagnosed with liver emphysema (Burrows et al., 2000). TMAO improved the surface expression of mutant podocin with native functions in transformed human kidney embryonic epithelial (HEK) 293 cells (Ohashi et al., 2003). Moreover, sodium 4-phenylbutyrate could improve the expression and/or surface trafficking of several mutant proteins, such as ΔF508 cystic fibrosis transmembrane regulator (CFTR) protein in bronchial epithelial cells (Lim et al., 2004; Singh et al., 2008), apoptosis-inducing mutant of carbonic anhydrase IV in HEK293 cells (Bonapace et al., 2004a) and trafficking defective nephrin mutant in COS7 cells (Liu et al., 2004). More work using chemical chaperones was done to improve the surface expression of various mutant proteins, reduce ER stress and apoptosis in different cell types, such as in fibroblasts from spinal muscular atrophy patients (Andreassi et al.,

2004). TMAO was reported to reverse ER perturbation induced by the overexpression of iodide transporter pendrin (Shepshelovich et al., 2005a), to rescue mutant vasopressin V2 receptor in the treatment of congenital nephrogenic diabetes insipidus (Cheong et al., 2007). NaPB was found to reduce the aggregation of Pael receptors and the associated ER stress (Kubota et al., 2006), restore the functionality of the misfolded mutant low-density lipoprotein receptor (Tveten et al., 2007), enhance the cell surface expression and the transport capacity of mutated bile salt export pumps (Hayashi et al., 2007), prevent mutant HFE aggregate formation in the ER (de Almeida et al., 2007) as well as alleviate the severity of lysosomal storage disorder (Wei et al., 2008).

Other than the classical studies of retrieving the misfolded proteins, chemical chaperones were reported to improve cell survival. TMAO reduced the aggregation of keratin in primary EBS cells with keratin mutations (Chamcheu et al., 2009). A study of primary human skin fibroblasts from patients of torsion dystonia revealed the enhanced function of torsin A by chemical chaperones (Cao et al., 2010). In Globoid cell leukodystrophy (GLD) (Krabbe disease), the D528N mutation causes hyperglycosylation and protein misfolding, which was alleviated after the treatment of α-lobeline on D528N mutant and GALC activity was significantly increased (Lee et al., 2010). DMSO and glycerol could rescue the activity of T49M mutant retinol dehydrogenase 12 in HEK293 cells (Lee et al., 2011).

In the model of eye diseases, chemical chaperones also play an important role in correcting misfolded proteins and resulting in cell rescue. In diabetic retinopathy, NaPB reduced the expression of inflammation markers in human retinal endothelial cells (Li et al., 2009). It also reduced myocilin aggregates, attenuated the ER stress and improved the survival of human trabecular meshwork cell in primary open angle glaucoma (Yam et al., 2007; Jia et al., 2009). In congenital cataract, TMAO alleviated the aggregation and the ER stress caused by G98R αA-crystallin, and sodium 4-phenylbutyrate corrected the mistrafficking of G165fsX8 γD-crystallin and suppressed the apoptosis (Gong et al., 2009; Gong et al., 2010).

1.4.3.2.3 Animal and clinical trials of chemical chaperone-assisted therapy

As chemical chaperones can rescue cells experiencing different kinds of stress and apoptosis, this suggests that chaperone-assisted therapy might hold novel treatment for stress-related diseases. As early in 1990s, there were some human studies on chemical chaperones suggesting potential treatments on some diseases. In the clinical trial of anemia including sickle cell anemia and β-thalassemia, NaPB treatment for not more than 20 grams/day induced the production of F-reticulocyte, hence increasing the hemoglobin production (Dover et al., 1992; Dover et al., 1994; Collins et al., 1995). In 1998, there was a clinical trial for oral NaPB (Buphenyl). In this study, eighteen patients with cystic fibrosis caused by homozygous ΔF508 mutated cystic fibrosis transmembrane regulator (CFTR) were tested randomly, double-blinded and placebo-controlled. Results showed that oral NaPB (19 grams) three times daily for a

week had significant improvement of nasal potential response, indicating an improvement of lung and bronchial activity (Rubenstein et al., 1998).

In recent years, studies of chemical chaperones were focused on human diseases such as cancer, leukemia, cystic fibrosis as mentioned, and diabetes. Most results showed the treatment efficacy or improved response to the conventional clinical treatment (Carducci et al., 2001; Gore et al., 2002; Zeitlin et al., 2002; Maslak et al., 2006; Kars et al., 2010; Xiao et al., 2011). Also, chemical chaperones were studied in animal models of various human diseases, including liver emphysema, cystic fibrosis, stroke, diabetes, ischemia reperfusion injury, familial intrahepatic cholestasis type II, Parkinsonism, multiple epiphyseal dysplasia, and Wilson's diseases. All of them showed the potential capacity of chemical chaperones to alleviate the stress and improve the functions of cells and proteins (Burrows et al., 2000; Fischer et al., 2001; Qi et al., 2004; Fu et al., 2005; Vilatoba et al., 2005; Özcan et al., 2006; Hayashi et al., 2007; Jafarnejad et al., 2008; Kurokawa et al., 2009; Ono et al., 2009; Alvarez et al., 2010; Budas et al., 2010; Luo et al., 2010; Nundlall et al., 2010; Bromati et al., 2011; Rahmanpour et al., 2011).

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In 1990's, there were three clinical studies on anemia (sickle cell anemia and beta-thalassemia), showing the transcriptional regulation effect of NaPB. It increased F-reticulocyte and hemoglobin production, and the maximal dose of NaPB used was 20 g/day, 14 to 460 days (Dover et al., 1992; Dover et al., 1994; Collins et al., 1995).

So, NaPB was approved by the US Food and Drug Administration (FDA) to treat β-thalassemia. In cystic fibrosis, NaPB promoted the surface expression of ΔF508 mutant CFTR protein in primary nasal polyp epithelial cells, and this improved the nasal potential difference response on the patients significantly. The highest dose of oral NaPB used was 60 g/day (20 g, 3 times daily) and the patients tolerated well (Rubenstein et al., 1997; Rubenstein et al., 1998). NaPB is also reported to have anti-caner effect. It induced cell differentiation, and also enhanced clinical response to chemotherapy. Although a high dose intravenous NaPB up to 410 mg/kg/day caused some toxicity effects, such as excessive somnolence, confusion, hypokalemia, hyperuricemia and hyponatremia, NaPB induced tumor cell differentiation and regression of tumor volume (Carducci et al., 2001; Gilbert et al., 2001).

In eye diseases, chemical chaperones such as sodium 4-phenylbutyrate, TUDCA, and TMAO were studied in the animal models of glaucoma, cataract and diabetic retinopathy. They were shown to delay the formation of hypermature cataract in rats (Mulhern et al., 2007). NaPB could reduce the loss of retinal thickness and RGCs in SD rats with ocular hypertension, and reduced inflammation in diabetic mice (Jeng et al., 2007; Li et al., 2009). In addition, in animal model of MYOC-caused glaucoma, NaPB prevented IOP rise in *Tg-MYOC*^{Y437H} mice by promoting the secretion of mutant myocilin in the aqueous humor and by decreasing intracellular accumulation of myocilin in the ER, thus rescue TM cells from death (Zode et al., 2011). Although chemical chaperones showed promising effect on some eye diseases, these studies

have not been performed on human eyes and results are pending.

1.4.3.2.4 Examples of chemical chaperones

1.4.3.2.4.1 Osmolytes: including glycerol, TMAO, DMSO

1.4.3.2.4.1.1 Characterization, literature review and reported actions

Osmolytes are compounds which affect osmosis (Street et al., 2006). They are soluble in the solution in and around the cells. For example, the plasma osmolytes are present in circulation and they maintain the blood cell volume and fluid balance. During osmotic stress conditions, the cells swell. The opening of cell membrane channels allows the outflow of cellular osmolytes, which carry water molecules, hence restoring the original cell volume (Yancey, 2005).

In general, osmolytes, including glycerol, trimethylamine N-oxide (TMAO), dimethylsulfoniopropionate (DMSO), and amino acid derivatives (such as sarcosine and proline), are common chemical chaperones. Natural osmolytes like proline and glycine betaine can act as osmoprotectants to defend the cells against osmotic stress (Ma et al., 2010). Osmolytes are also the ancient members of stress responses, as they regulate the free movement of proteins by improving the solubility, thus preventing the accumulation of misfolded proteins. Many studies have proven the ability of osmolytes in reducing the frequency of switching to non-productive folding pathways for various mutant proteins associated with conformational diseases (Ghumman et al., 1998; Bennion et al., 2004).

Among the conformational diseases, the most frequently examined target is the cAMP-activated chloride ion channel protein, cystic fibrosis transmembrane regulator protein (CFTR) and the most common mutation ΔF508 CFTR causes cystic fibrosis (Brown et al., 1996; Sato et al., 1996; Fischer et al., 2001). This mutation causes the protein to degrade rapidly. With treatment of glycerol and TMAO, the maturation of mutant CFTR protein was increased and this rescued the cAMP-activated chloride conductance of mutant cells (Sato et al., 1996; Fischer et al., 2001). DMSO was also shown to have chemical chaperone activity, since it facilitated the mutant CFTR transport in a cell culture model (Bebök et al., 1998).

The development of nephrogenic diabetes insipidus is associated with the mutation of water channel, aquaporin-2 in which the folding deficiency was correctable by chemical chaperones, such as glycerol, TMAO and DMSO (Tamarappoo et al., 1998). The mutation of a vasopressin receptor, V2R can cause diabetes insipidus. With the help of glycerol, mutated V2R proteins escaped from the quality control machinery, integrated to the membrane, and acted as a normal functional vasopressin receptor (Tan et al., 2003). It was proven that the sequestration of mutant α1 anti-trypsin Z variant is involved in emphysema and liver diseases. The solubility and transport was enhanced by glycerol and TMAO (Burrows et al., 2000). Maple syrup urine disease is mainly caused by the mutation of branched-chain

a-ketoacid decarboxylase (BCKD) complex, and the cells were rescued by TMAO treatment (Song et al., 2001).

Because the regenerating ability of neuron is poor, the central nervous system is sensitive to folding diseases. This makes the neurodegenerative diseases a relevant topic in the research of chemical chaperone-assisted protein rescue. In Alzheimer's disease, the beta amyloid plaque formation causes neuronal cell death, which result in mental deterioration. Both glycerol and TMAO successfully inhibited the formation of beta amyloid plaques (Yang et al., 1999). Prion disease such as Creutzfeldt-Jacob disease is caused by prion protein folding disorder, that is, the mutation of PrP(Sc). DMSO and glycerol could reverse the harmful effect of mutated PrP(Sc), which gave a possible treatment for such disease (Gu et al., 2004). Machado-Joseph disease, also called Azorean disease, is a heritage neurodegenerative disease triggered by the misfolding of ataxin-3 protein. This misfolded protein was correctable by glycerol, TMAO and DMSO (Yoshida et al., 2002).

Cancer is also an important field of study in chemical chaperone research. Usually, the formation of tumors is due to the gene mutations. The mutation of tumor suppressor protein p53 and viral oncogene protein pp60src are the two key proteins in the onset and progression of up to half of the tumor types. The active osmolytes, glycerol, TMAO, DMSO, as well as deuterated water (D_2O) can correct the mislocalization of p53 mutants, pp60src, or the ubiquitin-activating enzyme E1

(Brown et al., 1996). It was also proven that mutated glucocorticoid receptor which is involved in cancer was corrected by glycerol, TMAO and D_2O (Baskakov et al., 1999).

Chemical chaperones such as glycerol, TMAO, and DMSO are also reported to improve the efficiency of immune system. They can enhance the antigen presentation by promoting the folding of major histochompatability complexes (Ghumman et al., 1998).

Recently, the research of osmolytes was extended to other diseases including steroid-resistant nephrotic syndrome, scrapie, Pendred syndrome, diabetes, lysosomal storage disorders, pulmonary arterial hypertension, epidermolysis bullosa simplex, polycystic kidney disease, hereditary fructose intolerance and Huntington's disease (Ohashi et al., 2003; Bennion et al., 2004; Shepshelovich et al., 2005b; Cheong et al., 2007; Sobolewski et al., 2008; Wei et al., 2008; Chamcheu et al., 2009; Ma et al., 2010; Borwankar et al., 2011; Stopa et al., 2011). Among these osmolytes, TMAO was studied on cataract. In 2007, TMAO was reported to suppress the lens epithelial cell death and cataract formation not only on lens epithelial cells, but also on mouse model (Mulhern et al., 2007). In congenital cataract, TMAO alleviated the aggregation and the ER stress caused by G98R αA-crystallin (Gong et al., 2009).

1.4.3.2.4.2 Sodium 4-phenylbutyrate

1.4.3.2.4.2.1 Characterization, literature review and reported actions

The sodium 4-phenylbutyrate molecule contains a hydrophobic part, which can be dissolved in fluids and then binds to proteins. The lysophosphatidic acids and/or butyrate derivatives were found to mask the mutations of proteins and stabilize their structure in a native conformation. The suggested mechanism is that they bind to the hydrophobic part that remains surface-exposed in the unfolded proteins. The binding prevents the aggregation and may assist in the degradation of misfolded proteins (Papp et al., 2006).

Sodium 4-phenylbutyrate (NaPB) is an aromatic fatty acid with reported histone deacetylase inhibition activity and chaperone activity and thus it could affect local gene transcription and chaperoning action to stabilize protein folding. It is permeable through tissue cells and metabolized rapidly through beta-oxidation into phenylacetate, which conjugates with glutamine to form acetylglutamine and is excreted in urine (Hommes, 1999). The metabolites of NaPB provide two nitrogen atoms for the defective urea disorders for urinary excretion of waste nitrogen products, so NaPB was originally used as an ammonia-scavenging agent in urea metabolism disorders (Brusilow et al., 1984).

NaPB is reported to have actions on various human diseases, including its chaperoning effect, transcriptional regulatory, anti-cancer, neuro-protective, and

anti-stress. The chaperone effect of NaPB is the most commonly known. As early as 1997 and 1998, a cell model and a clinical study on cystic fibrosis revealed the chaperone effect of NaPB. It promoted the surface expression of ΔF508 mutant CFTR protein in primary nasal polyp epithelial cells, and this improved the nasal potential difference response on the patients significantly (Rubenstein et al., 1997; Rubenstein et al., 1998). Recently, there were more studies reporting the use of NaPB in cellular and animal models as well as in clinical trials. These studies showed the chaperoning effect of NaPB and the effect of NaPB on reducing the ER stress and protecting the cells from apoptosis. They were related with diseases including liver emphysema, cystic fibrosis, α1AT deficiency, nephrological disorder, diabetes, ischemia reperfusion injury, and Parkinsonism (Burrows et al., 2000; Zeitlin et al., 2002; Lim et al., 2004; Liu et al., 2004; Teckman, 2004; Fu et al., 2005; Vilatoba et al., 2005; Kubota et al., 2006; Özcan et al., 2006).

In addition, there are studies reporting the chaperoning effect of NaPB in diseases, like Fabry's disease, familial hyper-cholesterolemia, familial intrahepatic cholestasis type II, hereditary hemochromatosis, hypoparathyroidism, cystic fibrosis, pulmonary arterial hypertension, obesity, cholera, diabetic nephropathy, spinal cord ischemia (Datta et al., 2007; de Almeida et al., 2007; Hayashi et al., 2007; Prulière-Escabasse et al., 2007; Tveten et al., 2007; Sobolewski et al., 2008; Basseri et al., 2009; Mizukami et al., 2010; Qi et al., 2011; Taylor et al., 2011).

NaPB is also reported to act as a transcriptional regulator, and this effect was reported earlier than that reported as a chaperone. By 1995, there were three clinical studies on anemia (sickle cell anemia and beta-thalassemia), showing the transcriptional regulation effect of NaPB. It increased F-reticulocyte and hemoglobin production (Dover et al., 1992; Dover et al., 1994; Collins et al., 1995). After that, the studies on NaPB's action as a transcriptional regulator were reported on cystic fibrosis, spinal muscular atrophy, β-thalassemia, leukemia, sickle cell anemia (Rubenstein et al., 2000; Andreassi et al., 2004; Singer et al., 2005; Maslak et al., 2006; Hines et al., 2008).

NaPB is also reported to have anti-caner effect. It induced cell differentiation, and also enhanced clinical response to chemotherapy. Two clinical studies were carried out on the oral and intravenous administration of NaPB in solid tumor and lymphoma, respectively. Although a high dose intravenous NaPB up to 410 mg/kg/day caused some toxicity effects, such as excessive somnolence, confusion, hypokalemia, hyperuricemia and hyponatremia, NaPB induced tumor cell differentiation and regression of tumor volume (Carducci et al., 2001; Gilbert et al., 2001). Moreover, other cellular and clinical studies also demonstrated the therapeutic effect of NaPB on malignant and recurrent gliomas, leukemia, liver cancer, and solid tumors (Baker et al., 2002; Gore et al., 2002; Svechnikova et al., 2003; Phuphanich et al., 2005; Camacho et al., 2007).

Because of the poor regenerating ability of neuron, it is generally difficult to solve the problem of the degeneration of central nervous system (CNS). However, NaPB has been reported to have neuroprotective effect. In a stroke model in mouse, NaPB was shown to protect neurons and delay the death of neurons, providing a new hope for the treatment of CNS degeneration (Qi et al., 2004). Moreover, NaPB exhibited neruoprotective effect on diseases like Alzheimer's disease and Parkinsonism (Inden et al., 2007; Ono et al., 2009; Ricobaraza et al., 2009).

NaPB is also shown to be cardioprotective and is beneficial for the treatment of cardiac injury, Huntington's disease, drug sensitization, amyotrophic lateral sclerosis (Daosukho et al., 2007; Hogarth et al., 2007; Burkitt et al., 2008; Cudkowicz et al., 2009).

In ophthalmic research, NaPB has been shown to exhibit its chaperone and neruoprotective activity in retinitis pigmentosa, cataract, glaucoma and diabetic retinopathy (Bonapace et al., 2004b; Jeng et al., 2007; Mulhern et al., 2007; Yam et al., 2007; Jia et al., 2009; Li et al., 2009; Gong et al., 2010). Among them, there were three significant studies of effect of NaPB on glaucoma. In the IOP induction model of Sprague-Dawley (SD) rats, intra-peritoneal injection of NaPB at 100 mg/kg reduced loss of retinal thickness and retinal ganglion cells (Jeng et al., 2007). Another two important studies of NaPB on glaucoma were on myocilin-transfected HTM cells and CHO-K1 cells. Treatment with 1 mM NaPB improved mutant MYOC trafficking,

solubility, secretion and cell survival (Yam et al., 2007; Jia et al., 2009). In animal model of MYOC-caused glaucoma, 10 mg/day of NaPB prevented IOP rise in *Tg-MYOC*^{Y437H} mice by promoting the secretion of mutant myocilin in the aqueous humor and by decreasing intracellular accumulation of myocilin in the ER, thus rescue TM cells from death (Zode et al., 2011).

1.4.3.2.4.2.2 triButyrate® – FDA approved applications

The commercially available product for sodium 4-phenylbutyrate (NaPB) is triButyrate®, which was originally developed in the 1980's by Triple Crown Co., US, at the request of Johns Hopkins Hospital for the treatment of inborn urea-cycle disorder in children. It was approved by the US Food and Drug Administration (FDA) to treat urea-cycle disorder and β-thalassemia. Inborn errors of urea synthesis (urea-cycle disorder) are rare and are due to the disorders of amino acid metabolism, which are hereditary and caused by enzyme defects. Among the disorders of amino acid metabolism, phenylketonuria (PKU) is the most well known. PKU is characterized by an accumulation of phenylalanine, and if it is untreated, it often results in mental and psychomotor retardation. The disorders of inborn errors of urea synthesis are less known, but they are more life threatening. Inborn errors of urea synthesis are associated with hyperammonemia or high levels of plasma glutamine, and frequently leading to coma and death in infants, if not treated immediately.

The metabolism of NaPB provides two nitrogen atoms for urinary excretion of waste nitrogen products, so it can be used to treat the urea-cycle disorders. The treatment effect of NaPB on urea-cycle disorder was first reported in 1984 (Brusilow et al., 1984). Since then, triButyrate® became a choice of treatment, and the recommended dosage is 450 to 500 mg/kg/day. It has been proved by the toxicity studies derived from long-term continuous use and treatment at these dosage levels on infants and children cases that the application of triButyrate® is safe and efficacious, even at the high dosage given over their individual lifetime (Maestri et al., 1995; Maestri et al., 1996). Only at levels of five to ten times larger than the commonly used dosages, or up to 3 grams/kg bodyweight/day (about 200 gm/adult/day), the toxicity effect starts to appear (Praphanproj et al., 2000). Nowadays, because of triButyrate®, approximately 1500 children with such kind of diseases are alive and living well.

In US, sickle cell anemia affects lots of people and the prevalence is approximately 0.02% of total US population (Prabhakar et al., 2010). Mostly, sickle cell anemia affects blacks such as Sub-Saharan African descent, and also, variations of it can be seen in whites of Mediterranean, Middle-Eastern and East Indian descent (Rees et al., 2010). It is known that, hemoglobin is a protein, which carries oxygen of erythrocyte. Sickle cell anemia is caused by the homozygocity for the mutation that causes HbS (hemoglobin S, sickle hemoglobin). In this disease, the abnormal erythrocytes turn rigid, thus form a sickle shape and block circulation within vessels (Zhou et al., 2011). Among the patients suffering from sickle cell anemia, those who

have a naturally higher level of fetal hemoglobin, which normally diminishes with age, have fewer painful episodes (Coleman et al., 2007).

Phenylacetate, a naturally occurring chemical in our body, has been found to increase the production of fetal hemoglobin significantly, and it is known to have no toxicity effect, even at high doses (Fibach et al., 1993). Because of this promising effect, doctors at Johns Hopkins Hospital, Baltimore, US, conducted an initial clinical trial of phenylbutyrate (to produce phenylacetate in the body) on anemia patients. The effect on increased levels of fetal hemoglobin in the blood circulation of 15 children after receiving oral NaPB was studied for five to 65 months (Dover et al., 1992). Since then, there were more clinical studies reporting the efficiency and safety of triButyrate® treatment in sickle cell anemia and β-thalassemia (Dover et al., 1994; Collins et al., 1995; Singer et al., 2005; Hines et al., 2008).

1.4.3.2.4.3 Other chemical chaperones

1.4.3.2.4.3.1 Characterization, literature review and reported actions

Among other chemical chaperones, tauroursodeoxycholic acid (TUDCA) is more commonly studied. It is a hydrophilic bile acid which is the taurine conjugate form of ursodeoxycholic acid. TUDCA has anti-inflammatory and cytoprotective effects towards the liver (Neuman et al., 1995; Lim et al., 2010). It has been used for the treatment of gallstones and liver cirrhosis and it is approved by US FDA for the treatment of primary biliary cirrhosis (Portincasa et al., 1996; Setchell et al., 1996). As

a chemical chaperone, it has the effect to reduce the ER stress and apoptosis, and hence, it shows a therapeutic potential in the management of diabetes, hereditary hemochromatosis, cardiac failure, Huntington's disease, Parkinson's disease, stroke and lysosomal storage disorders (Özcan et al., 2006; de Almeida et al., 2007; Rivard et al., 2007; Wei et al., 2008).

In recent years, TUDCA has been reported to have protective effect on ocular diseases, such as retinal degenerative disorders (Boatright et al., 2009). In a rat model of retinitis pigmentosa expressing P23H mutant *rd10,* TUDCA treatment rescued retinal cell death and showed anti-angiogenesis effect, which may be related to its anti-inflammatory activity (Phillips et al., 2008; Woo et al., 2010; Fernández-Sánchez et al., 2011; Oveson et al., 2011).

Moreover, TUDCA was reported to suppress the death of lens epithelial cells, and delay hypermature cataract formation in the rat model of cataract (Mulhern et al., 2007).

1.5 Objectives and research impacts

Glaucoma is the second leading cause of blindness and is affecting tens of thousands of people worldwide. Topical and systemic use of corticosteroids may cause a rise of intraocular pressure (IOP), leading to secondary open angle glaucoma. Although not all subjects taking corticosteroids will develop high IOP, patients will remain

undiagnosed with their high IOP, and this can result in glaucomatous type of optic nerve damage and requires medical intervention. The main mechanism of steroid induced ocular hypertension is the increased resistance of aqueous humor outflow in the trabecular meshwork region. It has been shown previously that steroids modify the gene expression profile of human trabecular meshwork cells in both primary and cell line culture. In this study, I hypothesize that steroids application induces cell stress of trabecular meshwork region, which might be mediated by altered gene expression, and, if unattended, this will affect trabecular meshwork cell survival and hence, the homeostasis of the trabecular meshwork region. As a whole, this can lead to a higher resistance of the aqueous humor outflow and cause a rise of IOP. The sustained high IOP will be transmitted to the back of eye, compressed on the retina and this can result in the optic neuropathy and optic nerve head damage.

My study was aimed to find a novel therapeutic intervention for steroid-induced glaucoma. Conventionally, the management of steroid-induced ocular hypertension is controversial. The patients on continuous steroid treatment usually respond suboptimally to argon laser trabeculoplasty and are resistant to standard anti-glaucoma eye drop medication. Many of them may require the invasive filtration surgery. As the side effects of existing medication and the risk of infection or failure of surgeries, the exploration of a new and safe medication that can use in parallel with steroids will be beneficial to reduce ocular hypertension and maintain the steroid-associated anti-inflammatory effect.

Small molecule chemical chaperones are known to stabilize and facilitate the folding of a variety of proteins, thus alleviating cell stress and promoting cell survival. They are potential therapeutic agents for the treatment of conformational diseases and stress-related diseases. The Heat-Shock stress response protein HSP90 is an important regulator for GRα as it binds to GRα and blocks its interaction with Dex to activate GRE-mediated gene expressions (Tago et al., 2004). Also, HSP90 is an essential molecular chaperone for nuclear transport of GRβ (Zhang et al., 2006). As mentioned, myocilin (MYOC) which is in glaucoma loci was reported as a delayed secondary GRE-mediated gene in (Shepard et al., 2001). Since HSPs are chaperones which regulate incorrectly folded proteins, and NaPB was reported to reduce stress in cell and animal model of MYOC-caused glaucoma, it is worthwhile to investigate whether small molecule chaperones such as sodium 4-phenylbutyrate (NaPB, triButyrate®) will have preventive or reduction effect on Dex-induced ocular hypertension.

