# Glaucoma Treatment with the Aqueous Extract of Prunella vulgaris in Rats Experimental Model

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**Abstract**: Glaucoma is one of the world's leading causes of blindness. Prunella vulgaris is a well-known traditional Chinese herbal medicine widely used for a long time. The aim of our study is to evaluate the activity of lowering intraocular pressure through the use of aqueous extracts of prunella vulgaris (AEPV) in an experimental glaucoma model. The rats used in the study were divided into six groups: one sham group, two positive control groups with topical brimonidine instillation and oral acetazolamide therapy, and three groups treated with AME (low, medium and high dosage). The antioxidant activity of AEPV was accessible by MDA and GPx levels. The ability to lower intraocular pressure (IOP) signified the efficiency of treating glaucoma. The results revealed that AME may decrease the MDA production and restore the GPx level in the periocular blood. This extremely beneficial effect may be by the same as that of brimonidine. Furthermore, AEPV also showed the ability to significantly lower IOP as is the case with brimonidine and acetazolamide. AEPV is a relatively safe Chinese herbal medicine with no observed side effects such as body-weight loss, or pathological change. In conclusion, the aqueous extract of prunella vulgaris is beneficial in treating glaucoma during the development of progression of this disease due to its significant IOP and antioxidant activities.

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## 1. Introduction

Glaucoma is one of leading causes of ocular diseases. It comprises a prevalent group of retinal and optic neuropathies that currently renders approximately 67 million people worldwide at risk for developing significant vision loss including blindness. Elevated intraocular pressure (IOP) is a major factor for glaucomatous optic nerve damage. Although other factors are postulated as playing a role in glaucoma, IOP remains the best documented〔1,2〕. Thus, nearly all of our current glaucoma therapy is directed toward lowering IOP.

Laser and surgical procedures for glaucoma have being evaluated according to new treatment guidelines. In addition, medical therapy has been used to lower IOP for many years；the mechanism of the medications include two major groups to control IOP by either decreasing the production or enhancing the outflow drainage of aqueous humor〔3〕. Because of most drugs have different side effects, plant extracts have now been applied as one of the most attractive sources for medicinal purposes now〔4〕.

*Prunella vulgaris*, common as "self-heal", is a low-growing perennial herb with worldwide distribution. The herb is a member of the mint family Lamiaceae. Salves, teas, and extract made from the plant have been used to treat wounds, inflammation and other minor body disorders by both the Chinese and Native American. Various bioactive constituents have been identified in extracts of prunella vulgaris. These included phenolic constituents, complex carbohydrates and more hydrophobic metabolites such as triterpenes. The abundant polysaccharides, cyandin, oleanolic acid, ursolic acid and caffeic acid present in prunella vulgaris are readily extracted by water and several of the triterpenes have been identified with anti-flammatory activity〔5〕. However, relatively little scientific information has been obtained concerning on the role of prunella vulgaris in curing ocular diseases such as glaucoma. The purpose of our study is to determine whether the effects of the prunella vulgaris include a reduction of IOP and a solution to its associated problems.

## 2. Material and Methods

### *2.1. Material*

The Prunella vulgaris was purchased from an oriental drug store (Kaohsiung, Taiwan) and was then prepared for the aqueous extraction of prunella vulgaris (AEPV). At first, the dried prunella vulgaris samples (200g) were immersed in a 10-fold volume of $dH_2O$, boiled at 80℃ for one hour, and then the water extract was collected. The process was repeated once. The fluid refluxed for one hour in a reflux extraction apparatus. The aqueous extract solution was then

filtered using filter paper and a filter funnel. The filtered extract was dried *lyophilizely* and then stored in an electronic dry cabinet for subsequent study.

*2.2 Test animals*

Thirty-six male Sprague-Dawley (SD) rats, mean age about 8 weeks, were enrolled in this experiment. Among them, 6 male SD rats belonged to the control group (group 1). The other SD rats received the subconjunctival betamethasone injection (4mg) weekly (total of 3 doses for 3 weeks). It can cause a significant increase in IOP after 3 treatments. The elevated IOP varied 30-40 mmHg in this experimental model (so the SD rats were identified as glaucoma). The other 30 SD rats with glaucoma were divided into 5 groups (group 2 to group 6 separately received various treatments). All the SD rats were maintained under standard laboratory conditions (12 h light/dark cycle, temperature (22 ± 2) ℃). Standard meals (contents of more than 25% crude protein, more than 4.5% crude fat, less than 12% water, and less than 9% ash) and sterilized water were available ad libitum. The rats were procured one week before the experiments to acclimatize them to the laboratory environment. All protocols followed the guidelines of the institutional ethics committee at Kaohsiung Armed Forced General Hospital. We measured the body weight body every day and observed the daily activity and external appearance every day during the entire study. At the same time, we collected and recorded the daily total urine output for further evaluation.

