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Study on the Prevalence and Proteomic Analysis of the Aqueous Humor in Canine Primary Glaucoma

개의 원발성 녹내장 발생 현황과 안방수 **Proteomic** 분석에 대한 연구

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ABSTRACT

This study investigated the prevalence of breed, age, and sex of canine primary glaucoma patients, and analyzed the aqueous humor (AH) of canines with primary angle-closure glaucoma (PACG).

In chapter 1, the prevalence of canine primary glaucoma by breed, age, and sex was compared with previous reports in Korea. We included the medical records of dogs diagnosed with primary glaucoma who visited the veterinary medical teaching hospital of

Seoul National University (SNU) from January 2011 to December 2020 and investigated their breed, age, and sex. All the patients underwent a full ophthalmic examination. We analyzed the results using a binary logistic regression analysis based on the Jindo dog, which was close to the mean value of the primary glaucoma incidence rate. Of the 14,587 dogs treated at the veterinary medical teaching hospital of SNU, 107 (0.73%) were diagnosed with primary glaucoma. Glaucoma occurred in 14 breeds, including American Cocker Spaniel, Shih Tzu, Maltese, Pomeranian, Jindo dog, Mixed Breed, Pekinese, Toy Poodle, Samoyed, Shiba Inu, Miniature Pinscher, Boston Terrier, Labrador retriever, and Yorkshire Terrier. The mean age of onset of primary glaucoma was 7.8 ± 2.3 years old. Primary glaucoma was observed in 53 spayed females, 11 females, 38 castrated males, and 5 males. Regardless of neutralization, the ratio of females to males was 1.5:1. This study showed that primary glaucoma was significantly higher in American Cocker Spaniels and higher in Shih Tzus than other breeds in Korea; they had the highest incidence of primary glaucoma at 7 and 8 years of age, respectively. Therefore, the two breeds should be carefully monitored for the occurrence of primary glaucoma when they approach 7 years of age.

In chapter 2, proteomic profiles of the aqueous humor (AH) of canines with primary angle-closure glaucoma (PACG) was analyzed and associated protein alterations was identified. Six American Cocker Spaniels with PACG and six American Cocker Spaniels without ocular disease. Total AH protein concentration was determined by the bicinchoninic acid (BCA) assay. AH protein samples were quantified by liquid chromatography-mass spectrometry (LC-MS/MS) and the Gene Ontology (GO)

enrichment analysis was performed using ClueGO. The AH protein concentration in the PACG group (10.49 ± 17.98 μg/μl) was significantly higher than that of the control group (0.45 ± 0.11 μg/μl; $p < 0.05$). A total of 758 proteins were identified in the AH. Several proteins both significantly increased (n=69) and decreased (n=252) in the PACG group compared to those in the control group. GO enrichment analysis showed that the "response to wounding", "negative regulation of endopeptidase activity" and "cell growth" pathways were the most enriched terms in the PACG group compared to the control group. The top 5 proteins that were significantly increased in the AH of the PACG group were secreted phosphoprotein 1 (SPP1), peptidoglycan recognition proteins 2 (PGLYRP2), tyrosine 3-monooxygenase (YWHAE), maltase-glucoamylase (MGAM), and vimentin (VIM). Gene Ontology enrichment analysis using the proteomic data showed that proteins and pathways related to inflammation were significantly upregulated in the various stage of PACG. Proteomic analysis of the AH from the PACG may provide valuable insights into PACG pathogenesis.

In conclusion, Shih Tzu and Cocker Spaniel should be concerned about primary glaucoma at the age of seven and the aqueous humor analysis of primary glaucoma patients, and identified proteins which are potential biomarkers for assessing increased risk.

Keywords: American Cocker Spaniel, angle-closure glaucoma, aqueous humor, dog, LC-MS/MS, primary glaucoma, proteomics

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CONTENTS

GENERAL INTRODUCTION

Glaucoma is a complex group of optic neuropathy that causes progressive retinal ganglion cell and their axons apoptosis and irreversible vision loss, associated with atrophy of retina and optic nerve (Komáromy *et al.*,2019). Glaucoma is classified as congenital, primary, or secondary glaucoma according to the etiology of the disease. Congenital glaucoma (characterized by goniodysgenesis with iridocorneal angle abnormalities) occurs at a relatively early age (<1 year of age) and has a low occurrence in dogs (Strom *et al.*, 2011). In addition, congenital glaucoma has been reported at the age of 21 days in the breed Dogue de Bordeaux, 28 days in Jack Russell Terrier, 35 days in German Hunting Terrier, and 6.5 months in Kooikerhondje in Switzerland (Strom *et al.*, 2011). Unlike secondary glaucoma, primary glaucoma develops because of primary abnormalities rather than other ocular diseases. It occurs bilaterally, and breed predisposition has been reported (Park *et al.*, 2019). Although the exact mechanism of primary glaucoma is still unknown, genes that develop glaucoma have been documented (Park *et al.*, 2019). Primary glaucoma is divided into primary open-angle glaucoma (POAG) and primary angle-closure glaucoma (PACG). In POAG, the ICA is normal at the beginning of the disease, but gradually becomes closed; it has been reported in juvenile beagles or middle-aged-to-older Petit Basset Griffon Vendéens and Norwegian

Elkhounds (Kuchtey *et al.*, 2013; Ahonen *et al.*, 2015; Bedford, 2017). POAG often has a mutation gene, such as ADAMTS10, that affects trabecular meshwork and raises the IOP (Kuchtey *et al.*, 2013). It is more common in humans than in dogs (Kuchtey *et al.*, 2013). However, PACG is characterized by structural abnormalities, such as a narrowed ICA and ciliary cleft. It is also closely associated with pectinate ligament dysplasia (PLD), and high IOP-related clinical signs usually occur in middle-to-old age (Pearl *et al.*, 2015). The breed predispositions for PLD are English Springer Spaniel, Flat-coated retriever, Great Dane, and Samoyed (Ekesten and Torrång, 1995; Wood *et al.*, 2001; Bjerkås *et al.*, 2002). The prevalence of PACG in primary glaucoma is approximately 26% and 87% in humans and dogs, respectively (Miller, 2018; Dubey *et al.*, 2019). Among congenital, primary, and secondary glaucoma, PACG progresses most rapidly and even causes vision loss with severe pain within a day in dogs (Miller, 2018).

The mechanism underlying intraocular pressure (IOP) elevation in human POAG is yet to be elucidated; however, the mechanism underlying IOP elevation was identified in Beagle and Basset Hound by ADAMTS10 and nebulin (NEB), respectively. ADAMTS17 mutation has also been identified as a causative mutation in POAG in the breeds Shar Pei and Petit Basset Griffon Vendeen (Ekesten and Torrång, 1995; Heo *et al.*, 2018; Dubey *et al.*, 2019). In humans, optic neuropathy can develop even with normal IOP, and it has been shown that an increase in IOP can further exacerbate glaucoma (Weinreb *et al.*, 2014). While nebulin mutations exert a strong effect on the anterior segment, ADAMTS10 mutations affect the elasticity of the scleral and optic nerve head lamina; therefore, the potential effect of these mutations on the optic nerve cannot be

considered independent of IOP elevation (Dubey *et al.*, 2019). Controlling IOP through medication or surgical methods is known as the best treatment for glaucoma (Houston and Moore, 2009; Heo *et al.*, 2018). Early diagnosis of glaucoma is important for a better prognosis and appropriate treatment to prevent vision loss.

The optically clear fluid, aqueous humor (AH) is crucial as it supplies nutrients to the avascular ocular structures and removes metabolic waste from these structures (Kato *et al.*, 2006). The AH maintains the IOP, has antioxidant properties, and plays an important role in the immunity of the eye (Kato *et al.*, 2006; Kim *et al.*, 2016). AH is secreted by the ciliary body epithelium and contains various proteins (Kato *et al.*, 2006). Depending on ocular diseases, the AH displays different protein expression patterns (Kuchtey *et al.*, 2013). Some proteins appear or increase, while some proteins disappear or decrease depending on the mechanism of various ocular diseases (Kunz *et al.*, 2015).

Mass spectrometry (MS)-based proteomics is a key technology for large-scale protein identification and quantification (Aslam *et al.*, 2017). Understanding the protein expression levels will help in the prevention, diagnosis, treatment, and prognosis of glaucoma (Li *et al.*, 2017). Proteomic analysis has been applied in human ophthalmology to understand the pathogenesis of glaucoma (Sharma *et al.*, 2018). Previous studies suggested that some AH proteins could be closely related to the development of glaucoma. In veterinary ophthalmology, while AH proteomic studies have not been conducted for canine glaucoma, a proteomic study has been performed on the tear film in canine glaucoma, and elevated levels of myocilin, CD44, metalloproteinase 2, endothelin 1, nitric oxide, and nebulin were detected in the AH of dogs with glaucoma using

Coomassie staining, enzyme immunoassay, and Western blot (Källberg *et al.*, 2007; Weinstein *et al.*, 2007; MacKay *et al.*, 2008; Dina *et al.*, 2015; Graham *et al.*, 2021). Peptidoglycan recognition protein 2 (PGLYRP2) plays a very important role in the innate immune response and has antibacterial activity. However, its role in veterinary ophthalmology is not well known (Brandley and Schnaar, 1986; Ranjita *et al.*, 2015). Proteomic analyses of AH have been performed in animals, such as rabbits and rats, as well as in humans. However, to the best of our knowledge, proteomic analysis of AH has not been performed in dogs.

The purpose of this study was to determine the prevalence of breed, age, and sex of canine primary glaucoma by comparing previous reports in Korea (Chapter 1) and perform proteomic analysis of the AH of dogs with PACG and identify associated protein alterations. Proteomic analysis may provide valuable insights into glaucoma pathogenesis, thereby identifying proteins that are potential biomarkers for assessing increased risk (Chapter 2).