In this study, several commonly reported small molecule chemical chaperones prepared in eye drop formulation were attempted to treat steroid-induced ocular hypertension in an experimental rabbit model. Among them, NaPB showed the most effective prevention of rise of intraocular pressure caused by corticosteroids. I hypothesized that the cell stress caused by altered gene expression due to steroid effect could be alleviated or reversed by sodium 4-phenylbutyrate. This gives a healthy and functional trabecular meshwork population in the aqueous human outflow

pathway, thereby maintaining a constant or low intraocular pressure level. This can protect the eyes against the onset of steroid-induced glaucoma.

Results of this study will throw light on a new design of potential medication that can be used in parallel with steroids, without causing ocular hypertension. The eye drop application of sodium 4-phenylbutyrate (NaPB, triButyrate®) may be safe and effective to prevent or treat steroid induced glaucoma.

Chapter 2: Materials and Methods

2.1 *In vivo* experiments

2.1.1 Establishment of steroid-induced ocular hypertension model in rabbits

2.1.1.1 Animal maintenance

A model of steroid-induced ocular hypertension was established New Zealand albino rabbits. The holding and experimental protocols were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Experimental Ethics Committee of The Chinese University of Hong Kong. All personnel in this project obtained valid Animal License from the Hong Kong SAR Government.

Male rabbits of 7-week-old (body weight ranging from 1 to 1.5 kg) were recruited from the Laboratory Animal Service Center, The Chinese University of Hong Kong. The rabbits were transferred to the rabbit holding room of the Department, and maintained with regular feeding with rabbit chow, dry grass and water. The bedding was changed every day and cages were washed every week. The temperature of holding room was maintained at 20-22°C.

2.1.1.2 Topical steroid treatment

The rabbits received topical application of Maxi-Dex® (0.1% dexamethasone ophthalmic suspension, Alcon), 4 times daily for a continuous 14 to 53 days. Each ml

Maxi-Dex® contains 0.1% dexamethasone as active ingredient, 0.01% benzalkonium chloride as preservative, 0.5% hypromellose as vehicle and sodium chloride, dibasic sodium phosphate, polysorbate 80, disodium edetate, citric acid, sodium hydroxide (for pH 6-8) and purified water. Alternatively, the rabbits received topical Ocu-Pred Forte® (0.5% prednisolone acetate ophthalmic suspension, Allergan); Vexol® (1% rimexolone ophthalmic suspension, Alcon) each ml Vexol® contains 1% rimexolone as active ingredient, 0.01% benzalkonium chloride and carbomer 974P, polysorbate 80, sodium chloride, disodium edetate, sodium hydroxide and/or hydrochloric acid and purified water; or FLAREX® (0.1% fluorometholone acetate ophthalmic suspension, Alcon) each ml FLAREX® contains 0.1% fluorometholone acetate as active ingredient, 0.01% benzalkonium chloride and sodium chloride, monobasic sodium phosphate, disodium edetate, hydroxyethyl cellulose, tyloxapol, hydrochloric acid and/or sodium hydroxide (for pH 6-8) and purified water. The topical treatment was performed on both rabbit eyes 4 times daily (at 9 am, 12 noon, 3 pm and 6 pm). The rabbits were removed from the cage and laid calmly on the bench. The eye was maintained open by pressing slightly on the upper and lower eyelids. One drop of steroid ophthalmic suspension was applied to the conjunctival sac and the eyelid was closed with gentle pressing for 1-2 times to evenly distribute the eye drop on the corneal surface. The eyelash was cleaned afterwards before the rabbit returned back to the cage.

2.1.1.3 Drug treatment

TriButyrate® (sodium 4-phenylbutyrate powder, from Fyrklovern Scandinavia, Sweden) is weighed to 18.6 gm in a sterile container. It is dissolved in 90 ml distilled water with stirring at 100 rpm in dark (covered with tin foil) at room temperature. The pH was adjusted to pH 7.0. The final volume was adjusted to 100 ml with distilled water, to make a 1 M stock solution. The solution was then filter-sterilized through millipore filter (0.22 μm pore size) to a sterile container. The sterile solution was diluted in normal saline (0.9% sodium chloride, B. Braun Medical Industries, US) or balanced salt solution (BSS, B. Braun Medical Industries) to the appropriate 0.01-15 mM concentration. The pH was again checked and adjusted to pH 7. The final solution was sterilized by millipore filtration (0.22 μm pore size) and aliquot to 5 ml eye drop bottles, which were labeled properly and stored at 4°C until use.

Alternatively, a stock concentration of 5 M trimethylamine N-oxide (TMAO) was prepared in distilled water, adjusted to pH 7 and sterilized by millipore filtration (0.22 μm pore size). The sterile solution was further diluted in normal saline (0.9% sodium chloride, B. Braun Medical Industries, US) or balanced salt solution (BSS, B. Braun Medical Industries) to the appropriate 50-300 mM concentrations. The pH was again checked and adjusted to pH 7. The final solution was sterilized by millipore filtration (0.22 μm pore size) and aliquot to 5 ml eye drop bottles, which were labeled properly and stored at 4°C until use.

For topical treatment procedure, the drug eye drop was applied at least 10 minutes after Maxi-Dex® or other steroids eye drop. The treatment was carried out one to four times daily. The contralateral eyes received steroids and vehicle only.

2.1.1.4 Measurement of intraocular pressure

The intraocular pressure (IOP) of rabbits was measured solely by me and I was masked from the experimental details: eye drop treatment to the rabbit and eye drop treatment to the eyes. The IOP measurement was done by using TonoPen XL in the first 2 experiments and TonoVet for the rest of experiments. In every time of IOP measurement, 10 consecutive readings were obtained. With the highest and lowest values removed, the mean and standard deviation was calculated using the remaining 8 readings. To avoid the diurnal fluctuations of IOP level, IOP was measured twice with the first measurement performed at 10 am and second measurement at 2 pm. The average of the two mean IOP was considered as the IOP on that day.

Prior to eye drop application (day 0), the baseline IOP was obtained, which was the mean of 2 measurements taken at the same time of -1 and -2 day. During the drug treatment, IOP was measured twice a week with an interval of 3 to 4 days between each measurement.

For the use of TonoPen XL, calibration was conducted every time before IOP measurement. It was performed by bringing the TonoPen upside down. For the use of

TonoVet, the probe was loaded just before IOP measurement.

2.1.1.5 Monitoring of animal behavior and other changes

The rabbits were weighed when they arrived at the holding facility. They were weighed regularly every week during the treatment.

Before and during topical treatments, the rabbits were closely monitored every day for their behaviors, including diarrhea, depilation, and other abnormal activity. For the ocular responses, they were checked before and after every eye drop treatment for eye redness, inflammation, ulcer and other abnormalities.

Since these young rabbits were 7 weeks old, 3 rabbits were put in a cage to reduce their behavioral stress. However, when there was diarrhea or depilation, they were transferred to separate cages and the former cage was washed immediately to prevent any spreading of germs or bacteria.

2.1.2 Histochemistry analysis of trabecular meshwork region

2.1.2.1 Sample collection and fixation

The rabbits were sedated by intramuscular injection of ketamine (75 mg/kg)/ xylazine (7.5 mg/kg). The eye surface was flushed by BSS and dried.

Sample of aqueous humor was collected by 1 ml syringe with 30-G needle. After the corneal incision, the aqueous humor fluid was removed from the anterior chamber, and 50-100 μl per sample was collected and stored temporally on ice.

Sample of vitreous humor was collected by 3 ml syringe with 25-G needle. At 1 mm below the limbus position, the needle was inserted through the sclera into the posterior chamber. About 0.5 ml per sample was collected and stored temporally on ice.

Both aqueous and vitreous samples were centrifuged at 10000 rpm for 10 minutes at 4°C. Clear supernatant was collected to a new sterilized 1.5 ml eppendorf tube, which was properly labeled. The samples were stored at -80°C until analysis.

The rabbit was then sacrificed by intravenous overdosed sodium pentobarbital. The eyes were enucleated, washed extensively with PBS to remove blood trace. A small piece of cornea-limbus-sclera tissue of 2 x 4 mm^2 was finely trimmed and immersed into 4% glutaldehyde in 0.01 M phosphate buffer (PB) at pH 7.4 for electron microscopy. The remaining eye globe was put to 10% neutral buffered formalin for light microscopy.

2.1.2.2 Light microscopy

2.1.2.2.1 Paraffin processing and sectioning

The formalin-fixed eyeballs were cut to two pieces along the midline from pupil to optic nerve head (PO position). After rinses in PBS, one eyecup was transferred to plastic cassette. The sample was dehydrated through a series of graded ethanol baths with increasing concentration (30%, 70%, 95% and absolute ethanol) in a processing machine (Shandon Excelsior, Thermo). After the clearing in xylene, the sample was infiltrated with paraffin. The infiltrated tissue was then embedded (with the open edge facing downwards) as a wax block using the Embedding Center (EG1160, Leica, Germany). A mold that best corresponds to the size of rabbit eyeball was chosen before the embedding procedure.

Paraffin sectioning was done with microtome (RM2135, Leica, Germany) and thin sections (5 μm in thickness) were serially obtained. Each section was extended on 30% alcohol followed by floating on 42°C water bath and collected on the glass slide (HistoBond, Marienfeld). The sections were dried thoroughly and stored at room temperature until staining.

2.1.2.2.2 Histochemical staining

The paraffin sections were placed in a 60°C oven for 1 hour for wax melting and secure attachment of sample on glass surface. The sample was then deparaffinized in xylene solution and rehydrated using a graded series of alcohol baths from absolute to

95% and 70% ethanol concentration, and finally to water.

For haematoxylin and eosin (H&E) staining, the section was immersed in CAT haematoxylin solution (Biocare Medical) for 10 minutes for nuclei staining. After rinsing in running tap water, the staining was differentiated by putting the section in 1% acid alcohol for 3 seconds, rinsed immediately in running tap water and then put in Scott's tap water for two minutes until blue coloration appeared. The sample was then put in 1% aqueous eosin for 10 minutes for cytoplasm staining.

For Periodic acid Schiff (PAS) staining, the section was treated with 1 % periodic acid for 10 minutes and washed. It was then covered with Schiff's reagent for 10 minutes. After this, the section was rinsed with running tap water for 10 minutes. It was then immersed in CAT haematoxylin solution for 30 seconds to counterstain nuclei, rinsed and in Scott's tap water for 1 minute for color differentiation.

For Masson trichrome staining, which is commonly used to identify muscle and collagen, the section was put in Weiger's iron haematoxylin (freshly prepared with the mix of equal volume of haematoxylin solution and iron solution) for 12 minutes for nuclear staining. After washes in running tap water, the color was put in 1% acid alcohol briefly and rinsed with water immediately. After blue color differentiation in Scott's tap water for two minutes, the sample was left in 0.5% acid fuchsin solution

for 3 minutes to stain the cytoplasm and muscle. It was then put in 1% phosphotungstic acid for 8 minutes to remove the excessive color from the structures not belonged to cytoplasm and muscle. Then, it was put in 0.5 % light green for 1.5 minutes for the staining of collagen.

After the staining procedure, the sections were brought to water, and then dehydrated through a series of ethanol baths (70%, 95% to absolute ethanol) and cleared in xylene. They were then mounted with Canada Balsum and dried.

2.1.2.2.3 Image acquisition

The mounted slide was put to a light microscope (Leica, Wetzlar, Germany) equipped with SPOT RT color system (Diagnostic Instruments, Serling Heights, MI, USA). TM region was located and images were captured and properly labeled for further analysis.

2.1.2.2.4 Quantification analyses of TM tissue changes

The TM region was identified as spongy tissue below the scleral veins. Using Image J software (from National Institutes of Health, NIH, US), the TM area was measured. Haematoxylin stained cell nuclei within this area were quantified. The density of cells in TM region was calculated by number of nuclei in TM area (mm^2). The data was compared among control, treatment with steroids and treatment with steroids and drugs. Statistical significance was calculated by independent Student's *t*-test.

2.1.2.3 Electron microscopy

2.1.2.3.1 Sample processing

After 2 hour fixation at 4°C, the glutaldehyde-fixed tissue was washed in 0.01 M PB. It was trimmed to small pieces of 1 x 1 x 1 mm^3 in size. The samples were then post-fixed in 1% aqueous solution of osmium tetroxide for 1 hour at room temperature and were rinsed in distilled water extensively. They were then dehydrated in a graded series of alcohol of increasing concentration (70%, 85%, 95% and absolute alcohol).

2.1.2.3.2 Epon 812 infiltration and embedding

The dehydrated samples were put to Epon 812 infiltration with gentle agitation:

1. Epon 812 : absolute ethanol = 1 : 1 vol/vol for 1 hour

2. Epon 812 : absolute ethanol = 2 : 1 vol/vol for 3 hours

3. Pure Epon 812 for overnight

4. Pure Epon 812 for 1 hour

The samples were then put embedding mold and overlaid with pure Epon 812 and left in a 60 °C oven for at least 18 hours for complete solidification.

2.1.2.3.3 Ultrathin sectioning

Ultrathin sections (~70 nm in thickness) of the TM region were obtained using an ultra-microtome (EM UC6, Leica, Germany). The sections were collected on copper

grids and dried completely.

2.1.2.3.4 Contrast-staining

To identify the microstructures of TM region more clearly, contrast staining should be performed. Before starting the contrast staining, distilled water was boiled for 10 minutes to remove carbon dioxide (CO_2) and cooled to room temperature.

The section was floated on 3% aqueous solution of uranyl acetate for 40 minutes at room temperature in dark. It was then rinsed with water and air-dried for 10 minutes. It was transferred to 0.2 N freshly prepared sodium hydroxide for 1 second, lead citrate solution (42% lead nitrate, 42% sodium citrate and 16% 1 N sodium hydroxide, freshly prepared) for 30 seconds, and then to 0.2 N sodium hydroxide. After rinsed with water, the section was air-dried.

2.1.2.3.5 Transmission electron microscopy and image acquisition

The section was examined using a transmission electron microscope (Hitachi H7100FA) in the School of Biomedical Sciences, The Chinese University of Hong Kong (CUHK). The TM region was located and images of different magnifications were captured.

2.1.3 Drug safety study

2.1.3.1 Behavioral changes

Before and during experiment, the behavior of rabbits was monitored every day, including diarrhea, depilation, and any abnormal behavior. The rabbit eyes were also checked before and after each eye drop application for any redness, inflammation and other abnormalities.

2.1.3.2 Cornea surface changes

2.1.3.2.1 Slit lamp biomicroscopy

The rabbits were sedated by intramuscular injection of ketamine (75 mg/kg)/ xylazine (7.5 mg/kg). The eye surface was flushed by BSS and dried. The examination of cornea surface, limbus and conjunctiva regions as well as corneal epithelium condition by fluorescein staining were performed using a slit lamp biomicroscope (Carl Zeiss), and pictures were captured using SPOT RT color system (Diagnostic Instruments, Serling Heights, MI, US).

2.1.3.2.2 Corneal endothelial cell count

The sedated rabbits were transferred to specular microscopy (SP-3000P, Topcon) and corneal endothelial cells were visualized. The density of corneal endothelial cells were recorded and compared among rabbits receiving different treatment or controls.

2.2 *In vitro* experiments

2.2.1 Human trabecular meshwork cell line culture

Immortalized human trabecular meshwork (HTM) cells were kindly provided by Dr. Thai D. Nguyen (Polansky et al., 1979; Nguyen et al., 1998). The cells were grown in DMEM low glucose (1,000 mg/l D-glucose, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gibco), sodium pyruvate (110 g/ml), and antibiotics (100 units/ml penicillin G and 100 g/ml streptomycin sulfate, Invitrogen) and incubated at 37°C in a humidified chamber with 5% CO_2 balanced with air. One day before treatment, HTM cells were trypsinized and plated on tissue culture dishes (60 mm in diameter, Nunc).

2.2.2 Dexamethasone and small molecule chemical chaperone treatment

Small molecule chemical treatment was started at least 12 hours after cell seeding. HTM cells were washed once with sterile phosphate buffered saline (PBS, 0.1 M, Invitrogen) and refreshed with medium (DMEM low glucose supplemented with 10% charcoal-stripped FBS (Invitrogen) and antibiotics. Dexamethasone (Dex, 100 nM, water-soluble, Sigma), and/or sodium 4-phenylbutyrate (triButyrate®, NaPB, 1 mM, Fyrklovern Scandinavia, Sweden), was added to the culture. Fresh medium containing specified drug was replenished every other day.

2.2.3 Microarray analysis

2.2.3.1 RNA Preparation and Qualification

Total RNA enriched and purified by RNeasy extraction kit and RNase-free DNase on-column digestion was determined for their high quality to meet the requirement for microarray experiment.

2.2.3.1.1 Size distribution

This RNA quality control analysis was performed by Agilent 2100 bioanalyzer (detection for RNA degradation and DNA contamination) with an Agilent RNA 6000 Nano LabChip® Kit (for qualitative measurement of RNA).

2.2.3.1.2 Determination of RNA concentration

The RNA concentration was measured by UV absorbance at 260 nm using Nanodrop ND-1000 UV-Vis spectrophotometer spectrophotometer. An A260 of 1 equals RNA concentration of ~40 ng/μl.

2.2.3.2 Synthesis of Cyanine 3- and Cyanine 5-labeled cDNA

The procedure followed the instruction supplied by Agilent Fluorescent Direct Label Kit. In brief, total RNA (10 μg) was required per labeling reaction and it was adjusted to a volume of 24 μl with nuclease-free water. DNA primer (1 μl) was added to reach reaction and the mixture was incubated at 70°C for 10 minutes to denature the primer and template RNA. After cooling on ice for 5 minutes, cyanine 3-dCTP or

cyanine 5-dCTP (1 mM, 1.25 µl) was added, together with the cDNA master mix (23.75 µl). The cDNA synthesis reaction was performed at 42°C for 60 minutes, followed by heating to 70°C for enzyme inactivation. The sample was then put on ice for 5 minutes with brief spinning. RNase 1A (1 µl) was added to the sample at room temperature for 30 minutes to digest the remaining RNA.

Preparation of cDNA master mix

	Volume (µl) / reaction
Nuclease-free water	6
5x First Strand buffer	10
0.1 M DTT	5
dNTP (no dCTP)	0.5
5 mM dCTP	0.25
MMLV-RT	2

2.2.3.3 Purification of labeled cDNA

The Cy3 and Cy5-cDNA reactions were combined to ~100 µl volume and mixed with five volume of PBS. The sample was loaded to a QIAquick spin column in a 2 ml collection tube and spun at 10,000 g for 1 minute. The flow-through was discarded and the column was washed two times with 0.4 ml PE buffer (Qiagen). After spinning, the column was transferred to a new collection tube. EB buffer (30 µl, Qiagen) was applied to the column to elute the labeled cDNA.

2.2.3.4 Concentration of labeled cDNA

The solution was dried under vacuum in a rotary desiccator and the dry sample was stored at -80°C until hybridization.

2.2.3.5 Hybridization

The dried cDNA targets were reconstituted in 100 µl nuclease-free water and heat denatured at 98°C for 3 minutes, then cool to room temperature. It was then mixed with 25 µl 10x control targets and 125 µl 2 x hybridization buffer (Agilent Oligonucleotide Microarray Hybridization kit). Using a 1 ml syringe with 25-G needle, the mixture was slowly injected into the septum installed in the chamber. The hybridization chamber was placed on the rotator rack in the hybridization oven and incubated with rotation at 60°C for 17 hours. A bubble was formed during incubation and move about in the solution to aid the mixing during hybridization.

2.2.3.6 Washings and slide drying

Three staining dishes were prepared before the disassembly of hybridization chamber. The chamber was immersed in Wash Solution 1 and disassembled to release the glass slide, which was placed immediately to the second staining dish with Wash Solution I and washed for 10 minutes with stirring at room temperature. The slide was then transferred to Wash Solution II on ice and washed for 5 minutes with stirring. The slide surface was dried by nitrogen blowing.

2.2.3.7 Array data acquisition

The fluorescence intensity was measured by loading the dried slide into the Agilent's dual-laser Microarray Scanner (G2565BA) using 532 nm laser line for Cy-3 and 633 nm for Cy-5 signal collection.

2.2.3.8 Data extraction and normalization

The signal image was visualized and digitalized by Feature Extraction software v 9.5.3.1 (Agilent Technologies), in which the background signal, non-uniform signal and the average raw signal on each probe were automatically calculated. The resulting data files were generated and transferred to GeneSpring GX version 7.3.1 (Agilent Technologies) for further analysis. In order to compare samples on different arrays and obtain meaningful data, several normalization methods in GeneSpring were applied including per gene (normalize to median) normalization and per chip (normalize to 50^{th} percentatile) normalization. The cut-off value for the differential change of gene expression was set as 2 fold for the data analysis. Gene ontology (GO term) and pathway analysis was performed with references to the pathway information downloaded from KEGG database (ftp://ftp.genome.jp/pub/kegg/).

2.2.4 Gene expression analysis

2.2.4.1 Sample collection

HTM cells without treatment, HTM cells treated with 100 nM Dex and HTM cells treated with both 100 nM Dex and 1 mM NaPB were collected at different time points: 0, 10 and 30 minutes, 1, 2, 6 and 12 hours, 1, 7 and 10 days post-treatment. Cells were cultured in tissue culture dish (60 mm diameter, Nunc) and the treatment was performed. At specific time points, the medium was removed and cells were added with 350 µl RLT buffer (RNeasy Extraction kit, Qiagen) freshly added with 1% β-mercaptoethanol (Sigma). The cell lysate was collected in a RNase/DNase-free 1.5 ml eppendorf tube and immediately stored at -80°C until RNA extraction.

2.2.4.2 Total RNA extraction and purification

Total RNA was extracted using a commercially available extraction kit (RNeasy extraction kit) following manufacturer's protocol. In brief, RLT-extracted lysate was homogenized through a Qiashredder column (Qiagen) under a full-speed centrifugation for 2 minutes at room temperature for the release of RNA and DNA. The lysate solution was mixed with 350 µl 70% ethanol and put to the RNeasy column with spinning at 8000 rpm for 15 seconds at room temperature. The flow-through was discarded. The column-bound RNA was then washed with 350 µl RW1 buffer (RNeasy extraction kit) and flow-through was discarded. On-column DNA digestion was carried out by putting RNase-free DNase (10 µl DNase stock diluted in 70 µl RDD buffer, Qiagen, according to manufacture's instruction) to the

column and incubated for 15 minutes at room temperature. The reaction was stopped by washing the column with 350 µl RW1 buffer. The RNA bound in column was then cleaned by washes with RPE buffer (ethanol-added, according to manufacture's instruction in RNeasy kit) twice with centrifugations at 8000 rpm for 15 seconds. The purified RNA was then eluted with 30 µl RNase-free water. The quantity (ng/µl) and quality (absorbance at 260 nm wavelength / absorbance at 280 nm wavelength) of the purified RNA was determined by Nanodrop ND-1000 UV-Vis spectrophotometer (NanoDrop technologies, Wilmington, DE, USA) and NanoDrop 3.1.0 sofware, with the zero level referenced to the RNase-free water used for elution. The RNA samples were immediately stored at -80°C until reverse transcription.

2.2.4.3 Reverse transcription

The purified total RNA (1 µg) was used to synthesize cDNA by using SuperScript III cDNA synthesis kit (Invitrogen), following the manufacturer's instructions. RNA was diluted in RNase-free water added with 1 µl of dNTP mixture (10 mM, Roche, Basel, Switzerland), 1 µl random hexamer (250 ng/µl; Qiagen). The mixture was made up to a volume of 13 µl and heated to 65°C for 5 minutes, followed by ice incubation for 2 minutes. The mixture was then added with 4 µl 5x First-Strand Reaction Buffer (Invitrogen), 1 µl $_{DL}$-DTT (0.1 M), 1 µl RNaseOUT™ recombinant RNase inhibitor and 1 µl SuperScript™ III reverse transcriptase. The reaction condition was set to 25°C for 5 minutes for primer annealing, at 50°C for 60 minutes for strand elongation, at 70°C for 15 minutes for enzyme denaturation, and finally at 4°C in a thermal cycler

(BioRad). The synthesized cDNA was collected and stored at -20°C until PCR analysis.

2.2.4.4 Candidate gene expression analysis

2.2.4.4.1 Polymerase chain reaction

Expression of genes of interest and housekeeping GAPDH was analyzed by semi-quantitative PCR. In brief, the PCR mixture was composed of 10x PCR buffer (Qiagen), $MgCl_2$ (optimized between 1 to 3 mM final concentration, Qiagen), 1 mM dNTP mix (Roche), 1 μM forward primer, 1 μM reverse primer, 1 U platinum *Taq* DNA polymerase (Qiagen) and cDNA from 50 ng total RNA. The reaction volume was brought to 25 μl with autoclaved milliQ water. The PCR was performed in a thermal cycler (iCycler; BioRad, Hercules, CA) and the condition was set as initial denaturation at 95°C for 2 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58 to 61°C (optimized by preliminary runs) for 30 seconds to 1 minute and extension at 72°C for 1 to 2 minutes, and a final extension at 72°C for 5 minutes. The primer sequences, amplification conditions and number of cycles were listed in **Table 2**.

2.2.4.4.2 Agarose gel electrophoresis

The PCR product (10 μl) was mixed well with 2 μl 6x DNA loading buffer (Invitrogen) and resolved by agarose gel electrophoresis. Agarose gel (2%) was made by dissolving 1.5 gm agarose (BioRad) in 75 ml 0.5x TAE (Tris-acetic acid-EDTA)

buffer (BioRad) using microwave heating (900 W until complete dissolution). After a brief cooling down to 50 to 60°C, the gel solution was added with 0.01% of ethidium bromide (EB) and poured to the agarose gel holder and comb was inserted. The gel solution was allowed to solidify for 30 minutes at room temperature. The gel was then placed in the gel tank containing 0.5x TAE buffer and the wells were rinsed.

