의학박사 학위논문

Development of drug delivery system using albumin nanoparticles for sustained intraocular release of brimonidine

지속적 안구내 약물 방출을 위한 알부민 나노입자 기반 약물 전달 시스템 개발

2017 년 2 월

서울대학교 대학원

의학과

김 고 은

Abstract

Purpose: Eye drops are one of the most common types of glaucoma medication notwithstanding their limitations in terms of bioavailability and low compliance. Albumin-based nanoparticles have shown potential for effective and continuous drug release. Thus, we investigated the potential of human serum albumin nanoparticle (HSA-NP) as a tool for sustained delivery of neuroprotective agent in optic nerve crush (ONC) injury model.

Methods: Albumin nanoparticles loaded with brimonidine (HSA-BRM-NPs) were prepared by ethanol precipitation, including 0.18% brimonidine (BRM) and 3.5% human serum albumin (HSA) in HSA-BRM-NP solution. In vitro release profile of BRM was measured using Spectra/por dialysis membrane and assessed by high-performance liquid chromatography. We performed ONC and intravitreal injection in Sprague-Dawley rats, which were divided into Normal, balanced salt solution (BSS)-injected ONC, HSA-NP-injected ONC, BRM-injected ONC, and HSA-BRM-NP-injected ONC groups. The survival of RGC was compared at 5 and 14 days after procedures.

Results: The HSA-BRM-NP released 95% BRM in a sustained manner for 3 days, and the rest until 5 days. The percentages of RGC survival in the HSA-NP (52.6 ± 3.3%), BRM (58.0 ± 4.2%), and HSA-BRM-NP (63.5 ± 7.1%) groups relative to Normal (100%) were significantly higher than in the BSS group (29.2 ± 3.3%) 5 days after ONC ($P < 0.001$). At 5 days, no significant difference was found in the percentage of RGC survival between BRM and HSA-BRM-NP groups ($P = 0.014$).

However, the HSA-BRM-NP (38.1 ± 3.6%) group showed significantly higher RGC density than the BRM (18.6 ± 3.9%, $P = 0.006$) group at 14 days.

Conclusions: Albumin nanoparticles showed sustained therapeutic effect with the combined neuroprotective agent. Our results suggest the potential of albumin nanoparticles as a promising tool for sustained intraocular drug release.

Keywords: Human serum albumin-nanoparticle; Brimonidine; Sustained drug release; Retinal ganglion cell; Optic nerve crush injury model

Student Number: 2015-30584

Contents

List of Figures

Introduction

Glaucoma is a progressive optic neuropathy that involves retinal ganglion cell (RGC) death and subsequent axonal degeneration, eventually leading to visual loss.(1) To date, intraocular pressure (IOP) is the only treatable factor and thus, IOP lowering medications are crucial for reducing the risk of disease progression.(2, 3) Eye drops are usually considered as the first choice for IOP lowering glaucoma treatment in current clinical practice. However, undesirable side effects such as ocular irritation, allergic reaction, systemic side effects, and limited bioavailability can be the main reasons for poor adherence and persistence, resulting in unsuccessful treatment outcome.(4, 5) Although considerable progress has been made for various types of medical treatments to reduce the number of eye drops and to raise compliance, efficient sustained drug delivery system are still far from being applicable.

Nanoparticles have been reported as an effective intravitreal drug delivery system.(6-8) Among the several types of nanoparticles, human serum albumin-based-nanoparticle (HSA-NP) has been identified as a promising drug transporter for intraocular disease treatment, due specifically to its superior capability in penetrating deeper retinal structures.(6, 8) In addition to the advantage of nanoparticles, human serum albumin (HSA) can conjugate to bioactive proteins or to low-molecular-weight drugs and form self-aggregates, thus facilitating the secure release of intraocular drugs.(9)

Brimonidine (BRM) is a highly selective α2-adrenergic receptor agonist that is well known for its neuroprotective effect against RGC death, which has been confirmed by a large number of experimental studies.(10-16) BRM is known to exert this effect by reducing intraocular excitotoxins (e.g. aspartate, glutamate) elevated secondary to ischemic insult,(17) upregulating neurotrophic growth factors (e.g. brain-derived neurotrophic factor, fibroblast growth factor),(15, 18, 19) or activating anti-apoptosis cascades,(20, 21) ultimately leading to prevention of RGC death. It is still one of the most commonly used anti-glaucoma medications. However, it is recommended to use 2 or 3 times per day due to its low bioavailability.(22) Therefore, considering the life-long treatment required in glaucoma patients, continuous and effective delivery of BRM would aid in achieving successful persistence in glaucoma therapy as well as longer neuroprotective effect.