CHAPTER I

A Retrospective Study of Canine Primary Glaucoma (2011-2020)

ABSTRACT

The prevalence of breed, age, and sex of canine primary glaucoma was determined by comparing previous reports in Korea. The medical records were included dogs diagnosed with primary glaucoma who visited the veterinary medical teaching hospital of Seoul National University (SNU) from January 2011 to December 2020 and investigated their breed, age, and sex. All the patients underwent a full ophthalmic examination. We analyzed the results using a binary logistic regression analysis based on the Jindo dog, which was close to the mean value of the primary glaucoma incidence rate. Of the 14,587 dogs treated at the veterinary medical teaching hospital of SNU, 107 (0.73%) were diagnosed with primary glaucoma. Glaucoma occurred in 14 breeds, including American Cocker Spaniel, Shih Tzu, Maltese, Pomeranian, Jindo dog, Mixed Breed, Pekinese, Toy Poodle, Samoyed, Shiba Inu, Miniature Pinscher, Boston Terrier, Labrador retriever, and Yorkshire Terrier. The mean age of onset of primary glaucoma was 7.8 ± 2.3 years old. Primary glaucoma was observed in 53 spayed females, 11 females, 38 castrated males, and 5 males. Regardless of neutralization, the ratio of females to males was 1.5:1. This study showed that primary glaucoma was significantly higher in American Cocker Spaniel and higher in Shih Tzu than other breeds in Korea; they had the highest incidence of primary glaucoma at 7 and 8 years of age, respectively. Therefore, the two breeds should be carefully monitored for the occurrence of primary glaucoma when they approach 7 years of age.

INTRODUCTION

Glaucoma is a painful, progressive, and leading cause of irreversible blindness in humans and dogs (Dees *et al.*, 2014; Medeiros *et al.*, 2015). In glaucoma, apoptosis of retinal ganglion cells and axons results from heterogeneous causes, such as inhibition of aqueous humor drainage, leading to an increase in intraocular pressure (IOP) (major risk factor) (Seon *et al.*, 2020). Congenital glaucoma (characterized by goniodysgenesis [GD] with iridocorneal angle [ICA] abnormalities) occurs at a relatively early age (<1 year of age) and has a low occurrence in dogs (Seon *et al.*, 2020). In addition, congenital glaucoma has been reported at the age of 21 days in the breed Dogue de Bordeaux, 28 days in Jack Russell Terrier, 35 days in German Hunting Terrier, and 6.5 months in Kooikerhondje in Switzerland (Strom *et al.*, 2011). Unlike secondary glaucoma, primary glaucoma develops because of primary abnormalities rather than other ocular diseases. It occurs bilaterally, and breed predisposition has been reported (Park *et al.*, 2012). Although the exact mechanism of primary glaucoma is still unknown, genes that develop glaucoma have been documented (Park *et al.*, 2019). Primary glaucoma is divided into primary open-angle glaucoma (POAG) and primary angle-closure glaucoma (PACG). In POAG, the ICA is normal at the beginning of the disease, but gradually becomes closed; it has been reported in juvenile beagles or middle-aged-to-older Petit Basset Griffon Vendéens and Norwegian Elkhounds (Quigley, 1993; Kaeslin *et al.*, 2016; Plummer *et al.*, 2021a). POAG often has a mutation gene, such as ADAMTS10, that affects trabecular meshwork and raises the IOP (Kuchtey *et al.*, 2013). It is more common in

humans than in dogs (MacKay *et al.,* 2008). However, PACG is characterized by structural abnormalities, such as a narrowed ICA and ciliary cleft. It is also closely associated with pectinate ligament dysplasia (PLD), and high IOP-related clinical signs usually occur in middle-to-old age (Pearl *et al.,* 2015). The breed predispositions for PLD are English Springer Spaniel, Flat-coated retriever, Great Dane, and Samoyed (Bjerkås *et al.,* 2002). The prevalence of PACG in primary glaucoma is approximately 26% and 87% in humans and dogs, respectively (Miller *et al.,* 2018; Dubey *et al.,* 2019). Among congenital, primary, and secondary glaucoma, PACG progresses most rapidly and even causes vision loss with severe pain within a day in dogs (Miller *et al.,* 2018). Primary glaucoma has been reported to occur in 42 predisposed breeds (Gelatt and MacKay, 2004). The American Cocker Spaniel, Basset Hound, Chow Chow, Shar-Pei, and Boston Terrier in North America; Siberian Husky, mixed breed, Entlebucher Mountain Dog, Vizsla, and Newfoundland in Switzerland; Shiba Inu Dog, Shih Tzu, Mixed breed, American Cocker Spaniel, and Beagle in Japan; and American Cocker Spaniel, Shih Tzu, and mixed breed in Korea, are breeds with a high prevalence of primary glaucoma (Gelatt and MacKay, 2004; Kato *et al.,* 2006; Strom *et al.,* 2011; Park *et al.,* 2012). The prevalence of primary glaucoma is affected by nation, region, and breed preferences. Primary glaucoma is diagnosed using gonioscopy, ultrasound biomicroscopy (UBM), high-resolution ultrasonography (HRUS), or optical coherence tomography (Plummer *et al.,* 2021b). The ICA can be identified as open, narrow, occluded, or GD. This study investigated the prevalence of breed, age, and sex of canine primary glaucoma by comparing previous reports in Korea.

MATERIALS AND METHODS

In this study, we included the medical records of dogs diagnosed with primary glaucoma who visited the veterinary medical teaching hospital of Seoul National University (SNU) from January 2011 to December 2020. We investigated breed, age, and sex of patients diagnosed with primary glaucoma. All patients underwent a full ophthalmic examination. Schirmer tear test-1 (STT-1; Schering-Plough Animal Health, Union, NJ, USA), tonometry (TonoVet®, iCare, Helsinki, Finland), fluorescein staining (Flu-Glo®, Akorn Pharmaceuticals, Decatur, IL, USA), neuro-ophthalmic testing, slit-lamp biomicroscopy (SL-D7®, Topcon, Tokyo, Japan), gonioscopy (Volk Optical, Mentor, OH, USA), indirect ophthalmoscopy (Vantage Indirect Ophthalmoscope® , Keeler, Windsor, UK) with a 30D condensing lens, and b-mode ocular ultrasonography were performed. The ICA and ciliary cleft were identified using gonioscopy and UBM (MD-320W; MEDA Co., Ltd, Tianjin, China). The diagnostic criteria for primary glaucoma were an IOP of >30 mmHg measured using rebound tonometer and accompanied by clinical signs of glaucoma, including episcleral injection, mydriasis, corneal edema, buphthalmos, optic disc cupping, retinal hyperreflectivity, ocular pain, and reduced or loss of vision. We excluded patients with secondary glaucoma with a history of lens luxation, uveitis, intraocular tumors, ocular trauma, or those who had undergone intraocular surgery, such as phacoemulsification. The results of this study were compared with those of a previous study on American Cocker Spaniels and Shih Tzus, the breeds with the highest prevalence of primary glaucoma in Korea (Park *et al*, 2012). We compared and analyzed

the data obtained using binary logistic regression analysis based on the results of analysis on the Jindo dog, which were close to the mean value of the primary glaucoma incidence rate.

RESULTS

Of the 14,587 dogs treated at the veterinary medical teaching hospital of SNU, 107 (0.73%) were diagnosed with primary glaucoma. Glaucoma occurred in 14 breeds, including American Cocker Spaniel, Shih Tzu, Maltese, Pomeranian, Jindo dog, Mixed Breed, Pekinese, Toy Poodle, Samoyed, Shiba Inu, Miniature Pinscher, Boston Terrier, Labrador retriever, and Yorkshire Terrier (Table 1). In this study, we identified nine new breeds, including Pomeranian, Jindo dog, Toy Poodle, Samoyed, Shiba Inu, Miniature Pinscher, Boston Terrier, Labrador retriever, and Yorkshire Terrier, which were not reported in previous domestic studies. Compared with previous studies in Korea, primary glaucoma was also significantly higher in American Cocker Spaniels and higher in Shih Tzus in this study (Park *et al*, 2012). The mean age of onset of primary glaucoma was 7.8 ± 2.3 (mean ± SD) years old. The top three breeds, American Cocker Spaniel, Shih Tzu, and Maltese, had a mean age of 7.8 ± 2.6, 8.1 ± 2.6, and 8.9 ± 2.0 years old, respectively. The sexes of primary glaucoma breeds were 53 spayed females, 11 females, 38 castrated males, and 5 males. Regardless of neutralization, the ratio of females to males was 1.5:1. The ratios of females to males in the American Cocker Spaniel, Shih Tzu, and Maltese breeds were 1.3:1, 1.4:1, and 1.8:1, respectively. The mean IOP was 44.0 ± 21.4 (mean ± SD) mmHg at the time of hospital visit. The mean IOPs of the American Cocker Spaniel, Shih Tzu, and Maltese breeds were 47.4 ± 25.4 mmHg, 48.4 ± 18.0 mmHg, and 47.6 ± 24.6 mmHg, respectively. Primary glaucoma occurred in 60 right eyes and 47 left eyes.

Tabel 1. Patients summary with primary glaucoma. Binary logistic regression analysis based on the Jindo dog

No	Breeds	Individual	Primary glaucoma	(%) Incidence rate	Age	Sex (F:M)	Direction (OD/OS)	p value
1	American Cocker Spaniel	656	36	5.5	7.8±2.6	19:17	21:15	0.002*
2	Shih Tzu	1698	39	2.3	8.1±2.6	23:16	20:19	0.057
3	Maltese	2977	11	0.37	8.9±2.0	8:3	6:5	0.353
4	Pomeranian	930	4	0.43	7.0±2.6	3:1	2:2	0.553
5	Jindo dog	444	3	0.68	5.7±2.5	2:1	2:1	-
6	Mixed Breed	789	2	0.25	7.5±3.5	0:2	2:0	0.282
7	Pekinese	570	2	0.35	11.5±0.7	2:0	0:2	0.472
8	Toy Poodle	1461	2	0.14	10.0±4.4	1:1	2:0	0.080
9	Samoyed	303	2	0.66	6.0±1.4	2:0	0:2	0.980
10	Shiba Inu dog	313	2	0.64	3.0±2.8	1:1	1:1	0.951
11	Miniature pinscher	476	1	0.21	6	1:0	1:0	0.311
12	Boston Terrier	309	1	0.32	9	1:0	1:0	0.523
13	Labrador Retriever	582	1	0.17	8	0:1	1:0	0.235
14	Yorkshir Terrier	1,399	1	0.07	11	1:0	1:0	0.051
15	Others	1,680	0	0				
Total		14,587	107	0.73	7.8±2.3	64:43	60:47	

***** p value < 0.05

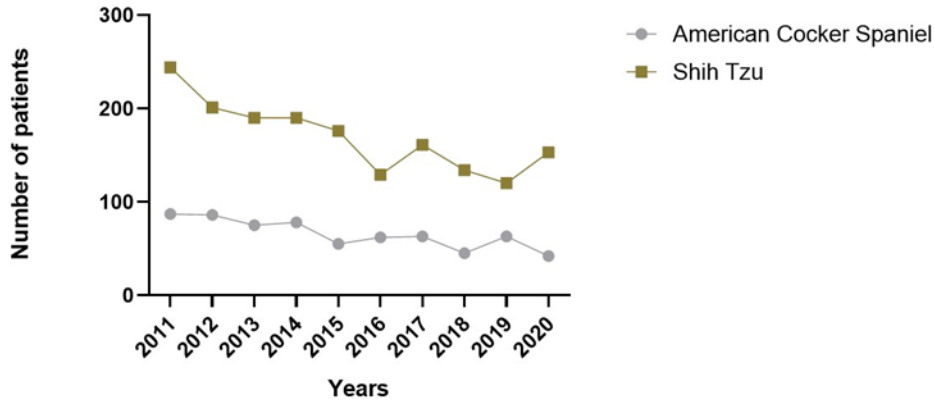


Fig. 1. Presentation number of ocular examinations by years for the American Cocker Spaniel and Shih Tzu in VMTH SNU.