PCR products were mixed well with 6x DNA loading dye (Invitrogen) in a ratio of DNA:dye = 5:1 vol/vol. The mixtures were loaded to appropriate wells. DNA size standard (50 base-pair) markers (BioRad) were also loaded to one well for size determination. The gel was run at a constant 135 voltage (V) for 20-25 minutes until the frontier reached the lower one-third of the gel length. Signal bands were visualized under UV transillumination and images were captured using Gel-Doc (BioRad) and analyzed.

2.2.4.4.3 Statistical analyses

Specific band intensity and the corresponding background intensity were measured by Quantity One® Image Analysis software (BioRad). The relative expression of gene of interest was normalized by housekeeping GAPDH. It was then compared between control and treated samples and mean and standard deviation were obtained for statistical analysis using independent Student's *t*-test. P value below 0.05 was considered to be significant.

Table 2. Expression primer sequences.

Gene	Specific primer sequences (5' to 3')	Product size (bp)	Annealing T	Cycle number
APOD	F: CTGCATCCAGGCCAACTAC R: TGGATGATGCAGGTACAGG	237	58 ℃	35
CA12	F: GCGGCAGGACTGAGTCTGGG R: CAAGCGTGGGCCTCAGTCTC	175	58 ℃	30
Caspase3	F: TATTCTTGGGGAAATTCAAAGGAT R: AAAGTAGCGTCAAAGGAAAAGGA	158	58 ℃	30
c-fos	F: AGACAGACCAACTAGAAGATGA R: AGCTCTGTGGCCATGGGCCCC	457	58 ℃	32
c-jun	F: GTGACGGACTGTTCTATGACTG R: GGGGGTCGGCGTGGTGGTGAT	297	58 ℃	30
HSP70	F: AAGTACAAAGCGGAGGACG R: GATGGGGTTACACACCTGC	249	58 ℃	30
HSP90a	F: ACCCAGACCCAAGACCAACCG R: ATTTGAAATGAGCTCTCTCAG	141	58 ℃	30
IGF2	F: ACACCCTCCAGTTCGTCTGT R: GGGGTATCTGGGGAAGTTGT	211	58 ℃	30
IGFBP4	F: AGCCCCTGTAGCGCCCATGA R: ACTTGCCACGCTGCCCATCC	292	58 ℃	30
IL1B	F: ACGCTCCGGGACTCACAGCA R: TGAGGCCCAAGGCCACAGGT	234	58 ℃	35
SAA2	F: TGGCGAGGCTTTTGATGGGGC R: AGTCCTCCGCACCATGGCC	205	58 ℃	30
TGFB3	F: CCAGCTCTAAGCGGAATGAG R: ATTGGGCTGAAAGGTGTGAC	230	58 ℃	35
ZNF343	F: CCAGAAAAAGGAGGGAAAGG R: CAGAAGGCAGGAGGAACAAG	563	58 ℃	30
GAPDH	F: GAAGGTGAAGGTCGGAGT R: GAAGATGGTGATGGGATTTC	225	58 ℃	25

2.2.5 Protein analyses

2.2.5.1 Sample collection and processing

Total protein from HTM cells without treatment, HTM cells treated with 100 nM Dex and HTM cells treated with both 100 nM Dex and 1 mM NaPB was collected at different time points: 1, 7 and 10 days post-treatment. In brief, cells were cultured in tissue culture dish (60 mm diameter, Nunc) and the treatment was performed. At specific time points, the medium was collected in a 15-ml centrifuge tube. Cells were washed with ice-cold PBS (0.01 M) and the washing buffer was also collected in the same 15-ml tube. The cells on dish were added with radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris-HCl (Sigma), 150 mM sodium chloride, 1% Nonidet P-40 (Sigma), 0.25% sodium deoxycholate (Sigma), protease inhibitor cocktail (Roche) and 1 mM phenylmethyl sulfonylfluoride (PMSF, Sigma) at a concentration of $2.5x10^6$ cells/ml. The cells were scrapped immediately and collected in a sterile 1.5-ml eppendorf tube on ice. The medium and PBS wash in 15-ml tube was spun at 1500 rpm for 5 minutes at 4°C. The supernatant was removed and cell pellet was resuspended in the RIPA lysis buffer containing the same sample. When cells of the same treatment were collected, the lysis was performed on ice for 30 minutes. After centrifugation at 4°C for 15 minutes, the supernatant was collected and denatured in sample buffer with a final concentration of 50 mM Tris HCl (pH 6.8), 2% sodium dodecylsulfate (SDS, BioRad), 10% glycerol, 0.002% bromophenol blue (Sigma), 1% β-mecaptoethanol (Sigma), and 50 mM DL-dithiothreitol (DTT; Sigma) at 95°C for 5 minutes. All protein samples were stored at -80°C until electrophoresis.

2.2.5.2 Denaturing gel electrophoresis and protein transfer

PROTEAN III SDS-polyacrylamide gel electrophoresis system (SDS-PAGE, BioRad) was used to resolve the denatured protein. In brief, a resolving gel was made up of 10 to 15% acrylamide: 0.3% bis-acrylamide, 0.375 M Tris.HCl (pH 8.8), 0.1% SDS, 0.05% ammonium persulfate (Sigma) and 0.02% TEMED (Sigma), and the gel mixture was set for polymerization at room temperature for 30 minutes. A stacking gel with a final concentration of 4% acrylamide: 0.15% bis-acrylamide, 0.125 M Tris.HCl (pH 6.8), 0.1% SDS, ammonium persulfate and TEMED were allowed to polymerize on top of the resolving gel for another 30 minutes.

Total protein sample equivalent to $7.5x10^4$ cells was loaded to each well. Five µl of size standard marker (Precision plus protein™ dual color standards, BioRad) was applied to indicate protein sizes during separation. The samples were mobilized through the gel encompassed in a Tris/glycine/SDS running buffer (BioRad) at 70 V for 30 minutes and then 150 V for 60 minutes. After electrophoresis, the gel was equilibrated in chilled transfer buffer, which consisted of 25 mM Tris, 192 mM glycine and 10% methanol, for 5 minutes before the blotting of proteins to a pre-wet nitrocellulose/ECL membrane (GE Healthcare, Pittsburgh, PA) at a constant 100 V for 1.5 hour on ice.

2.2.5.3 Immunostaining

After transfer, the membrane was rinsed once with distilled water and then transferred to TBST buffer (Tris buffered saline with 0.05% Tween-20; Sigma) added with 5% blocking powder (Santa Cruz) for blocking the non-specificities at room temperature for 1 hour. Primary antibody solution was prepared by diluting the antibody stock to the appropriate concentration in TBST buffer. The membrane was then transferred to the primary antibody solution and incubated with rotation for 2 hours at room temperature or overnight at 4°C. After TBST washes for 4-5 times, the membrane was incubated in TBST added with secondary Ig antibody conjugated with horseradish peroxidase (HRP) (Jackson Immuno. Res., West Grove, PA) for 1 hour at room temperature. All antibodies and concentrations are listed in **Table 3**.

2.2.5.4 Signal detection, data acquisition and statistical analysis

After TBST washes, the staining signal was revealed by enhanced chemiluminescence (ECL, GE Healthcare) using a ChemiDoc™ System (BioRad). Gel imaging and band densitometry was analyzed by Quantity One® 4.6.2 (BioRad). The specific protein expression was normalized with housekeeping GAPDH from the same sample. Statistical significance was determined by independent Student's *t* test.

Table 3. Concentrations of antibodies used in Western Blotting

Antibodies	Company	Origin	Concentration
Anti-GAPDH	Sigma	Mouse	1:2000
Anti-caspase3	Imgenex	Mouse	1:500
Horseradish peroxidaseconjugated IgG	Jackson Immuno. Res., West Grove, PA	Goat anti mouse	1:5000

2.2.6 Cell viability/proliferation assay

Cells ($2x10^5$ cells/ml) were seeded to 24-well plate (Corning) one day before the treatment. On day 1, 5 and 10 post-treatment, the cells were assayed by MTT cell viability/proliferation test. At time of testing, 20 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) working solution (5 mg/ml in culture medium and filter-sterilized) was added to the medium in each well and incubate at 37 °C for 4 hours. The medium was removed without disturbing the cells and the purple crystals were resolved by 150 µl MTT solvent (4 mM HCl, 0.1% Nonidet-40 in isopropanol) in dark with shaking at 100 rpm. The plate was transferred to a plate reader (Powerwave XS, Bio-Tek Instruments) and the signal was measured by absorbance at wavelength 570 nm with reference to 630 nm. The experiment was done in quadriplicate and means and standard deviations were obtained. The percentage of cell viability was calculated as: mean OD_{570} in treated sample / mean OD_{570} in control x 100% and analyzed using independent Student's *t* test.

2.2.7 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay

The assay was performed using *In Situ* Cell Death Detection Kit (Roche), following the manufacturers' protocol. In brief, cells grown on glass coverslips were fixed with a freshly prepared 4% neutral buffered paraformaldehyde for 1 hour at room temperature. The sample was rinsed with PBS and put to permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate in PBS) for 2 minutes on ice. After washes,

the permeabilized sample was incubated in 50 μl TUNEL reaction mixture (TdT enzyme solution: label solution = 1:9 vol/vol) in a humidified atmosphere for 1 hour at 37 °C in dark. DNase I-treated cells were used as positive control (cells incubated with 30 U/ml DNase I in 50 mM Tris-HCl, pH 7.5 and 1 mg/ml BSA for 10 minutes at room temperature to induce DNA strand breaks) and sample incubated without TdT enzyme was used as negative control. After washes, the nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI, Sigma, 100 ng/ml) for 45 minutes. The sample was washed and mounted with Fluoromount G on a piece of glass slide. The staining signal was observed using a Leica DMRB fluorescence microscope (Leica, Wetzlar, Germany) equipped with SPOT RT color system (Diagnostic Instruments, Serling Heights, MI, US). Terminal apoptosis rate was represented as the percentage of TUNEL positive cells. For each experiment (n=3), 10 random images (x20 objective) were analyzed.

Chapter 3: Results

3.1 *In vivo* treatment on rabbit model of steroid-induced ocular hypertension

3.1.1 Steroid-induced IOP elevation in rabbits

To adopt an animal model of steroid-induced ocular hypertension, I studied our previous work on the age-dependency of steroid effect in rabbits (Qin et al., 2010). IOP changes caused by different steroids on 7-week-old, 6-month-old and 1-year-old New Zealand albino rabbits were compared. It was shown that corticosteroids, in particular Maxi-Dex® induced the most drastic IOP rise in 7-week-old rabbits. In a two-week treatment with Maxi-Dex®, these rabbits about 40% IOP rise (from mean baseline 10.7 mmHg to 14.7 mmHg).

This model was repeated in my experiment by topical Maxi-Dex® treatment to male New Zealand albino rabbits of 7-week-old. Before drug administration, the baseline IOP was measured for two consecutive days (same time of each day) using Tonopen XL or Tonovet. In each time, ten IOP measurements were obtained and the mean IOP and standard deviation were calculated after the removal of the highest and lowest IOP values and were represented as mmHg. Topical Maxi-Dex® (0.1% dexamethasone, Alcon), Pred Forte® (0.5% prednisolone acetate, Alcon) eye drop was then applied on both eyes 4 times daily for a minimum of 14 days. IOP was measured in a blind mode (from the details of eye drop treatment) every 3 or 4 days at 2 standard time points: 10 am and 2 pm. This was to minimize the diurnal effect of IOP fluctuation in rabbits. Again, after removal of the highest and lowest IOP values

of each time point, the mean IOP and standard deviation of IOP of the day were calculated.

Like human, rabbits also exhibit different levels of steroid responsiveness. In the experiments, the tested rabbits were categorized into non-responder (<20% IOP change), mild responder (20 to 50% IOP rise) and severe responder (>50% IOP rise) (Knepper et al., 1978; Knepper et al., 1985). All were testified after 14 days of topical steroid treatment. In our animal experiments, topical Maxi-Dex® or Pred Forte® raised up the IOP in 75% of rabbits, which defining a successful establishment of a rabbit model of steroid-induced ocular hypertension (**Figure 7**).

In my study, I recruited 150 rabbits, but not all of them were included in the result analyses. That is because some were non-steroid-responders (IOP rise less than 20% in 14 days of steroid treatment), and some died during the course of experiments. One leading cause of death was diarrhea, and others were fighting injury, paralysis and minor bleeding (not in ocular region). The death were not due to the drug treatment (steroids or NaPB/small molecule chemicals), as those rabbits which survived until the end of experiments did not show any systemic adverse effect. And most of the rabbits could be alive until they were sacrificed for further studies.

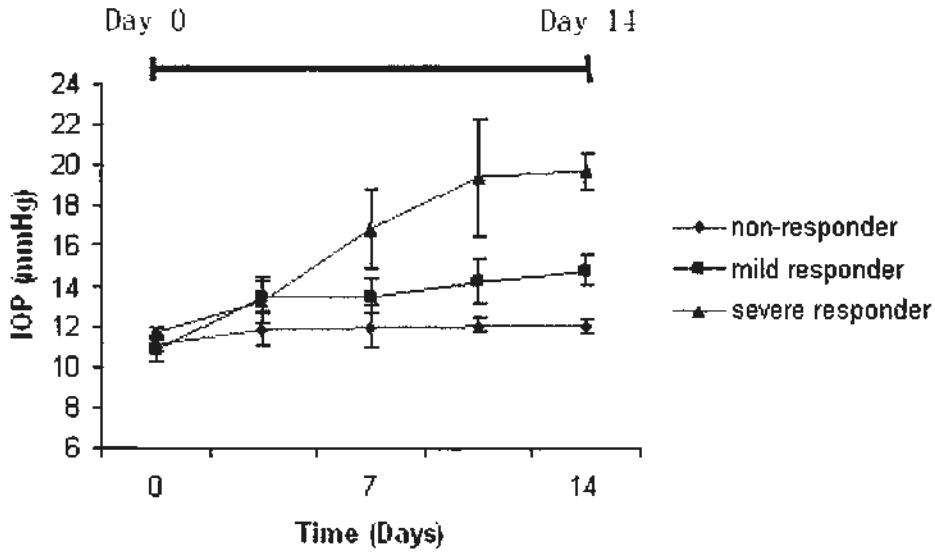

Figure 7. Intraocular pressure trend of rabbit eyes treated with dexamethasone of non-responder, mild responder and severe responders. After 14 days treatment of Dexamethasone, for the non-responder, IOP rise within 20%. For mild responder, IOP rise between 20%-50%. IOP rise more than 50% for the severe responder.

3.1.2 Effect of small molecule chemicals on steroid-induced ocular hypertension

TMAO (Sigma) and NaPB are small molecule chemicals that are commonly reported to have chaperoning activity for defective protein biosynthesis and protein misfolding in eye diseases (Yam et al., 2007; Gong et al., 2009; Jia et al., 2009; Gong et al., 2010). Both have been shown to alleviate biosynthetic stress in cells, induce heat-shock response and ultimately promote cell survival. As shown by *in vitro* study, Dex treatment on HTM cells caused elevated cell death, which was subdued by the co-treatment with NaPB, indicating a possible cell rescue effect. Furthermore, patients with ocular hypertension-associated glaucoma were found to have fewer functional TM cells, suggesting a cause of increased resistance to the aqueous humor outflow (Fuchshofer et al., 2009).

To examine if small molecule chemicals could have the any effect of experimental steroid-induced ocular hypertension on rabbits, sterile eye drop of TMAO (50 to 300 mM) or NaPB (0.01 to 15 mM) was prepared in normal saline (NS) or balanced salt solution (BSS) as vehicle. The drug was topically applied 4 times daily on the right eye of rabbit at 10 minutes after topical Maxi-Dex®. Left eye received vehicle only after Maxi-Dex®. The treatment was carried out for a minimum of 14 days. IOP was measured every 3 or 4 days at 10 am and 2 pm of the day. After removal of the highest and lowest IOP values of each time point, the mean IOP and standard deviation were calculated. IOP ratio between Maxi-Dex®/drug-treated and Maxi-Dex® only eyes

was calculated and referenced to the different dose of small molecule chemicals.

TMAO, a natural osmolyte with reported chemical chaperoning activity to stabilize protein folding, was attempted to evaluate its effect on Dex-induced IOP changes. It was reported that TMAO ranging from 25 to 300 mM could alleviate ER stress and apoptosis on G98R CRYAA-transfected human lens epithelial B3 cells, and this effect was dose-dependent (Gong et al., 2009). Hence, 3 doses (50, 100 and 300 mM) of TMAO were selected to prepare eye drop in normal saline.

In this experiment, 15 rabbits were divided into 3 groups and 3 doses (50, 100 and 300 mM) of TMAO were tried. Five rabbits for each group. The baseline IOP was 10.8 ± 0.9 mHg. At day 14 of treatment, eyes combined treatment with TMAO of 50, 100 and 300 mM did not reduce Dex-induced IOP rise, and showed similar IOP trend (**Figure 8**). Compared with contralateral Dex-treated rabbit eyes, the p-values were 0.846 for 50 mM TMAO (paired Student's *t*-test, n=4), 0.862 for 100 mM TMAO (paired Student's *t*-test, n=4), and 0.922 for 300 mM TMAO (paired Student's *t*-test, n=3), respectively. All rabbits had no sign of ocular and systemic discomfort.

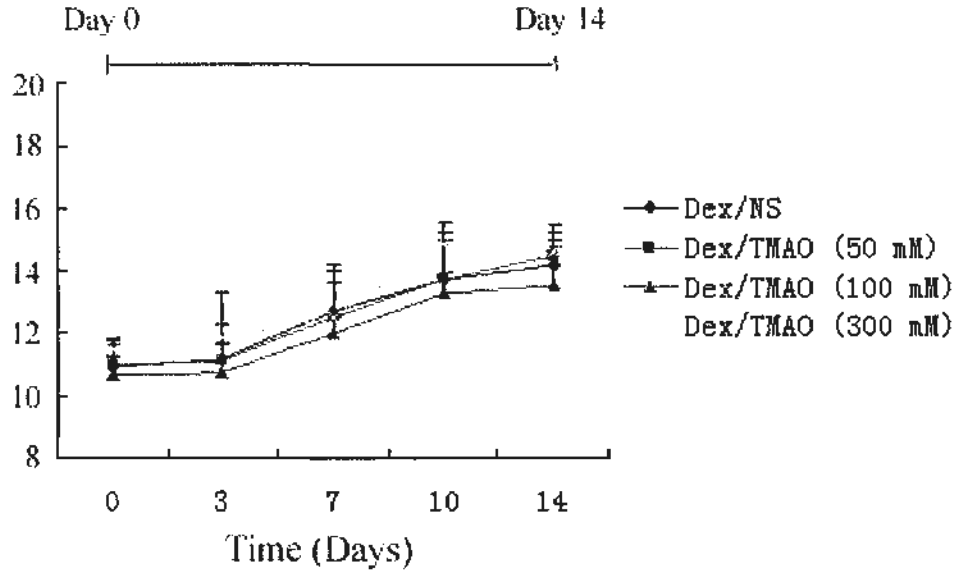

Figure 8. Effect of trimethylamine N-oxide on dexamethasone-induced ocular hypertension. IOP trend of Dex/TMAO-treated and Dex-treated rabbit eyes for 50 mM, 100 mM and 300 mM of TMAO 4 times daily. The IOP levels were not significantly changed.

As NaPB optimized at 1 mM was reported to improve the detergent solubility of a congenital cataract-causing G165fsX8 mutant CRYGD in COS-7 cells and attenuate the ER stress and apoptosis (Gong et al., 2010). In this experiment, 9 rabbits were divided into 3 groups and 3 doses (1, 5, 15 mM) of NaPB prepared in normal saline were tried. The eye drop was applied 4 times daily to the right eye of rabbit at least 10 minutes after the topical Maxi-Dex®. This allowed sufficient uptake of dexamethasone by the eye before the possible dilution by NaPB. The left eye received normal saline only, at 10 minutes after Maxi-Dex®. The eye drop was carried out for 49 days.

In the representative experiment, 9 rabbits were divided into 3 groups and 3 doses (1, 5 and 15 mM) of NaPB were tried. Three rabbits for each group. The baseline IOP was 11.9±0.6 mmHg. At day 14 of treatment, eyes combined treatment with NaPB of 1, 5 and 15 mM reduced Dex-induced IOP rise significantly and showed similar IOP trend. The IOP reduction percentages were 14.7±1.5% ($p=0.049$, paired Student's *t*-test, n=3), 13.4±1.9% ($p=0.004$, paired Student's *t*-test, n=3), 13.6±1.3% ($p=0.001$, paired Student's *t*-test, n=3), respectively, compared to contralateral Dex-treated rabbit eyes, respectively (**Figure 9**). In this experiment, the IOP reduction percentage of the 3 groups did not have significant difference. The p-values were 0.587 compared between 1 and 5 mM NaPB groups, 0.922 between 5 and 15 mM NaPB groups, and 0.582 between 1 and 15 mM NaPB groups (paired Student's *t*-test, n=3). The IOP reduction effect of 1, 5 and 15 mM NaPB did not have significant difference. So,

NaPB had reduction effect of Dex-induced IOP rise, whereas TMAO did not affect the IOP level. During the experiment, all rabbits had no sign of ocular and systemic discomfort.

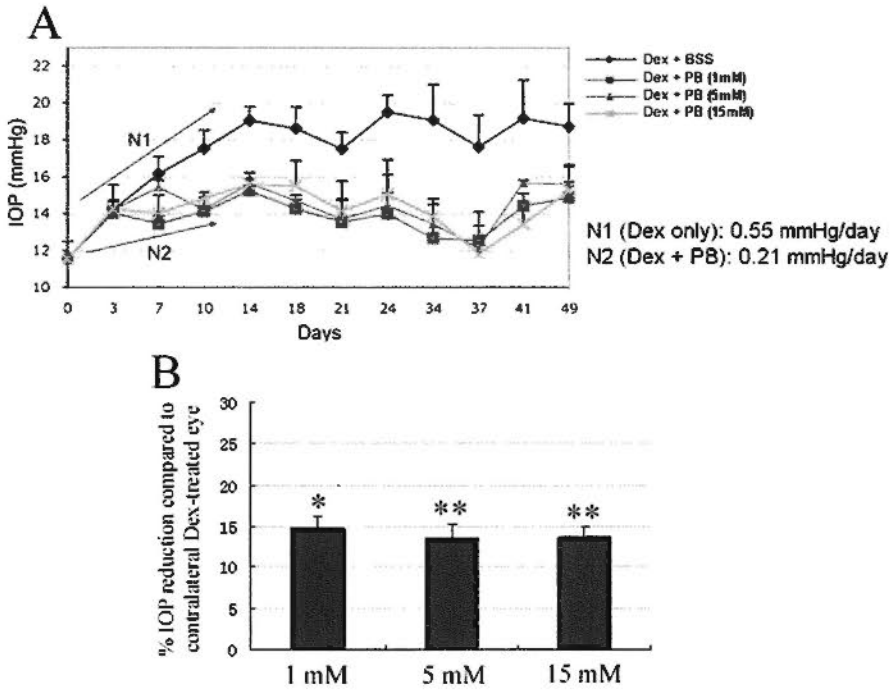

Figure 9. Effect of sodium phenylbutyrate on dexamethasone-induced ocular hypertension. (A) IOP trend of Dex/NaPB-treated and Dex-treated rabbit eyes for 1, 5 and 15 mM NaPB 4 times daily. **(B)** IOP reduction percentage on day 14 compared with contralateral Dex-treated eyes. NaPB reduced Dex-caused IOP rise, but there were no significant difference between 1, 5 and 15 mM NaPB. * indicates $p<0.05$; ** indicate $p<0.01$, paired Student's *t*-test (n=3), compared with contralateral Dex-treated eyes.

3.1.3 Optimal dose and frequency of triButyrate® on steroid-induced ocular hypertension

As the reduction effect of 1, 5 and 15 mM NaPB on experimental steroid-induced ocular hypertension showed no distinct dose-dependent relationship. It was reasonable to hypothesize that the minimum effective dose of NaPB is below 1 mM. Hence, NaPB of 0.01, 0.1, 0.5 and 1 mM in concentration were attempted and topically applied to the right rabbit eyes randomly to search for the minimum effective dose.

Summarized from this experiment and the representative study (27 rabbits), NaPB between 0.01 and 15 mM all showed reduction effect on Dex-induced IOP rise, the baseline IOP was 11.9±0.5 mHg. On day 14, compared with contralateral Dex-treated eyes, the IOP reduction percentages were 17.1±8.9% ($p=0.229$, paired Student's *t*-test, n=3) for 0.01 mM, 24.7±4.3% ($p=0.004$, paired Student's *t*-test, n=3) for 0.1 mM, 27.7±3% ($p=0.025$, paired Student's *t*-test, n=3) for 0.5 mM, 20.4±6.6% ($p=0.036$, paired Student's *t*-test, n=6) for 1 mM, 13.4±1.9% ($p=0.004$, paired Student's *t*-test, n=3) for 5 mM, 13.6±1.3% ($p=0.001$, paired Student's *t*-test, n=3) for 15 mM (**Figure 10**). Among them, the IOP reduction percentage of 0.5 mM NaPB was the highest. Hence, this suggested the optimal dose of NaPB was 0.5 mM. All rabbits had no sign of ocular and systemic discomfort.

To examine the effect of frequency of NaPB treatment on Dex-caused IOP rise, NaPB of 0.5 mM was applied for different frequencies (1, 2 or 4 times daily). On day

14, compared with contralateral Dex-treated eyes, the IOP reduction percentages were 1.9±0.7% (p=0.866, paired Student's *t*-test, n=3) for NaPB applied once a day, 9.1±4.8% (p=0.289, paired Student's *t*-test, n=4) for 2 times daily, and 18.5±4.8% (p=0.048, paired Student's *t*-test, n=6) for 4 times daily (**Figure 11**). Among them, the IOP of 0.5 mM NaPB treatment applied 4 times daily was the most significantly lowered on day 14 (p=0.048, paired Student's *t*-test, n=6). So, the optimal frequency of 0.5 mM NaPB should be 4 times daily. All rabbits had no sign of ocular and systemic discomfort.

