Effect of Chrysin on Experimentally Induced Choroidal Neovascularization in Rats

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Effect of Chrysin on Experimentally Induced Choroidal Neovascularization in Rats

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<ABSTRACT>

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Purpose: The aim of this study is to evaluate the effect of chrysin on laser-induced experimental choroidal neovascularization (CNV) in rat model.

Methods: Male Brown Norway rats were anesthetized to receive diode laser to break the Bruch's membrane. 5 $\mu\ell$ of 15 mg/ml chrysin was given through intravitreal injection on 1 week after laser delivery. The development of CNV was determined by fluorescein angiography performed on week 2 and angiograms were graded into 4 scales.

Results: 2 weeks after administration of laser, the intensity of fluorescein leakage from the photocoagulated lesions decreased significantly compared to the control group (P=0.044). When the lesions were categorized into low-leakage or high-leakage group, there was a significant correlation between chrysin treatment and degree of leakage (P=0.028) and the relative risk of developing high-leakage lesion of the control group to the chrysin-treated group was 3.18.

Conclusions: Chrysin exerts an inhibitory effect on choroidal neovascularization in experimental rat model. It should be further evaluated for its potential as a therapy for CNV in age-related macular degeneration.

Key words : Choroidal neovascularization, Chrysin, Fluorescein angiography, Age-related macular degeneration

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I. INTRODUCTION

Age-related macular degeneration (AMD) is the principal cause of legal blindness among those over 65 years of age in the developed countries.[1-4] The development of choroidal neovascularization (CNV) in AMD represents a severe stage of the disease and usually damages central vision.[3-5] There are limited choices of treatment for this kind of disease and visual prognosis in patients with AMD is still poor, despite current treatment modalities such as laser photocoagulation, photodynamic therapy, macular translocation. [6-10] Recently introduced anti-vascular endothelial growth factor (VEGF) antibodies such as pegaptanib, ranibizumab, or bevacizumab which is off-label used for AMD, bring new hope to the treatment of CNV, but these therapy still have limitations.[11-18] Therefore, there is a need for an alternative treatment modality for this condition.

CNV is a result of pathologic angiogenesis. Angiogenesis is an invasion process that requires proteolysis of the extracellular matrix, proliferation and migration of endothelial cells, and simultaneous synthesis of new matrix components.[19] Antiangiogenesis therapies have thus far focused on inhibitors of vascular endothelial growth factor (VEGF)[20, 21] and proteolytic enzymes such as matrix metalloproteinases (MMPs).[22-26] An essential element in the growth of CNV is the rupture of Bruch's membrane and the proliferation of blood vessels through breaks in the membrane. As the pathogenesis of angiogenesis becomes better understood, pharmacologic inhibition of angiogenesis becomes a novel approach in the treatment of CNV[11] and new drug targets are emerging. However, the pathogenesis of CNV is not completely understood.[27]

Flavonoids are present in fruits, vegetables, and beverages derived from plants and in many dietary supplements or herbal remedies.[28] Flavonoids have been described as health-promoting, disease-preventing dietary supplements and having activity as cancer-preventive agents.[28] It was also found that some flavonoids markedly inhibited the proliferation, and, to a lesser degree, the migration of endothelial cells, and capillary formation in vitro.[29] Additionally, they are extremely safe and associated with low toxicity, making them excellent candidates for chemo-preventive agents. Flavonoids comprise several classes, including flavones, flavanones, flavanols, and flavans.

Chrysin (5,7-dihydroxyflavone) is a natural flavonoid and commonly found in fruits and vegetables (Figure 1.). It has been used as a dietary supplement and has become an attractive compound in the cancer research community because of its antitumor properties.[30] Several studies in recent years have shown that chrysin has multiple biological activities, such as anti-inflammation, anaticancer, and antioxidation effects.[31-33] The molecular mechanism of chrysin biologic effect was not well understood, but recently, it was found that chrysin can inhibit vascular endothelial growth factor (VEGF) transcriptional activation in vitro and it also inhibits VEGF expression in the human tumor tissue, as well as angiogenesis in nude mice, using the Matrigel assay.[34]

Figure 1. Chrysin is a solid substance with the molecular formula $C_{15}H_{10}O_4$. It is also known as 5,7-dihydroxyflavone and 5,7-dihydroxy-2-phenyl-4H-1-benzopyran-4-one.