*2.3 Effect of SCE on IOP in the Glaucoma Model*

In accordance with the various treatments, all subjects were divided into 6 groups. Group 1 (sham group, received no special therapy) was made up of rats with normal intraocular pressure. Groups 2 to 6 consisted of SD rats with glaucoma. Group 2 received topical 0.15% brimonidine instillation two times a day (Alhagan-P, Allergan, Inc., Texas, USA). Groups 3 to 6 were administered acetazolamide (Diamax, Taiwan Veterans Pharmaceutical Co Ltd., Taiwan) (78.13mg/kg/day), AME-100 (100 mg/kg/day), AME-200 (200 mg/kg/day) and ACE-500 (500 mg/kg/day) by gastric gavages, respectively. Checking IOP and periocular blood tapping were assessed via ethanol anesthesia. All procedures in the experiment were performed in a very short time when the rats were semi-conscious and cooperative. When the study started at week 1, the IOP of the left eye of the rats was measured with the Tono-pen XL hand-held applanation tonometer (Reichert, USA). The tip of this tonometer was exactly touched the central cornea of the rats. This procedure was completed under the light-microscopy in order to avoid interfering with the inaccurate central corneal thickness reading. The results would be read automatically and the mean IOP was determined after five times. In addition, blood samples were tapped from the periocular region of each right eye using microcapillary tubes. The total collected blood was 1 CC from every SD rat. The blood was applied to glutathione peroxidase (GPx) and TBARS (thiobartituric acid reactive species) tests at once. At the same time, one normal rat and one glaucoma rat were sacrificed. The livers and kidneys of these rats were excised and the specimens were embedded in 20% formalin for further histological analysis. From week 2 to 5 during the experiment, the IOP of the left eye of each rat was checked. No further blood was necessary. In addition to IOP checking, the periocular blood tapping was also carried out at the end of week 5. All the mice were sacrificed, and kidneys and livers were all dissected carefully for biopsy.

*2.4 Determination of malondialdehyde (MDA) level*

In the case of MDA analysis, the modified thiobartituric acid reactive species (TBARS) assay was used to measure lipid peroxide. MDA, produced by the oxidant of polyunsaturated fatty acids, reacts with two molecules of TBA. At first, we used the above solution and shook the solution to mix it well. Then the solution was bathed under 37℃ water for one hour. Each tube was given 500 μL 0.1 N HCl and 200 μL 9.8% SDS. Then 900μL pure water was poured in and mixed well: 2 mL 0.6% TBA was mixed in a 95℃ hot-water bath for one hour. The solution was then cooled to room temperature for about 10 min. The n-butanol was added in the amount of 5 mL and mixed well by centrifugation at a speed of 3000 rpm for 25 min at the temperature of 25℃. The upper clean solution was taken and loaded in a 200 μL/well. The layer of n-butanol yielded a pink-red chromogen with an absorbance maximum at 532 nm measured by the ELISA reader. We can analyze the changes of TBARS in each group before and after treatment.

*2.5 Determination of glutathione peroxidase (GPx) activity*

The agent RS 504 ( Randox Laboratories, Antrim, UK ) was purchased from the market. Blood samples were collected and only the upper layer was added with a buffer diluted to 2%. We mixed it well and heated it at 37℃ for 3 minutes. Then the fluid was added with 200μl 1.25 mM $H_2O_2$ and mixed well at 37℃ in a water-bath for 3 minutes again. Then 4mL metaphosphate was added to the sediment protein for mixing and centrifuged under 3000 rpm at 4℃ for 10 minutes. The upper clear fluid of 100μl was poured in 100μl 0.4 M $Na_2HPO_4$ and 50μl 0.4 mg/ml DTNB. Finally, we put the mixture inside an oven at 37℃ for 3 minutes in boiling water. At 422nm, absorbance was measured and in contrast with

GPX, a standard curve was introduced to calculate the specific activity of $GP_X$ (U/mL) in each extract solution. Then the levels of GPX in each group were determined.