Next, I examined if NaPB possessed similar effect of IOP rise caused by other steroids. NaPB of 0.5 mM was applied for different frequencies (1, 2 or 4 times daily) after topical Pred Forte® (0.5% prednisolone acetate, Alcon). On day 14, compared with contralateral prednisolone-treated eyes, the IOP reduction percentages were 3.2±1.9% (p=0.607, paired Student's *t*-test, n=3) for NaPB applied once a day, 12±4.4% (p=0.412, paired Student's *t*-test, n=3) for 2 times daily, and 14.1±3.6% (p=0.011, paired Student's *t*-test, n=4) for 4 times daily (**Figure 12**). Among them, the IOP of 0.5 mM NaPB treatment applied 4 times daily was the most significantly lowered on day 14 (p=0.011, paired Student's *t*-test, n=4). So, the IOP reduction effect of NaPB could be replicated in prednisolone-treated eyes. All rabbits had no sign of ocular and systemic discomfort.

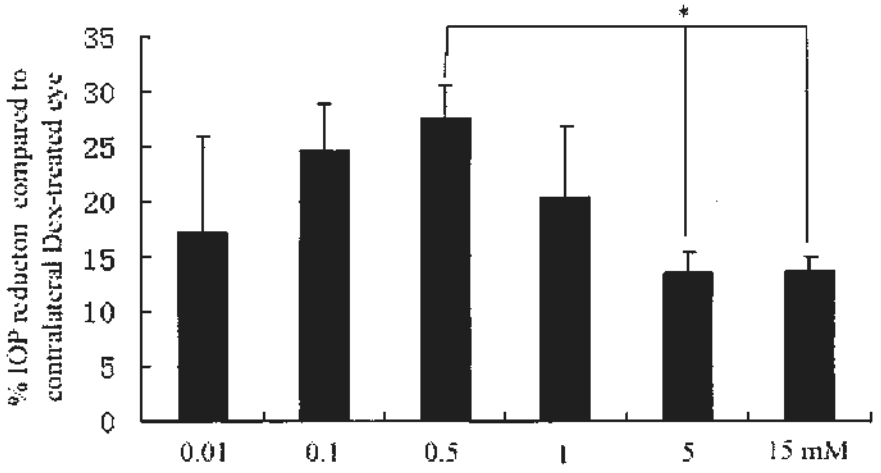

Figure 10. Effect of sodium phenylbutyrate on dexamethasone-induced intraocular pressure changes. IOP reduction percentage compared with contralateral Dex-treated eyes on day 14 of 0.01, 0.1, 0.5, 1, 5 and 15 mM NaPB, 4 times daily. 0.5 mM, 4 times daily of NaPB achieved the highest IOP reduction percentage compared with contralateral Dex-treated eyes. * indicates $p<0.05$, paired Student's *t*-test.

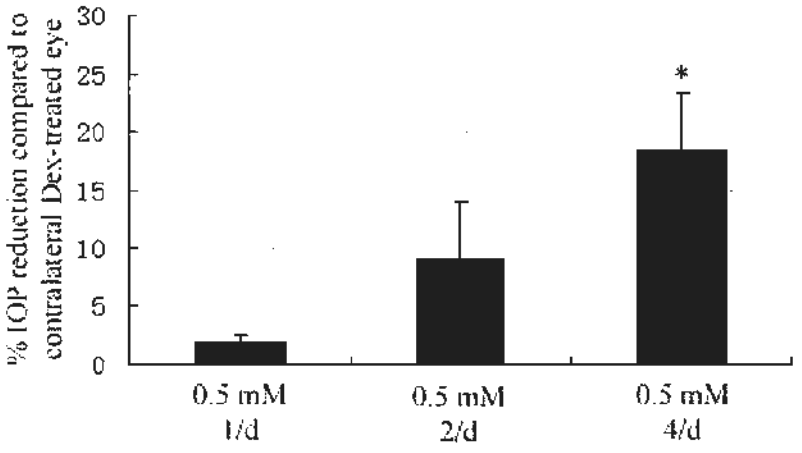

Figure 11. Effect of topical frequencies of sodium phenylbutyrate on dexamethasone-induced intraocular pressure changes. IOP reduction percentage compared with contralateral Dex-treated eyes on day 14 of 0.5 mM NaPB for 1, 2 and 4 times daily. NaPB applied 4 times daily achieved the highest IOP reduction percentage compared with contralateral Dex-treated eyes. * indicates $p<0.05$, paired Student's *t*-test, compared with contralateral Dex-treated eyes.

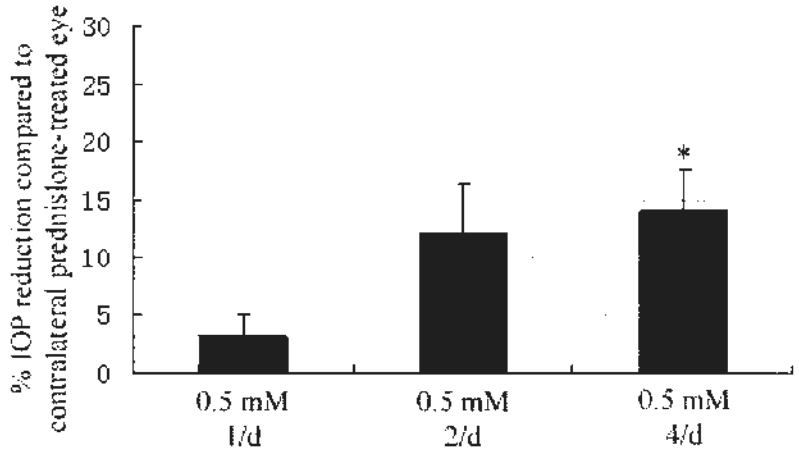

Figure 12. Effect of topical frequencies of sodium phenylbutyrate on prednisolone-induced intraocular pressure changes. IOP reduction percentage compared with contralateral prednisolone-treated eyes on day 14 of 0.5 mM NaPB for 1, 2 and 4 times daily. NaPB applied 4 times daily achieved the highest IOP reduction percentage compared with contralateral prednisolone-treated eyes. * indicates $p<0.05$, paired Student's *t*-test, compared with contralateral Dex-treated eyes.

3.1.4 Effect of triButyrate® and NaPB (Sigma) on steroid-induced ocular hypertension

As sodium 4-phenlybutyrate (NaPB) reduced steroid-induced ocular hypertension, to examine the effect of NaPB of different companies, triButyrate® and NaPB (Sigma) of 0.5 mM, 4 times daily were applied at least 10 minutes after Maxi-Dex®. On day 14, compared with contralateral Dex-treated eyes, the IOP reduction percentages were 13.5±3.6% ($p=0.213$, paired Student's *t*-test, $n=4$) for triButyrate®, and 13.7±2.1% ($p=0.080$, paired Student's *t*-test, $n=3$) for NaPB (Sigma) (**Figure 13**). The p-value was 0.973 compared between these two groups (paired Student's *t*-test, $n=3$), so the IOP reduction effect of triButyrate® and NaPB (Sigma) of 0.5 mM, 4 times daily were similar on steroid-induced ocular hypertension. During the experiment, all rabbits had no sign of ocular and systemic discomfort.

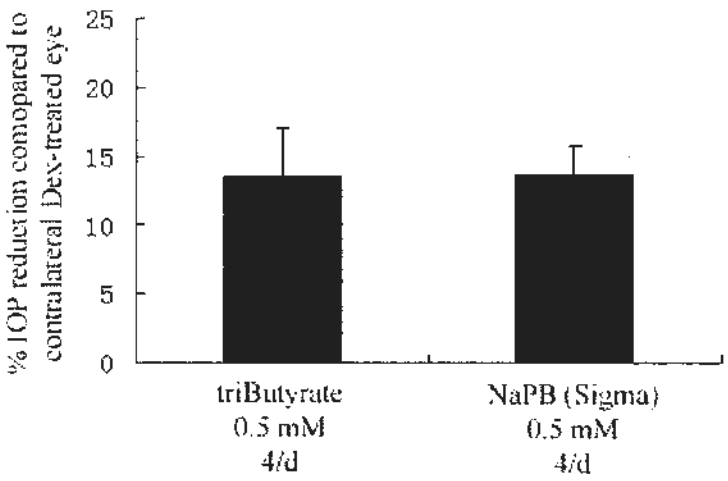

Figure 13. Effect of sodium phenylbutyrate (triButyrate® and Sigma) on dexamethasone-induced intraocular pressure changes. IOP reduction percentage compared with contralateral Dex-treated eyes on day 14 of 0.5 mM NaPB (triButyrate® or Sigma) for 4 times daily. The IOP reduction effect did not have significant difference between triButyrate® and NaPB (Sigma)

3.1.5 Effect of Ammonul® on steroid-induced ocular hypertension

Sodium 4-phenylbutyrate (NaPB) is permeable through tissue cells and metabolized rapidly through beta-oxidation into phenylacetate, which conjugates with glutamine to form acetylglutamine and is excreted in urine (Hommes, 1999). The metabolites of NaPB provide two nitrogen atoms for the defective urea disorders for urinary excretion of waste nitrogen products, so NaPB was originally used as an ammonia-scavenging agent in urea metabolism disorders (Brusilow et al., 1984).

To examine if phenylacetate, the metabolite of NaPB has similar effect on steroid-induced ocular hypertension as NaPB, Ammonul® (sodium phenylacetate and sodium benzoate, Ucyclyd) and triButyrate® of 0.5 mM, 4 times daily were applied at least 10 minutes after Maxi-Dex®. On day 14, compared with contralateral Dex-treated eyes, the IOP reduction percentages were 13.5±3.6% ($p=0.213$, paired Student's *t*-test, n=4) for triButyrate®, and 10.7±2.5% ($p=0.568$, paired Student's *t*-test, n=5) for Ammonul® (**Figure 14**). The p-value was 0.541 compared between these two groups (paired Student's *t*-test, n=4). Although it was not statistically significant, the IOP reduction by Ammonul® was not as effective as that of triButyrate®. During the experiment, all rabbits had no sign of ocular and systemic discomfort.

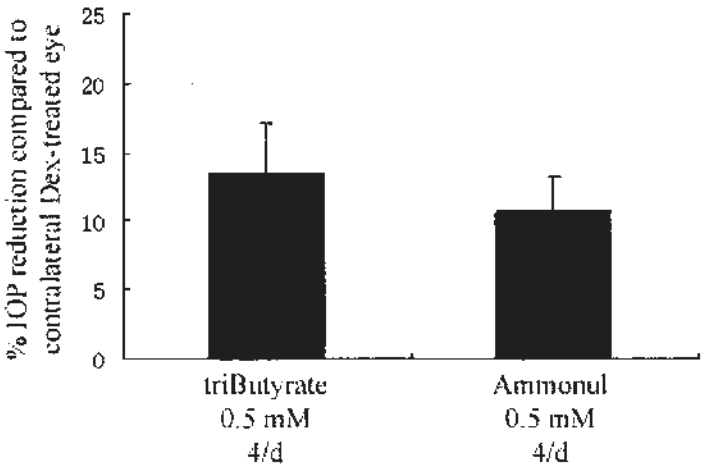

Figure 14. Effect of sodium phenylbutyrate and sodium phenylacetate on dexamethasone-induced intraocular pressure changes. IOP reduction percentage compared with contralateral Dex-treated eyes on day 14 of 0.5 mM NaPB (triButyrate®) and Ammonul® for 4 times daily. The IOP reduction effect of Ammonul® was not as good as that of triButyrate®.

3.1.6 Toxicity effect of triButyrate® on eye surface

Because NaPB lower than 1 mM had reduction effect on steroid-induced ocular hypertension, to investigate the safety of NaPB eye drop on cornea surface of the rabbits, the rabbit eyes which received 1 or 5 mM of NaPB or Maxi-Dex® combined with 1 mM of NaPB 4 times daily for 8 weeks were examined by a slit lamp biomicroscope and corneal endothelial cell density was recorded.

During topical treatment of steroid and small molecules, the rabbits were healthy and they gained weight during holding and treatment. Also, they did not exhibit any abnormal behavior. From the slit lamp examination, the rabbit eyes received Maxi-Dex® and NaPB showed no observable corneal opacity, ulcer, and neovascularization. In the pictures, the cornea was smooth and transparent. The anterior chamber was clear and no kerato-precipitates or empyemata were observed (**Figures 15A, 16A, 17A, and 18A**). To examine if there was any corneal epithelial defect, fluorescence examination was performed and the pictures were captured. And in these pictures, the fluorescence showed blue and no obvious yellow and green colour can be observed. It showed no corneal epithelial defects and inflammation (**Figures 15B, 16B, 17B, and 18B**).

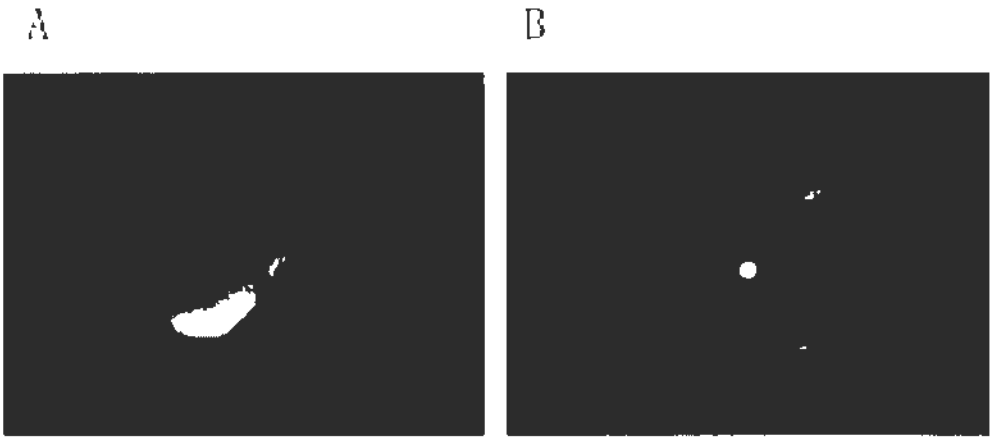

Figure 15. Effect on sodium phenylbutyrate (1 mM)-treated rabbit eye surface. **(A)** Slit lamp examination showed no observable corneal opacity, ulcer, and neovascularization. **(B)** Fluorescence examination showed no corneal epithelial defects.

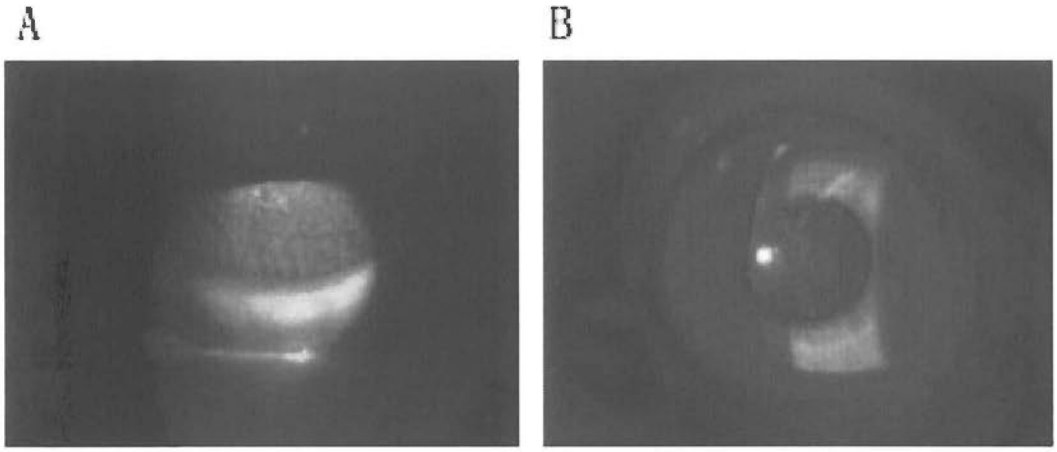

Figure 16. Effect on sodium phenylbutyrate (5 mM)-treated rabbit eye surface. **(A)** Slit lamp examination showed no observable corneal opacity, ulcer, and neovascularization. **(B)** Fluorescence examination showed no corneal epithelial defects.

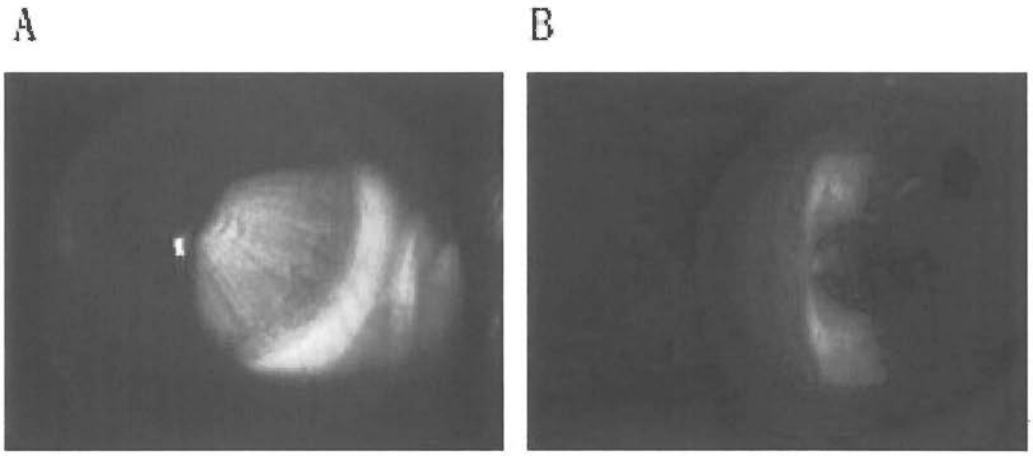

Figure 17. Effect on dexamethasone/sodium phenylbutyrate (1 mM)-treated rabbit eye surface. (A) Slit lamp examination showed no observable corneal opacity, ulcer, and neovascularization. **(B)** Fluorescence examination showed no corneal epithelial defects.

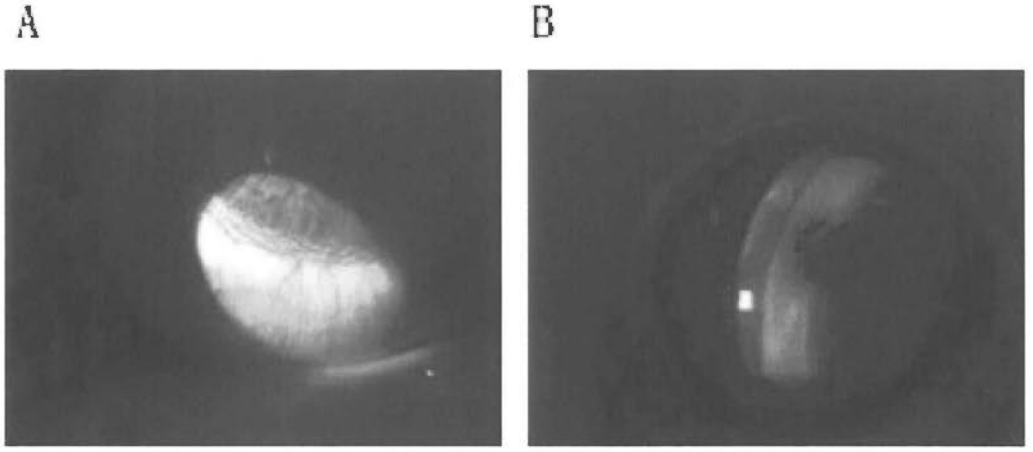

Figure 18. Effect on dexamethasone-treated rabbit eye surface. (A) Slit lamp examination showed no observable corneal opacity, ulcer, and neovascularization. **(B)** Fluorescence examination showed no corneal epithelial defects.

Corneal endothelial cell count is a standard way to test the drug-induced toxicity in the anterior segment. In this work, the toxicity effect of Dex/NaPB treatment was monitored by corneal endothelium number changes. The sedated rabbits were transferred to specular microscopy and corneal endothelial cells were visualized. The density of corneal endothelial cells were recorded and compared among rabbits receiving different treatment or controls. In the pictures, the corneal endothelial cells were hexagon in shape, and they were orderly lined up (**Figure 19A**). The average numbers of corneal endothelium cells of the rabbit eyes treated with NaPB (1 mM), NaPB (5 mM), Dex/NaPB (1 mM) and Dex only were more than 2000. This suggested no endothelial cell loss/gain, and the numbers of corneal endothelial cells were similar (**Figure 19B**).

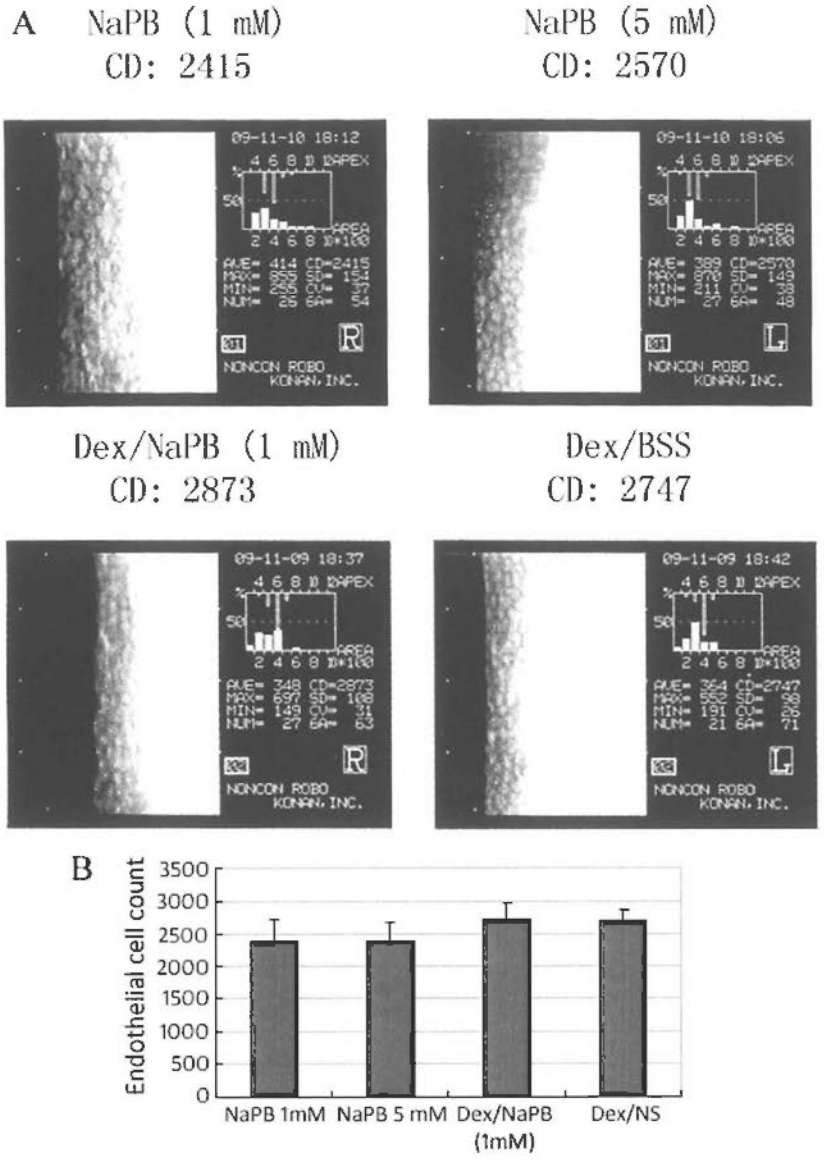

Figure 19. Effect of sodium phenylbutyrate on corneal endothelial cell count. (A) Corneal endothelial cell counting showing no loss/gain of corneal endothelial cells after the treatment of Dex/NaPB. **(B)** The numbers of corneal endothelial cells of different treatments were similar.

3.1.7 Effect of steroid on TM region of rabbit eyes

Steroid-induced ocular hypertension has long be postulated to associate with the change of extracellular matrix composition in the trabecular meshwork region and this blocks or resists the aqueous humor outflow, causing a rise of intraocular pressure. It was until recently that in steroid-treated bovine eyes, an accumulation of plaque-like materials in the extracellular matrix (Tektas et al., 2010). They were shown under electron microscopy to contain fibrillar basement membrane-like material and were located underneath the endothelium of the inner wall of the outflow loops. The deposition thus could block the aqueous humor outflow and a rise of IOP. In rabbits, topical steroid treatment for as long as 14 days could substantially thicken the extracelluar matrix materials, and reduce cellularity in the TM region (Qin et al., 2010).

In my experiment, the effect of dexamethasone on the morphological changes of rabbit TM region was examined. After sedation, the rabbits were sacrificed by overdosed pentobarbital. The eyes treated with Maxi-Dex® or normal saline for 14 days were enucleated and fixed immediately in 10% neutral buffered formaldehye. After processing for paraffin infiltration and embedding, serial paraffin sections were obtained. Consecutive sections were stained with H&E (hematoxylin and eosin), PAS (periodic acid-Schiff) and masson trichrome histochemistry. Located by the scleral vein, behind the anterior chamber, the cells in the TM region were quantified by counting number nuclei stained with hematoxylin and the area was measured by

Image J analysis (NIH). The cell density was calculated by the number of nuclei in TM region in unit area (represented as mm^2). The cell density was compared between steroid-treated and control groups. In normal rabbit eyes, the TM cell density was $3666\pm359/mm^2$ (mean ± standard deviation) (**Figure 20**). After 14 days of Maxi-Dex® topical treatment, the TM cell density was dropped to $1636\pm186/mm^2$. By independent Student's *t*-test test, the cell reduction was found to be significant ($p=0.007$, $n=3$).