In light of these, we hypothesized that HSA-NPs could have a potential drug-delivery capability. Moreover, if HSA-NPs can conjugate with the neuroprotective agent, enhanced long-term release of drug accomplishing less number of instillation can be expected. In this regard, we investigated the potential of HSA-NPs as a tool for sustained delivery of neuroprotective agent in optic nerve crush (ONC) injury model.

Materials and Methods

Materials

HSA, BRM (powder type), ethanol, glutaldehyde solution, and dimethylsulfoxide were obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification. Dextran tetramethylrhodamine (DTMR) and Alexa Fluor® 555 carboxylic acid succinimidyl ester were purchased from Life Technologies (Carlsbad, CA). Rat Aβ peptide (1-42) was obtained from AnaSpec (Fremont, CA). A Spectra/por dialysis membrane (molecular weight cut-off: 1,000) was purchased from Spectrum Labs (Rancho Dominguez, CA), and DAPI was obtained from Invitrogen (Eugene, OR).

Preparation of human serum albumin nanoparticles loaded with brimonidine

HSA-BRM-NPs were synthesized via the ethanol precipitation method.(23) Specifically, HSA was dissolved in 1 ml of distilled water to a final concentration of 20 mg/ml, and BRM in dimethylsulfoxide to a final concentration of 10 mg/ml. Subsequently, 200 μl of the BRM solution was added to 1 ml of HSA solution (20 mg/ml). Ethanol (1.2–3 ml) was added dropwise to the resulting mixture, which was then stirred for 1 hour at 550 rpm. Twenty microliters of 4% glutaraldehyde solution was added to the mixture, which was then stirred overnight at room temperature. After 24 hours of stirring, the HSA-BRM-NPs were purified 3 times by centrifugation (13,200 rpm, 10 min, 4℃) and then resuspended in distilled

water. Finally, the suspension was centrifuged (3,000 rpm, 5 min, 4℃) to remove aggregates.

To prepare HSA-BRM-NPs with conjugated NHS-fluorescein, 5 μl of NHS-fluorescein was added to 1 ml of HSA-BRM-NP solution, which mixture was maintained at room temperature for 1 hour. To remove the remaining free NHS-fluorescein, the solution was centrifuged (3,000 rpm, 5 min, 4℃) and resuspended twice in a phosphate-buffered solution (PBS; pH 7.4).

To prepare HSA-BRM-NPs with conjugated Alexa 555, HSA-BRM-NPs were resuspended in PBS (pH 7.4), and Alexa Fluor 555 carboxylic acid succinimidyl ester was dissolved in methanol. Subsequently, 5 μl of Alexa Fluor 555 carboxylic acid succinimidyl ester solution was added to 1 ml of HSA-BRM-NP solution. The mixture underwent reaction at room temperature for 30 minutes. To remove the remaining dye, the mixture was centrifuged (3,000 rpm, 5 min, 4℃) and resuspended twice in PBS (pH 7.4).

Characterization of human serum albumin nanoparticles loaded with brimonidine

The size distribution and zeta potential of the HSA-BRM-NPs that had been suspended in PBS (pH 7.4) at 37℃ were measured 3 times on a Malvern Zetasizer Nano ZS 3000HAs (Malvern Instrument Ltd., Worcestershire, UK). Their morphologies were observed by transmission electron microscopy (TEM; CM30, Philips, CA). The HSA-BRM-NPs were then dissolved in distilled water and

applied to a 300-mesh copper grid coated with carbon. After 2 minutes, the grid was tapped with filter paper to remove the distilled water and air-dried. Afterward, each grid was stained with a droplet of 2% (w/v) uranyl acetate.

To test the in vitro release of BRM from HSA-BRM-NPs, a Spectra/por dialysis membrane (molecular weight cut-off:1,000) was applied. Two hundred microliters of the HSA-BRM-NPs was added to the dialysis membrane, both ends of which were sealed with clips. The HSA-BRM-NP-loaded membrane was then inserted into a 50 ml tube filled with 50 ml of PBS (pH 7.4), and placed in a 37℃ shaking incubator. Samples were obtained from the tube at various time points. The amount of BRM released from the HSA-BRM-NPs was assessed by high-performance liquid chromatography (YL9100 HPLC system, Younglin, Gyeonggi-do, South Korea). Zorbax Eclipse Plus C18 (4.6 x 250 mm, 5 μm; Agilent Technologies, Santa Clara, CA) was employed for separation. The flow rate was 1.0 ml/min, and elution of the mobile phase of ACN:buffer (10:90; consisting of 10 mM triethylamine adjusted to pH 3.2 with phosphoric acid) was isocratic. The UV detector was set to 248 nm, and the retention time was 6 minutes.