*2.6 Histopathological evaluation*

All tissue specimens were buried within 20% formalin. After 24 hours, the fixative was replaced with the appropriate buffer and subsequently embedded in hydroxyethyl methylacrylate. We obtained series of sections (at an interval of 5μm), and they were all stained with haematoxylin and eosin stain (H&E stain). The results were observed and taken pictures were taken under a light microscope (Nikon, Japan).

*2.7 Statistical analysis*

All values are shown with a mean ± standard deviation (SD). The changes of GPx activity, TBARS level, IOP, and body weight before and after treatment were analyzed by an ANOVA (analysis of variance) test. If the measured IOP was less than 18 mmHg, we suggested that the period time of treatment had reached the target IOP, and "success" was defined according to the criteria of the Advanced Glaucoma Intervention Study (AGIS). A *p value* of less than 0.05 was considered significant.

## 3. Results

*3.1 The changes of the external appearance and body weight after 5 weeks of treatment*

The body-weight changes of six groups after treatment for 5 weeks of are shown in table 1. The mice in group 3 had mild, relatively retarded growth; the other groups experienced normal weight gained. In addition, the appearance of SD rats that took oral acetazolamide had poorer quality of hair and daily exercise performance, as well as an unstable gait. The results indicated that the rats that received oral acetazolamide treatment experienced a reverse effect on body weight, external appearance and daily activity.

*3.2 Effect of AEPV on IOP in the Glaucoma Model*

The IOP of glaucoma mice in all groups (except group 1) before and after 5 week of treatment all had significant differences statistically by ANOVA test (Table 1). It showed that the SD rate with high IOP treated five weeks by topical brimonidine use, oral acetazolamide, and any doses of AEPV all experienced "success" (IOP < 18 mmHg)(Table 2). However, only group 3 and 6 had reached the target IOP beginning at week 4. This means that oral acetazolamide and H-AEPV showed the better efficacy. In the meantime, AEPV-500 even revealed stronger ability to reduce IOP apparently than the AEPV-100 and AEPV-200. It may show the dose-dependent relationship in which a higher dose of AEPV may reduce the IOP more quickly. Furthermore, any dose of AEPV all should reach the target IOP after 5 weeks of treatment. The ability to reduce IOP in all AEPV was not any less than that of the groups that used brimonidine and acetazolamide.

*3.3 The total urine output before and after treatment*

The total urine output in each group before and after various treatments for 5 weeks was shown (Table 3). Significant increases in daily urine output were found in group 3 (oral acetazolamide) and group 4 to 6 (any doses of AEPV). This demonstrated that acetazolamide may have a diuretic effect. At the same time, it is not surprising that AEPV has marked diuretic effect.

*3.4 The GPx level in the periocular blood before and after treatment*

Table 4 shows the GPx level in the periocular blood before and after treatment for 5 weeks was shown (Table 4). The GPx level in groups 1 and 3 revealed no significance for 5 weeks. Before the treatment in group 2, an average GPx of 6.1± 0.4 (u/mg protein) was noted. After treatment with topical brimonidine instillation, it rose to 19.2± 1.4 (u/mg protein) ($p<0.01$). In group 4 and 5, the GPx level apparently changed after AME-100 and AME-200 ($p<0.01$). However, the GPx level before and after AME-500 in group 6 were noted more significantly (5.9± 1.0 u/mg protein vs 24.5 ± 4.9 u/mg protein; $p<0.001$). This means that the treatment with topical brimonidine instillation and any doses of AME may restore the GPx and enhance the associated antioxidant activities in the periocular circulation.

*3.5 The TBARS level around the periocular blood before and after treatment*

Table 4 shows the TBARS level in the periocular blood before and after treatment for 5 weeks was shown (Table 4). The TBARS level in group 1 revealed no significant change for 5 weeks. Before the treatment in group 2, an average TBARS of 0.8 ± 0.1 nmul/mg protein was noted. After treatment of topical brimonidine instillation, it reduced to 0.4 ± 0.2 nmul/mg protein ($p<0.05$). In group 3, the level of TBARS revealed no remarkable change after oral acetazolamide. The TBARS level before and after any doses of AME in group 4 to 6 were all noted as be significant ($p < 0.05$). This means that the treatment of the glaucoma mice with topical brimonidine instillation, and any doses of AME may decrease the MDA production in the periocular blood.

*3.6 The histological results of the livers and kidneys after oral acetazolamide for 5 weeks*

We observed that the kidney and liver of glaucoma rats treated for 5 weeks with AME all had normal morphologic features (data not shown).