The possibility of using chrysin in treating angiogenesis in eye disease has not yet been tested. In this study, we hypothesized that chrysin may inhibit CNV because of its inhibitory effect on angiogenesis. To test this hypothesis, we wanted to determine whether chrysin can inhibit the angiographic activity of experimental CNV in rat model.

The objective of this study was to evaluate the efficacy of chrysin when administered by single intravitreal injection in experimental rat models after induction of CNV.

II. MATERIALS AND METHODS

1. Materials

Chrysin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the purity of chrysin was >96%. Sodium fluorescein was purchased from Alcon laboratories, Inc. (Fort Worth, TX, USA).

2. Laser-induced CNV Rat Model

Seven or eight-week old male Brown Norway (BN) rats, weighing 200-250 g, were used in the study, in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rats were anesthetized for all procedures with intramuscular injection of ketamine (20mg/kg) and xylazine (5mg/kg). The pupils were dilated with 1% tropicamide and 2.5% phenylephrine. The fundus was visualized with slide cover glass instead of Volk super pupil Xl Biomicroscopy Lens, and with 2.5% hydroxypropyl methylcellulose solution (Methocel: Ciba Vision, Wessling, Germany). Frequency-doubled, diode-pumped, solid state laser (VISULAS 532s; Carl Zeiss Meditech Inc., Dublin, CA) with a wavelength of 532nm was used. Laser parameters were 100 μm spot size, 100 ms exposure, and 150mWpower. A pattern of eight lesions was concentrically placed at approximately equal distance around the optic disc of both eyes. Acute vapor bubbles suggested the rupture of Bruch's membrane. Only laser spots with bubble formation were included in the study. If lesions with subretinal hemorrhage interfered with the evaluation of the lesions, they were excluded. If adjacent lesions merged together, they were also excluded from evaluation of lesion.

3. Administration of Chrysin and Control

After 1 week of laser delivery, chrysin was dissolved in DMSO and Balanced Salt Solution(BSS) at a concentration of 15mg/ml. Under anesthesia, 5 μl of this

solution was given through an intravitreal injection with 30 gauge needle in right eye of all BN rats. 5 $\mu\ell$ of solvent solution alone was injected intravitreally in left eye as control.

4. Fluorescein Angiography (FAG)

Fluorescein angiography (FAG) was performed on 2 weeks after laser treatment with a confocal scanning laser ophthalmoscope (Heidelberg Retinal Angiogrph 2, Heidelberg Engineering, Heidelberg, Germany) to evaluate CNV development and its activity. Each rat was injected with 0.3ml of 10% fluorescein sodium intravenously through the hypoglossal vein and both early (<2 min) and late (>7 min) phase angiogram photographs were taken. The formation of CNV was evaluated according to the size and the presence or absence of dye leakage. The lesions were considered as "having leakage" if hyperfluorescence showed in the early stage with fluorescein leakage continuing through the FAG procedure, and the size and intensity of leakage increased in the late stage. Each photocoagulated lesion was classified as score 1 to 4 according to the intensity of fluorescein leakage by author. The guideline for CNV scoring was as follows: minimum leakage or a staining of tissue with no leakage (score 1); small but evident leakage (score 2); moderate intensity and medium sized (less than 1/2 disc diameter) leakage (score 3); large evident leakage (score 4). A typical photograph of each CNV score is shown in Figure 2. Two examiners judged the scores in a masked fashion, and when the two scores given for a particular lesion did not coincide, the higher score was used for the analysis. For the further analysis of relationship between chrysin treatment and degree of angiographic leakage, each photocoagulated lesion was categorized to low or high leakage group. Score 1 and 2 lesions were grouped to low leakage, whereas score 3 and 4 to high leakage group.
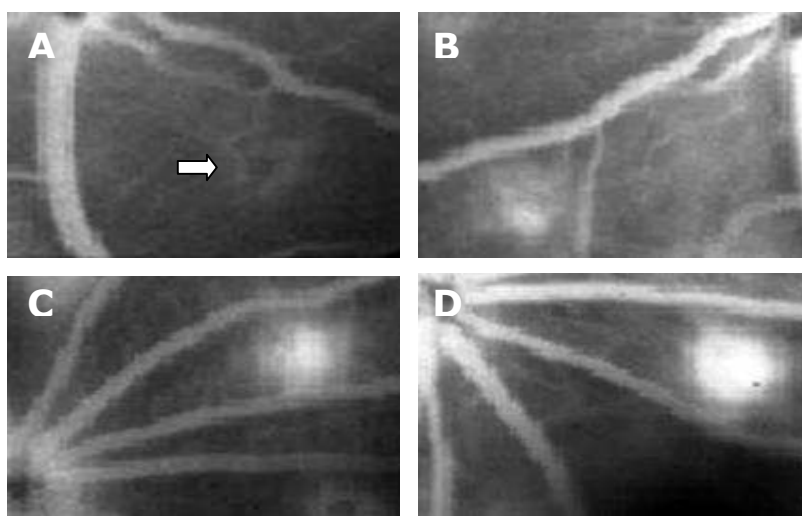
Figure 2. Typical examples of each CNV score in the fluorescein angiogram after laser-induced CNV in rat retina. Each laser spot was scored from 1 to 4 according to the intensity and the size of fluorescein leakage on the early and late phase angiogram photographs. The guideline of the CNV scoring was as follows: (A) minimum leakage or a staining of tissue with no leakage (score 1, arrow); (B) small but evident leakage (score 2); (C) moderate intensity and medium sized (less than 1/2 disc diameter) leakage (score 3); (D) large evident leakage (score 4).