Optic nerve crush injury model

All of the procedures involving animals adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Additionally, they were approved by the Animal Research Committee of Seoul National University Hospital. The rodent model of ONC injury,

which is one of the most commonly used animal models to investigate the process and general mechanism of RGC death and survival with a high degree of reproducibility and predictability, has been used for the investigation.(24) Specifically, male Sprague–Dawley (SD) rats purchased from Koatech (Pyeongtaek, South Korea) and weighing 250 to 300 g were housed with adequate care. Only the right eye of each SD rat was used for all of the procedures. The animals were maintained under alternating dark–light cycles of 12 hours at room temperature. After anesthetization by intramuscular injection of xylazine (1 mg/kg; Rompun, Bayer, Germany) and Zoletil (10 mg/kg; Virbac Animal Health Ltd., Carros, France), the superolateral conjunctiva was incised with scissors. The subconjunctival space was exposed, and the other tissues were gently separated by dissection under surgical microscopy. The optic nerve was exposed, and an aneurysm clip (5.2 mm blade length, 4.0 mm maximal opening, 110 g force; MINI Aneurysm-Clips; Aesculap AG, Tuttlingen, Germany) was used to clip 2 mm behind the globe for 60 seconds, without damaging the retinal perfusion. After removing the clip, an antibiotic ointment was applied and the animals were allowed to recover.(25)

Intravitreal injection of test agents

The animals were classified into 5 groups according to the intravitreally administered agent: Normal (normal negative control), BSS (balanced salt solution-injected ONC), HSA-NP (HSA-NP-injected ONC; 35 μg/μl), BRM (BRM-

solution-injected ONC; 1.76 μg/μl), and HSA-BRM-NP (HSA-BRM-NP-injected ONC; 35 μg/μl of HSA and 1.76 μg/μl of BRM) rat groups. Each group comprised at least 5 rats. The calculated concentration of BRM in the HSA-BRM-NP solution was 0.18% (w/v). Immediately following the ONC procedure, an equal amount (5 μL) of each agent was administered intravitreally by Hamilton syringe (Hamilton Company, Reno, NV).

Evaluation of retinal ganglion cell survival

To evaluate the RGC count 5 days after the ONC procedure and injection, retrograde labeling with DTMR (Molecular Probes, Eugene, OR) was performed one day prior to enucleation. DTMR was applied to the proximal cut surface of the optic nerve. Five days post-ONC and injection, the eyes were enucleated and fixed with 4% paraformaldehyde solution at 4°C for 120 minutes ($n \geq 5$ per group). The retinas were dissected, flattened with 4 radial cuts, and flat-mounted whole on glass slides. The slides were maintained in the dark and air-dried overnight. The DTMR-labeled RGCs were examined under fluorescence microscopy (BX-61; Olympus, Tokyo, Japan), and fluorescence micrographs were obtained with a microscope eyepiece reticle (400× final magnification) 1, 2, and 3 mm from the center of the optic nerve along the centerline of each retinal quadrant. Labeled RGCs in the fluorescence micrographs were counted in a masked fashion by 3 independent investigators (I.J., K.E.K., and Y.J.K.), and the results were averaged. To calculate the mean density of labeled RGCs per square millimeter of each retina, the number

of labeled cells in the 12 photographs was divided by the area of the region. The same procedures were repeated for evaluation of RGC survival 14 days post-ONC and intravitreal injection.

In vivo retinal distribution of human serum albumin nanoparticles loaded with brimonidine

Five microliters of HSA-BRM-NPs labeled with Alexa 555 in PBS (35 μg/μl of HSA and 1.76 μg/μl of BRM, pH 7.4) were intravitreally injected using a Hamilton syringe (Hamilton Company). Six, 24, and 72 hours after injection, the eyes were enucleated and fixed in 4% paraformaldehyde for 24 hours ($n \geq 5$ per group). They were then embedded in 7% agarose gel with 0.2% sodium azide and sliced to 200 μm thickness using a vibratome section. The slices were stained with 0.01% DAPI/PBTA solution (0.5% BSA, 0.05% sodium azide, and 0.1% Triton-X 100) overnight. After staining, the slices were washed with fresh PBTA 3 times and then mounted with Vectashield® mounting medium (Vector Laboratories Inc.). The retinal distribution of the HSA-BRM-NPs was observed under confocal laser scanning microscopy (Leica Microsystems GmbH).