Meanwhile, when the SD rats with glaucoma were treated with oral acetazolamide, the lipidosis were detected (Fig. 1). The results indicated that glaucoma mice treated with AME showed no remarkable complications in hepatic and renal tissue. In addition, we still have to pay attention to the side effects of systemic CAIs such as acetazolamide in treating glaucoma rats for an extended time.

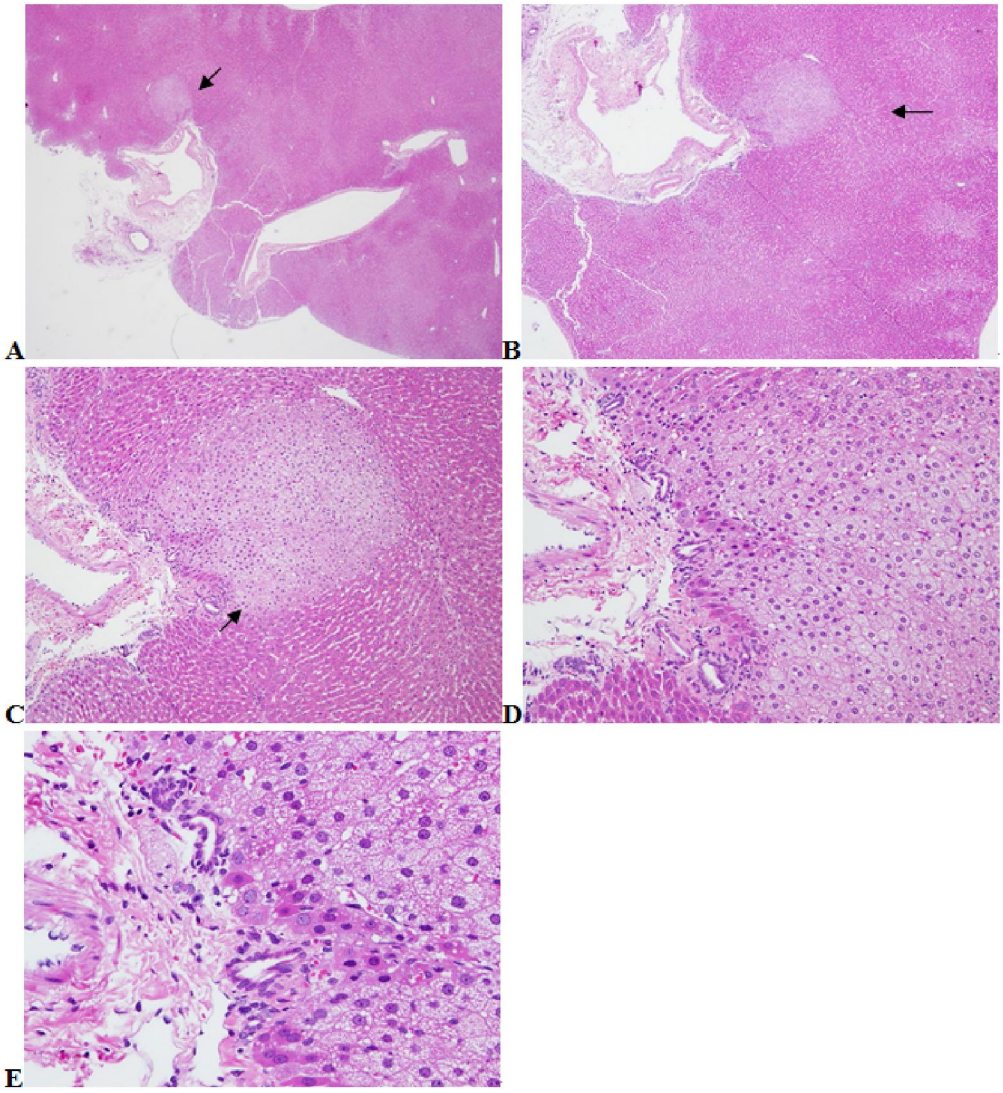


Fig.1. The rats treated with oral acetazolamide in group 3 all showed abnormality of the cells of liver. We found that the nodules lesion with collection of abundant cytoplasm; containing micro-vesicles, consistent with fatty liver. (A. 20x, B. 40x, C. 100x, D. 200x, E. 400x). H& E stain.