5. Statistical Analysis

Both eyes of each rat were used in the experiment, with right eye as study eye and left eye as control eye. The result data was expressed as mean ± standard error (SE) when the mean CNV score was indicated. The Student unpaired t test was used for the comparison of the mean CNV scores. The chi-square test was used for analysis of categorized groups. For all analysis, SPSS statistical software (version12.0; SPSS Inc., Chicago, IL) was used. P values less than 0.05 were considered statistically significant.

III. RESULTS

1. The Mean CNV Score

Fluorescein angiography, using sodium fluorescein, showed early hyperfluorescence of the laser lesions, which increased in size and intensity in the late phase. The leaky spots were considered as angiographically defined CNV. Each photocoagulated lesion was scored as 1 to 4 according to the intensity of fluorescein leakage, and number of laser lesions of each 1-4 CNV scores in both groups is shown in Table 1 and Figure 3. As shown Figure 4, the mean CNV scores in chrysin-treated and contriol (vehicle-treated) eyes were 2.40 ± 0.21 and 2.97 ± 0.18, respectively. There was a statistically significant difference between these two groups (P = 0.044, unpaired t test)

Table 1. Number of laser lesions and the mean CNV score of chrysin-treated and control group

	CNV score					The mean CNV score[1]
	1	2	3	4	Total	
Chrysin-treated group	9	7	7	7	30	2.40 ± 0.21
Control group	5	4	12	13	34	2.97 ± 0.18
P« value						0.044

[1] Data was expressed as mean ± standard error (SE).
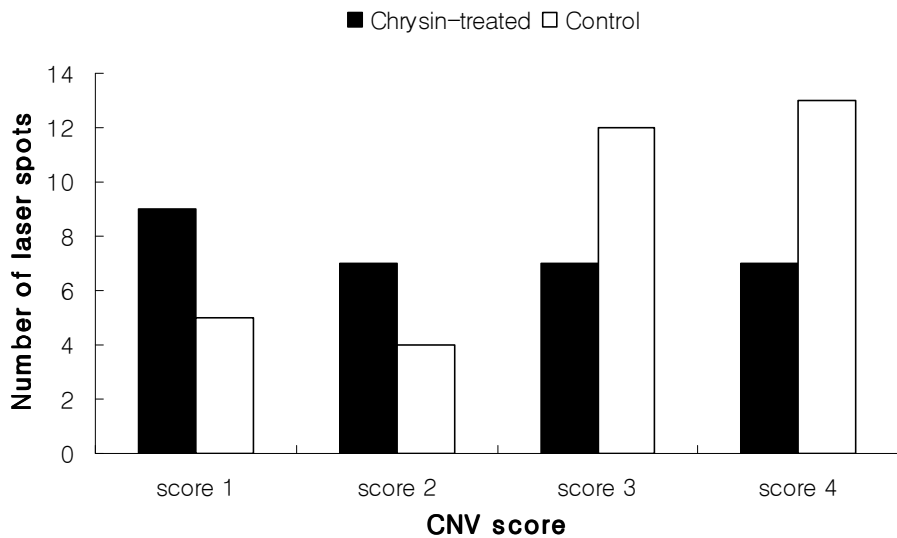
« Student unpaired *t* test