Immunohistochemistry for amyloid-β

The SD rats were divided into 3 groups: normal, ONC model, and HSA-NP-injected ONC model (n = 4 per each group). Right eyes were enucleated 14 days after the procedures and then immersed in 4% paraformaldehyde for 24 hours.

They were then embedded in 7% agarose gel with 0.2% sodium azide and sliced to 200 μm thickness using a vibratome section. The slices were treated with anti-Aβ (1-40), a rabbit antibody (Merck Millipore, Darmstadt, Germany), which had been diluted to 1/100 in PBTA. After 24 hours, the slices were washed with PBTA 3 times and treated with a 1:100 solution of Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and 0.01% DAPI in PBTA. After 24 hours, the samples were washed with PBTA 3 times and mounted with Vectashield® mounting medium (Vector Laboratories Inc.). The presence of Aβ in the retinal layers of the ONC model was examined under confocal laser scanning microscopy (Leica Microsystems GmbH).

Cell viability assay for human serum albumin inhibition of amyloid-β aggregation

To identify the mechanism of HSA inhibition of Aβ neurodegeneration, cell viability was evaluated by reduction assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Roche Applied Science, Indianapolis, IN). RGC-5 cells (2 x 10^4 per well) were seeded in 96-well plates. Twenty-four hours after plating, the cells were separately treated with Aβ (20 μM), HSA-NP (30 μg/μl), and Aβ (20 μM) mixed with HSA-NP (30 μg/μl; 5 wells per each group). After incubations for various durations (12, 24, and 48 hours), the wells were washed with 1× DPBS, and 30 μl of MTT and distilled water (3 mg/ml) were added. The wells were then incubated for 4 hours at 37˚C, after which 100 μl of

DMSO was added prior to measurement with a plate reader (EL800; BioTek Instruments Inc., Winooski, VT).

Statistical analysis

The data are presented as means with standard deviations of at least 3 independent experiments. One-way ANOVA with post-hoc Tukey test was conducted to compare the groups' RGC densities in both in vivo and cell viability experiments. All of the statistical analyses were performed using SPSS version 21.0 for Windows (IBM Corp., Armonk, NY). Statistical significance was determined to be $P < 0.05$.

Results

Characterization of nanoparticles

A narrow size distribution of round shaped nanoparticles was observed by TEM. Zetasizer measurements revealed that the HSA-BRM-NPs had a uniform size distribution (152.8 ± 51.1 nm diameter) and highly negative potential (−29.7 ± 7.5 mV). The HSA-BRM-NPs released about 95% BRM (i.e., 454.84 μg BRM per mg particles) in a sustained manner for 3 days, after which the rest 5% BRM was released slowly until 5 days (Fig. 1).

In vivo retinal distribution of human serum albumin nanoparticles loaded with brimonidine

Figure 2 shows the retinal distributions of HSA-NPs in normal control (Fig. 2A) and ONC model (Fig. 2B) at baseline and 6, 24, and 72 hours after injection ($n > 5$). Their persistent time and distribution patterns in the retinal layer did not differ between the 2 groups. DAPI and Alexa 555 images reveal the locations of the retinal layers and HSA-BRM-NPs, respectively. Merged images proved that the HSA-BRM-NPs were located within the retinal layers. The magnified images in Figure 2 further demonstrate that the HSA-BRM-NPs were well located within the ganglion cell layer (GCL).

Sustained therapeutic effect of human serum albumin nanoparticles loaded with brimonidine on retinal ganglion cells

Figure 3A shows the remaining RGCs in the groups at 5 (first row) and 14 (second row) days following the procedure and Table 1 summarizes their densities. The percentages of RGC survival relative to the RGC density in the Normal group (100%) were 29.2 ± 3.3, 52.6 ± 3.3, 58.0 ± 4.2, and 63.5 ± 7.1% in the BSS, HSA-NP, BRM, and HSA-BRM-NP groups, respectively, at 5 days, and 10.3 ± 5.6, 30.7 ± 11.7, 18.6 ± 3.9, and 38.1 ± 3.6%, respectively, at 14 days (Fig. 3B). The RGC densities at 5 and 14 days after ONC injury and intravitreal injection were then compared among the BSS, HSA-NP, BRM, and HSA-BRM-NP groups to evaluate their neuroprotective effects. Five days after the procedure, the RGC densities in the HSA-NP ($P < 0.001$), BRM ($P < 0.001$), and HSA-BRM-NP ($P < 0.001$) groups were significantly higher than that in the BSS group. However, 14 days after the procedure, only the HSA-NP ($P = 0.002$) and HSA-BRM-NP ($P < 0.001$) groups showed significantly higher RGC densities than the BSS group. In comparing the BRM and HSA-BRM-NP groups, there was found to be no significant difference at 5 days ($P = 0.014$); however, at 14 days, the HSA-BRM-NP group showed a significantly higher RGC density ($P = 0.006$).