Table1: Variation in body weight and intraocular pressure (IOP) before and after various treatments for 5 weeks

| Group | Before | | After | |
|---|---|---|---|---|
| | Body weight(g) | IOP(mmHg) | Body weight(g) | IOP(mmHg) |
| 1(n=6) | 195.5±3.5 | 18.5±2.6 | 358.6±7.8*** | 19.2±3.5 |
| 2(n=6) | 202.7±2.7 | 36.6±2.7 | 375.5±20.5*** | 19.4±4.5* |
| 3(n=6) | 204.7±2.5 | 35.5±3.4 | 278.9±8.1* | 15.7±5.5** |
| 4(n=6) | 208.9±3.5 | 32.5±4.5 | 358.7±2.5*** | 20.6±4.7* |
| 5(n=6) | 198.7±4.6 | 33.5±4.7 | 370.9±15.5*** | 19.6±5.5* |
| 6(n=6) | 204.5±4.6 | 32.2±3.5 | 349.7±8.5*** | 16.5±3.5** |

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, Significantly differed before and after treatment for 5 weeks by ANOVA

Table 2: Different intraocular pressures (IOP) of each group within each week

| | Group | n | IOP ( mmHg) |
|---|---|---|---|
| Week 1 | 1 | 6 | 12.4 ± 2.7 |
| | 2 | 6 | 38.4 ± 2.1 |
| | 3 | 6 | 36.6 ± 1.9 |
| | 4 | 6 | 32.5 ± 2.3 |
| | 5 | 6 | 35.8 ± 4.5 |
| | 6 | 6 | 39.2 ± 5.4 |
| Week 2 | 1 | 6 | 14.4 ± 3.2 |
| | 2 | 6 | 31.5 ± 2.4 |
| | 3 | 6 | 32.5 ± 3.3 |
| | 4 | 6 | 31.5 ± 1.5 |
| | 5 | 6 | 29.6 ± 2.6 |
| | 6 | 6 | 33.6 ± 3.8 |
| Week 3 | 1 | 6 | 15.6 ± 2.8 |
| | 2 | 6 | 28.6 ± 4.8 |
| | 3 | 6 | 22.5 ± 3.2 |
| | 4 | 6 | 34.2 ± 3.4 |
| | 5 | 6 | 33.5 ± 3.5 |
| | 6 | 6 | 35.6 ± 3.8 |
| Week 4 | 1 | 6 | 16.8 ± 3.4 |
| | 2 | 6 | 20.5 ± 5.2 |
| | 3 | 6 | 17.6 ±3.2 |
| | 4 | 6 | 24.6 ± 3.6 |
| | 5 | 6 | 23.6 ± 3.5 |
| | 6 | 6 | 17.5 ± 3.6 |
| Week 5 | 1 | 6 | 15.5 ± 2.7 |
| | 2 | 6 | 16.4 ± 1.5 |
| | 3 | 6 | 12.8 ± 1.3 |
| | 4 | 6 | 14.8 ± 4.5 |
| | 5 | 6 | 13.6 ± 1.9 |
| | 6 | 6 | 13.6 ± 2.5 |

Table 3: Variation in total urine output before and after various treatments for 5 weeks

| Group | Before(ml/24 hr) | After(ml/24hr) |
|---|---|---|
| 1(n=6) | 142.5±11.2 | 158.4±9.5 |
| 2(n=6) | 156.5±7.7 | 156.4±9.6 |
| 3(n=6) | 153.4±9.5 | 238.8±5.6** |
| 4(n=6) | 147.5±8.5 | 198.8±10.6* |
| 5(n=6) | 153.6±13.5 | 237.5±7.4** |
| 6(n=6) | 150.4±9.6 | 249.5±8.5** |

* $P<0.05$, ** $P<0.01$, Significantly differed before and after treatment for 5 weeks by ANOVA

Table 4: Variation in GPx and TBARSm level before and after various treatments for 5 weeks

| Group | Before | | After | |
|---|---|---|---|---|
| | GPx(u/mg Pro) | TBARS(nmul/mg Pro) | GPx(u/mg Pro) | TBARS(nmul/mg Pro) |
| 1(n=6) | 6.2±1.8 | 0.2±0.2 | 6.5±2.2 | 0.3±0.1 |
| 2(n=6) | 6.4±0.4 | 0.8±0.1 | 18.2±2.4** | 0.4±0.2* |
| 3(n=6) | 5.9±2.2 | 0.7±0.3 | 8.5±2.4 | 0.5±0.2 |
| 4(n=6) | 6.4±2.6 | 0.8±0.2 | 18.5±5.4** | 0.3±0.1* |
| 5(n=6) | 6.5±2.8 | 0.8±0.1 | 21.2±1.2** | 0.4±0.1* |
| 6(n=6) | 5.8±1.5 | 0.8±0.5 | 25.5±4.9*** | 0.5±0.1* |