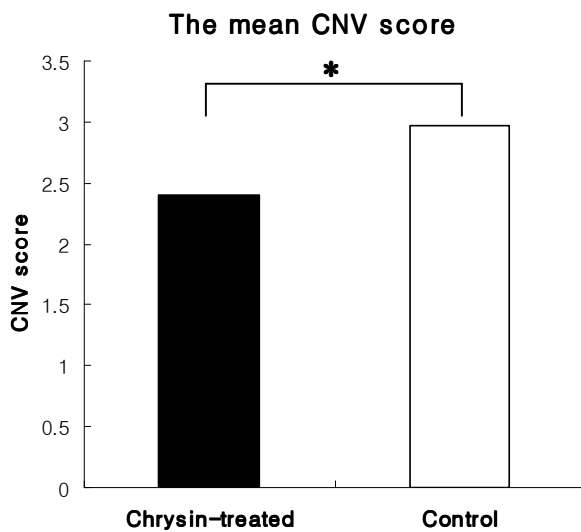Figure 3. Number of laser lesions of each 1-4 CNV scores in chrysin-treated and control group.

Figure 4. Effect of chrysin on CNV score on 2 weeks after laser induced CNV in rat retina. The mean CNV score in chrysin-treated and control group. 〃 , $P<0.05$ compared with control group.

2. Analysis of Categorized Groups

When each photocoagulated lesion was categorized to low or high leakage group, such as grade 1 or 2 lesions to low leakage and grade 3 or 4 to high leakage group, number of laser lesions of each group is shown in Table 2 and Figure 5. Analysis of chrysin treatment and degree of angiographic leakage was performed with chi-square test. After 2 weeks after laser administration, the number of lesions which belonged to low leakage group was significantly more in the chrysin-treated group (P=0.028). Relative risk of control group for developing high leakage lesion to chrysin-treated group was 3.18 (95% confidence interval, 1.12-9.04).

Table 2. Number of lesions in each categorized group

	Low leakage group	High leakage group	Total
Chrysin-treated group	16	14	30
Control group[1]	9	25	34
P＜ value			0.028

[1] Relative risk (control/chrysin-treated) = 3.18

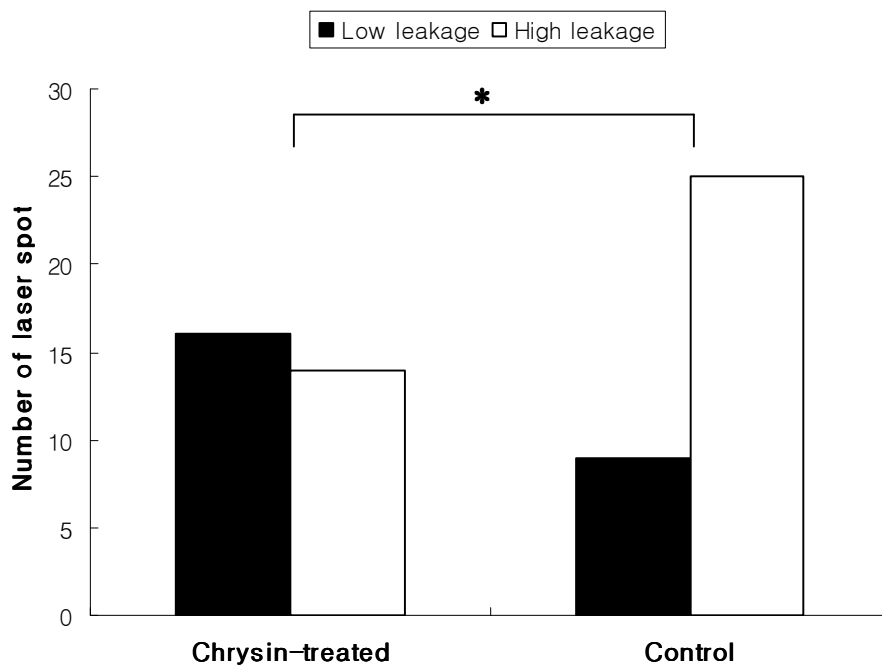　 95% confidence interval, 1.12-9.04

＜ Pearson chi-square test

Figure 5. Number of laser lesions of low leakage and high leakage in chrysin-treated and control group. ⁎ *P* < 0.05 with Pearson chi-square test