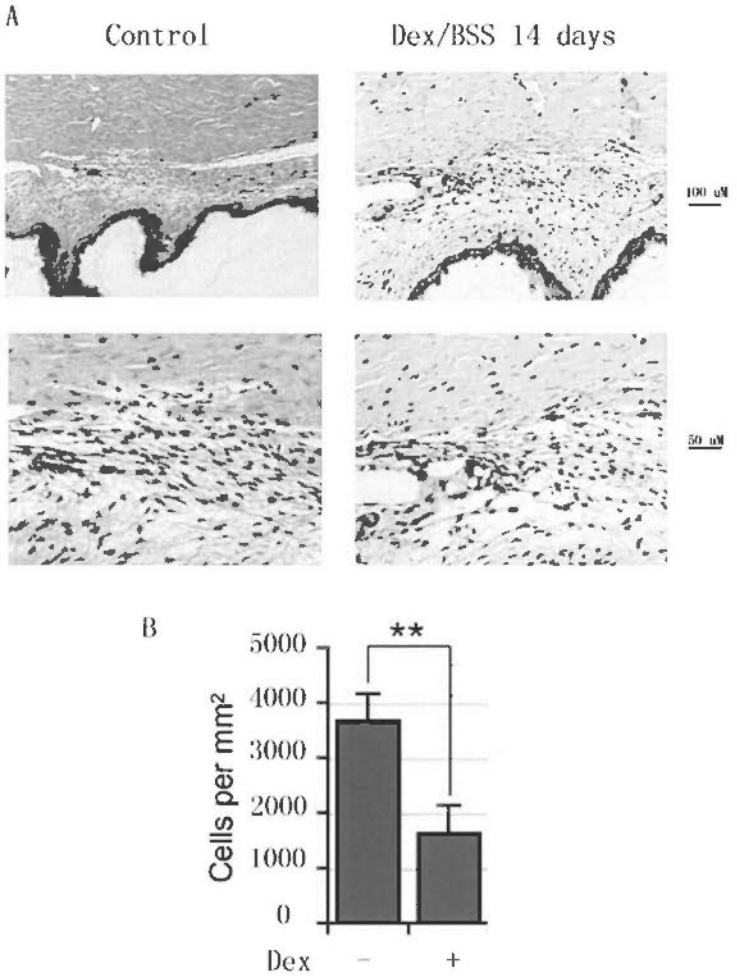

Figure 20. Light micrographs of rabbit trabecular meshwork region. (A) H&E staining of TM regions of no drug and Dex-treated rabbit eyes. **(B)** TM cell density calculation showed TM cell loss on Dex-treated eyes. ** indicates $p<0.01$ by independent Student's *t*-test, n=3.

Periodic acid-Schiff staining is commonly used to reveal glycogen, glycoprotein, and proteoglycans content in the tissue. In the TM region of normal rabbit eyes, it showed clear subendothelial region and uniform glycogen content in the intercellular space (**Figure 21A**). At day 14 of Maxi-Dex® topical treatment, more extracellular materials within the cribriform layers and glycogen loss could be observed (**Figure 21B**). There were plaques underneath the endothelium of all outflow loops, and they were compact. The plaques underneath the outer wall of the loops were smaller, fewer and more irregular distributed (**Figure 21B**).

Masson trichrome histochemistry is commonly used to reveal collagen content in the tissue. On the slides, the collagen stained green, the plasma was grey, and the cell nuclei were dark brown to black. In the TM region of normal rabbit eyes, it showed mild collagen content in the intercellular space (**Figure 22A**). At day 14 of Maxi-Dex® topical treatment, the TM extracellular matrix had obvious deposition of collagen-like materials. It also showed thickening of extracellular matrix materials like a dark line in the drainage pathway (**Figure 22B**).

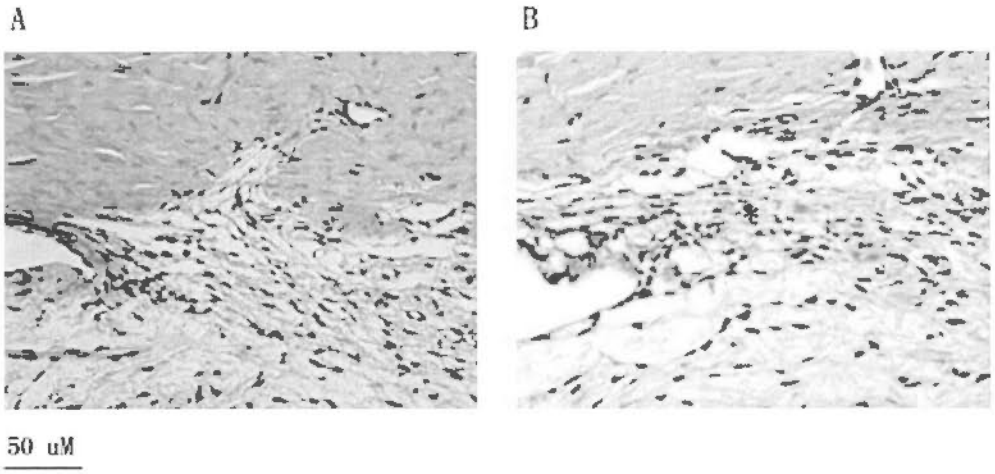

Figure 21. Light micrographs of rabbit trabecular meshwork region with Periodic Acid schiff staining. (A) No drug treated TM region of rabbit eyes. No ECM thickening and glycogen loss. **(B)** Dex-treated TM region of rabbit eyes 14 days. It showed ECM deposition, plaques and glycogen loss (*).

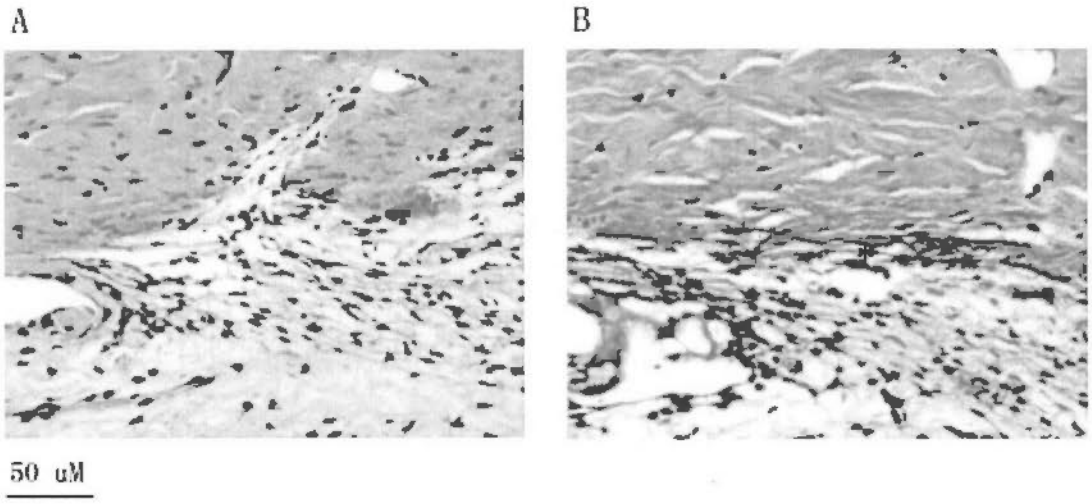

Figure 22. Light micrographs of rabbit trabecular meshwork region with Masson trichrome staining. (A) No drug treated TM region of rabbit eyes. No ECM thickening and glycogen loss. **(B)** Dex-treated TM region of rabbit eyes 14 days. It showed ECM deposition and collagen accumulation (*).

When study the ultrastructural morphology, it was reported that there was a loss of glycogen in the cytoplasm of TM cells, an increase in basement membrane-like material, and enlarged cisterns of rough ER of TM cells in steroid-treated bovine eyes (Tektas et al., 2010).

To examine the TM ultrastructural change caused by steroid in our rabbit model, the anterior chamber angle region of 2 x 2 mm^2 were collected and fixed with 4% glutaldehyde, and ultrathin sections were obtained and stained by uranyl acetate-lead citrate method. In untreated control eyes, clear extracellular matrix region and unblocked TM channels were shown. It showed that the TM region was connect to the outflow pathway of aqueous humor via fiber net (**Figure 23**). In the TM region after treatment with Maxi-Dex® 4 times daily for 42 days, there was an accumulation of plaques and irregularly arranged basement membrane-like materials and blocked channel region (**Figure 24A**). The extracellular matrix materials were increased such as microfilament and microtubules, cytoplasmic vacuolation, and intercellular amorphous materials. The fibers were thickened, and crosslinked (**Figure 24A, B**). Moreover, enlarged rough endoplasmic reticulum (ER) lumen was observed (**Figure 24B**).

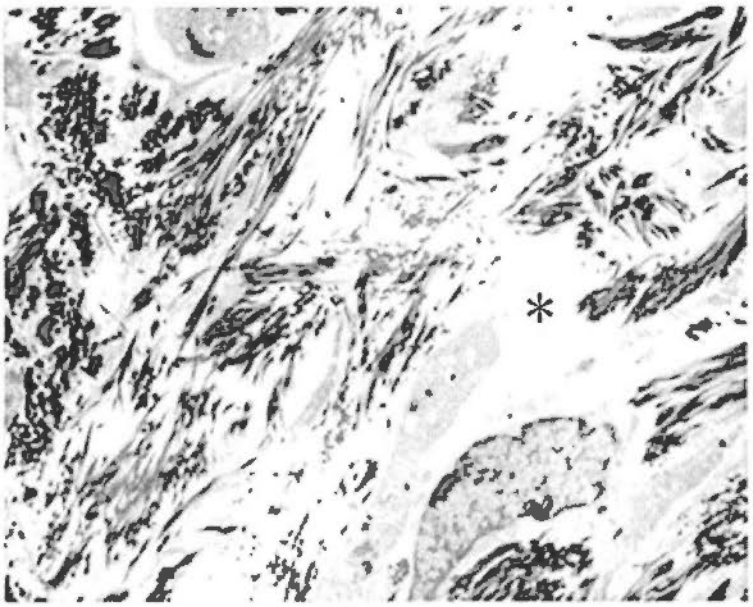

Figure 23. Transmission electron micrograph of trabecular meshwork region of control eyes. Clear extracellular matrix regions and unblocked TM channels (*) were shown.

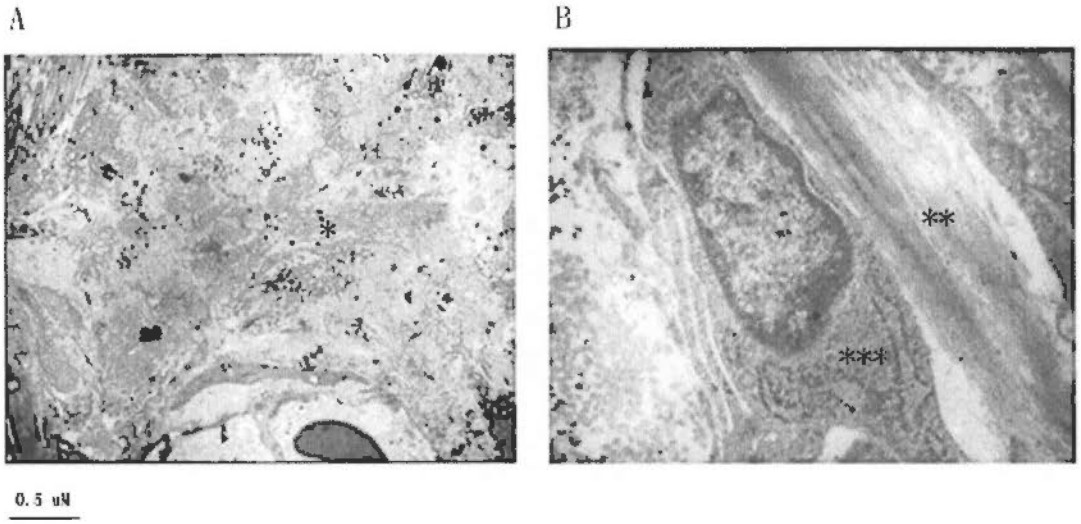

Figure 24. Transmission electron micrographs of trabecular meshwork region of dexamethasone-treated rabbit eyes. (A) It showed unclear TM channel and more accumulation of plaques (*). **(B)** It showed basement membrane-like materials in the extracellular region (**) and dilated ER inside cell (***).

3.1.8 Effect of triButyrate® on TM region of rabbit eyes

To illustrate the effect of NaPB on the morphological changes of TM region in rabbit eyes, PAS staining were also performed on the eyes treated with Dex and NaPB. After 14 days of combined treatment with NaPB, PAS staining showed a much mild extracellular materials within the cribriform layers and glycogen loss (**Figure 25B**). The plaques underneath the endothelium of all outflow loops were not as compact as that of Dex-treated eyes. The plaques underneath the outer wall of the loops were not obvious compared with Dex-treated eyes (**Figure 25B**).

Masson trichome staining was also performed on the TM region of Dex/NaPB-treated eyes. On the slides, the collagen stained green and the plasma was grey. On Dex/NaPB-treated eyes, it showed a much mild staining representing collagen content in the TM region. Also, the extracelluar materials were not as thick as that of Dex-treated eyes (**Figure 26B**). These findings suggest a reduced amount of ECM material deposition.

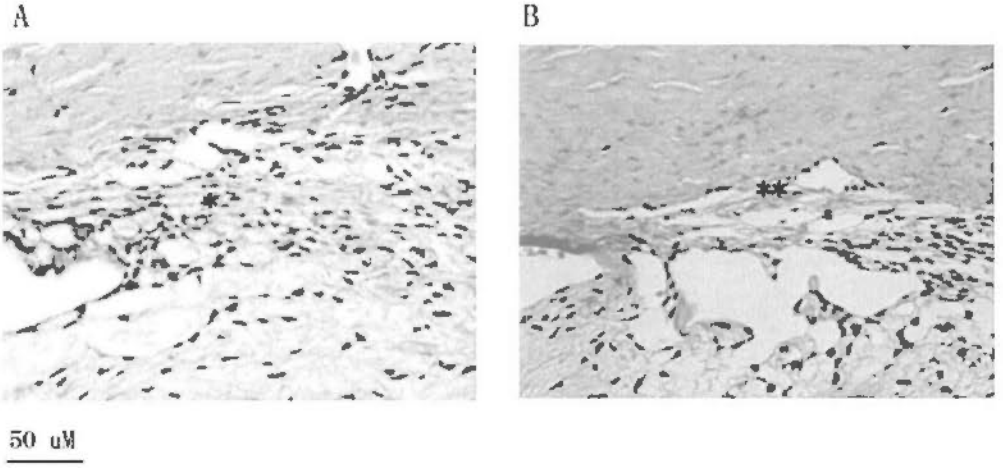

Figure 25. Light micrographs of rabbit trabecular meshwork region after topical sodium phenylbutyrate by Periodic Acid schiff staining. (A) Dex-treated TM region of rabbit eyes 14 days. It showed ECM deposition and glycogen loss (*). **(B)** Dex/NaPB-treated TM region of rabbit eyes 14 days. It showed alleviated ECM thickening and glycogen loss (**).

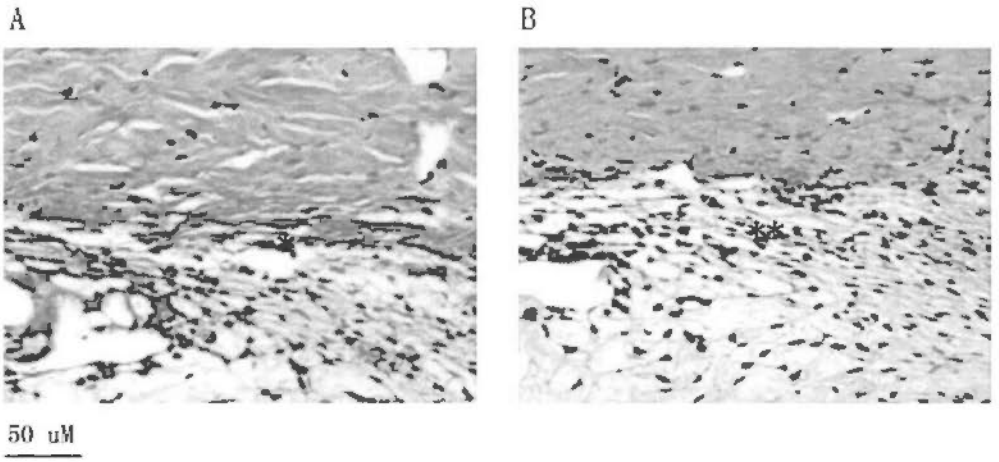

Figure 26. Light micrographs of rabbit trabecular meshwork region after topical sodium phenylbutyrate by Masson trichrome staining. (A) Dex-treated TM region of rabbit eyes 14 days. It showed ECM deposition and collagen accumulation (*). **(B)** Dex/NaPB-treated TM region of rabbit eyes 14 days. It showed alleviated ECM deposition and collagen accumulation (**).

I further examined the ultrastructural changes in the TM region of eyes receiving topical triButyrate® and Maxi-Dex® treatment. Compared with TM region of normal rabbit eyes, the Dex-treated samples had the accumulation of plaques and basement membrane-like materials and blocked channel region, and also ER enlargement as well (**Figure 27A**). With combined treatment with triButyrate®, the reduction of plaques and alleviated ER enlargement was observed (**Figure 27B**). In addition, more clear TM channels were seen, although extracellular fibre-like structures with interconnections were observed.

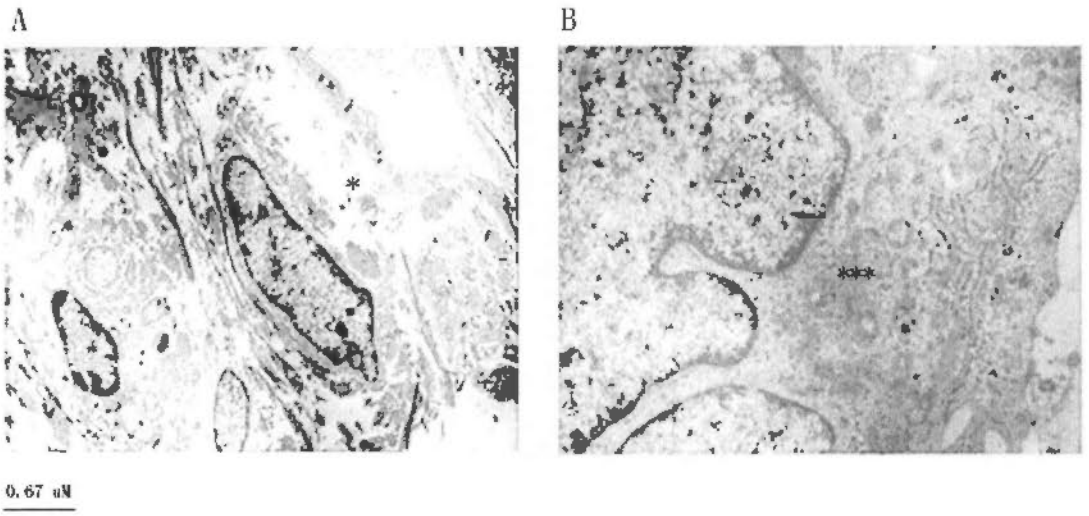

Figure 27. Transmission electron micrographs of trabecular meshwork region of dexamethasone/sodium phenylbutyrate-treated rabbit eyes. (A) It showed reduction of plaques (*) and more clear TM channels. **(B)** It showed alleviated ER enlargement (***) and more clear TM channels.

3.1.9 Effect of triButyrate® on the cell loss in drainage pathway

With the combined topical treatment of Maxi-Dex® and NaPB, the cell density in TM region was 2272±180/mm^2 for 0.02 mM, 2556±199/mm^2 for 0.1 mM, 2656±49/mm^2 for 0.5 mM NaPB, respectively. Topical NaPB (0.02 to 0.5 mM) 4 times daily was shown to reduce Dex-caused cell loss in the TM drainage pathway. By independent Student's *t*-test, NaPB application of 0.1 mM (p=0.04) and 0.5 mM (p=0.03) significantly reduced the cell loss (independent Student *t*-test, n=3) (**Figure 28**).

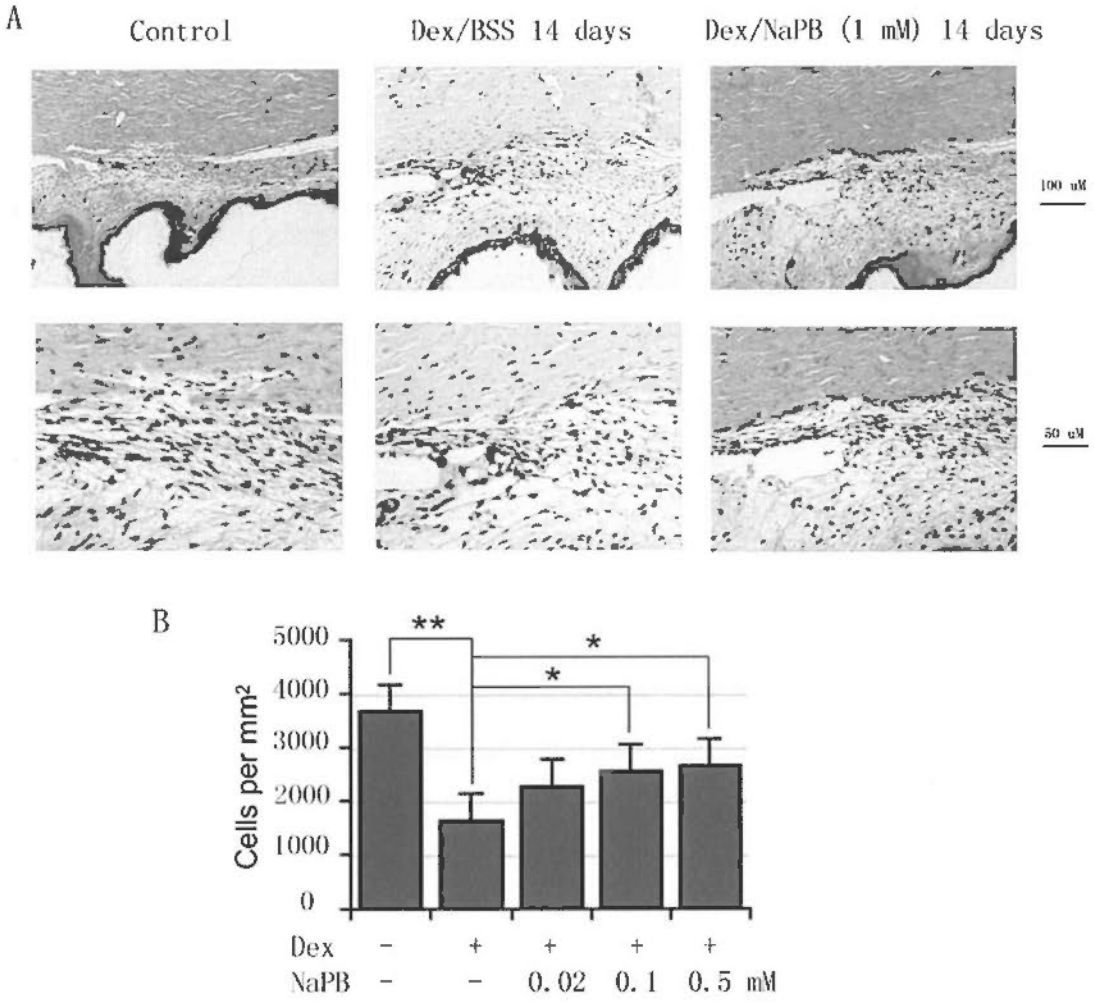

Figure 28. Effect of sodium phenylbutyrate on dexamethasone-caused trabecular meshwork cell loss. (A) H&E staining of TM regions of no drug, Dex, and Dex/NaPB-treated rabbit eyes **(B)** TM cell density calculation showed TM cell loss on Dex-treated eyes, and NaPB could reduce Dex-caused TM cell loss. * indicates $p<0.05$ and ** indicate $p<0.01$ by independent Student's *t*-test, n=3.

3.2 *In vitro* treatment on human trabecular meshwork cell line

3.2.1 Cell viability and apoptosis study

To investigate the mechanism of NaPB action on steroid-induced ocular hypertension, I examined the effect of NaPB on human trabecular meshwork cell lines to search for the mechanism of NaPB action on preventing steroid-induced ocular hypertension. Immortalized human trabecular meshwork (HTM) cell line was kindly given by Dr. Nguyen TD (Polansky et al., 1979; Nguyen et al., 1998). The cells were maintained in Dulbecco's modified Eagle's medium (with 1000 μg/ml glucose) supplemented with 10% fetal bovine serum and antibiotics. The cells were grown to about 70% confluence and treated with 100 nM Dex and/or 1 mM NaPB. They were slender in shape when viewed under phase contrast light microscopy and formed monolayer (**Figure 29**).