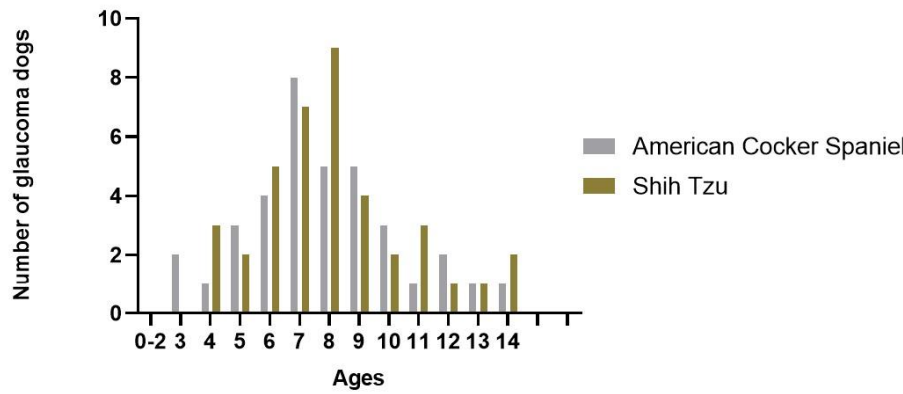


Fig. 2. Presentation number of primary glaucoma by ages for the American Cocker Spaniel and Shih Tzu in VMTH SNU.

DISCUSSION

This study investigated breed prevalence, age, and sex, of canine primary glaucoma in Korea from 2011 to 2020. The breeds with primary glaucoma vary in different countries and regions and may change with time for numerous reasons. Therefore, the occurrence of primary glaucoma may also change. In this study, primary glaucoma occurred at a high rate in the following order of breeds: American Cocker Spaniel, Shih Tzu, Maltese, and Pomeranian. It did not occur in the Maltese breed at a significantly higher rate, even though it is a very popular breed in Korea. A large number of primary glaucoma cases were confirmed because of the large study population. In the current study, compared with a previous study, we identified nine new breeds, including Pomeranian, Jindo dog, Toy Poodle, Samoyed, Shiba Inu, Miniature Pinscher, Boston Terrier, Labrador retriever, and Yorkshire Terrier. Cases of primary glaucoma in the Pomeranian, Toy Poodle, Samoyed, Shiba Inu dog, Boston Terrier, Labrador retriever, and Yorkshire Terrier breeds have been reported in the United States, Japan, and Switzerland (Gelatt and MacKay, 2004; Kato *et al.*, 2006; Strom *et al.*, 2011). Among these breeds, Shiba Inus had the highest incidence (3.4%) of glaucoma in Japan, and it is gradually increasing in Korea. Although the incidence rate was low, Jindo dogs had no report of primary glaucoma in other countries and regions. The reason for this is because the Jindo dog is a preferred breed in Korea, just as the West Highland White Terrier in North America, the Entlebucher Mountain Dog in Switzerland, and the dachshund in Japan (Gelatt and MacKay, 2004; Kato *et al.*, 2006; Strom *et al.*, 2011). Another reason for this was

probably the lack of reports on the prevalence of primary glaucoma in these breeds. In this study, the prevalence of primary glaucoma was higher (0.73%) than in a previous study in Korea (0.55%) (Park *et al*, 2012). This observation was similar to that in a study on primary glaucoma in North America, which showed a steady increase in over 40 years (1964-73: 0.29%, 1974-1983: 0.46%, 1984-1993: 0.76%, 1994-2002: 0.89%) (Gelatt and MacKay, 2004). In Korea, as in North America, the diagnosis of primary glaucoma has increased with the distribution of ophthalmic equipment, such as tonometers, slit-lamp biomicroscopes, gonioscopes, and the increasing number of private specialty practices. American Cocker Spaniels and Shih Tzus demonstrated a high prevalence of primary glaucoma, which was similar to results of a previous study in Korea (Park *et al.,* 2012).

Primary glaucoma in American Cocker Spaniels is characterized by a narrowed ICA and ciliary cleft with PLD (Plummer *et al.,* 2021b). This breed has a high prevalence of primary glaucoma worldwide, including in North America, Japan, Switzerland, and South Korea (Gelatt and MacKay, 2004; Kato *et al.,* 2006; Strom *et al.,* 2011; Park *et al*, 2012). The mean ages of the American Cocker Spaniels at the onset of primary glaucoma in North America, Japan, and the previous domestic study were 6.4 ± 1.3, 7.4 ± 2.5, and 6.8 ± 2.1 years old, respectively (Gelatt and MacKay, 2004; Kato *et al.,* 2006; Park *et al.,* 2012), and that of the present study was 7.8 ± 2.6 years old. The females to males ratio in North America, Japan, and the previous domestic study was 1.5:1, 1:1, and 2.6:1, respectively, and 1.3:1 in this study. The prevalence of primary glaucoma in the American Cocker Spaniel was as follows: 5,984 (1.39%; 1964-1973); 15,440 (2.07%; 1974-1983); 25,205 (3.95%; 1984-1993); and 10,591 (5.52%; 1994-2002) in North

America (Gelatt and MacKay, 2004). The number of American Cocker Spaniels increased steadily (1964-1993) and declined sharply in the last period (10,591; 1994-2002). However, the prevalence of primary glaucoma in American Cocker Spaniels increased more in the last period (5.52%; 1994-2002) (Gelatt and MacKay, 2004). In this study, the number of American Cocker Spaniels steadily decreased over the past decade (Fig. 1) and is expected to decrease further in the future, similar to that in North America. The preference for the American Cocker Spaniel breed showed a decreasing trend due to various diseases, such as external otitis and intervertebral disc disease, as well as ocular diseases, including cataract and glaucoma in Korea (Sapienza and van der Woerdt, 2005; Zur *et al.,* 2011; Scott *et al.*, 2013; Kunz *et al.,* 2015).

Primary glaucoma in Shih Tzu is characterized by a closed ICA (Park *et al.,* 2012). Previous studies reported that secondary glaucoma associated with hyphema, vitreous degeneration, and retinal detachment were common in Shih Tzus (Kato *et al.*, 2006; Park *et al.*, 2012). Shih Tzu was the second most common breed with primary glaucoma after American Cocker Spaniels in Japan and Korea. The age of onset of primary glaucoma in this breed was 7.0 ± 1.4 years (1994-2002) and 8.3 ± 2.1 years old in North America and Japan, respectively, and 8.1 ± 2.6 years old in this study. The ratio of females to males in North America, Japan, and our study was 1.2:1, 1.1:1, and 1.4:1, respectively. Similar to the American Cocker Spaniel, the popularity of Shih Tzu has also been decreasing, and its population has steadily decreased over the past 10 years. However, the incidence rate of primary glaucoma has increased nearly twice as high in this breed compared to the previous domestic study. Shih Tzu is predisposed to many chronic diseases, such as

calculus, hyperadrenocorticism, mammary gland tumors, and ocular diseases, including primary glaucoma and corneal disease. (Houston and Moore, 2009; Kim *et al.*, 2016; O'Neill *et al.*, 2017; Heo *et al.*, 2018).

The age of onset of primary glaucoma varies by breed and usually occurs in middle-to-old age. In this study, the mean age of onset of primary glaucoma in canines was 7.8 ± 2.3 (mean ± SD) years old (Fig. 2). A previous study reported that the mean ages of onset of canine primary glaucoma in North America, Switzerland, and Japan were 6.39 ± 1.30, 7.25 ± 0.70, and 7.54 ± 1.54 years old, respectively (Gelatt and MacKay, 2004; Kato *et al.*, 2006; Strom *et al.*, 2011). It has been reported that the reason for the occurrence of primary glaucoma in middle age is the position of the lens, which is relatively anterior near the pupil, and prevents the drainage of aqueous humor and increases the length and thickness of the lens axis (Kato *et al.*, 2006).

Primary glaucoma occurred at a higher rate in females than in males in several studies, including English Cocker Spaniel (7.4:1), Welsh Terrier (2.5:1), Newfoundland (2:1), Vizsla (1.7:1), Basset Hound (1.7:1), and American Cocker Spaniel (1.5:1) (Gelatt and MacKay, 2004; Strom *et al.*, 2011). In the present and Japanese studies, the ratio of females to males with primary glaucoma prevalence showed a similar trend, with a mean of 1.5:1 and 1.3:1, respectively. This tendency is mainly observed in human PACG, especially in Asian people (Plummer *et al.*, 2021b). A recent study using HRUS and ultrasound reported a decrease in ICA, small-angle open distances, and angle recess area in female PACG (Tsai *et al.*, 2012).

Conclusion

This study confirmed that primary glaucoma was significantly higher in American Cocker Spaniels ($p < 0.002$) and higher in Shih Tzus than other breeds in Korea. American Cocker Spaniels and Shih Tzus had the highest incidence of primary glaucoma at 7 and 8 years of age, respectively. Therefore, the two breeds should be carefully monitored for the occurrence of primary glaucoma as they approach 7 years of age.