Neuroprotective mechanism of human serum albumin nanoparticles

To investigate the possible pathogenic role of Aβ in RGC apoptosis and the therapeutic effect of HSA-NP against Aβ, we examined the presence of Aβ aggregation in the ONC model and performed an MTT assay. The immunohistochemistry results showed elevated expression of Aβ in the GCL of the

ONC model relative to the normal rats at 14 days following injury (Fig. 4). Merged DAPI and FITC images revealed the locations of Aβ in the retinal layers, and a magnified image indicated that Aβ was specifically located in the GCL. Interestingly, the amount of Aβ deposition was lower in the GCL of the HSA-NP-injected ONC model than in that of the ONC-only model.

An in vitro experiment showed that at 48 hours, the RGC viability was significantly decreased in the Aβ group (51.9 ± 7.6%) relative to the non-treated control (100%; $P < 0.001$) and HSA-NP (89.9 ± 4.9%; $P < 0.001$) groups; however, the Aβ and HSA-NP co-treated group (71.9 ± 8.5%) showed significantly higher RGC viability than the Aβ group (51.9 ± 7.6%; $P = 0.012$).

Table 1. Retinal ganglion cell survival densities (the mean number of cells/mm^2 ± SD) in each of the (1) Normal (normal control), (2) BSS (balanced salt solution-injected optic nerve crush [ONC]), (3) HSA-NP (human serum albumin-nanoparticle-injected ONC), (4) BRM (brimonidine-injected ONC), and (5) HSA-BRM-NP (HSA-NP loaded with BRM-injected ONC) rat groups.

Groups	**Retinal ganglion cell densities**	
	5 days after ONC	14 days after ONC
Normal	1501.3 ± 98.1	2236.2 ± 258.3
BSS	438.3 ± 69.7	232.4 ± 126.0
HSA-NP	810.9 ± 45.8	688.3 ± 262.3
BRM	862.7 ± 18.4	415.0 ± 86.8
HSA-BRM-NP	908.8 ± 51.4	851.2 ± 80.6

Figure 1. Release profile of briminodine (BRM) from human serum albumin nanoparticles loaded with BRM (HSA-BRM-NP). In vitro data showing accumulated amount of BRM released from HSA-BRM-NP in a sustained manner.

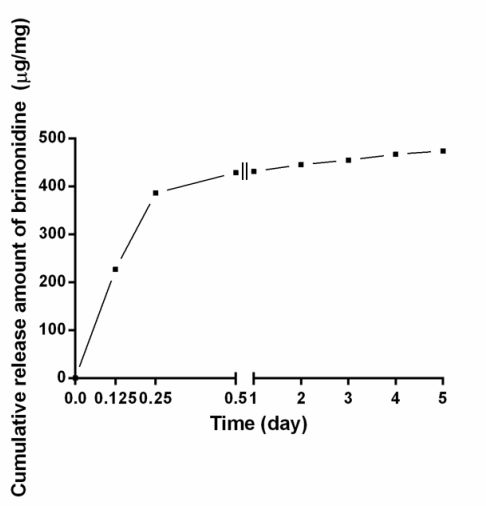


Figure 2. In vivo retinal distribution of human serum albumin nanoparticles loaded with brimonidine (HSA-BRM-NPs) in (A) normal rat and (B) ONC model at baseline and 6, 24, and 72 hours after intravitreal injection of HSA-BRM-NPs ($n \geq 5$ per group at each time point). DAPI and Alexa 555 images respectively represent the location of the retinal cells' nucleus and the location of the HSA-BRM-NPs. Magnified ganglion cell layer (GCL) images confirmed that the HSA-BRM-NPs were co-localized within the GCL among the intraretinal layers at 6, 24, and 72 hours after injection. INL = inner nuclear layer; ONL = outer nuclear layer. All of the images were taken at the same final magnification level (150x).