IV. DISCUSSION

AMD is a leading cause of irreversible blindness among people who are 50 years of age or older in the developed world.[35-37] The neovascular form of the disease usually cause severe vision loss and is characterized by the abnormal growth of new blood vessels under or within the macula, the central portion of the retina responsible for high-resolution vision. The etiology of AMD is obscure and the age-related changes that stimulate pathologic neovascularization are incompletely understood, but vascular endothelial growth factor (VEGF) – a diffusible cytokine that promotes angiogenesis and vascular permeability – has been implicated as an important factor promoting neovascularization.[38-43]

Vascular endothelial growth factor (VEGF) is the fundamental regulator of angiogenesis and plays an important role in development and progression of CNV. Increased expression of VEGF was noted in both laser-induced experimental CNV and CNV in AMD patient. Vascular endothelial growth factor (VEGF) is mainly regulated by HIF-1α at transcriptional level.[44]

Hypoxia-inducible factor-1(HIF-1) is a heterodimeric transcriptional factor composed of α and β subunits. HIF-1 is overexpressed in many human cancers[45] and the levels of its activity in cells correlate with tumorigenicity and angiogenesis.[46] HIF-1α is induced by hypoxia, growth factors, and oncogenes.[47, 48]

HIF-1 activates the transcription of many genes, including VEGF. It activates the expression of the VEGF gene by binding to the hypoxia response element (HRE) in the VEGF promoter.[44] HIF-1 is over-expressed in many human cancers and experimental CNV[44, 45] and levels of its activity in cells correlated with tumorigenicity and angiogenesis.[46]

Therefore, an antiangiogenic therapy that targets the HIF-1α/VEGF system is a promising strategy for the treatment of CNV. The discovery of a new agent that targets HIF-1α and VEGF is a potentially effective chemotherapeutic treatment for CNV in AMD.

Epidemiologic studies have suggested that the dietary intake of fruits and vegetables can reduce incidence of many types of cancer[49-51] and regular dietary

intake of antioxidants was associated with lower incidence of AMD and slow progression to advanced AMD.[52, 53] The preventive effects of plant-based diets on tumorigenesis and other chronic diseases have been well documented.[54] Flavonoids, which are present in many fruits and vegetables, were found to inhibit VEGF transcriptional activation and VEGF expression recently. It also inhibits endothelial proliferation and migration, and thus is proved to inhibit angiogenesis in tumor tissue.[34, 55, 56]

Chrysin, a common flavonoid, has been proposed as an antitumor agent.[57-59] This study evaluated the effect of chrysin on experimental CNV in rats and the possibility of using chrysin as a pharmacologic preventive or treatment modalities in CNV in AMD. Using laser-induced CNV model, the chrysin-treated group had significantly less fluorescence leakage compared with the vehicle-treated group on fluorescein angiography. The results demonstrated that, compared with controls, the chrysin injected eyes had a lower CNV score (P=0.044). There was a significant relationship between chrysin treatment and lower incidence of high leakage lesion (P=0.028).

In this study, chrysin could inhibit the fluorescein leakage after 2 week of laser delivery and, 1 week of chyrsin treatment. This suggests that chrysin interferes with the procedure of CNV development. However, the molecular mechanisms involved have been not well understood.

As mentioned earlier, vascular endothelial growth factor (VEGF) plays a fundamental role in development CNV in AMD and VEGF is mainly regulated by HIF-1α at transcriptional level.[44] Recent studies demonstrated that chrysin inhibits VEGF expression in tumor tissue at the transcriptional level through HIF-1α expression. Expression of HIF-1α is regulated via degradation and protein synthesis. A most recent study showed that chrysin reduced the half-life of HIF-1α and HIF-1α stability.[34] Chrysin inhibited the binding of HIF-1α to Heat shock protein (HSP) 90, suggesting that chrysin inhibits HIF-1α expression via interfering with the interaction between HIF-1α and HSP 90.[34]

This study demonstrated here for the first time that chrysin inhibits experimental CNV in rats. This novel finding provides new insight into the

possibilities of the anti-angiogenic propertied of chrysin. Based on the daily dietary consumption of flavonoids, the concentration of chrysin used in this work is nontoxic and physiologically relevant in humans.[60] Molecular targeting of the VEGF by chrysin may be a useful and novel strategy for pharmaco-prevention and/or treatment of CNV in AMD.