CHAPTER 2

Proteomic analysis of aqueous humor in canine primary angle-closure glaucoma in American Cocker Spaniel dogs

ABSTRACT

This study was performed to analyze proteomic profiles of the aqueous humor (AH) of canines with primary angle-closure glaucoma (PACG) and identify associated protein alterations. Animals were studied with six American Cocker Spaniels with PACG and six American Cocker Spaniels without ocular diseases. Aqueous humor samples were collected from six American Cocker Spaniels with PACG at Seoul National University, VMTH, and six healthy Cocker Spaniels without ocular disease at Irion Animal Hospital. For the PACG group, AH samples were obtained by anterior chamber paracentesis prior to glaucoma treatment. For the AH control group, AH samples were collected from patients anesthetized for other reasons. Total AH protein concentration was determined by the bicinchoninic acid (BCA) assay. AH protein samples were quantified by liquid chromatography-mass spectrometry (LC-MS/MS). Raw MS spectra were processed using MaxQuant software 30, and the Gene Ontology (GO) enrichment analysis was performed using ClueGO. The AH protein concentration in the PACG group (mean ± SD; 10.49 ± 17.98 μg/ μl) was significantly higher than that of the control group (0.45 ± 0.11 μg/μl) ($p < 0.05$). A total of 758 proteins were identified in the AH. Several proteins both significantly increased (n = 69) and decreased (n = 252) in the PACG group compared to those in the control group. GO enrichment analysis showed that the "response to wounding", "negative regulation of endopeptidase activity" and "cell growth" pathways were the most enriched terms in the PACG group compared to the control group. The top 5 proteins that were significantly increased in the AH of the

PACG group were secreted phosphoprotein 1 (SPP1), peptidoglycan recognition proteins 2 (PGLYRP2), tyrosine 3-monooxygenase (YWHAE), maltase-glucoamylase (MGAM), and vimentin (VIM).

Gene Ontology enrichment analysis using the proteomic data showed that proteins and pathways related to inflammation were significantly upregulated in the various stage of PACG. Proteomic analysis of the AH from the PACG might provide valuable insights into PACG pathogenesis.

INTRODUCTION

Glaucoma is a multifactorial optic neuropathy that causes progressive retinal ganglion cell damage and loss of the visual field (Plummer *et al.,* 2021b). Glaucoma occurs at similar rates in humans and dogs, around 1%–2% and 1.8% in humans and dogs, respectively (Quigley, 1993; Gelatt and MacKay, 2004). Glaucoma is classified as congenital, primary, or secondary glaucoma according to the etiology of the disease. There are two types of primary glaucoma: open-angle glaucoma (POAG) and angle-closure glaucoma (PACG). While POAG most often occurs in humans, PACG predominantly occurs in dogs. Moreover, canine PACG is more acute and causes irreversible vision loss faster than it does in humans. Breeds with PACG vary from country and region (Park *et al.,* 2012). While any dog can experience PACG, the most common breed worldwide is the American Cocker Spaniel (Gelatt and MacKay, 2004; Park *et al.,* 2012). In Korea, the breed with the second-highest prevalence of glaucoma after the American Cocker Spaniel is the Shih Tzu (Park *et al.*, 2012). The mechanism underlying intraocular pressure (IOP) elevation in human POAG is yet to be elucidated; however, the mechanism underlying IOP elevation was identified in Beagle and Basset Hound by ADAMTS10 and nebulin (NEB), respectively. ADAMTS17 mutation has also been identified as a causative mutation in POAG in the breeds Shar Pei and Petit Basset Griffon Vendeen (Joel *et al.,* 2013; Dina *et al.,* 2015; Emily *et al*., 2019). In humans, optic neuropathy can develop even with normal IOP, and it has been shown that an increase in IOP can further exacerbate glaucoma (Weinreb *et al.,* 2014). While nebulin

mutations exert a strong effect on the anterior segment, ADAMTS10 mutations affect the elasticity of the scleral and optic nerve head lamina; therefore, the potential effect of these mutations on the optic nerve cannot be considered independent of IOP elevation (Joel *et al.,* 2013). Controlling IOP through medication or surgical methods is known as the best treatment for glaucoma (Weinreb *et al.,* 2014). Early diagnosis of glaucoma is important for a better prognosis and appropriate treatment to prevent vision loss.

The optically clear fluid, AH, is crucial as it supplies nutrients to the avascular ocular structures and removes metabolic waste from these structures (To *et al.,* 2002). The AH maintains the IOP, has antioxidant properties, and plays an important role in the immunity of the eye (To *et al.,* 2002; Izzotti *et al.,* 2010). AH is secreted by the ciliary body epithelium and contains various proteins (To *et al.,* 2002). Depending on ocular diseases, the AH displays different protein expression patterns (Kaeslin *et al.,* 2016). Some proteins appear or increase, while some proteins disappear or decrease depending on the mechanism of various ocular diseases (Kliuchnikova *et al.,* 2016).

Mass spectrometry (MS)-based proteomics is a key technology for large-scale protein identification and quantification (Aslam *et al.,* 2017). Understanding the protein expression levels will help in the prevention, diagnosis, treatment, and prognosis of glaucoma (Li *et al.,* 2017). Proteomic analysis has been applied in human ophthalmology to understand the pathogenesis of glaucoma (Sharma *et al.,* 2018).

Previous studies suggested that some AH proteins could be closely related to the development of glaucoma (MacKay *et al,* 2008). In veterinary ophthalmology, while AH

proteomic studies have not been conducted for canine glaucoma, a proteomic study has been performed on the tear film in canine glaucoma, and elevated levels of myocilin, CD44, metalloproteinase 2, endothelin 1, nitric oxide, and nebulin were detected in the AH of dogs with glaucoma using Coomassie staining, enzyme immunoassay, and Western blot (Källberg *et al.*, 2007; Weinstein *et al.*, 2007; MacKay *et al.*, 2008; Dina *et al.*, 2015; Graham *et al.*, 2021). Peptidoglycan recognition protein 2 (PGLYRP2) plays a very important role in the innate immune response and has antibacterial activity. However, its role in canine eye is not well known (Brandley and Schnaar, 1986; Ranjita *et al.*, 2015). Proteomic analyses of AH have been performed in animals, such as rabbits and rats, as well as in humans. However, to the best of our knowledge, proteomic analysis of AH has not been performed in dogs. The purpose of this study was to perform proteomic analysis of the AH of dogs with PACG and identify associated protein alterations. Proteomic analysis may provide valuable insight into glaucoma pathogenesis, thereby identifying proteins that are potential biomarkers for assessing increased risk.

MEMATERIALS AND METHODS

1. Animal studied and preparation

The experiment complied with the guidelines of the Institutional Animal Care and Use Committee of Seoul National University (SNU-180625-5). AH samples were collected during glaucoma treatment at the veterinary medical teaching hospital (VMTH), Seoul National University (SNU), and Irion Animal Hospital. Consent was obtained from each patient's owner in normal group. The study period was from December 2016 to February 2019. Aqueous humor samples were obtained from six American Cocker Spaniel dogs with PACG in SNU and from six healthy American Cocker Spaniels without any ocular disease (control) in Irion Animal Hospital. The mean duration of glaucoma in the PACG group was 2.95 ± 1.04 months (Table 1) per the medical records. Ophthalmic examinations were performed, including slit-lamp biomicroscopy (Topcon Model SL-D7®, Topcon Corp.), indirect ophthalmoscopy (Vantage plus®, Keeler Instruments Inc.) with a 30-diopter indirect lens (Classic BIO lens®, Volk, Volk opti - cal Inc.), and rebound tonometer (TonoVet®, Icare). The dogs with glaucoma were all diagnosed with PACG (PACG group). The diagnosis of PACG was based on ultrasound biomicroscopy (MD-320W; MEDA Co., Ltd) and IOP. Patients with POAG and secondary glaucoma were excluded from this study. The control group consisted of dogs without clinical signs

of glaucoma on full ocular examination, including gonioscopy and ultrasound biomicroscopy.

2. Aqueous humor collection

All AH samples obtained from dogs in the PACG group were collected immediately before intravitreal cidofovir injection in dogs who no longer responded to glaucoma medication or at the discretion of the dog owner. Control group samples were collected from dogs under general anesthesia for unrelated reasons. AH samples were collected using a 30-gauge hypodermic needle syringe without a plunger through the cornea near the limbus, and the insert site was blocked with a cellulose swab to prevent leakage after removing the syringe. In both the PACG and control groups, 200 µL of AH per eye was collected and immediately stored at −80°C in a deep freezer for further analysis.

3. Total protein quantification

Total AH protein concentration was analyzed by bicinchoninic acid (BCA) assay (Thermo Fisher) according to the manufacturer's protocol.

4. Sample preparation and labeling

Proteomic sample preparation was performed as described in a previous study (Ju *et al.*, 2016). Briefly, AH samples (60 μg of total protein) from dogs in the PACG and the control groups were subjected to in-solution digestion. Proteins were reduced with 10 mM dithiothreitol (DTT) in 25 mM am - monium bicarbonate and alkylated with 50 mM iodoacet - amide. The DTT was diluted to 1 mM to digest proteins with trypsin (1:50) at 37℃ for 16 h in a thermo-mixer at 600 rpm. The next day, 32% trifluoroacetic acid was added to the elute to stop the reaction, and a C18 tip column was used to remove salts. A total of 4 μg/10 μL of the digested peptide were used for liquid chromatography-mass spec - trometry (LC-MS/MS).

5. Liquid chromatography-mass spectrometry analysis

Liquid chromatography-mass spectrometry analysis was performed as described in a previous study (Seon *et al.*, 2020). Briefly, raw spectra were acquired on an Orbitrap Fusion Lumos (Thermo Fisher) with EASY-nLC 1200 (Thermo Fisher). An autosampler was used to load 10 μL aliquots of the peptide solutions into an EASY column; Acclaim PepMap™ 100 of i.d. 75 μm, length 2 cm, and particle size of 3 μm (Thermo Fisher). The trapped peptides were separated on an EASY-Spray Column, C18 analytic-column (Thermo Scientific i.d. 75 μm, length 500 mm, and 2 μm particle size, 100 Å). The

mobile phases were composed of 0.1% formic acid water and acetonitrile. The LC gradient was initiated with 5% B, increased to 8% B for 1 min, 10% B for 16 min, 40% B for 79 min, and then maintained at 80% B for 9 min and 2% B for an additional 15 min at a flow rate of 250 nL/min. During chromatographic separation, Orbitrap Fusion Lumos was operated in a data-dependent acquisition mode. Full survey scans were acquired in the mass range of –400– 1600 m/z, maximum injection time of 100 ms, automatic gain control target 2e5 ions with a resolution of 120 000 and analyzed using the Orbitrap. MS/MS precursors were selected from top N intense ions for 3 s between survey scans, which were fragmented by 37.5% higher-energy collisional dissociation. MS/MS was acquired at a maximum injection time of 54 ms, automatic gain control 5e4 ions with a resolution of 30 000 and analyzed using the Orbitrap software. Previously fragmented precursors were excluded for 30 s.