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, Significantly differed before and after treatment for 5 weeks by ANOVA

**4. Discussion**

Glaucoma is a disease characterized by a specific pattern of optic head and visual field damage. Although several risk factors for glaucoma have been identified, elevated IOP is the best known. If failed to effectively control IOP, *the progression of glaucoma may* further cause the death of the retinal ganglion cells, resulting in loss of vision. Although there is no doubt that glaucoma has been traditionally associated with high IOP, glaucoma is now considered as a multi-factorial disease. However, IOP is still the most important risk factor for the development of glaucomatous optic nerve damage. Because the higher IOP directly leads to mechanical compression and associated optic nerve damage, the ophthalmologists may try to reduce IOP at once. Now for patients with glaucoma, medical, laser, and surgical therapy are used to decrease the formation of aqueous humor or to enhance its outflow. Medical treatments which included β-adrenergic receptor antagonists (eg. Timolol), α-adrenergic receptor agonists (eg. brimonidine), carbonic anhydrase inhibitors (CAIs) (eg. dorzolamide and acetazolamide) and prostaglandin analogues (eg. lantanoprost) are popular in treating various types of glaucoma. Until now, topical instillation of anti-glaucoma drugs is still the primary and first choice for treatment.

Most methods for induction of the glaucoma involved partial destruction of the aqueous humor outflow to abruptly raise IOP. For example, rabbits subjected to acute water loading were employed and induced the ocular hypertension model successfully. Some researchers had injected the alpha chymotrypsin into the posterior chamber of the eyes of normal rabbits. Some 70% of the animals may produce prolonged elevation in IOP because of the obstruction of the iridocorneal angle with zonular and inflammatory debris, angle closure, and peripheral anterior synechiae. Recent studies exposed animals to steroids for a long time and at a high dosage level (via eye drops, injection under the cornea or drug administration) to increase aqueous humor inflow and high IOP〔6〕. Galassi et al. used 0.1% dexamethasone eye drops 3 times a day for 5 weeks to induce the occurrence of glaucoma in New Zealand albino rabbits〔7〕; Agarwal et al. tried to use 1% prednisolone twice a day by topical instillation for 40 days to cause a rise in IOP in young rabbits. A rise of 31% -58% was observed at the end of the 40-day periods〔8〕. In our study, we used the method of subconjunctival betamethasone injection (4mg dose in each week, total 3 weeks) to create the experimental glaucoma model. Indeed, the rats offer advantages of availability, low purchase and maintenance costs, and ease of handling without general anesthesia which can affect the actual IOP. Therefore, we could measure the IOP quickly and correctly. At the same time, the development of the Tonopen tonometer with a small applanation tip, which was used in our study is very popular in the world〔9〕.

The principal therapy used in the treatment of glaucoma is to reduce the IOP at first. Therefore, it is sometimes successful in halting the progression of this disease. However, the elucidation of the other prospective factors would provide us with additional targets for the development of a novel treatment. Clinically, ophthalmologists found that although IOP was well controlled in glaucoma patients, the visual field damage or optic nerve head excavation also progressed step by step. Hence, many researchers had tried to focus on other factors that impacted the glaucoma. For example, Craig and his staff stated that the possible etiology of glaucoma may be due to genetics. Anderson et al. believed that the blockage of axoplasmic transport in the optic nerve is one of the causes of glaucoma. Quigley et al. proposed that the poor nutrition may contribute to glaucoma. Flammer and his workers demonstrated that vascular dys-regulation should be a principal risk factor for glaucomatous damage.

Recent knowledge about glaucoma shows that we can not seek for the explanation of some dilemmas exclusively in ophthalmology and related medical disciplines, but also in the fundamental scientific disciplines of genetics and biochemistry, leading us to the genetic and molecular definition of the etiology of glaucoma. Intensive investigations of oxidative stress in glaucoma have now been done. The relationship

between free radicals, oxidative stress and glaucoma is now well discussed now. Free radials are known to occur as the natural by-products under physiological conditions. Oxyradical-induced cytotoxicity arises from both acute and chronic increases in reactive oxygen species (ROS), which give rise to subsequent lipid peroxidation. For example, Welge-Lüssen et al. stated the pathogenesis of primary open-angle glaucoma (POAG) may be the formation of oxidative stress in the trabecular meshwork 〔10〕. Therefore the use of antioxidants and IOP-lowering drugs could help to reduce the progression of POAG. Yildrim and his coworkers contested the links of the pathogenic mechanism of glaucoma and oxidative stress by indicating a higher level of myeloperoxidase and catalase of patients with POAG〔11〕.