In this study, the score for CNV was determined using fluorescein angiography. A presence or absence of CNV could be clearly determined. However, fine grading of CNV lesion was difficult. This is consistent with the scoring system used by other investigators.[61, 62] Although efforts to minimize the bias involved in scoring each CNV lesion were made, some confounding factors in this procedure might confuse results of this study. Furthermore, relatively small sample size and using only one dosage of investigated drug are obvious limitations of this study. The inhibitory effect of chrysin on experimental CNV is expected to be due to inhibition of expression of HIF-1α and VEGF. However, the mechanism of the effect of chrysin was not evaluated in this study. Succeeding research is scheduled to investigate immunohistochemical stain of HIF-1α and VEGF in chrysin-treated CNV compared with control group. It should be also investigated whether inhibitory effect is dose-dependent or not, using multiple dose of chrysin.

Current treatment options for AMD are limited and to date have had little impact on the progression of visual loss. The results of this study suggest that chrysin, a common natural flavonoid, may be a candidate of pharmacologic treatment modality for CNV in AMD. Moreover, prevention of CNV formation may be important in maintaining visual function. Current preventive advice includes a suggestion for not to smoke, and possibly information regarding various supplementations; however, further preventive strategies are needed. The data of this study also suggest that the use of chrysin could be helpful in preventing CNV development in AMD patients. Further evaluations of chrysin seem justified to definitely address the question of the effect on CNV in AMD.

It is exciting to find that chrysin can reduce the angiographic leakage of CNV in laser-induced rat model. These results indicate that chrysin could be a drug

candidate to inhibit ocular neovascularization in AMD. Further study on the possible mechanism of effect of chrysin in CNV development is needed. Its anti-angiogenic effect in CNV rat model may be related to decreased HIF-1α expression and, thus inhibition of VEGF. Further research on the efficacy and the mechanism of this compound is warranted.

V. CONCLUSION

Chrysin, a member of the flavone family that is present at high levels in many vegetables, may have its priority in the treatment of patients with AMD. It can inhibit the angiographic leakage, namely, the activity of CNV in laser-induced animal models. Furthermore it is relatively safe and already available, such as dietary component. Thus, the role of chrysin in prevention or treatment of AMD warrants further investigation.

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< ABSTRACT(IN KOREAN)>

실험적으로 유발된 맥락막신생혈관에서 Chrysin에 의한
억제 효과

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송 지 훈

　Chrysin은 과일과 야채 등에 흔히 포함되어 있는 자연상태의
flavonoid이다. 본 연구에서는 쥐를 이용한 모델에서 레이저에
의해 실험적으로 유발된 맥락막신생혈관에 대한 chrysin의
효과에 대해 알아보고자 하였다.

　수컷 Brown Norway rat을 전신마취 후 양안에 다이오드
레이저를 이용하여 브루크막의 파열을 유발하였다. 레이저 조사
1주 후, 15 mg/ml 농도의 chrysin 5 $\mu\ell$를 레이저를 시행한 모든
쥐의 우안에 유리체내 주입하고, 좌안에는 동량의 용매만을
주입하여 대조군으로 하였다. 레이저 조사 2주 후
형광안저혈관조영을 시행하여 맥락막신생혈관의 발생을
확인하고, 형광안저촬영 상 나타나는 형광누출의 정도에 따라
각 레이저 조사 병변을 4 단계로 구분하였다.

　Chrysin 투여 1주 후, 맥락막신생혈관의 형성이 확인되는
병변들로부터 관찰되는 형광누출이 chrysin 투여 군에서
대조군에 비하여 유의하게 낮게 나타났다 (P=0.044). 또한 각

병변들을 형광안저촬영 상 저누출과 고누출로 분류하였을 때, chrysin 투여여부와 누출 정도와는 유의한 상관성을 보였으며 (P=0.028), 대조군에서 chrysin 투여군에 비하여 고누출이 발생할 상대위험도는 3.18 이었다 (95% 신뢰구간; 1.12-9.04).

결론적으로 chrysin은 쥐 모델에서 레이저에 의해 실험적으로 유발된 맥락막신생혈관에 대한 억제효과를 나타내었으며, 연령관련 황반변성에서 발생하는 맥락막신생혈관의 치료로서의 이용가능성에 대해 추가적인 연구가 필요할 것으로 생각된다.

핵심되는 말 : 맥락막신생혈관, Chrysin, 형광안저혈관조영, 연
                     령관련 황반변성