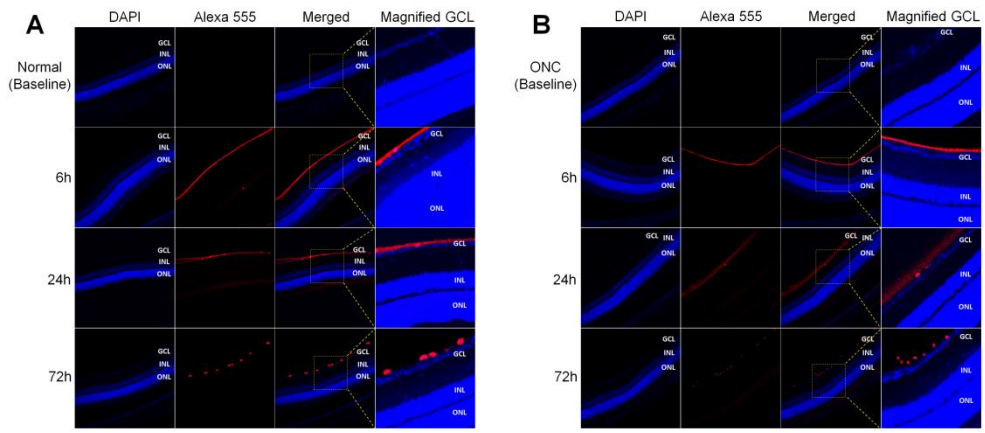


Figure 3. Sustained therapeutic effect of human serum albumin nanoparticles loaded with brimonidine (HSA-BRM-NPs) on survival of retinal ganglion cells (RGCs) in optic nerve crush (ONC) injury model. Animals were classified into 5 groups according to the intravitreally administered agents: (1) Normal (normal control), (2) BSS (balanced salt solution-injected ONC), (3) HSA-NP (HSA-NP-injected ONC), (4) BRM (brimonidine solution-injected ONC), and (5) HSA-BRM-NP (HSA-BRM-NP-injected ONC) rat groups. The RGC density in each group was evaluated 5 and 14 days after the ONC procedure and intravitreal injection ($n \geq 5$ per group at each time point). (A) Fluorescent retinal flat mount images showing remnant RGCs in each group at 5 and 14 days. All of the images were taken at the same final magnification level (400x). (B) In comparing the RGC densities among the groups, the HSA-NP ($P < 0.001$), BRM ($P < 0.001$), and HSA-BRM-NP ($P < 0.001$) groups showed higher RGC densities than the BSS group 5 days post-ONC. However, 14 days after the procedure, only the HSA-NP ($P = 0.002$) and HSA-BRM-NP ($P < 0.001$) groups showed significantly higher RGC densities than the BSS group. Additionally, when comparing between BRM and HSA-BRM-NP groups, no significant difference in RGC density was found at 5 days ($P = 0.014$), but a significantly higher RGC density was found in the HSA-BRM-NP group at 14 days ($P = 0.006$). All comparisons were performed using one-way ANOVA with post-hoc Tukey test (*$P < 0.013$; 0.05 divided by 4).