Oxidative stress occurs in the chain of some acute inflammatory reactions to the ocular tissues in humans. Under certain circumstances, the level of antioxidants and associated enzymes of the aqueous humor may change. For example, superoxide dismutase (SOD) will increase in some chronic diseases including HIV infection and Alzheimer's disease. The effects on the glaucoma patients may be chronic, gradually damaging, with a cumulative effect. In patients with glaucoma, the oxidants have an important role in phagocytosis, and the oxidants damaged the blood vessels' endothelial cells, and adjoining neural tissue. Additionally, they also disturb the structure and function of numerous bio-molecules and cellular organelles by increasing the accumulation of an extra-cellular matrix, resulting in a change in the cytoskeleton and cellular senescence〔12〕. Futhermore, the excessive free radicals will give rise to the lipid peroxidation and influence the modulation of the cellular signaling pathway and the ion transport mechanism. Zhou and his colleague mentioned that extensive oxidative stress may result in reduced trabecular meshwork cells, leading to cell loss, compromised trabecular meshwork integrity, and pathologic consequences〔13〕. To our knowledge, the hydrogen peroxide ($H_2O_2$) and superoxide anion ($O_2^-$ • ) in the anterior chamber will compromise the function of the trabecular meshwork and play an important role in the pathogenesis of glaucoma under oxidative stress. According to the previous reports, the patients with chronic glaucoma have a higher level of peroxidation. For example, Vendemiale et al. found the ability of antioxidant effects will dramatically decrease in case of glaucoma〔14〕. The damaged structures will decrease the drainage of aqueous humor and then cause IOP elevation in patients with glaucoma.

However, there are many antioxidant enzymes that help to reduce oxidative damage and also help to scavenge lipid hydroperoxides and protect against oxidative stress. One of them is glutathione peroxidase (GPx) that catalyze the breakdown of peroxides. It is widely accepted and experimentally proven the catabolic process can generate oxygen-free radicals and other ROS. Total antioxidant status was significantly decreased in the glaucoma group. For example, Majsterek et al. had reported that the activity of antioxidant enzymes such as GPx may decrease in POAG〔15〕. Thus, we believed that increased activity of GPx in the periocular blood flow may help to remove the free radicals in patients with glaucoma. Malondialdehyde (MDA), which is the end product of lipid peroxidation, not only indicates the level of lipid peroxidation, but also reflects the extent of oxygen-free radical formation. For example, Ghanem et al. tapered the aqueous humor of glaucoma patients and determined obtained that the MDA level showed an increase〔16〕. Chang et al demonstrated that increased levels of oxidative stress products such as MDA may be associated with primary angle-closure glaucoma 〔17〕. Thus, the decreased MDA level presents the good antioxidant capabilities.

Large qualities of anti-oxidants are known to be present in aqueous prunella extracts with the polyphenolic compound, rosmarinic acid, being one of the most abundant of these constituents〔18〕. In our study, we found that any doses of AEVP and topical brimonidine might increase the GPx level and decreased the MDA production significantly in the periocular circulation. Now GPx are thought to be responsible for decreased oxidative stress〔19〕. As we know, MDA production presents the accumulation of lipid peroxidation. Thus, we can conclude that AEVP be beneficial in the treatment of glaucoma. In recent years, natural products with antioxidant activity have drawn the most attention. AEVP is widely used for prevention of ROS-mediated injury in pathological situation through its antioxidant properties. A literature review had revealed that the antioxidant effects may come from rosmarinic acid, which is one of the main active components. It is a potent free radical scavenger capable of reducing both superoxide and hydroxyl radials〔20〕. Rosmarinic acid also may attenuate the MDA after ischemia and restored the tissue levels of GPx〔21〕. The biochemical process has been suggested in which the phenyl hydroxyl and cyclic propane groups are likely effective for their antioxidant properties. In our study, topical brinomidine has the same effect. On the other hand, oral acetazolamide did not show reduced MDA and increased GPx levels after five weeks. Recently, it was even reported that topical administration of dorzolamide markedly diminished oxidative stress and lowered the MDA level in patients with glaucoma

〔22〕. Why the systemic use of acetazolamide did not show the same effect needs further investigation.