6. Protein identification

To examine and visualize the overall expression pattern of proteins from each sample, a heatmap was generated using the Perseus software. Raw expression data were converted using the Log2 scale, further adjusted using z-score, and visualized. Proteins expressed in the AH were classified appropriately according to the differences in the levels between the PACG and control groups (Fig. 1). To identify the proteins upregulated in each sample, a volcano plot was generated (Fig. 2). In the heatmap and volcano plot, red and

blue indicate proteins with significantly increased and decreased expression in the PACG group compared to that in the control group, respectively.

7. Data analysis for protein profiling

Raw MS spectra were processed with MaxQuant software (Cox *et al*., 2009) (version 1.5.8.3) at default settings to identify proteins from ≥1 unique peptide(s) with a minimum of seven amino acids. Identified peaks were searched against a database of Canis lupus familiaris from Uniprot (https://www.uniprot.org/). Output files generated from MaxQuant were subjected to Perseus to perform unsupervised hierarchical clustering. Bioinformatic analysis was performed using ClueGO tools (Gabriela *et al.*, 2009).

8. Peptidoglycan recognition proteins 2 detection by Western blot

For verification of PGLYRP2 protein in AH of the PACG group, Western blotting was performed on the PACG and control groups. An equal volume of AH was used for western analysis. Western blot analysis was performed as described in a previous study (Dabin *et al*., 2018). Briefly, samples were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% skim milk in 0.1% Tween 20 in Tris-buffered saline (TBST). Membranes were then incubated with a PGLYRP2 antibody (Abcam®)

overnight at 4℃. The membrane was washed three times with TBST and then incubated with the horseradish peroxidase conjugated secondary antibodies (1:2000) for 1 h at room temperature. The blots were developed using ECL Western blotting Substrate (Thermo Fisher Scientific).

9. Statistical analysis

GraphPad Prism software (version 7.01, GraphPad Software, Inc.) was used for statistical analyses. Comparisons were performed using Student's t test. $p < 0.05$ was considered as statistically significant.

RESULTS

In this study, a total of 12 canine AH samples were subjected to proteomic analysis with six samples each from the PACG and control group. The mean ages of the PACG and control group were 12.66 ± 2.07 (mean ± SD) years and 13.17 ± 1.94 (mean ± SD) years, respectively, and the sex ratios were equal in both groups (Table 2).

1. Ophthalmic examination of primary angle-closure glaucoma group

One canine patient in the PACG group had weak visual ability, as indicated by menace response and maze test, which disappeared upon re-examination 2 weeks later. The remaining five patients had no vision at all. PLR and dazzle reflex were absent in the PACG group. Corneal edema was found in five of six patients. However, their severity was not sufficient to prevent anterior and posterior segment examinations, and the edemas were resolved in the following appointments. Two cases of mild flare and a case of moderate flare were identified in the anterior chamber in subjects from the PACG group. Retinal vessel attenuation and retinal hyper-reflectivity were observed in three patients with PACG, and optic nerve cupping was confirmed in two patients. Posterior synechia of the iris was detected in the right eye in one canine patient in this study.

Ultrasound biomicroscopy confirmed that the both iridocorneal angle and the ciliary cleft were closed in all canine patients with PACG.

Table 1. Ophthalmic examination of PACG group

No	Glaucoma duration (months)	Vision	PLR/ Dazzle reflex	Corneal edema	Flare	Synechia	Retinal vessel attenuation/ Retinal hyper reflectivity	Optic disc cupping	Iridocorneal angle grade by Ultrasoud biomicroscopy*
1	3.3	-	-	+	+	-	+	+	1
2	2.5	-	-	+	-	-	-	-	1
3	4	-	-	+	+	-	+	-	1
4	1.3	-	-	+	-	-	-	-	1
5	4	-	-	+	+	-	+	+	1
6	2.6	+	-	-	-	+	-	-	2
Mean±SD	2.95±1.04								

*Iridocorneal angle grade; grade 1, closed; grade 2, very narrow; grade 3, medium width; grade 4, open.

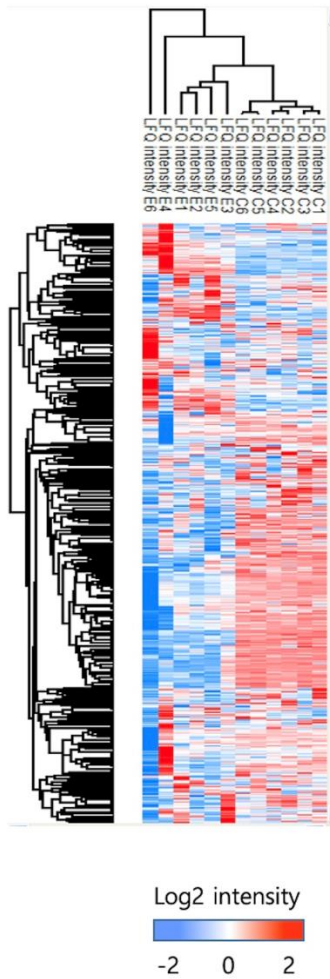


Fig. 1. Heatmap of the primary angle-closure glaucoma (E1-E6) and control (C1-C6) groups. Red and blue indicate significantly increased and decreased proteins in the primary angle-closure glaucoma group (PACG) compared to the control group, respectively. The heatmap value is the z-score normalized after converting the proteomic intensity of each sample to Log2.

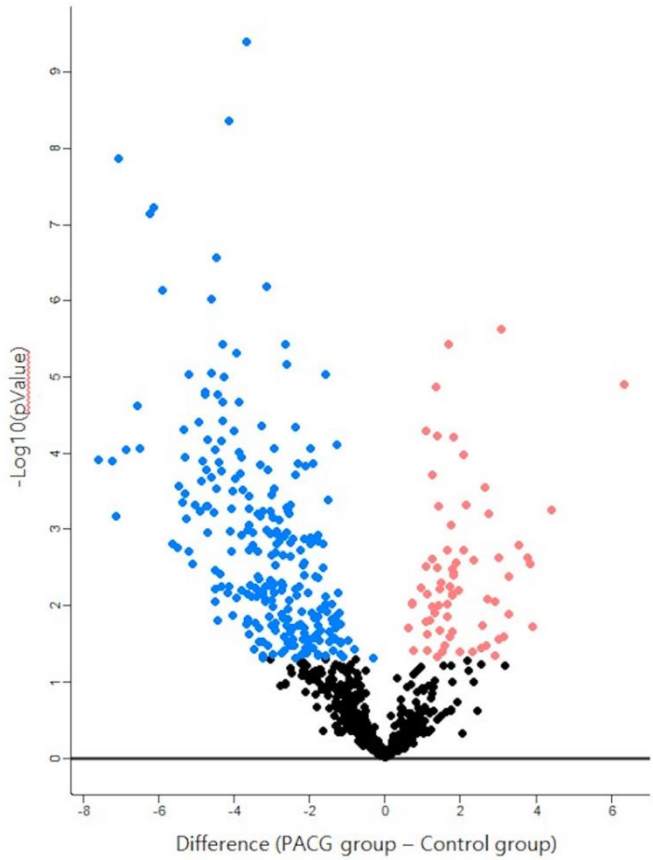


Fig. 2. Volcano plot of the primary angle-closure glaucoma (PACG) and control groups. Red and blue indicate significantly upregulated and downregulated proteins, respectively, in the PACG group compared to the control group. The horizontal axis indicates the difference between the two groups, and the vertical axis indicates the p-value.

Table 2. Clinical information of PACG dogs and control group

Group	No	Age	Sex	IOP(mmHg)
Patient	1	10	F	49
	2	15	MC	61
	3	15	MC	57
	4	11	MC	59
	5	13	FS	63
	6	12	FS	57
Mean±SD		12.6±2.0	M:F=3:3	57.6±4.8
Control	1	13	MC	12
	2	13	MC	13
	3	14	FS	11
	4	13	FS	16
	5	16	MC	12
	6	10	F	13
Mean±SD		13.2±1.9	M:F=3:3	12.8±1.7

2. Aqueous humor protein concentrations in the PACG and control groups

The mean total protein concentrations of the AH from the PACG group and control group were 10.49 ± 17.98 (mean ± SD) μg/ μL and 0.45 ± 0.11 (mean ± SD) μg/μL, respectively. The PACG group showed significantly higher total protein concentration in the AH than that of the control group ($p < 0.05$; Fig. 3).

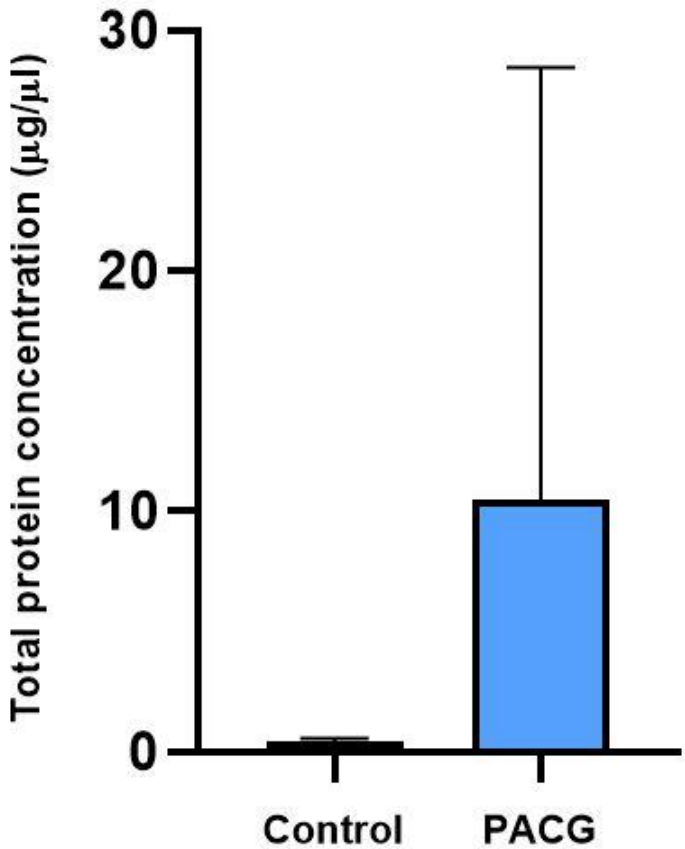


Fig. 3. Bar graph showing the total protein concentration of the aqueous humor of the primary angle-closure glaucoma (PACG) and control groups. The mean protein concentrations of the PACG and control groups were 10.49 ± 17.98 µg/µL and 0.45 ± 0.11 µg/µL, respectively.