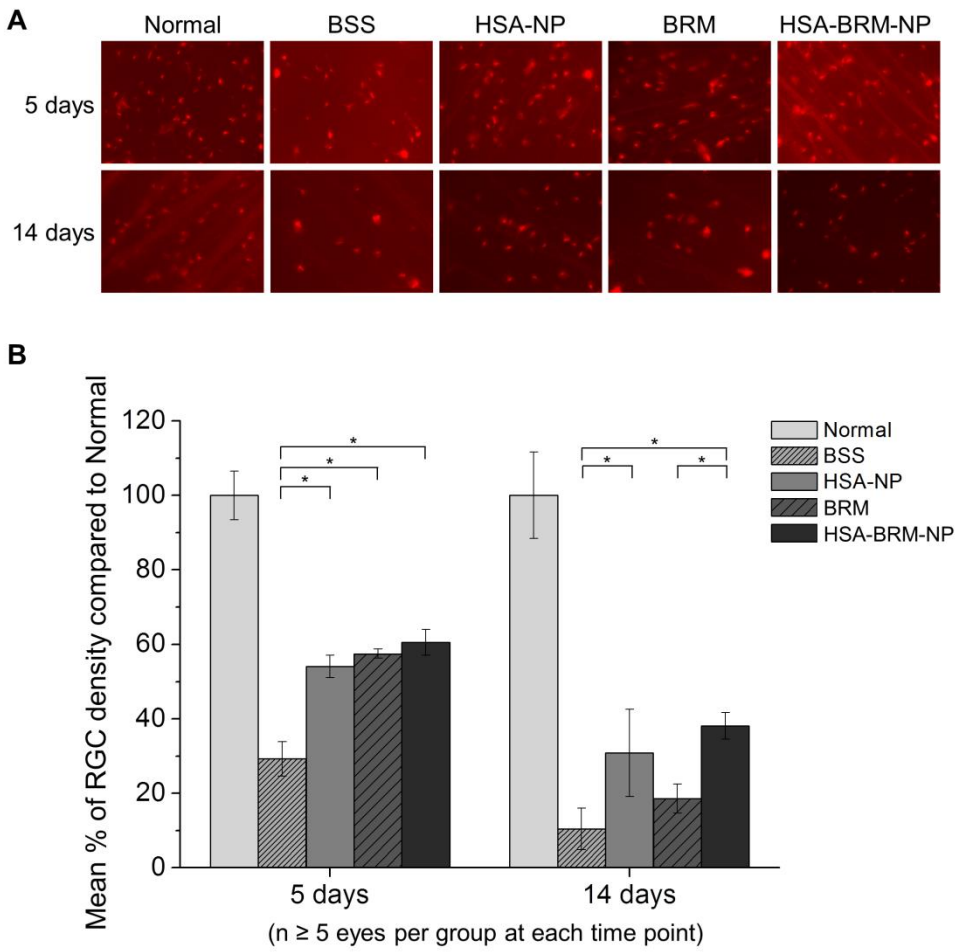
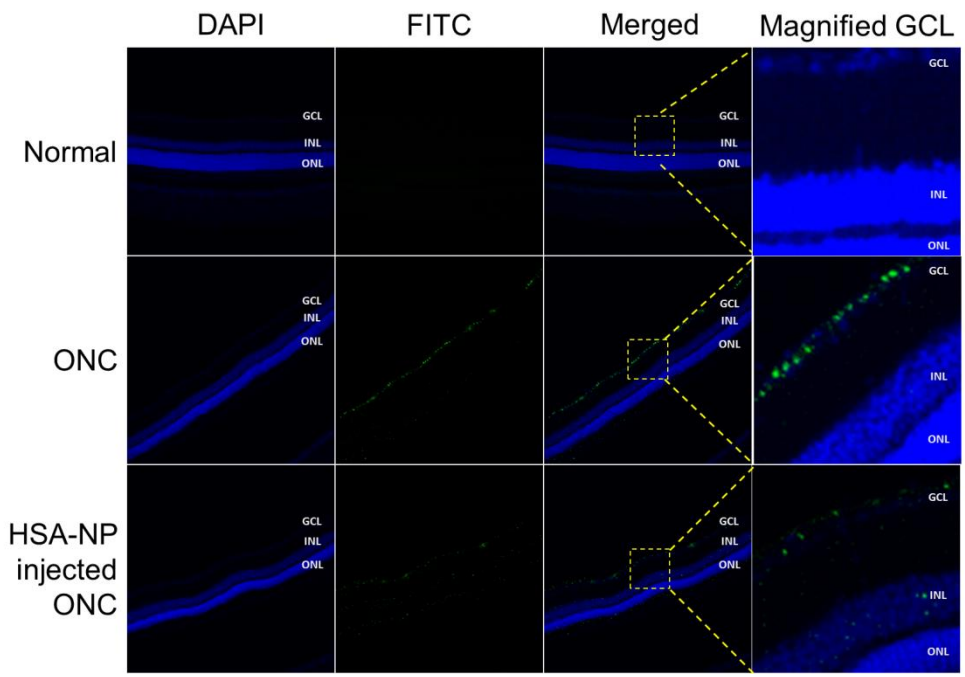

A
Normal
BSS
HSA-NP
BRM
HSA-BRM-NP
5 days
14 days
B
Mean % of RGC density compared to Normal
120
100
80
60
40
20
0
*
*
*
*
*
*
Normal
BSS
HSA-NP
BRM
HSA-BRM-NP
5 days
14 days
(n ≥ 5 eyes per group at each time point)

Figure 4. Immunohistochemistry for amyloid-β (Aβ) in the retinal layers of normal rat, optic nerve crush (ONC) model, and ONC model injected with human serum albumin nanoparticles (HSA-NPs) 14 days after ONC injury and intravitreal injection (all n = 4). The DAPI and FITC images represent the location of the retinal cells' nucleus and of Aβs, respectively. Merged images show the location of Aβs in the retinal layers. The magnified ganglion cell layer (GCL) images also confirm that the Aβs were expressed in the GCL of the ONC model. According to the magnified GCL images, the amount of Aβ deposition was decreased in the HSA-NP-injected ONC model than relative to the ONC-only model. All of the images were taken at the same final magnification level (150x).