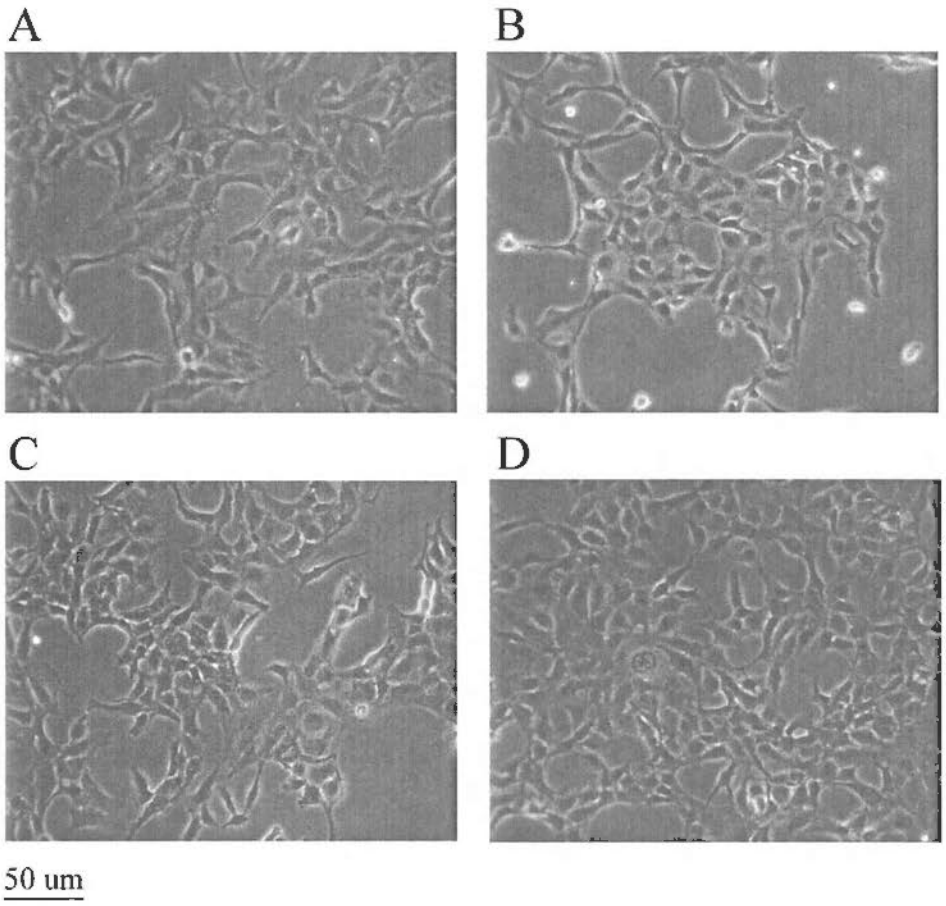

Figure 29. Dex and/or NaPB-treated human trabecular meshwork (HTM) cells. **(A)** HTM cells with no drug treatment/before drug treatment **(B)** HTM cells treated with 100 nM Dex for 3 days **(C)** HTM cells treated with 100 nM Dex and 1 mM NaPB for 3 days **(D)** HTM cells treated with 1 mM NaPB for 3 days

It was reported that TM cell migration, proliferation, and retraction can be inhibited by dexamethasone exposure (Clark et al., 1994; O'Brien et al., 1996; Matsumoto et al., 1997). To investigate the effect of Dex and/or NaPB treatment on HTM cell growth, MTT cell viability/proliferation assay was performed. The cells were treated with 100 nM Dex and /or 1 mM NaPB for 1, 5 and 10 days. When compared to the untreated control, the ratio of cell viability in Dex-treated cells was found to be slightly reduced which were 0.9±0.04-fold, 0.9±0.05-fold and 0.9±0.04-fold for 1, 5 and 10 days Dex treatment, respectively. However, the difference was not statistical significant (**Figure 30**). For NaPB treatment with or without Dex, an increase of cell viability ratio was observed at day 5 and 10 of treatment. Compared to the untreated control, they were 1.1±0.06-fold and 1.2±0.1-fold at day 5 for Dex/NaPB and NaPB treatment, respectively. And at day 10, they were 1.3±0.1-fold and 1.4±0.1-fold for Dex/NaPB and NaPB treatment, respectively. The viability rate of cells in the treatment group with Dex and NaPB were significantly higher than that of Dex-treated HTM cells ($p=0.037$ at day 5, $p=0.035$ at day 10, $n=3$, independent Student's *t*-test).

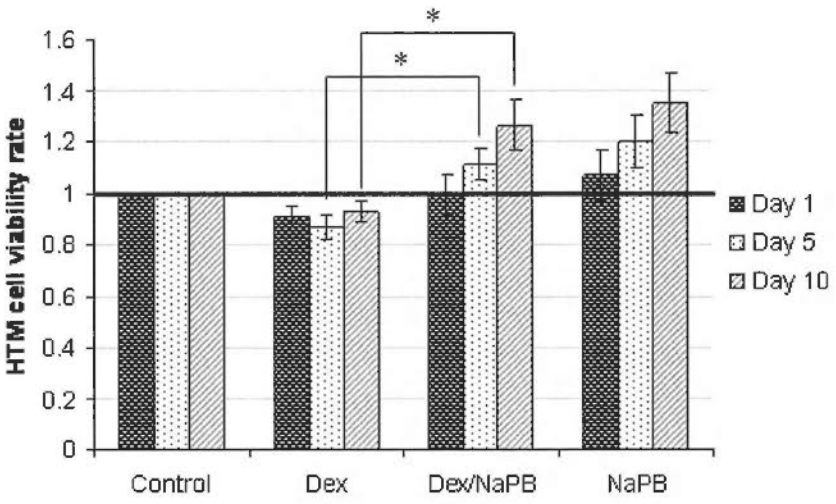

Figure 30. Effect of dexamethasone/sodium phenylbutyrate on the cell viability of human trabecular meshwork (HTM) cells. The cells were treated with 100 nM Dex and / or 1 mM NaPB for 1, 5 and 10 days and collected for MTT assay. Dex was found to slightly suppress HTM cell viability whereas NaPB promoted the cell number, indicative of higher viability and proliferation. * indicates $p<0.05$ by independent Student's *t*-test.

To investigate if the reduced cell viability in Dex-treated HTM cells could be recovered by NaPB treatment, I examined HTM cell apoptosis in the presence of Dex and/or NaPB by TUNEL apoptosis assay and the expression analysis of caspase-3. By western blotting and densitometry analysis, followed by normalization with the housekeeping GAPDH, expression of caspase-3 was increased by 20 % in HTM cells exposed to Dex (100 nM) for 5 days, when compared to untreated control. However, the difference was not statistical significant (**Figure 31**). It was reduced when the cells were treated with 1 mM NaPB for 5 days (**Figure 31**). The reduction was 23.1 % of the Dex-treated cells ($p=0.021$, $n=3$, independent Student's *t*-test).

Further investigation by TUNEL assay revealed similar reduction of apoptosis rate by NaPB treatment. At day 5 of Dex-treatment, the HTM cells were fixed and processed for TUNEL apoptosis assay. There were more TUNEL positive cells found in Dex-treated sample (6.4±0.9 %) than in the control group (3.2±0.5 %) ($p=0.036$, $n=3$, Student's *t*-test) (**Figure 32**). This was reduced when the cells were treated with 1 mM NaPB (1.6±0.4 %) and also in combined Dex (100 nM) and 1 mM NaPB (2.6±0.2 %) ($p=0.015$, $n=3$, independent Student's *t*-test).

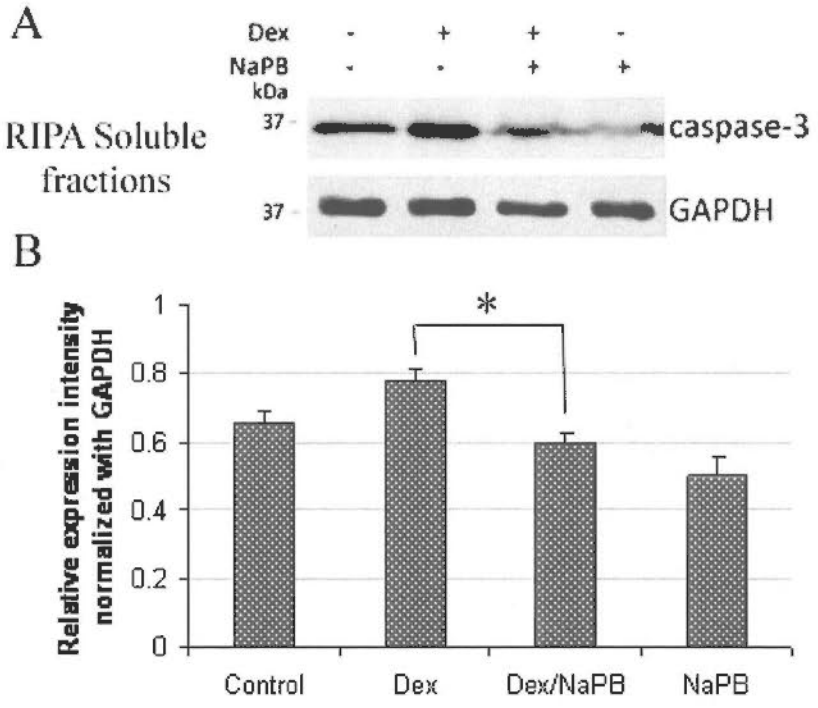

Figure 31. Caspase-3 expression of human trabecular meshwork HTM cells. (A) Western blot analysis of RIPA soluble lysate fraction for caspase 3 expression. The cells were treated with 100 nM Dex and / or 1 mM NaPB for 5 days. They were collected and lyzed in RIPA buffer freshly added with 1 mM PMSF and spun to obtain clear supernatant, which was denatured and resolved by 12.5 % SDS-PAGE. The samples were then immunoblotted for caspase-3 expression. **(B)** Densitometry of specific caspase-3 band at 32 kDa molecular size, followed by normalization with the housekeeping GAPDH. Caspase-3 expression was increased in HTM cells treated with Dex and was reversed after NaPB treatment. * indicates $p<0.05$ by independent Student's *t*-test.

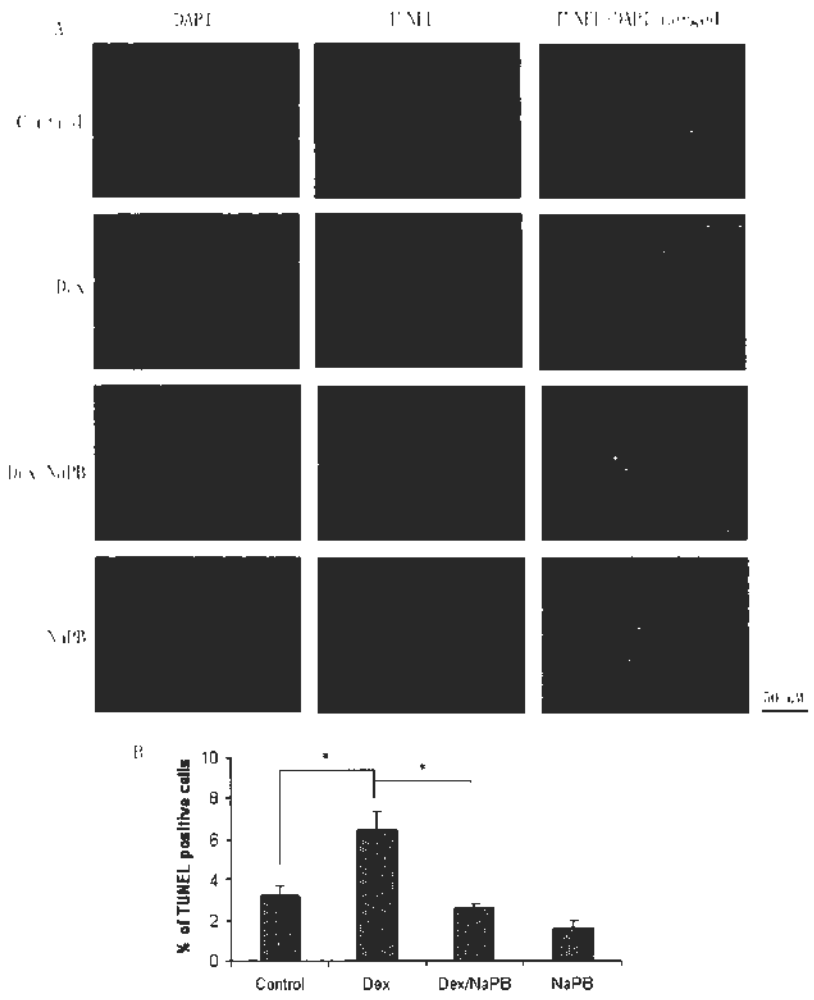

Figure 32. Effect of dexamethasone/sodium phenylbutyrate on HTM cell apoptosis. **(A)** Fluorescence micrographs showing the HTM cells positive to DAPI (nuclei staining), TUNEL assay for apoptotic cells and merged pictures of TUNEL-positive cells and DAPI staining. **(B)** Quantification of TUNEL-positive cells showed a 2-fold increase of apoptosis rate in HTM cells after Dex treatment for 3 days. This elevated apoptosis rate was lowered by NaPB treatment and returned to the levels similar as the control. * indicates $p<0.05$ by independent Student's *t*-test.

3.2.2 Microarray analysis

There were 5 microarray studies on Dex-treated human TM cells. In 2002, Ishibashi and colleagues showed 30 out of 2400 human genes analyzed in cultured human TM cells were up-regulated for more than two folds after treatment with dexamethasone (100 nM) for 7 days (Ishibashi et al., 2002). Lo and his group reported multiple genes differentially up-regulated in dexamethasone (100 nM, treated for 8 to 10 days)-treated human TM cells including myocilin (TIGR/MYOC), α1-antichymotrypsin (a serine protease inhibitor), pigment epithelium-derived factor (PEDF), cornea-derived transcript 6, and prostaglandin D2 synthase (Lo et al., 2003). Fifteen of the 249 differentially regulated genes with four or more fold changes were mapped to glaucoma-linked loci. For long-term dexamethasone treatment (for 21 days) on human TM cells, among 1260 genes analyzed using U133A GeneChip, 23 genes were up-regulated and 18 were down-regulated by more than three folds (Rozsa et al., 2006). Confirmed by quantitative PCR, genes with significant changes were *SLP1, SAA2, ANGPTL7, MYOC, SAA1, SERPINA3* and *ZBTB16*. Our laboratory also reported gene expression profile in steroid-treated human TM cells by using microarray and RT-PCR analyses. After treatment with Dex at 100 nM for 10 days, genes including *GAS1, CDH4, MT1L, CST3, ATF4, ASNS/TS11, CHOP, HSPA5* were expressed at levels more than 1000 times than at normal condition, and *TSC22, LDHA, IGFBP2, TAGLN, SCG2, WARS* more than 100 times (Leung et al., 2003). Several of them were reported to function in anti-inflammatory response and humor outflow resistance. In TM cells treated with either Dex or TA, *MYOC* and *GAS1* were

up-regulated (Fan et al., 2008). In contrast, *SENP1*, *ZNF343* and *SOX30* were down-regulated. Among them, *MYOC* and *GAS1* were located in known primary open-angle glaucoma (POAG) loci.

In my study, to examine if NaPB could alter the gene expression changes on Dex-treated HTM cells, gene ontology (GO term) analysis was performed. Compared to Dex treatment and no drug control, Dex-treated HTM cells were enriched for extracellular region (p=0.031, *RSPO1, GALNT13, WFDC13, ANGPT1, DEFB119, EPGN, FGL1, IL1F9, NPPA, Klkbl14, PRSS33, PROS1, SAA1, SAA2*), negative regulation of defense response (p=0.0061, *IRAK3, SAA1, SAA2*), acute inflammatory response (p=0.04, *CR1, SAA1, SAA2*). And also down-regulation of genes related to extracellular region (p=0.2, *SSPO, INSL4, MIA2, PECAM1*) (**Table 4**). At day 10, Dex-treated HTM cell was found to be enriched for defense response (p=0.00052, *BLNK, S100A8, ANKRD53, DEFB103A, CCR2, LILRB3, SAA1, SAA2, SAA4*), inflammatory response (p=0.0032 *BLNK, S100A8, CCR2, SAA1, SAA2, SAA4*), extracellular space (p=0.021, *TIMP4, ANGPT1, SCGB3A1, LIPG, SAA1, SAA2, SAA4*), response to hormone stimulus (p=0.027, *TIMP4, ACTA1, ANGPT1, GNG3, LCT*), and cell migration (p=0.01, *SOX1, NRCAM, PRKG1, SAA1, SAA2*). Also down-regulation of genes related to localization of cell (p=0.029, *IL1B, TNP2, IL8*) (**Table 5**). This suggested that Dex may mainly interfere with the extracellular matrix metabolism, inflammatory response and defense response in HTM cells.

Table 4. GO term analysis of HTM gene expression after Dex versus control at day 1

Go terms (genes/fold changes)	Count	P-value
(A) Upregulated genes in Dex treatment versus control		
Cell defense response (IRAK3/43.3, SAA1/31.4, SAA2/16.8)	3	0.0061
Wound healing (POU2F3/28.4, PROS1/10.0, SAA1/31.4, SAA2/16.8)	4	0.024
Extracellular region (RSPO1/26.0, GALNT13/30.2, WFDC13/64.9, ANGPT1/10.2, DEFB119/41.5, EPGN/10.0, FGL1/17.1, IL1F9/16.7, NPPA/30.7, Klkbl14/34.9, PRSS33/13.1, PROS1/10.0, SAA1/31.4, SAA2/16.8)	14	0.031
Acute inflammatory response (CR1/12.9, SAA1/31.4, SAA2/16.8)	3	0.04
Cell-cell signaling (TBX5/26.3, GRIA2/13.1, GRM5/10.1, IL1F9/16.7, NPY5R/10.5, VIPR2/16.7)	6	0.044
Ligand-gated channel activity (GRIA2/13.1, KCNK1/22.1, RYR3/28.1)	3	0.061
Cation homeostasis (RHCG/12.5, RYR3/28.1, SAA1/31.4, SAA2/16.8)	4	0.066
Regulation of secretion (NPY5R/10.5, SAA1/31.4, SAA2/16.8)	3	0.14
Cell migration (SAA1/31.4, SAA2/16.8, ROCK1/20.1)	3	0.23
Peptidase activity (CAPN11/23.0, Klkbl14/34.9, PRSS33/13.1)	3	0.55
Phosphorus metabolic process	3	0.84

(EPGN/10.0, IRAK3/43.3, ROCK1/20.1)		
Golgi apparatus (B3GNT6/20.7, GALNT13/30.2, ROCK1/20.1)	3	0.85
Cytoskeleton (CTAG2/31.3, GRIA2/13.1, LMOD3/17.8, ROCK1/20.1)	4	0.9

(B) Down-regulated genes in Dex treatment versus control		
Cell adhesion (SSPO/13.2, PECAM1/11.0, PCDH10/14.3)	3	0.11
Extracellular region (SSPO/13.2, INSL4/12.4, MIA2/28.0, PECAM1/11.0)	4	0.2

Table 5. GO term analysis of HTM gene expression after Dex versus Control at day 10

Go terms (genes/fold change)	Count	P-value
(A) Upregulated genes in Dex treatment versus Control		
Defense response (BLNK/13.4, S100A8/15.9, ANKRD53/27.6, DEFB103A/13.8, CCR2/21.0, LILRB3/19.7, SAA1/216.8, SAA2/131.5, SAA4/15.0)	9	0.00052
Inflammatory response (BLNK/13.4, S100A8/15.9, CCR2/21.0, SAA1/216.8, SAA2/131.5, SAA4/15.0)	6	0.0032
Response to extracellular stimulus (TIMP4/10.7, ACTA1/12.4, ANGPT1/11.0, GNG3/13.7, LCT/18.6)	5	0.0047
Contractile fiber system (TIMP4/10.7, ACTA1/12.4, ANKRD1/14.9, SYNE1/14.7)	4	0.0056
Cholesterol transport (ABCG5/29.3, CD36/12.6, LIPG/10.2)	3	0.0059
Response to nutrient (ABCG5/29.3, ANGPT1/11.0, LCT/18.6, LIPG/10.2)	4	0.0093
Cell migration (SOX1/12.7, NRCAM/15.2, PRKG1/34.1, SAA1/216.8, SAA2/131.5)	5	0.01
Chemical homeostasis (ABCG5/29.3, CCR2/21.0, GNG3/13.7, LIPG/10.2, SAA1/216.8, SAA2/131.5)	6	0.02
Extracellular space (TIMP4/10.7, ANGPT1/11.0, SCGB3A1/12.2, LIPG/10.2, SAA1/216.8, SAA2/131.5, SAA4/15.0)	7	0.021
Response to hormone stimulus (TIMP4/10.7, ACTA1/12.4, ANGPT1/11.0, GNG3/13.7, LCT/18.6)	5	0.027

Acute inflammatory response (SAA1/216.8, SAA2/131.5, SAA4/15.0)	3	0.037
Intracellular signaling cascade (BLNK/13.4, CD36/12.6, SHC4/13.5, CCR2/21.0, CNKSR1/11.3, FPR1/12.2, GNG3/13.7, MTNR1B/61.8, NPR3/22.2)	9	0.037
Regulation of cell adhesion (CD36/12.6, SAA1/216.8, SAA2/131.5)	3	0.067
Insoluble fraction (ABCG5/29.3, CD36/12.6, LCT/18.6, NPR3/22.2, SCNN1A/11.3, SLC18A3/10.2)	6	0.13
ATPase activity (ABCG5/29.3, DDX53/72.8, DNAH3/10.0)	3	0.24
Cytoskeleton (S100A8/15.9, ACTA1/12.4, DNAH3/10.0, SYNE1/14.7)	4	0.26
Cell cycle (PRDM9/15.9, USH1C/15.2, PRUNE2/12.8)	3	0.7

(B) Down-regulated genes in Dex treatment versus control		
Subcellular localization (IL1B/15.7, TNP2/50.0, IL8/11.5)	3	0.029
Cytoplasmic vesicle (CPA3/12.0, FLG/10.2, IL1B/15.7)	3	0.058
Response to organic substance (AQP1/13.3, COL3A1/13.2, IL1B/15.7)	3	0.13
Regulation of cell proliferation (GATA4/12.8, IL1B/15.7, IL8/11.5)	3	0.15
Intracellular signaling cascade (EFS/14.3, IL1B/15.7, IL8/11.5)	3	0.31
Non-membrane-bounded organelle	3	0.51

(FLG/10.2, TNP2/50.0,TNNT3/17.3)		
Intracellular non-membrane-bounded organelle		
(FLG/10.2, TNP2/50.0,TNNT3/17.3)	3	0.51
Regulation of transcription		
(GATA4/12.8, ASXL3/10.2, IL1B/15.7)	3	0.7

Compared with Dex treatment, Dex/NaPB-treated HTM cells were enriched for extracellular region (p=0.00089, *C1QTNF3, SPINLW1, WFIKKN2, BMP7, CPB1, CBLN1, F2, F7, COL24A1, CRISP1, DEFA1, DEFB106A, FGG, FNDC1, IL16, LCN 12, WNT11*), and cell growth (p=0.023, *SOX10, BMP7, CHRNA1, FOXP2*) at day 1. And also down-regulation of genes related to the response to bacterium (p=0.11, *BPI, DEFA3, DEFB119*) (**Table 6**). At day 10, Dex/NaPB-treated HTM cells were enriched for immune response (p=0.024, *CD1A, SLAMF7, CDSN, DSC1, FGFBP2, IMPG1, KLK10, HLA-DRB3, TMED4*), extracellular region (p=0.39, *CDSN, FGFBP2, IL33, IMPG1, KLK10*), and defense response (p=0.26, *SLAMF1, AFAP1L2, MNDA*). And down-regulation of genes related to integral to plasma membrane (p=0.029, *HTR3B, CCR2, F2RL3, LILRB3, MTNR1B, PCDH12, SLC18A3, SLC22A3*) and inflammatory response (p=0.16, *S100A8, CCR2, CCL23*) (**Table 7**). This suggested that NaPB mainly regulates the effect of Dex through extracellular matrix metabolism, inflammatory response and defense response in HTM cells.

Table 6. GO term analysis of HTM gene expression after Dex/NaPB versus Dex treatment at day 1

GO terms (genes/fold changes)	Count	P-value
(A) Upregulated genes in Dex and NaPB treatment versus Dex treatment		
Extracellular region (C1QTNF3/11.6, SPINLW1/56.5, WFIKKN2/11.1, BMP7/11.5, CPB1/18.2, CBLN1/24.1, F2/19.2, F7/18.7, COL24A1/18.1, CRISP1/16.5, DEFA1/13.5, DEFB106A/13.3, FGG/10.1, FNDC1/12.8, IL16/10.1, LCN 12/17.4, WNT11/10.5)	17	0.00089
Extracellular structure organization (CHRNA1/16.4, DNM3/14.6, MUSK/10.5)	3	0.017
Growth (SOX10/19.4, BMP7/11.5, CHRNA1/16.4, FOXP2/12.6)	4	0.023
Wound healing (CDH3/32.3, F2/19.2, F7/18.7, FGG/10.1)	4	0.025
Cell adhesion (CDH3/32.3, CDH9/10.9, COL24A1/18.1, NLGN4X/13.2, PCDHGB1/23.9)	5	0.081
Transcription regulator activity (SOX10/19.3, TLX3/11.0, ATOH7/14.5, FOXP2/12.6, HOXB13/10.8, SKAP1/15.3, TFCP2L1/13.6, ZNF192/32.4)	8	0.21
Cell junction (CDH3/32.3, CBLN1/24.1, CHRNA1/16.4, DMD/13.3)	4	0.27
Cell motility (TLX3/11.0, DNAH11/10.0, IL16/10.1)	3	0.27
Peptidase activity (CPB1/18.2, F2/19.2, F7/18.7, USP47/26.5)	4	0.28
Cell-cell signaling	4	0.32

(CBLN1/24.1, CHRNA1/16.4, DMD/13.3, MUSK/10.5)		
Extracellular matrix (BMP7/11.5, COL24A1/18.1, WNT11/10.5)	3	0.34
Cellular chemical homeostasis (CHRNA1/16.4, F2/19.2, KCNA5/16.7)	3	0.36
Positive regulation of macromolecule biosynthetic process (SOX10/19.3, BMP7/11.5, F2/19.2, SKAP1/15.3)	4	0.37
Cell surface receptor linked signal transduction (WFIKKN2/11.1, BMP7/11.5, F2/19.2, KLRK1/11.2, MUSK/10.5, NPBWR2/12.1, SKAP1/15.3, WNT11/10.5)	8	0.42
Protein dimerization activity (CBLN1/24.1, FOXP2/12.6, NLGN4X/13.2)	3	0.53
Defense response (F2/19.2, DEFA1/13.5, DEFB106A/13.3)	3	0.61
Macromolecular complex assembly (FGG/10.1, MUSK/10.5, KCNA5/16.7)	3	0.66
Immune response (DEFA1/13.5, IL16/10.1, SKAP1/15.3)	3	0.68
Regulation of apoptosis (BMP7/11.5, F2/19.2, F7/18.7)	3	0.76
Golgi apparatus (B3GNT3/1554.0, B4GALNT2/10.1, KCNA5/16.7)	3	0.82
Phosphate metabolic process (BMP7/11.5, F2/19.2, MUSK/10.5)	3	0.84
Cell fraction (B4GALNT2/10.1, F2/19.2, DMD/13.3)	3	0.9
Mitochondrion (C1QTNF3/11.6, XAF1/39.7, HSDL2/12.4)	3	0.91