3. Protein identification

A total of 758 proteins were identified in both groups. Sixty-nine proteins were at significantly upregulated (Table 3), and 252 proteins were at significantly downregulated in the PACG group than those in the control group. Proteins in the PACG group were ranked by their relative expression level compared to that of the control group. The top 14 proteins were seven times higher than those in the control group (Table 3). In particular, SPP1 (osteopontin) was noticeably higher (81-fold) in the PACG group than in the control group. The following top 13 proteins were peptidoglycan recognition protein 2 (PGLYRP2), angiotensin I precursor (Q7M301), tyrosine 3-monooxygenase (YWHAE), carboxypeptidase N subunit 1 (CPN1), maltase-glucoamylase (MGAM), apolipoprotein C2 (APOC2), J9P7N3, Vimentin (VIM), LOC476104, paraoxonase 1 (PON1), Chemokine ligand 23 (CCL23), Crystallin beta A3 (CrybA3), and carboxypeptidase N subunit 2 (CPN2).

Among the 252 proteins that were significantly lower in PACG group, the lowest ten proteins in the AH were aldehyde dehydrogenase 3 family, member A1 (ALDH3A1), integral membrane protein 2B (ITM2B), tumorigenicity 1 (NBL1), prostaglandin D2 synthase (PTGDS), VIT, L7N0E5, Secretogranin V (SCG5), C5NM83, desmocollin 2 (DSC2), and serine proteases 35 (PRSS35). The lowest ten proteins were present at levels 50 times less than the control group.

Gene ontology (GO) enrichment analysis using ClueGO revealed that the regulation of response to wounding/response to wounding followed by negative regulation of endopeptidase activity and cell growth pathways were the most enriched terms in PACG group (Figure 4). PGLYRP2, chemokine C-C motif ligand 14 (CCL14), CCL23, beta cytoplasmic actin (ACTB), angiotensin (AGT), alpha-2- heremans-schmid glycoprotein (AHSG), apoprotein E (APOE), complement component 3 (C3), c4b-binding protein α-chain (C4BPA), F10, fibronectin (FN1), kallikrein (KLKB1), LOC476104, neurofilament light polypeptide (NEFL), and serpin family C1 (SERPINC1) were significantly upregulated proteins in the regulation of response to wounding/response to wounding pathway in PACG group. The negative regulation of endopeptidase activity pathway includes significantly upregulated proteins such as fetuin-B (FETUB), inter-alpha-trypsin inhibitor heavy chain 1 (ITIH1), ITIH2, SERPINC1, SERPINF2, tissue inhibitors of metalloproteinase 1 (TIMP1), and vitronectin (VTN). Upregulated proteins included in the cell growth pathway in PACG group were APOE, F2, and fibronectin (FN1). In the cell growth pathway, significantly downregulated proteins were also identified in PACG. These proteins included acute-phase protein (APP), core architecture data model 1 (CADM1), calsyntenin-1 (CLSTN1), CLSTN3, connective tissue growth factor (CTGF), insulin-like growth factor-binding protein 2 (IGFBP2), IGFBP5, IGFBP7, laminin subunit beta-2 (LAMB2), semaphorin-3F (SEMA3F), SEMA7A, stratifin (SFN), secreted frizzledrelated protein 1(SFRP1), transforming growth factor-beta 2 (TGFB2), tumor necrosis factor receptor superfamily member 12A (TNFRSF12A), tenascin R (TNR), vinculin (VCL), and palmitoyl-protein thioesterase 1(PPT1).

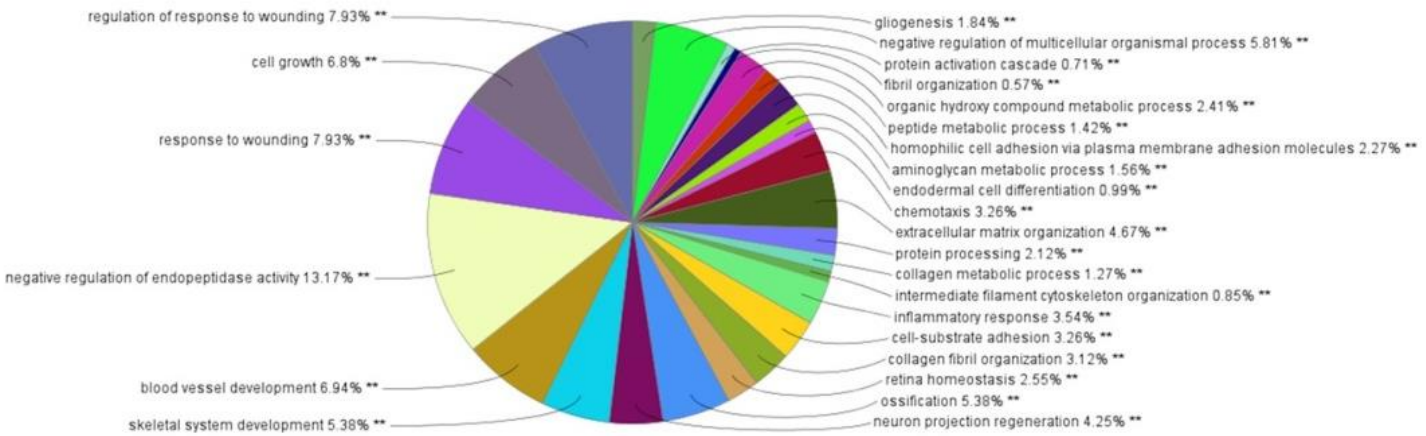
Table 3. Significantly elevated proteins of AH in Cocker Spaniels with PACG

Protein number	Protein IDs	Gene name	Fold Change (Patient/Normal)	pValue
1	E2R161	SPP1	81.18	0.00001277
2	F6XZU1	PGLYRP2	21.17	0.000553764
3	Q7M310		15.11	0.01919827
4	J9P9V0	YWHAE	14.49	0.002888052
5	E2RNG3	CPN1	13.61	0.002352396
6	E2RT38	MGAM	11.72	0.001605929
7	J9NWJ6	APOC2	9.66	0.004223022
8	J9P7N3		9.64	0.013005166
9	F1PLS4	VIM	8.89	0.025812005
10	J9NVK0	LOC476104	8.41	0.00000233
11	E2RPW3	PON1	8.17	0.002369716
12	J9PBF2	CCL23	8.16	0.027649322
13	A2IBY9	CrybA3	7.59	0.045786651
14	J9PAA4	CPN2	7.50	0.008953231
15	D3YJ60	CHI3L1	6.66	0.000616829
16	E2QZ98	ANGPTL3	6.63	0.008181336
17	E2QW82	MMP19	6.43	0.034055526
18	F1PJ74	APOE	6.32	0.000278832
19	A0A096P6K5	LOC100684663	5.96	0.018515115
20	F6Y6T8	PIGR	5.89	0.035543952

21	J9NYC0	MFAP4	5.07	0.002519961
22	J9P9J6		4.97	0.040686742
23	E2QUV3	AHSG	4.45	0.000473655
24	J9P430	TF	4.28	0.000104407
25	F1PDJ5	APOA1	4.27	0.001882584
26	E2RA67	CCL14	3.96	0.040907066
27	E2RK02	GPLD1	3.82	0.006353949
28	E2RFV9	C9	3.67	0.002791043
29	E2RES2	SERPINC1	3.54	0.00006302
30	F1PG39	ITIH2	3.52	0.004079669
31	J9NXE2	ACTB	3.50	0.003775388
32	E2R9D2	F12	3.45	0.003310878
33	A0A1S7J0A8	CD14	3.45	0.007151641
34	J9NYQ3	SMPDL3A	3.41	0.021875522
35	F1PAL5	AGT	3.41	0.007407498
36	F1PIX8	C3	3.40	0.000889004
37	F1PGM9	C4BPA	3.32	0.025275259
38	Q7M321		3.27	0.00565653
39	E2QYU2	CLU	3.25	0.0000037
40	F1PYM4	IGFALS	3.12	0.014169039
41	E2QZ19	AFM	3.12	0.001864203
42	F1P7J4		3.11	0.009594636

43	E2RJC2	C1QC	2.97	0.03416136
44	F1P685	ACTR3B;ACTR3C	2.85	0.040094882
45	E2RS79	C2	2.77	0.005033005
46	F1PQS2	TIMP1	2.74	0.021419809
47	F1Q1K9	HABP2	2.73	0.005990785
48	J9NSF9	F2	2.65	0.000501828
49	E2RS80	C2	2.65	0.009881472
50	E2R9B6	FETUB	2.64	0.003223688
51	G1K2D7	F9	2.62	0.000058896
52	F1Q4A3	F10	2.60	0.046973766
53	F1PNS6	PEPD	2.56	0.000013824
54	E2R4V3	SERPINF2	2.47	0.012452889
55	F1PZR4	HPX	2.37	0.002471263
56	E2R5W6	GC	2.37	0.000192023
57	F1PUM6	VTN	2.37	0.010481644
58	E2RG01	C7	2.28	0.015972762
59	E2R2F7	SHBG	2.18	0.007169384
60	E2RNL8	APOD	2.16	0.023488191
61	F1Q418	ITIH1	2.16	0.039323688
62	F1PNV5	KLKB1	2.14	0.016341387
63	F1PAF3	HGFAC	2.10	0.0000518
64	E2R7A3	CFI	2.10	0.003089366

65	F1PSK8	CTSC	1.91	0.005929718
66	F1P6H7	FN1	1.69	0.038360979
67	F1PWR2		1.66	0.009431632
68	E2QWN7	LCP1	1.64	0.009319686
69	F2Z4Q6	ALB	1.54	0.019580953



% genes for the PACG group compared to the control group

Fig. 4. Gene ontology (GO) enrichment analysis using ClueGO (http://apps.cytoscape.org/). Pie chart showed that regulation of response to wounding /response to wounding, negative regulation of endopeptidase activity, blood vessel development, and cell growth pathways were the most enriched terms in PACG group compared to the control group.