The results of this study show that topical use of brimonidine, oral acetazolamide, and any doses of AME all demonstrated significant IOP-lowering activity and reached the target IOP after 5 weeks. It is interesting to know how the mechanism of lowering IOP for prunella vulgaris works is. At first, the extracts from the root of prunella vulgaris was found to induce the vasodilation through the nitric oxide-guanosine 3',5'-cyclic monophosphate (NO-cGMP) pathway and reduce the $Ca^{++}$ concentration in vascular smooth muscle cells〔23〕. Some scholars have demonstrated that prunella vulgaris has the ability to up-regulate eNOS through the mechanism of vasodilation. The hypo-tensive function of AEVP is equal to that of atenolol. This is first supported by Xia〔24〕. In our opinion, the vaso-dilation of the smooth muscle of the afferent arterioles of the glomerulus may hence, increase the renal blood flow, the glomerular filtration rate (GFR), and the function of diuresis. Recently Memarzadeh and his coworkers identified that higher systolic and mean arterial blood pressure are associated with a higher prevalence of glaucoma. They concluded that lowering the blood pressure may have the benefit of reducing the developing glaucomatous damage〔25〕. Thus, it showed the indirect evidence that pruella vulgaris may lower the blood pressure and IOP at the same time with the effect of its component (rutin).

Another mechanism of the ability of prunella vulgaris in human to reduce IOP may be the diuretic effect which was ever described in ancient Chinese books, but its mechanism has not been identified. Definitely, the diuretics could increase the urine output and decrease the volume of interstitial fluid. For example, acetazolamide could lower the IOP because of the reduction of the formation of aqueous humor and the enhancement of the diuretic function. In our study, we could find the increased amount of daily total urine output after the treatment of acetazolamide and any doses of AEVP. This is good evidence about lowering IOP. A recent literature review also indicates that the aqueous extract of AEVP radix may facilitate human natriuresis and diuresis〔26〕. Their conclusion is that AME may induce the natriuresis by means of the enhancement of the renal response to atrial natriuretic peptide (ANP). However, the active component deserves further investigation.

In clinics, acetazolamide is always administered according to the patient's condition. In recent years, acetazolamide has been often used to quickly reduce IOP before surgery or for acute glaucoma. In our study, the experimental rats were given the dosage according to their own weight. However, poor external appearance, retarded growth, and weight gain were observed apparently in the group. The fact that acetazolamide may induced metabolic acidosis is well known now. Metabolic acidosis may be a mediating factor for growth failure. Sharan et al. demonstrated that chronic metabolic acidosis exerts as an anti-anabolic effect in bone growth centers, which is partly related to a state of resistance to growth hormone and insulin-like growth factor-1 (IGF-1)〔27〕. This phenomenon maybe could perhaps be used to explain the retarded growth and abnormal weight gain of the SD rats with oral acetazolamide. We also found the less shiny hair and an unstable gait in this group. The carbonic anhydrases are not only distributed at the ciliary body and renal tubular lumen but also at the endothelial cells of the capillary vessels. Therefore, orally administered acetazolamide will cause the loss of potassium ions resulting in rats with hypokalemia demonstrating muscle weakness. The hypokalemic patient being treated with acetazolamide, which induces muscle weakness, has ever been reported. Acetazolamide may reduce exercise capacity associated with increased perception of leg fatigue〔28〕. These articles are compatible with our findings.

At the same time, the liver biopsy in orally administrated acetazolamide group at the end of this experiment showed abnormal findings. We found nodule lesions with a collection of abundant cytoplasm containing micro-vesicles, consistent with fatty liver. However, we can not find out the associated problem through a Medline search. Further evaluation of the etiology and mechanism is needed. We also observed that the SD rats receiving different dosages of AME all have a normal external appearance and normal body-weight gain. We can conclude that any dosage of AME will provide glaucoma patients with a higher efficacy, and safer method of treatment without apparent complications.

## 5. Conclusion

Indeed, the use of natural herbal medicine to treat glaucoma has been of interest concerned in many countries now. For example, extracts of the seeds of Daucus carota, and the fruits of Aegle Marmelos were proved to reduce the IOP〔8〕. In China, Jue Ming Zi has been used for the treatment of "green blindness" for thousands of years. Our study supported that prunella vulgaris may help to control the IOP. In addition, it also enhances the antioxidant effect around the periocular region. Therefore, we concluded that AEVP has the theoretical and clinical bases to lower the IOP, and it could be used for the prevention and treatment of glaucoma in the near

future.

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