(B) Down-regulated genes in Dex and NaPB treatment versus Dex treatment		
Response to bacterium (BPI/15.1, DEFA3/10.8, DEFB119/42.8)	3	0.11
Channel activity (GRIA2/16.3, KCNK1/22.9, KCNS1/14.4, RYR3/23.4)	4	0.12
Intracellular signaling cascade (DMRT1/14.6, ELMO1/10.5, EPGN/10.6, GRM5/10.4, OPRM1/14.2, REN/16.0, RP1L1/12.0)	7	0.16
G-protein coupled receptor protein signaling pathway (GPR120/11.8, GRM5/10.4, NPY5R/10.8, OR6Y1/16.7)	6	0.24
Regulation of cell proliferation (TBX5/26.9, EPGN/10.6, NPY5R/10.8, OPRM1/14.2)	4	0.41
Multicellular organism reproduction (DMC1/11.8, MORC1/10.5, NPY5R/10.8)	3	0.42
Endoplasmic reticulum (OPRM1/14.2, NT5C1B/33.8, RYR3/23.4, SC5DL/11.3)	4	0.6
Cytoplasmic vesicle (GPR120/11.8, CAPN11/11.8, GRIA2/16.3)	3	0.62
Golgi apparatus (B3GNT6/21.6, GALNT13/31.3, OPRM1/14.2)	3	0.78
Cytoskeleton (CTAG2/31.8, ELMO1/10.5, GRIA2/16.3, LMOD3/18.3)	4	0.83
Cytosol (ELMO1/10.5, PDE11A/19.8, NT5C1B/33.8)	3	0.94
Nucleotide binding (DMC1/11.8, MORC1/10.5, NT5C1B/33.8)	3	0.99

Table 7. GO term analysis of HTM gene expression after Dex/NaPB versus Dex treatment at day 10

GO terms (genes/fold changes)	Count	P-value
(A) Upregulated genes in Dex and NaPB treatment versus Dex treatment		
Immune response (CD1A/14.2, SLAMF7/13.3, CDSN/14.1, DSC1/13.2, FGFBP2/11.1, IMPG1/11.1, KLK10/30.9, HLA-DRB3/17.4, TMED4/11.3)	9	0.024
Regulation of transcription (BARX1/20.4, IKZF4/58.1, ISL2/10.2, AFAP1L2/11.4, MNDA/13.6, SCRT1/15.1, ZBTB33/29.0, ZNF311/30.1)	8	0.11
Defense response (SLAMF1/13.3, AFAP1L2/11.4, MNDA/13.6)	3	0.26
Cell adhesion (SLAMF1/13.3, CDSN/14.1, DSC1/13.2)	3	0.32
Extracellular region (CDSN/14.1, FGFBP2/11.0, IL33/10.6, IMPG1/11.1, KLK10/30.9)	5	0.39
Endoplasmic reticulum (GIMAP1/38.2, C3orf57/10.0, TMED4/11.3)	3	0.45
Integral to membrane (CD1A/14.2, GIMAP1/38.2, KIAA1161/27.7, SLAMF7/13.3, AQP10/18.9, C3orf57/10.0, DSC1/13.2, HS6ST3/10.2, HLA-DRB3/17.4, TMED4/11.3)	10	0.46
Organelle membrane (CD1A/14.2, C3orf57/10.0, DAB2/20.2)	3	0.52
Plasma membrane (CD1A/14.2, KCNIP4/11.0, CDSN/14.1, DSC1/13.2, HLA-DRB3/17.4, ZBTB33/29.0)	6	0.75

(B) Down-regulated genes in Dex and NaPB treatment versus Dex treatment		
Integral to plasma membrane (HTR3B/13.3, CCR2/30.7, F2RL3/10.0, LILRB3/16.9, MTNR1B/90.3, PCDH12/28.8, SLC18A3/13.2, SLC22A3/17.4)	8	0.029
Cell surface receptor linked signal transduction (HTR3B/13.3, CCL23/11.5, CCR2/30.7, F2RL3/10.0, CNKSR1/11.9, DTX4/36.4, GNG3/20.1, LILRB3/16.9, MTNR1B/90.3)	9	0.045
Cell-cell junction (CLDN19/11.7, CNKSR1/11.9, PCDH12/28.8)	3	0.086
Cation homeostasis (CCL23/11.5, CCR2/30.7, GNG3/20.1)	3	0.13
Cell motility (SOX1/18.5, DNAH3/14.3, PRKG1/49.4)	3	0.15
Cell-cell signaling (HTR3B/13.3, CCL23/11.5, MTNR1B/90.3, SLC22A3/17.4)	4	0.15
Inflammatory response (S100A8/22.7, CCR2/30.7, CCL23/11.5)	3	0.16
ATPase activity (ABCG5/17.5, DDX53/103.4, DNAH3/14.3)	5	0.51
Insoluble fraction (ABCG5/17.5, SLC18A3/13.2, SLC22A3/17.4)	3	0.65
Cytoskeleton (DNAH3/14.3, DNALI1/14.2, KRTAP15-1/14.5)	3	0.89
Regulation of transcription, DNA-dependent (PRDM9/22.4, SOX1/18.5, HOXC13/11.1)	3	0.92
Intracellular non-membrane-bounded organelle (PRDM9/22.4, USH1C/20.9, DNAH3/14.3, DNALI1/14.2)	5	0.93

3.2.3 Gene expression

As it was reported, heat stress increases the production of ceramide, which activates the c-Jun N-terminal kinase (JNK), then promotes the expression of the c-jun and c-fos mRNAs to induce apoptosis (Kondo et al., 2000). HSP70 may activate JNK phosphatase to suppress the activation of JNK and kinase. This prevents apoptosis and provides the cells with thermotolerance (Gabai et al., 1997; Mosser et al., 1997; Gabai et al., 2000). And it was known that triamcinolone acetonide (TA), a commonly used steroid, of 0.1 and 1 mg/ml caused stress induction in HTM cell culture (Wang et al., 2008).

To examine the Dex-induced changes and increased apoptosis in HTM cells, the expression of stress-related genes and reported genes affected by Dex was examined by semi-quantitative RT-PCR analysis. A slight up-regulation of stress-related genes (c-jun and c-fos) was noted after Dex treatment for 24 hours (**Figure 33**). C-jun expression in Dex-treated cells was 1.1 ± 0.2 fold more than control cells whereas c-fos was 1.3±0.2 fold higher in Dex-treated cells than control cells. HSP90a was found down-regulated in Dex-treated cells (75% of control cells). HSP70 was not affected by Dex-treatment. These changes were reversed by the co-treatment with NaPB. C-jun and c-fos expression was reduced and returned to the control level (0.9±0.2 and 1.2±0.2 fold respectively). The Dex-caused low level of HSP90a was increased by NaPB by 70%. In addition, the caspase 3 expression was dramatically reduced by NaPB (48% change as Dex-treated cells, p=0.048, n=3, independent

Student's *t*-test).

Next, the effect of NaPB on the reported Dex-caused gene expression was investigated. A total of 8 genes were examined, including *CA12*, *ZNF343* reported by Leung et al., 2003 which may related with the formation of aqueous humor (Leung et al., 2003), *SAA2* which may related with endothelial function, *IL1B* which regulated the inflammatory response and involved in cell proliferation and apoptosis reported by Fan et al., 2008 (Fan et al., 2008), *APOD* which may related with endothelial function reported by Lo et al. 2003 (Lo et al., 2003). In addition, *IGFBP4* and *IGF2* were selected because it binds to insulin-like growth factor II. Also, the expression of *IGFBP4* consistently inhibits prostate and colon cancer cells *in vivo* and *in vitro* (Durai et al., 2007). It acts as an apoptotic factor and controls the growth of cancer. Because the extracellular matrix (ECM) deposition could cause the resistance of aqueous humor outflow, *TGFB3* which is involved in cellular adhesion and ECM formation was also examined.

In my gene expression analysis, among them, the expression of *IGFBP4* was induced for 11.5±0.5-fold by 24-hour Dex treatment ($p=2.22\times10^{-5}$, n=3, independent Student's *t*-test), whereas *CA12* and *IL1B* were down-regulated. Compared with non-treated control, the expression level of CA12 and IL1B were 0.4±0.4-fold and 0.4±0.1-fold (p=0.002, n=3, independent Student's *t*-test), respectively (**Figure 34**). In co-treatment with NaPB, the expression levels of *IGFBP4*, *CA12* and *IL1B* were

revered to control levels of 2.2±0.3-fold ($p=7.26x10^{-5}$, n=3, independent Student's *t*-test), 2.3±0.4-fold (p=0.031, n=3, independent Student's *t*-test), and 0.9±0.06-fold (p=0.010, n=3, independent Student's *t*-test) changes, respectively (**Figure 34**). For *ZNF343, TGFB3, APOD* and *IGF2*, they did not show much change after 24-hour Dex treatment. Compared with non-treated control, they were 2.4±0.5-fold, 0.7±0.07-fold, 1.3±0.3-fold and 0.9±0.05-fold, respectively (**Figure 35**). When combined treatment with 1 mM NaPB, there was still not much change in *ZNF343, TGFB3, APOD* and *IGF2*, which were 1.7±0.3-fold, 0.48±0.11-fold, 1.2±0.3-fold and 2.6±0.3-fold compared with control. Although *SAA2* had a 28.2±2.6-fold change after 24-hour Dex-treatment, which was very significant (p=0.0004, n=3, independent Student's *t*-test), NaPB treatment did not reverse it to the control level (20.3±2.2-fold).

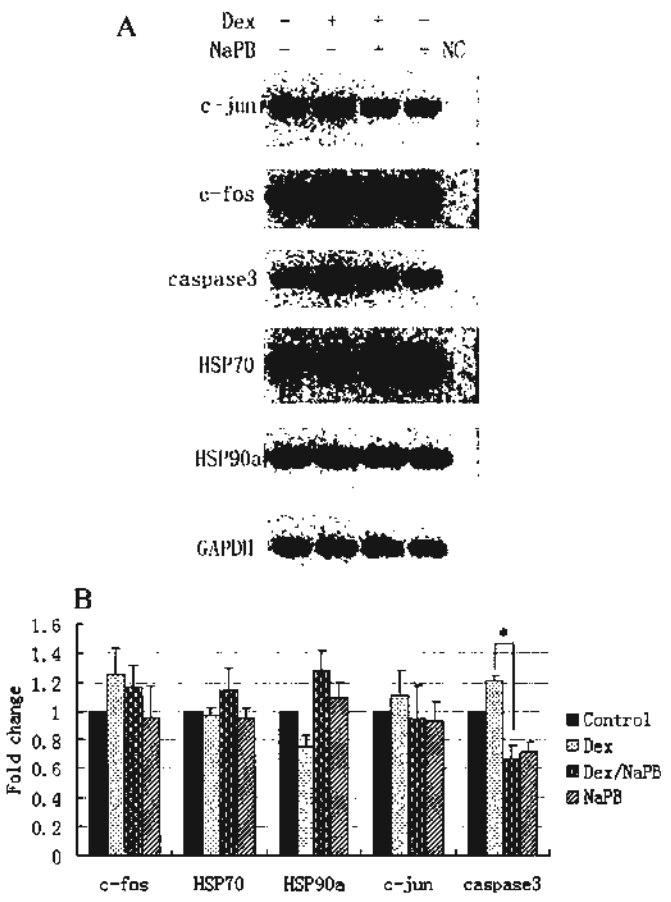

Figure 33. Stress/apoptosis-related gene expression of HTM cells (A) RT-PCR analysis for c-jun, c-fos, HSP70, HSP90a, and caspase 3 expression. The cells were treated with 100 nM Dex and / or 1 mM NaPB for 24 hours. **(B)** Densitometry of specific c-jun, c-fos, HSP70, HSP90a, and caspase 3 band, followed by normalization with the housekeeping GAPDH. A slight up-regulation of c-jun and c-fos was noted after Dex treatment. Hsp90a was found down-regulated in Dex-treated cells. HSP70 was not affected by Dex-treatment. These changes were reversed by the co-treatment with NaPB. In addition, the caspase 3 expression was dramatically reduced by NaPB. * indicates $p<0.05$ by independent Student's *t*-test.

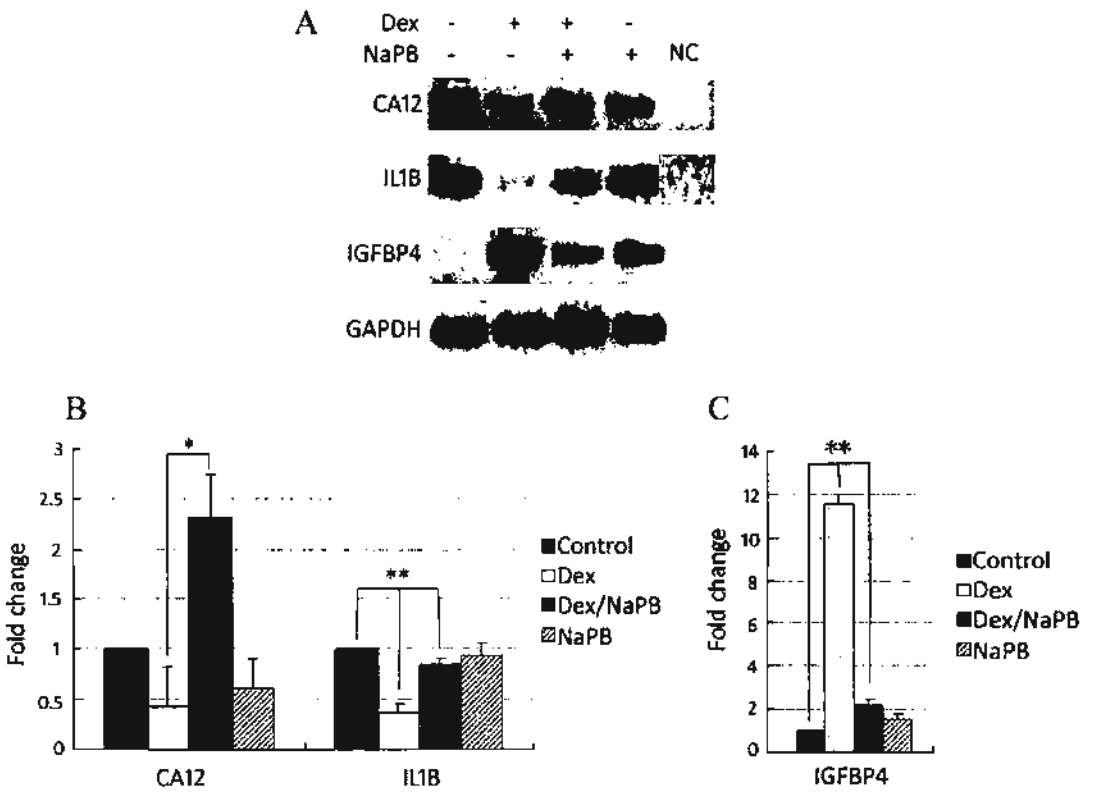

Figure 34. Candidate gene expression of HTM cells. (A) RT-PCR analysis for CA12, IL1B, IGFBP4 expression. The cells were treated with 100 nM Dex and / or 1 mM NaPB for 24 hours. **(B)** Densitometry of specific CA12, IL1B band, followed by normalization with the housekeeping GAPDH. The expression of CA12 and IL1B were down-regulated. They were all reversed by NaPB to control levels. **(C)** Densitometry of specific IGFBP4 band, followed by normalization with the housekeeping GAPDH. The expression of IGFBP4 was induced significantly. This was reversed by NaPB to control levels. * indicates $p<0.05$; ** indicate $p<0.01$ by independent Student's *t*-test.

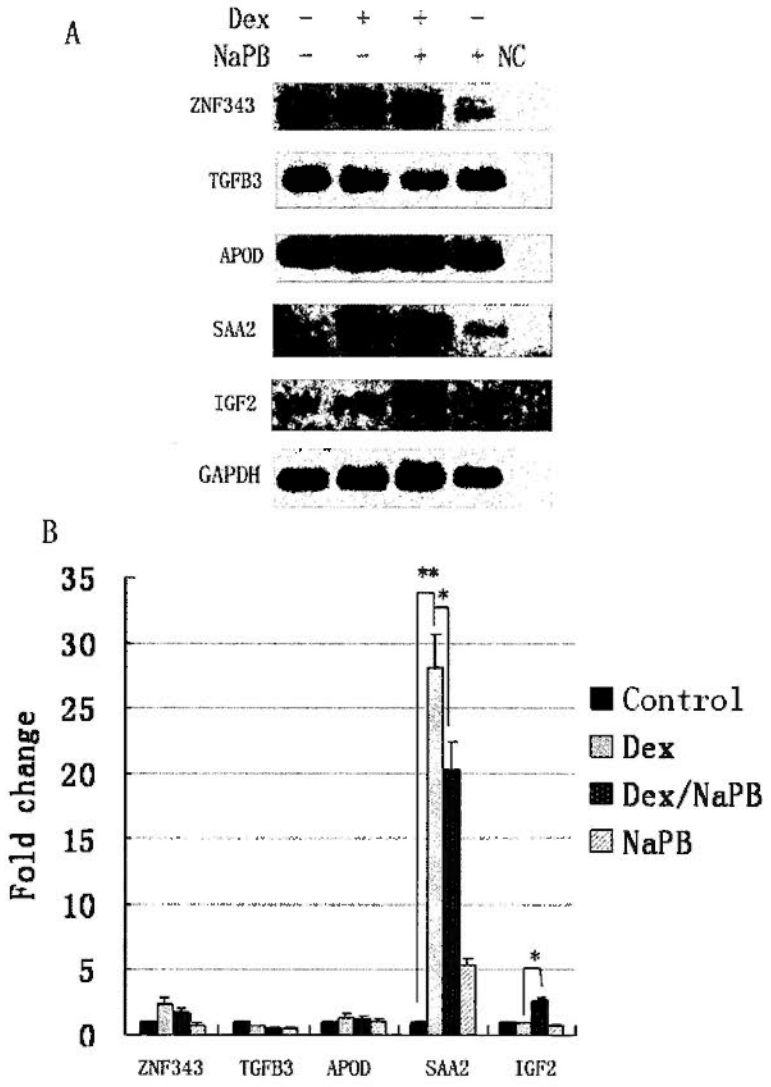

Figure 35. Candidate gene expression of HTM cells. (A) RT-PCR analysis for ZNF343, TGFB3, APOD, IGF2, SAA2 expression. The cells were treated with 100 nM Dex and / or 1 mM NaPB for 24 hours. **(B)** Densitometry of specific RT-PCR analysis for ZNF343, TGFB3, APOD, IGF2, SAA2 band, followed by normalization with the housekeeping GAPDH. They did not show much change after 24-hour Dex treatment with/without NaPB. SAA2 showed significantly upregulation after 24-hour Dex-treatment, but NaPB treatment did not reverse it to the control level. ** indicate $p<0.01$ by independent Student's *t*-test.

Chapter 4: Discussion

4.1 Regulation of human TM cells by dexamethasone/steroids *in vitro*

4.1.1 Cell stress response to dexamethasone/steroids

Steroids such as dexamethasone are known to induce IOP rise. Since year 2000, this was translated to cell culture to create a cell model of glaucoma using human trabecular meshwork (HTM) cells (Polansky et al., 2000). In this cell model, treatment of dexamethasone/steroids on HTM cells induced cell changes not only morphological, but also functional, including cytoskeletal changes, deposition of extracellular matrix materials, and inhibition of phagocytosis, cell migration, proliferation, and retraction, and also gene expression changes including stress/apoptosis-related genes (Shirato et al., 1989; Wilson et al., 1993; Clark et al., 1994; O'Brien et al., 1996; Matsumoto et al., 1997; Clark et al., 2005; Zhang et al., 2007; Wang et al., 2008).

In the *in vitro* part of this study, 100 nM of dexamethasone was used to treat HTM cells for different time points to examine the cell response to steroids. MTT assay showed that the cell viability rate was lowered by the exposure of dexamethasone. However, when compared to the untreated control, the difference of cell viability was not significant, even when the treatment time was up to 10 days. Although, gene and protein expression of caspase-3 was not up-regulated significantly by dexamethasone treatment, the result of TUNEL apoptosis assay showed a 2-fold increase of the apoptosis percentage of HTM cells, when compared to the control

group. Furthermore, insulin-like growth factor binding protein 4 (IGFBP4) was significant up-regulated after dexamethasone treatment, which might relate with apoptosis development. IGFBP4 is composed of an IGFBP domain and a thyroglobulin type-I domain (LaTour et al., 1990). It circulates in the plasma in both glycosylated and non-glycosylated forms and binds to both insulin-like growth factor (IGFs) I and II. The binding to IGF prolongs the half-life of IGFs and alters their interaction with cell surface receptors. The expression of IGFBP4 consistently inhibits prostate and colon cancer cells *in vivo* and *in vitro* (Durai et al., 2007). It acts as an apoptotic factor and controls the growth of cancer. Hence, the significant up-regulation of IGFBP4 by dexamethasone treatment might relate with the elevated cell death. This might indicate the suppressive effect of dexamethasone on HTM cell growth and proliferation, which is accordant to the other reports (Clark et al., 1994; Wang et al., 2008).

Interleukin 1β (IL1B) is a cytokine produced by activated macrophages (Mizutani et al., 1991). The pro-protein is proteolytically processed to its active form by caspase-1. IL1B is commonly known as an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis (Nishida et al., 1987; Bratt et al., 1997; Madge et al., 2000). It stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity (March et al., 1985; Van Damme et al., 1985). As it is known that TM cells possess macrophage-like

activity for the cleaning of aqueous humor which could beneficial to protect the aqueous humor drainage system from inflammatory responses (Takase et al., 2002). In my study, Dex down-regulated IL1B in HTM cells, suggesting its involvement in regulating the inflammatory response.

Carbonic anhydrase 12 (CA12) belongs to a large family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide, and it has two transcript variants encoding different isoforms (Türeci et al., 1998).

It is a type I membrane protein that is highly expressed in normal tissues, such as kidney, colon and pancreas, and ciliary bodies (Karhumaa et al., 2000; Karhumaa et al., 2001; Liao et al., 2003). It is found to participate in a variety of biological processes, including respiration, calcification, acid-base balance, bone resorption, and the formation of aqueous humor, cerebrospinal fluid, saliva and gastric acid (Karhumaa et al., 2000; Karhumaa et al., 2001; Liao et al., 2003). As it is known, aqueous humor is produced by ciliary body, pass into the anterior chamber through the pupil and drain out through the trabecular meshwork region next to the anterior chamber angle towards the scleral vein system (Tamm, 2009). The normal level IOP is maintained by the balance of the secretion and outflow of aqueous humor. In this study, down-regulation of CA12 expression in TM cells by dexamethasone might relate with altered aqueous humor production and IOP regulation.

It was known that triamcinolone acetonide (TA) of 0.1 and 1 mg/ml caused stress induction in HTM cell culture (Wang et al., 2008). This could be similar for dexamethasone treatment in HTM cells. In the gene expression study, it showed slight up-regulation of stress-related genes such as c-jun and c-fos in Dex-treated HTM cells, which indicate that dexamethasone might affect stress response in HTM cells.

4.1.2 Effect of sodium phenylbutyrate (NaPB, triButyrate®) on human TM cells and its action to steroid treatment

As mentioned, the main principal of cell stress management is to alleviate the cells from stress-caused damage and to improve cell survival. A group of small molecule chemicals were reported to act as a chemical chaperone, which can rescue cells experiencing different kinds of stress and apoptosis. For example, in eye-related studies, TMAO suppressed the lens epithelial cell death in the cell model of cataract (Mulhern et al., 2007).

Among them, sodium 4-phenylbutyrate was a common small molecule used in various protein folding diseases and stress conditions. It has been reported to have stress relieving effect in cell models of various eye diseases including retinitis pigmentosa, cataract, glaucoma and diabetic retinopathy (Bonapace et al., 2004b; Mulhern et al., 2007; Yam et al., 2007; Jia et al., 2009; Li et al., 2009; Gong et al., 2010). In human trabecular meshwork cells (a cell model of POAG), it was shown to reduce myocilin (MYOC) aggregates, attenuate the ER stress and improve the cell

survival (Yam et al., 2007; Jia et al., 2009).

In this study, 1 mM NaPB was applied to HTM cells with/without dexamethasone treatment to examine the effect of NaPB on cells and its action to steroid-induced stress. By MTT analysis on day 5 and 10 after treatment, significantly higher viability was found in HTM cells treated with dexamethasone and NaPB for 5 to 10 days than those treated with dexamethasone only. The group treated with NaPB only showed further better level of cell viability. Compared with dexamethasone-treated HTM cells, expression of caspase-3 in NaPB-treated cells was relatively low, irrespective to with or without Dex treatment. In addition, the increased IGFBP4 expression caused by dexamethasone was lowered by NaPB, indicative of a possible anti-apoptotic effect. Also, TUNEL apoptosis assay showed a lower apoptosis rate of HTM cells after NaPB treatment, even lower than that of control. These results indicate that NaPB can improve the cell proliferation ability and protect HTM cells from apoptosis.

Heat shock proteins, especially HSP70 and HSP90, can act as molecular chaperones to assist proper protein folding and prevent abnormal protein aggregation thus protecting the cells against the stress (Walter et al., 2002). Cells are protected from death with the HSP expression, and those failed to produce HSP die via apoptosis (Santoro, 2000). In my work, NaPB treatment up-regulated HSP70 and HSP90a, but it reduced c-jun and c-fos expression. This indicates that NaPB can alleviate steroid-induced stress in HTM cells.

In addition, GO term analysis found NaPB could have the potential to regulate inflammatory response, and NaPB treatment lowered the IL1B expression and suggesting that NaPB might influence the anti-inflammatory action of dexamethasone, however this is yet to be investigated. Also, NaPB treatment could maintain intraocular pressure through the possible regulatory action on CA12.

4.2 Animal models of steroid-induced ocular hypertension

4.2.1 Assessment of rabbit steroid-induced OHT model with clinical situation

In clinical situation, topical and systemic use of corticosteroids may cause an elevation of intraocular pressure (IOP), leading to the secondary open angle glaucoma (Sapir-Pichhadze et al., 2003). Those who will develop steroid-induced elevations in IOP (above 6 mmHg, nearly 30% of human baseline IOP) after 4 weeks of topical steroid 4 times daily are called steroid-responders, and those who developed more than 10 to 15 mmHg (50% of human baseline IOP) IOP rise with topical steroid use within 2 weeks are "super-responders" (Tripathi et al., 1999). Steroid-induced ocular hypertension can occur in both male and female, in any races and ages, especially children have been reported to have more drastic increase of IOP after using steroids (Fan et al., 2001; Fan et al., 2003). And previously, the IOP changes caused by different steroids on 7-week-old, 6-month-old and 1-year-old New Zealand albino rabbits were compared, and showed that Maxi-Dex® treatment could induce the most drastic changes on 7-week-old New Zealand albino rabbits (Qin et al., 2010).