4. Western blot analysis of peptidoglycan recognition proteins 2

Peptidoglycan recognition proteins 2 (PGLYRP2) was absent in all samples of the control group. However, PGLYRP2 was detected by Western blot in four of the six samples in the PACG group (Fig. 5).

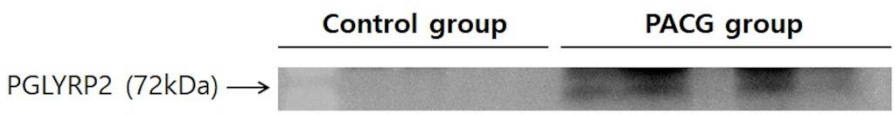


Fig. 5. Western blotting analysis of PGLYRP2 in the AH (n = 4). It was not confirmed in the left control samples but confirmed in four samples in the right PACG group.

DISCUSSION

The purpose of this study was to analyze the AH of canines with PACG. Several proteins, including myocilin, CD44, metalloproteinase 2, endothelin 1, nitric oxide, and nebulin, have been identified in a previous study of canine AH and ocular tissues using Coomassie staining, ELISA, and Western blot methods (Källberg *et al.,* 2007; Weinstein *et al*., 2007; MacKay *et al.,* 2008; Dina *et al.*, 2015). The present study used LC-MS/MS to comprehensively analyze canine AH. Label-free quantitative proteomic analysis identified 758 proteins in AH samples of the PACG group. Among the 758 proteins, 321 were differentially expressed, compared with the control group. Among these 321 proteins, 69 proteins were significantly higher, and 252 proteins were significantly lower in the PACG group than those in the control group. Of the 69 proteins that were significantly elevated, 42 were already studied in human glaucoma (Can *et al.*, 2015; Kaeslin *et al.,* 2016), whereas the remaining 27 proteins were novel proteins that were not found in human glaucoma patients yet.

According to this study, SPP1 was the most significantly increased protein in the PACG group and was 81 times higher than that of the control group. SPP1 is expressed by different types of immune cells, such as neutrophils, macrophages, T, and B cells (Wang *et al.,* 2008). SPP1 has been identified as a biomarker for inflammatory diseases and many types of tumors (Rangaswami *et al.,* 2006; Ramaiah and Rittling, 2008). It was also found to be increased in retinal ganglion cells (RGC) of rats with ischemic injury, and which might be related to the pathophysiology of glaucoma (Chidlow *et al.,* 2008).

Recently, SPP1 was reported as a new marker of trabecular meshwork (TM) differentiation as it was highly expressed in TM tissues (Sathiyanathan *et al.,* 2017). In this study, SPP1 was elevated in the AH of canine PACG patients. Inflammation in the TM increases the production of inflammatory markers in glaucoma (Li *et al.,* 2010), which also occurs in raised IOP. Therefore, this study suggests that SPP1 might be one of the inflammatory mediator of the TM in canine PACG.

Vimentin (8.90 fold change) was also markedly upregulated protein which was already been reported in human glaucoma study (Beutgen *et al.,* 2019). VIM is a type III intermediate filament protein found in mesenchymal cells. It has been used as a marker of various cancers, such as sarcoma tumors and colon cancer (Sho *et al.,* 2011). The importance of VIM expression is especially noted after an injury and is relevant to wound repair (Eckes *et al.,* 2000). VIM have reported that causes damage to the retinal nerve fiber layer in Müller cells and astrocytes in an experimental glaucoma model (Carter-Dawson *et al.,* 1998). A large amount of VIM have also found in Tenon's capsule of the glaucoma surgical model (Paola *et al.,* 2013).

Secreted phosphoprotein 1 and VIM have been extensively studied in human glaucoma (Ramaiah *et al.,* 2008; Beutgen *et al.,* 2019). This study suggests that SPP1 and VIM play a role as glaucoma-related proteins in PACG dogs as well as human glaucoma. In this study, PGLYRP2, YWHAE, and MGAM were detected in the AH of PACG dogs but not in the AH of human glaucoma patients. This was the first study to detect PGLYRP2, YWHAE, and MGAM in the AH of canine PACG. Proteins that are ten times higher than that of the control group may suggest a relation to the pathological

progression of canine PACG. If the elevated 69 proteins were found in AH, this dog might be considered as having a risk for PACG.

Biomarkers for ocular diseases are known to exhibit low reproducibility and repeatability (Hubens *et al.*, 2020). However, typical biomarkers for glaucoma, cataracts, and uveitis are myocilin, crystallin, and human leukocyte antigen (HLA)-B27, respectively (MacKay *et al.*, 2008; Keke *et al.*, 2018; Rosenbaum and Asquith, 2018). SPP1, PGLYRP2, YWHAE, MGAM, and VIM are proteins that are frequently expressed during inflammation. If glaucoma is diagnosed, lowering IOP is important, but treating the inflammation should proceed more intensively.

Gene Ontology enrichment analysis showed a high proportion of terms related to regulation of response to wounding, response to wounding, negative regulation of endopeptidase activity, and cell growth in the PACG group, when compared to the control group. In case of regulation of response to wounding/response to wounding pathway, upregulated proteins were PGLYRP2, CCL14, CCL23, ACTB, AGT, AHSG, APOE, C3, C4BPA, F10, FN1, KLKB1, LOC476104, NEFL, and SERPINC1. These proteins are known to be involved in inflammation and immunity, and they also serve as tumor markers.

Peptidoglycan recognition proteins 2 (21-fold change) was the second most upregulated protein in this study, and this gene has not yet been identified in the AH of canines and humans with PACG. PGLYRP2 is involved in the innate immunity and recognizes peptidoglycan, which is a component of the bacterial cell wall (Brandley and

Schnaar, 1986). PGLYRP2 has been reported to reduce inflammation and promote antibacterial activity in bacterial keratitis (Ranjita *et al.,* 2015). To verify the reproducibility of the proteomic analysis, Western blotting was performed for PGLYRP2 in the PACG and control groups. Four of six samples in the PACG group were reconfirmed. C3 is a protein involved in the immune system and inflammatory response (Hanhan *et al.,* 2020). Since increased C3 expression in the AH and serum is reportedly correlated with more aggressive progression of retina/optic nerve damage and increasing visual field deficit in human patients with primary glaucoma and in experimental models of glaucoma (Alejandra *et al.,* 2018; Hubens *et al.,* 2021), C3 is one of the potential therapeutic target molecules. Since this study was an analysis of the AH in severe glaucoma patients, it was presumed that these proteins might appear as a result of severe inflammation, or an alteration of the AH in response to reduced glaucoma-related inflammation.

The negative regulation of endopeptidase activity pathway was also upregulated in the PACG group. Endopeptidase is a protein that cleaves the peptide bond between amino acids in the target protein. Proteins in the pathway that negatively regulates endopeptidase activity include FETUB, ITIH1, ITIH2, SERPINC1, SERPINF2, TIMP1, and VTN. FETUB belongs to the cystatin superfamily and functions as a cysteine protease inhibitor. FETUB was reported previously in the AH proteomics of POAG (Sharma *et al.,* 2018). The ITIH family contributes to extracellular matrix stability and plays an important role in carcinogenesis and inflammation (Alexander *et al.,* 2008). Bioinformatic analysis of proteomic alterations of AH in patients with POAG shows that

SERPINF2, FETUB, and ITIH4 negatively regulate the endopeptidase activity pathway (Sharma *et al.,* 2018). These results were consistent with the results of a previous proteomic study of human glaucoma.

Proteins involved in the cell growth pathway, including APOE, F2, and FN1, were upregulated in PACG in this study. In the cell growth pathway, 17 proteins were significantly downregulated in PACG. These included APP, CADM1, and CLSTN1. Interestingly, compared to the response to wounding pathway, there were many more proteins that were significantly downregulated in the cell growth pathway. Since these alterations in AH result from progressive glaucoma status, it was assumed that developing cell activity had decreased in the cell growth pathway.

In the absence of ocular diseases, there are small alterations in the protein components of the AH, even in older dogs. However, in many ocular diseases such as glaucoma, cataracts, and uveitis, the protein content of the AH would be markedly changed. The converted protein components might be very closely related to the pathology of the disease (Duan *et al.,* 2010). Thus, the upregulation of proteins is very important. In this study, compared to the control group, ten proteins were markedly decreased in the AH of PACG dogs.

In human glaucoma patients, proteins such as prostaglandin D2 synthase (PTGDS), secretogranin 2 (SCG2) and putative serine protease 56 (PRSS56) have been reported to be downregulated (Yuk *et al.,* 2003; Bouhenni *et al.,* 2011; Cassandre *et al.,* 2020). PTGDS affects the IOP through a mechanism involving uveoscleral outflow, in which a

reduction in PTGDS in the AH affects small nonsubstrate lipophilic molecules, such as retinoic acid, thereby affecting the iridocorneal angle, which consequently promotes the uveoscleral outflow (Bouhenni *et al.,* 2011). SCG2 is a neuroendocrine marker expressed in the ciliary body. SCG2 upregulation in TM increases the IOP by promoting cellular infiltration and vascular permeability. SCG2 reduction in response to dexamethasone suppression in TM reportedly alleviates inflammatory symptoms (Yuk *et al.,* 2003). The absence of PRSS56 also reduces the ocular axial length, which is also an anatomical feature observed in patients with PACG and is responsible for IOP elevation (Cassandre *et al.,* 2020). In this study, PTGDS, SCG2, and PRSS56 were found to be significantly downregulated. Further investigation is needed to determine whether the expression of PTGDS, SCG2, and PRSS56 decreases in the AH of patients with PACG via the same mechanism reported in humans or via other mechanisms.