So, in this study, to establish an animal model of steroid-induced ocular hypertension, male New Zealand albino rabbits of 7-week-old were used. Before drug treatment, baseline IOP was measured twice using TonoPen XL. After topical treatment with dexamethasone/steroids on both eyes 4 times daily, IOP was measured every 3 or 4 days for at least 14 days. The rabbits which had an IOP rise of more than 25% were defined as responder, and those had elevated IOP of greater than 50% were defined as severe responders (Knepper et al., 1978; Knepper et al., 1985). In several animal experiments, topical treatment of Maxi-Dex® and 0.5% prednisolone acetate brought up the IOP to more than 75% of the rabbits, indicating a success of establishing the animal model of steroid-induced ocular hypertension.

In my study, IOP was measured using Tonopen XL and TonoVet. It was reported that Tonopen XL is subject to inaccurately estimate IOP and has higher deviation than TonoVet. It overestimated IOP at low levels and underestimated at high IOP levels (Kalesnykas et al., 2007). So, IOP measured by Tonopen XL should be adjusted to the measurement as TonoVet. It was reported by Jeong et al. in 2007 that the linear regression equation describing the relationship between Tonopen XL and TonoVet was $y=0.669x+4.194$ (x=Tonopen XL, and y=TonoVet) in normal Eurasian eagle owls (Jeong et al., 2007). Although this comparison between Tonopen XL and TonoVet was not on rabbit, the adjustment according to this comparison could reduce the deviation of Tonopen XL measurements and make them closer to TonoVet measurements.

4.2.2 Correlation of IOP levels with TM cell density

Trabecular meshwork is a spongy region, which is lined by trabeculocytes located around the base of cornea, below the scleral veins, and near to the ciliary body, overlying the Schlemm's canal (Soules et al., 2005; Chen et al., 2008). It allows fluid to drain into a set of tubes called Schlemm's canal (in human) flowing into the scleral venous system. The function of TM is thus to drain the aqueous humor from the anterior chamber and regulate the IOP level.

In human, TM region is composed of arrays of collagen beams covered by endothelium-like cells (HTM cells), with extracellular matrix occupying the spaces between the beams (Tian et al., 2000). TM cells are primarily involved in maintaining the extracellular matrix of the trabecular meshwork region through the deposition and degradation of proteoglycans, elastin, collagen and other extracellular matrix materials (Ryland et al., 2003). To facilitate the aqueous humor outflow, TM cells have the abilities of phagocytic and contractile (de Kater et al., 1990; Flügel et al., 1991; Gabelt et al., 1997)

Beside the humor synthesis, the fluctuation of IOP is also due to the outflow resistance from the anterior chamber through the trabecular meshwork region, which is regulated by trabecular meshwork cells (Tian et al., 2000). Steroid treatment *in vivo* and *in vitro* can cause increased production and release of glycosaminoglycans and

other proteins, as well as cytoskeletal changes, like actin cross-linking. This may cause cellular stress and morphological changes in trabecular meshwork cells and ultimately result in cell death (Clark et al., 1994; Clark et al., 1996; Wang et al., 2008). As a consequence, the disrupted trabecular meshwork tissue cells may have ineffective phagocytic ability and reduced pinocytosis of aqueous humor, causing resistance to humor flow and IOP rise (Shirato et al., 1989; Matsumoto et al., 1997; Zhang et al., 2007). In an animal model of steroid-induced ocular hypertension, an accumulation of ECM in the morphology of plaques containing fibrillar basement membrane-like material underneath the endothelium of the inner wall of the outflow loops could be observed in steroid-treated bovine eyes (Tektas et al., 2010). In the rabbit eyes, it also showed substantial thickening of extracellular matrix materials, and a drop of cellularity in TM region after topical Maxi-Dex®/steroid treatment (Qin et al., 2010). This resists the aqueous humor outflow, and causes an IOP elevation.

As mentioned, TM cells are primarily involved in maintaining the extracellular matrix of TM region through the deposition and degradation of proteoglycans, elastin, and collagens (Ryland et al., 2003). The TM tissue of rabbit eyes should be examined. The cells in TM region and the structures such as proteoglycans and collagens should be identified via different staining methods. So, in this study, serial paraffin sections of PO (pupil-optic nerve) position of the steroid-treated rabbit eyes showing high IOP were obtained for histochemistry examination. The cells were quantified by blue coloration of nuclei by haematoxylin and eosin (H&E) staining. The density of cells

in TM region was calculated by number of nuclei per mm^2 TM area. In my study, there was a significant TM cell loss caused by Dex treatment, as the result showed (*in vitro* result and H&E staining). It indicated that Dex-caused TM cell loss may further affect the function of TM cells and the deposition and formation of extracellular matrix.

As glucose residues can be oxidized by periodic acid and create aldehyde to react with the Schiff reagent and creates a purple-magenta color, PAS staining is used for identify the structures containing a high proportion of carbohydrate macromolecules including glycogen, glycoprotein, and proteoglycans (Huet et al., 1975). In addition, Masson's trichrome staining produces red keratin and muscle fibers, green collagen and bone, pink cytoplasm, and black cell nuclei. It is often used for the identification of collagen (Lanir et al., 1984). In Dex-treated eyes, it showed more extracellular materials within the cribriform layers, glycogen loss and more collagen accumulation. This may affect the function of TM cells to regulate the IOP level.

Compared with normal rabbit eyes, the ECM deposition, glycogen loss, collagen accumulation, and significant cell loss were observed in the TM region of steroid-treated eyes. As mentioned earlier, IOP level is mainly regulated by trabecular meshwork cells and the function of TM region, when the number and function of these cells were affected by steroid treatment, the IOP level rose.

4.2.3 Prevalence of rabbit steroid-induced OHT model in drug testing

As early as in 1978, weekly subconjunctival injections of 4 mg betamethasone for more than three weeks produced a sustained increase of IOP in 96% of the treated rabbits (Bonomi et al., 1978). This finding created an animal model of steroid-induced ocular hypertension on rabbits. After that, there are more and more animal models on steroid-induced ocular hypertension on rabbits. Before 1990, topical treatment of 0.1% dexamethasone 4 times daily was found to have the hypertensive effect on young New Zealand red rabbits. Approximately 75% of rabbits had more than 5 mmHg of IOP rise in 4 weeks (Knepper et al., 1978; Knepper et al., 1985). After two years, this model was used with New Zealand white rabbits to perform a drug test on 3α,5β-tetrahydrocortisol, which was considered as an inactive metabolite of cortisol (Southren et al., 1987). Also, subconjunctival injection of cortisone and triamcinolone were found to elevate the IOP significantly and consistently (Hester et al., 1987).

In the 1990s, repeated weekly subconjunctival injection of betamethasone over 4 weeks resulted in an IOP rise in New Zealand albino rabbits, and this elevated IOP was sustained in 7 weeks and prolonged to 11 weeks (Melena et al., 1997).

This finding showed that this animal model might be suitable for testing the short-term and long-term effects of anti-glaucoma drugs. In the next year, the same group used the same animal model to test the effect of diltiazem on betamethasone-induced ocular hypertension (Melena et al., 1998).

In recent years, rabbit model of steroid-induced ocular hypertension was performed using topical instillation of 1% prednisolone twice a day, and this elevated the IOP significantly (Agarwal et al., 2009). This model was used in testing the effect of topical *Aegle Marmelos* fruit extract.

Besides using the rabbits, there were also other types of animal models of steroid-induced ocular hypertension. Gerometta's group induced high IOP on cattle and sheep using topical prednisolone (Gerometta et al., 2004; Gerometta, et al., 2009; Candia et al., 2010; Gerometta et al., 2010; Tektas et al., 2010).

In addition, Sawaguchi's group successfully established steroid-induced ocular hypertension in rats and cats using topical dexamethasone (Gerometta et al., 2004; Sawaguchi et al., 2005; Shinzato et al., 2007; Miyara et al., 2008). In late 2010, implantation of osmotic mini-pumps delivering dexamethasone to C57BL/6J-*Tyr*$^{c\text{-}Brd}$x129S5/SvEvBrd (B6.129) mice successfully induced high IOP (Whitlock et al., 2010).

In my laboratory, ocular hypertension in rabbits was induced by topical steroids, and the IOP changes caused by different steroids on 7-week-old, 6-month-old and 1-year-old New Zealand albino rabbits were compared (Qin et al., 2010). We showed that Maxi-Dex® treatment induced the most drastic changes on 7-week-old rabbits.

We also used this model to show the suppressive effect of *Ginkgo biloba* extract (GBE) on topical Dex-induced ocular hypertension (Jia et al., 2008). So, to consolidate this result in my experiment, I repeated the topical Maxi-Dex® treatment to male New Zealand albino rabbits of 7-week-old. And as mentioned in the assessment of the animal model, it was successfully established and then used to examine the effect of small molecule chemicals on steroid-induced ocular hypertension.

4.3 Effect of sodium 4-phenylbutyrate (triButyrate®) on a rabbit model of steroid-induced ocular hypertension

4.3.1 Drug safety

TriButyrate® is a commercially available product of sodium 4-phenylbutyrate (NaPB), and it was originally developed in 1980's by Triple Crown Co., US. Because triButyrate® was proved to be safe and efficacious in treating urea-cycle disorder and β-thalassemia (Brusilow et al., 1984; Dover et al., 1992; Dover et al., 1994; Collins et al., 1995; Maestri et al., 1995; Maestri et al., 1996; Praphanproj et al., 2000; Singer et al., 2005; Hines et al., 2008), it was approved by Food and Drug Administration (FDA) of US to treat these two diseases. NaPB exhibits its chaperone and neruoprotective activity on several eye diseases including retinitis pigmentosa, cataract, glaucoma and diabetic retinopathy (Bonapace et al., 2004b; Jeng et al., 2007; Mulhern et al., 2007; Yam et al., 2007; Jia et al., 2009; Li et al., 2009; Gong et al., 2010).

It was reported that, the maximal dose used of NaPB was 60 g/day (20 g, 3 times daily) for oral form to treat cystic fibrosis (Zeitlin et al., 2002). And, for intravenous injection (iv), the maximal tolerated dose of NaPB was 410 mg/kg/day to treat lymphoma (Carducci et al., 2001). In animal study on MYOC-caused glaucoma, NaPB of 10 mg/day in drink water (~400 mg/kg/day) reduced ER stress and prevented IOP rise in *Tg-MYOC*^{Y437H} mice (Zode et al., 2011). The optimal dose of NaPB on steroid-induced ocular hypertension was 0.02 mg/day (0.013-0.02 mg/kg/day), which was 1/20500-31500 as reported tolerated iv dose and was 1/20000-30769 as oral dose used to prevent IOP rise in *Tg-MYOC*^{Y437H} mice. It should be safe on preventing steroid-induced ocular hypertension on rabbits.

Until now, there is no report on its effect on steroid-induced ocular hypertension. And sodium 4-phenylbutyrate (triButyrate®) has not yet been approved by FDA on the treatment of eye diseases, so it is indispensable to test the safety on rabbit eyes.

To examine the ocular safety of sodium 4-phenylbutyrate (triButyrate®), the rabbit eyes were checked before and after each eye drop application for any redness, inflammation and other abnormalities. And, the pictures of corneal surface, limbus and conjunctiva regions as well as corneal epithelium condition by fluorescein staining were captured and the density of corneal endothelial cells was recorded. It showed no distinct endothelial cell loss/gain in the rabbit eyes received NaPB, even up to the dosage of 5 mM, 4 times daily. And in slit lamp examination, no observable

corneal opacity, epithelial defects, neovascularization or inflammation was showed. So, in this study, the rabbits showed no adverse response to sodium 4-phenylbutyrate (triButyrate®). It can be considered as a safe application on rabbit eyes.

In my study, although I have recruited 150 rabbits to establish the animal model of steroid-induced ocular hypertension, my result did not include all of them. That was because some rabbits did not respond to steroid treatment (IOP rise less than 20% in 14 days), and some died during the experiments. The leading cause of rabbit death was diarrhea, and other reasons such as fighting injury, paralysis, and bleeding may also cause rabbit death. The rabbit death should not be due to the treatment of drugs (steroid or NaPB/triButyrate®), as the rabbits did not have systemic adverse effect after drug application and most of the rabbits could alive until they were sacrificed at the end of experiments.

4.3.2 Treatment ability of sodium 4-phenylbutyrate (triButyrate®) on developed high IOP

In my study, the proof-of-principal finding is the preventive effect of sodium 4-phenylbutyrate (NaPB, triButyrate®) on steroid-induced ocular hypertension. NaPB application reduced the IOP rise caused by steroid by lower the IOP ratio between steroid/NaPB-treated and steroid-treated rabbit eyes. This was replicated in several rabbit experiments including our pilot study, and the experiments on different dose and frequency of NaPB. From the results of our experiments on rabbits, the optimal

dose and frequency of topical NaPB should be 0.5 mM, 4 times daily.

In addition, the IOP reduction effect of Ammonul® was not as good as that of triButyrate®. As NaPB metabolized rapidly through beta-oxidation into phenylacetate, which conjugates with glutamine to form acetylglutamine and is excreted in urine (Hommes, 1999), it was originally used as an ammonia-scavenging agent in urea metabolism disorders (Brusilow et al., 1984). As the metabolism was in the urea-cycle, it may not affect IOP level. This indicated that there could be specific mechanism for the action of NaPB to prevent steroid-induced ocular hypertension.

Compared with NaPB, TMAO did not suppress the IOP rise caused by steroids. This also indicated a specific mechanism of NaPB on preventing steroid-induced ocular hypertension. For the suppression of steroid-induced IOP elevation by NaPB, is a preventive effect, because slower IOP rise was observed when dexamethasone-treated rabbit eyes were combined the treatment with NaPB. However, whether NaPB reduces the high IOP caused by steroids is yet to be investigated. In the rabbit model of steroid-induced ocular hypertension, Maxi-Dex®/steroid is applied for 2 to 3 weeks to see if steroid-induced IOP effect in the young rabbits. After the selection of steroid responders, NaPB is applied on one eye of them, and the other eye followed by normal saline. IOP will be measured regularly to see if the high IOP can be lowered. This is to examine whether NaPB has the treatment effect on steroid-induced ocular hypertension.

4.4 Proposed mechanisms of sodium 4-phenylbutyrate (triButyrate®) action on steroid-induced ocular hypertension

Aqueous humor is produced by ciliary body and it drains out of the eye through the trabecular meshwork. A constant IOP is maintained by the balance between the production and drainage of aqueous humor and violation of this causes the fluctuation of IOP.

As it is known, steroid-induced IOP elevation is mostly due to diverse effects of steroids on the TM, altering TM cell functions, gene expression, extracellular matrix metabolism and cytoskeleton structure, leading to the resistance of aqueous humor outflow (Steely et al., 1992; Clark et al., 1994; Clark et al., 1996; Dickerson et al., 1998; Zhou et al., 1998; Tane et al., 2007). For the alteration of TM cell gene expression of steroids, in 2002, Ishibashi's group showed 30 out of 2400 human genes analyzed in cultured human TM cells were up-regulated for more than two folds after treatment with dexamethasone (100 nM) for 7 days (Ishibashi et al., 2002). Lo and his group reported multiple genes differentially up-regulated in dexamethasone (100 nM, treated for 8 to 10 days)-treated human TM cells including myocilin (TIGR/MYOC), α1-antichymotrypsin (a serine protease inhibitor), PEDF, cornea-derived transcript 6, and prostaglandin D2 synthase (Lo et al., 2003). Fifteen of the 249 differentially regulated genes with four or more fold changes were mapped to glaucoma-linked loci. For long-term dexamethasone treatment (for 21 days) on human TM cells, among

1260 genes analyzed using U133A GeneChip, 23 genes were up-regulated and 18 were down-regulated by more than three folds (Rozsa et al., 2006). Confirmed by quantitative PCR, genes with significant changes were *SLP1, SAA2, ANGPTL7, MYOC, SAA1, SERPINA3* and *ZBTB16*. Our laboratory also reported gene expression changes in steroid-treated human TM cells by using microarray and RT-PCR analyses. After treatment with Dex at 100 nM for 10 days, genes including *GAS1, CDH4, MT1L, CST3, ATF4, ASNS/TS11, CHOP, HSPA5* were expressed at levels more than 1000 times than at normal condition, and *TSC22, LDHA, IGFBP2, TAGLN, SCG2, WARS* more than 100 times (Leung et al., 2003). Several of them were reported to function in anti-inflammatory response and humor outflow resistance. In TM cells treated with either Dex or TA, *MYOC* and *GAS1* which located in known primary open-angle glaucoma (POAG) loci were up-regulated (Fan et al., 2008). In contrast, *SENP1, ZNF343* and *SOX30* were down-regulated. TA up to 0.1 mg/ml and 1 mg/ml induced the expression of c-jun, c-fos, HSP90a ($p<0.005$) and caspase-3 ($p<0.05$). Also, TA suppressed the cell proliferation, and induced apoptosis in HTM cells (Wang et al., 2008).

From our results, dexamethasone treatment altered gene expression, which is related to aqueous humor production, apoptosis, and inflammatory response. Dexamethasone treatment also suppressed the viability and induced apoptosis of human trabecular meshwork cells. Moreover, from the ultrastructure of the TM region of Dex-treated rabbit eyes, ER dilation of TM cells, accumulation of plaques and

basement membrane-like materials and blocked channel region can be observed. The TM cell loss, deposition and degradation of proteoglycans and collagens could be detected under light microscopy. When the number and function of trabecular meshwork cells were affected by steroid treatment, TM cells became incapable of phagocytosis and contractile effect which caused the resistance of aqueous humor outflow, thus leading to IOP elevation (Matsumoto et al., 1997; Zhang et al., 2007).

Steroid affects TM cells and TM region not only structurally, but also functionally to result in steroid-induced ocular hypertension. The most important principal to lower the high IOP developed by steroids is to alleviate the structural and functional changes of TM cells and TM region including the rescue of TM cells from stress and apoptosis and reduction of ECM deposition.

The heat-shock stress response protein HSP90 is an important regulator for GRα as it binds to GRα and blocks its interaction with Dex to activate GRE-mediated gene expressions (Tago et al., 2004). Also, HSP90 is an essential molecular chaperone for nuclear transport of GRβ (Zhang et al., 2006). As mentioned, myocilin (MYOC) which is in glaucoma loci was reported as a delayed secondary GRE-mediated gene in (Shepard et al., 2001). In MYOC-caused glaucoma, NaPB was reported to reduce stress in both cell and animal models (Yam et al., 2007; Jia et al., 2009). In cell model, 1 mM of NaPB reduced myocilin aggregates and ER stress, leading to better cell survival in HTM cells. In animal model, oral NaPB of 10 mg/day reduced ER stress

and IOP rise in *Tg-MYOC*^{Y437H} mice (Zode et al., 2011). In my study, ER dilation of TM cells was observed in Dex-treated rabbit eyes and TM cell loss could be detected *in vitro* and *in vivo* after Dex treatment. HSPs are chaperones which regulate incorrectly folded proteins. NaPB was reported to reduce stress in cell and animal model of MYOC-caused glaucoma, and Dex could cause similar cell changes or apoptosis as myocilin. So, we hypothesize that NaPB could reduce steroid-induced IOP rise by reduce stress and apoptosis on TM cells.

In my study, GO term analysis found that NaPB might have the potential of regulate the extracellular matrix metabolism, inflammatory response and defense response changed by Dex. NaPB reversed the dexamethasone-caused CA12 down-regulation, which might relate with aqueous humor production. This indicated its possible effect on regulating intraocular pressure through controlling aqueous humor production. In cultured human trabecular cell line, NaPB also reversed dexamethasone-caused upregulation of IGFBP4, which is associated with cell apoptosis. Moreover, NaPB treatment suppressed caspase-3 gene expression indicating a possible cell rescue effect. MTT cell viability/proliferation assay and TUNEL apoptosis assay also supported these findings of gene expression study. In addition, NaPB application could reduce TM cell loss caused by dexamethasone in the rabbit model. Hence, NaPB could improve TM cell survival not only *in vitro*, but also *in vivo*. And this could preserve the TM function in the aqueous outflow pathway and alleviate steroid-induced IOP elevation. The reduction of extracellular matrix

deposition in TM region by NaPB was observed under both light microscopy and electron microscopy.

Ultrastructurally, it was reported that there was a loss of glycogen in the cytoplasm of TM cells, an increase in basement membrane-like material, and enlarged cisterns of rough ER of TM cells in steroid-treated bovine eyes (Tektas et al., 2010). In my study, the TM region of Dex-treated rabbit eyes showed more clear channels, less basement membrane-like material when in combined treatment with NaPB. Also, the ER enlargement was alleviated. However, prominent extracellular fibre-like structures with interconnections were observed. This structural change of TM region also suggested the potential of NaPB on extracellular matrix metabolism.

NaPB application might prevent steroid-induced IOP rise by reverse the gene expression changes caused by dexamethasone, reduce TM cell apoptosis, and reduce extracellular matrix deposition. All these findings supported that sodium 4-phenylbutyrate (NaPB/triButyrate®) prevented steroid-induced ocular hypertension.

Chapter 5: Conclusion and future prospects

Steroid-induced ocular hypertension is commonly encountered worldwide, particularly in children and patients who require long-term steroid treatment. In my project, through the examination of small molecule chemicals effect in a rabbit model of steroid-induced ocular hypertension, the investigation has been conducted *in vitro* to examine the effect of small molecule chemicals on steroid-induced cell responses, stress induction and cell death to investigate the mechanisms of small molecule chemical actions on TM cells.

Nowadays, the first step to treat high IOP is the medication. But for the existing anti-glaucoma medications, the IOP reduction percentage after treatment was usually not more than 25%. Also, they usually lower the IOP when high IOP has already developed (Rhee et al., 2001). In this study, a rabbit model of dexamethasone-induced ocular hypertension was successfully established as the rabbits showed a rise of intraocular pressure (IOP) by more than 20% after topical treatment of Maxi-Dex® (0.1% dexamethasone ophthalmic suspension, Alcon) 4 times daily for 14 days. Combined treatment of Maxi-Dex® and triButyrate® (NaPB) ranging from 0.01 to 15 mM showed preventive effect on Dex-induced IOP rise. The optimal dose of topical NaPB/triButyrate® was 0.5 mM, 4 times daily which showed the IOP reduction of nearly 30%. In addition, NaPB treatment could also suppress IOP rise caused by 0.5% prednisolone, indicating its effect was not restricted to dexamethasone. It showed favourable preventive effect of NaPB on steroid-induced ocular hypertension.

In addition, the morphological and structural changes in rabbit TM region after topical Maxi-Dex® and NaPB were studied by light microscopy and transmission electron microscopy. Histologically, NaPB reduced TM cell loss and extracellular matrix deposition caused by dexamethasone treatment. Under transmission electron microscopy, NaPB reversed dexamethasone-induced ECM plaque formation in the drainage pathway. Also, the TM cell change was normalized and the blockage of outflow channel was cleared, which supported the finding of the preventive effect of NaPB on steroid-induced ocular hypertension.

Moreover, the existing medical and surgical treatments may induce adverse effects and potential risks to the patients. For example, long term use of beta-adrenergic receptor antagonists may slow down the heart rate, and even result in atrioventricular block or asthma (Taira et al., 2008). Prostaglandin analogs may cause conjunctival hyperemia, elongation, and iris darkening, and they have abortive potential, which is the most dangerous (Cracknell et al., 2009). Alpha2- adrenergic agonists also have side effects like allergy in eyes, eye jumpiness, fast or atypical heartbeat and hypertension (Camras et al., 1999). And all surgeries may have the risk of infection and failure. In this study, the rabbit eyes with topical NaPB up to 5 mM had no ocular toxicity, no loss of corneal endothelial cell and no corneal epithelial defects. It showed the safety of NaPB on rabbit eyes.

From the *in vitro* study, dexamethasone (100 nM) treatment on immortalized human trabecular meshwork (HTM) cells resulted in a 2-fold increase of apoptosis compared to untreated control cells. For the combined treatment with dexamethasone and sodium phenylbutyrate (NaPB, triButyrate®, 1 mM), the apoptosis rate was reduced to the control level, which could be mediated by its suppression of insulin growth factor beta binding protein 4, a pro-apoptotic protein. GO term analysis found that NaPB might have the potential of regulate the extracellular matrix metabolism, inflammatory response and defense response changed by Dex. Further expression analysis showed dexamethasone-suppressed expression of carbonic anhydrase 12 could be reversed by NaPB treatment, suggesting a regulation to aqueous humor biosynthesis and intraocular pressure maintenance. However, NaPB treatment might interfere with anti-inflammatory effect of dexamethasone, probably through the regulation of interleukin 1β expression.

In summary, my work had utilized the HTM cell model and rabbit model of steroid-induced ocular hypertension and showed that NaPB protected human trabecular meshwork cells against apoptosis. In experimental rabbits, topical NaPB lowered the steroid-induced IOP rise and was safe for ocular use. My results indicate the possible mechanisms of NaPB action on steroid-induced ocular hypertension.

Although there were some important and meaningful findings in this study, the mechanism of NaPB/triButyrate® action on steroid-induced ocular hypertension is

still not fully understood and should be investigated. And, this study only showed the preventive effect of NaPB on steroid-induced ocular hypertension. For the developed IOP elevation after steroid treatment for a period of time, it is yet to be determined whether the IOP can be reduced by NaPB/triButyrate® treatment.

NaPB showed promising effect on preventing steroid-induced ocular hypertension, and it did not induce any ocular and systemic toxicity on rabbits. Since the major purpose of this work is to develop a useful medication to prevent/reduce steroid-induced glaucoma in patients. It is necessary and worthwhile for us to plan for clinical trials.

With this preventive effect on steroid-induced ocular hypertension, NaPB/triButyrate® could be a potential and novel anti-ocular hypertension agent. This study has demonstrated that stress alleviation in aqueous outflow pathway was a novel therapeutic strategy in treating hypertensive glaucoma. I hope that with further work in the near future, NaPB/triButyrate® may be recruited in the treatment of steroid-induced ocular hypertension in patients.

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