The general requirement for LS-MS/MS studies of the AH glaucoma proteome is their reproducibility and repeatability. However, almost identically designed studies would still produce a low level of similarities as they were affected by technical variability, including the number of samples, medications, and type of glaucoma surgery. Thus, proteomic studies must be conducted in dozens of samples in order to draw any meaningful conclusions (Hubens *et al.,* 2020). Another limitation of this study is the lack of adequate comparison groups. Since changes are of inflammatory nature, the adequate comparison groups should ideally include dogs with uveitis and dogs undergoing cataract surgery, which are positive for glaucoma genetic mutations/or dogs with closed angles while they are still normotensive. In this manner, one would be able to elucidate changes

that were the result of the inflammation and changes that were the result of the actual glaucoma status. A major limitation of this study is the use of samples derived from canines with primary glaucoma at almost all of them were in the end stage, which may not be representative of the early stage of ocular diseases in terms of proteomic changes. That said, the data from this study could provide foundational information in the future studies on glaucoma biomarkers. Another important limitation is that histopathology was not done to verify the presumed primary glaucoma observed in all PACG globes, since not all owners pursued enucleation.

The collection of AH is invasive. To prevent damage to the intraocular tissue, the syringe plunger was removed and the flowing AH was collected without aspiration. The amount of AH in dogs differs between breeds, but the usual amount of AH was approximately 1.6-times more than that in human studies (Murthy *et al*., 2015). In addition, other body fluids such as serum and tear protein could be obtained relatively more easily than obtaining AH protein for further study.

If proteomics is applied in veterinary ophthalmology, it will help future studies for diagnose of ocular diseases such as glaucoma, cataracts, and uveitis. No biomarkers for glaucoma have been commercialized for testing in veterinary ophthalmology. A biomarker for glaucoma in veterinary medicine would be developed based on the proteins found in this study. Further studies involving proteomic analysis of the AH of a large number of dogs with PACG and other ocular diseases, such as cataracts and uveitis, would be beneficial.

CONCLUSION

The current proteomic analysis identifying the AH proteins in normal and PACG American Cocker Spaniels showed that the composition of the AH in the PACG group was significantly different from that in the control group. There were significantly upregulated proteins in response to the wounding pathway related to inflammation. However, cell growth pathway proteins related to cell development were significantly downregulated. These results suggest that various proteins are associated with the development of canine PACG, and these proteins may provide insights on PACG biomarkers that may be identified in the future. In addition, the study of proteomics of AH in the canine PACG was thought to be helpful in elucidating the molecular mechanisms underlying the pathogenesis of PACG.

GENERAL CONCLUSIONS

Primary glaucoma was significantly higher in American Cocker Spaniels and higher in Shih Tzus than other breeds in Korea; they had the highest incidence of primary glaucoma at 7 and 8 years of age, respectively. Therefore, the two breeds should be carefully monitored for the occurrence of primary glaucoma when they approach 7 years of age.

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국 문 초 록

개의 원발성 녹내장 발생 현황과 안방수 Proteomic 분석에 대한 연구

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본 연구에서는 개의 원발성 녹내장 발생현황과 원발성 녹내장의 병인을 밝히기 위해 원발성 녹내장 개의 품종, 나이, 성별을 조사하고 안방수의 단백체를 확인하고 분석하였다.

제 1 장에서는 원발성 녹내장이 있는 개의 품종, 나이, 성별의 발생 현황을 한국에서의 이전 보고와 비교하였다. 2011 년 1 월부터 2020 년 12 월 까지 서울대학교 동물병원에 내원한 원발성 녹내장으로 진단된 개들의 품종, 나이, 성별 등의 의료 기록을 조사하였다. 모든 환자들에서 안과 정밀검사가 시행되었다. 분석은 원발성 녹내장의 평균 발생율과 가장 가까운 진돗개의 발생률을 기준으로 이항 로지스틱 회귀 분석을 통하여 분석되었다. 서울대학교 동물병원에 내원한 14,587 마리의 개 중에 107 마리가 원발성

녹내장으로 진단되었다. 원발성 녹내장은 아메리칸 코카 스페니얼, 시츄, 말티즈, 포메라니언, 진도견, 혼합견, 페키니즈, 토이 푸들, 사모이드, 시바이누, 미니핀, 보스턴 테리어, 라브라도 리트리버, 요크셔 테리어를 포함한 14 품종에서 발생하였다. 원발성 녹내장의 평균 발생 나이는 7.8 ± 2.3 세 였고, 53 마리의 중성화 암컷, 11 마리의 암컷, 38 마리의 중성화 수컷, 5 마리의 수컷에서 관찰되었다.

중성화와 관계없는 암컷, 수컷의 비율은 1.5:1 이었다. 본 연구에서 원발성 녹내장은 다른 품종보다 아메리칸 코카 스페니얼에서 유의적으로 높게 나타났으며, 시츄에서도 높게 확인되었으며, 7, 8 세에서 각각 높은 발생률을 보였다. 따라서, 두 품종은 7 세가 되면 원발성 녹내장 발생을 주의 깊게 관찰해야 할 것으로 판단된다.

제 2 장에서는 원발성 폐쇄우각 녹내장이 있는 개에서 안방수의 단백체를 분석하고 관련된 단백체의 변화를 확인하였다. 여섯 마리의 폐쇄우각 녹내장이 있는 아메리칸 코커 스페니얼과 안과 질환이 없는 여섯 마리의 아메리칸 코카 스페니얼의 안방수를 채취하였다. 비친코닌산(BCA) 분석으로 안방수의 총단백질 농도를 측정하였다. 안방수 단백질은 액체 크로마토그래피 질량 분석법 (LC-MS/MS)으로 분석하고 유전자 분석은 ClueGO 기법을 사용하여 수행되었다. 단백질 농도는 폐쇄우각 녹내장 그룹에서 (10.49 ± 17.98 μg/μl) 대조군 (0.45 ± 0.11 μg/μl) 보다 유의적으로 높았다

($p < 0.05$). 안방수에서 총 758 개의 단백질이 확인되었다. 여러 단백질들이 폐쇄우각 녹내장 그룹에서 유의적으로 증가 (n=69) 하거나, 감소 (n=252) 하였다. 상위 5 개 단백질은 인산단백질 1 (SPP1), 펩티도글리칸 인식단백질 2 (PGLYRP2), 티로신 3-모노옥시게나아제 (YWHAE), 말타아제-글루코아밀라아제 (MGAM), 비멘틴 (VIM) 이었다.

단백체 데이터를 이용한 유전자 분석을 통하여 폐쇄우각 녹내장의 마지막 단계에서 볼 수 있는 염증과 관련된 안방수 단백질들과 경로들이 유의적으로 높게 나타났음을 알 수 있었다. 폐쇄우각 녹내장 환자의 안방수 단백체 분석은 폐쇄우각 녹내장의 병인론에 대한 중요한 정보를 제공할 수 있을 것으로 생각된다.

결론적으로, 아메리칸 코커 스페니엘과 시츄는 7 세 때 원발성 녹내장의 발생을 고려해야 하며, 원발성 녹내장 환자의 안방수 분석으로 원발성 녹내장 진단에 필요한 잠재적인 바이오마커가 될 수 있는 단백체들을 확인할 수 있었다.

주요어: 아메리칸 코커 스페니얼, 폐쇄우각 녹내장, 안방수, 개,
액체 크로마토그래피 질량 분석법, 원발성 녹내장, 단백체학
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감사의 글

제가 박사학위를 할 마음이 없었던 시절 박사공부를 하라고 권하시며 용기를 주셨던 존경하고 사랑하는 아버지, 하늘나라에서 보고 계신가요? 막내 아들 이제 박사 되었습니다. 긴 시간이 흘렀네요. 늘 응원해 주셨던, 아버지, 감사합니다. 아버지 들리시게 크게 소리쳐 봅니다. "아버지 보고 싶습니다~~~!".

공부는 평생 하는 거라며 늘 말씀하시던 어머니, 어머니 말씀대로 되었습니다. 감사합니다. 앞으로도 공부하며 책 읽으며 살겠습니다. "사랑합니다. 어머니, 건강하세요!".

동생 자랑스러워 하는 어메리카 시애를에 계신 형님 고맙습니다. 건강 잘 챙기시고 나이 더 들면 서로 의지하면서 가족들과 함께 행복하게 살아봐요.

늘 신경 써 주시고 기도해 주시는 장인 어르신, 장모님, 감사 드립니다.

아버님, 어머님 덕분에 학업에 매진 할 수 있었습니다. 아버님, 어머님, 사랑합니다. 건강하세요. 저 대신 우리 아이들 좋은 구경 많이 시켜준 동서들, 처제들 고맙습니다.

안과연구회로 시작한 안과 공부가 이어져 박사공부를 하게 되었습니다. 너무도 많이 부족한 저를 끝까지 포기하지 않으시고 이끌어 주신 스승님,

고맙습니다. 수의학적 지식보다 더 소중한 것들을 몸소 보여주신 서강문 교수님, 가르쳐 주신 데로 올바른 수의사로 한 가정의 가장으로 성실히 살겠습니다.

10 년간 몸담았던 정 들었던 이리온 동물병원, 단백체 연구를 지원해 주신 대한제분에 감사 드립니다. 덕분에 학위 잘 마치고 임상경험, 인생경험 많이 하며 성장하였습니다.

대한민국 최고의 동물병원, 웨스턴 홍연정 원장님, 감사합니다. 심도 있는 진료, 수술들을 경험하고 있습니다. 웨스턴에서의 시간은 임상적으로나 인간적으로 저를 더 성숙하게 만드는 시간입니다.

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내 반쪽, 사랑하는 아내, 지연씨, 이 학위의 반 이상은 당신의 지분이네요. 가장 어려운 시간이 되니 당신은 정말 슈퍼우먼이 되더군요. 말로 다 할 수가 없어요. 고생 많았어요, 고맙습니다. 다시 태어나도 당신의 남편으로 살고

싶네요. 아빠를 많이 닮은 딸, 정원이, 우리 둘 다 예민한 시간이었네, 너는 중 2, 아빠는 논문 쓰는 진료수의사, 아빠가 많이 미안해, 앞으로 좋은 시간 보내자. 세상에서 가장 멋진 우리 아들 정호, 아빠랑 오마카세 먹으러 가자, 둘 다 아빠가 심하게 사랑해.

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추운 겨울날 자곡동에서