Discussion

Novel drug delivery system for sustained release of IOP lowering medication has long been recognized as a great need for patients with glaucoma. Nanoparticles have been expected to have promising roles as intraocular drug delivery system in neurodegenerative diseases, including glaucoma(26) and optic neuropathy.(27) Among the several types of nanoparticles, HSA-NP has been reported to have a beneficial potential as an intraocular drug transporter modality.(6, 8) Moreover, in consideration of HSA's neuroprotective effect by elimination of Aβ,(28, 29) we speculated that HSA-NP could synergistically enhance sustained release of neuroprotective drugs as well as neuronal cell survival. Hence, we investigated the potential of HSA-NP as an effective drug carrier when combined with BRM, a known neuroprotective glaucoma drug. The present study demonstrates that HSA-NPs attenuate RGC loss by improving the sustained delivery of BRM that can elongate the beneficial effect of BRM.

For intravitreally injected nanoparticles to fulfill their role as an intraocular drug delivery system, they need to overcome several barriers that hamper them from targeting the specific site of action. The important physical barriers against macromolecular penetration of the sub-retinal space are the vitreous and retinal layers, especially the internal limiting membrane and external limiting membrane.(30) First, the vitreous network has a negative potential, for it consists of collagen and hyaluronic acid.(31) Koo et al. reported that NPs with negative potential, such as HSA-NP and hyaluronic acid-NP, were observed

throughout the entire retinal layers, while polyethyleneimine-NP, with a positive potential, was trapped in the vitreous until 72 hours post-intravitreal injection.(6) Our HSA-BRM-NPs also had a negative surface charge (−29.7 ± 7.5 mV) similar to that of HSA-NP in a previous study,(6) enabling them to easily pass the vitreous. Second, the size of the HSA-BRM-NP is approximately 150 nm, which is relatively larger than the pore size of internal limiting membrane (10 to 20 nm)(32) and external limiting membrane (3 to 3.6 nm).(33) Therefore, it would be impossible for HSA-BRM-NP to overcome the retinal layers just by diffusion. We hypothesized that HSA-NPs would have to penetrate through the retinal layers by receptor-mediated endocytosis, as suggested elsewhere.(34) One of the known HSA-binding receptors is the transforming growth factor beta (TGFβ) receptor,(35, 36) which is also expressed on the surface of RGCs and müller cells. Previous results showing co-localization of müller cells and HSA-NPs might also support our theory.(6, 8) Therefore, we assumed that TGFβ could be one of the candidate receptors for the endocytosis of HSA-BRM-NPs in the retinal layers, though further experiments are warranted for confirmation. Nevertheless, intravitreal injection of HSA-NP is expected to surmount intraocular barriers to exhibit neuroprotection and to deliver targeting agent to the aiming site.

One of the primary goals of a nanoparticle drug-delivery system is to make possible continuous long-term treatment. In the present study, the therapeutic effect of the HSA-NP and HSA-BRM-NP groups lasted longer than those of the other groups. The RGC density was similar between the BRM and HSA-BRM-NP

groups at 5 days, but the HSA-BRM-NP group showed higher RGC density at 14 days. Interestingly, the HSA-BRM-NP group showed the strongest therapeutic effect at 14 days after ONC. We assumed that the HSA-NPs probably enabled the longer intraocular availability of BRM by supporting the sustained release of BRM, as in our in vitro release test. Moreover, the original neuroprotective effect of BRM probably has synergistically supported the therapeutic effect of HSA-NP, enabling a longer and stronger neuroprotective effect of HSA-BRM-NP than HSA-NP or BRM only. However, despite their therapeutic effect lasting up to 14 days, the HSA-BRM-NPs were detected for 3 days but not for 5 days (data not shown) in the retinal layers of either the normal control or the ONC model. For the discrepancy between the presence of HSA-BRM-NPs in the retinal layers and the effect time, we assumed that because the HSA-NPs' Aβ elimination effect was added to the therapeutic effect of BRM, the HSA-BRM-NPs could have exhibited an augmented initial neuroprotective effect relative to that of pure BRM. Therefore, even under the scenario of total HSA-BRM-NP degradation before 14 days, a latent period with respect to re-activation of the apoptotic pathways or accumulation of Aβ could have existed, consequently showing the reduced Aβ deposition amount until 14 days. Further efforts to extend the persistent time of HSA-NP are warranted for the purposes of their potential application to general treatments.

In this study, BRM was chosen as a neuroprotective drug to be loaded into the hydrophobic pockets of HSA-NPs, for the following reasons. First, it is one of the best known and most widely utilized neuroprotective drugs, and can benefit

from nanomedicine technology by increasing the contact time with the GCL.(37) Second, it is a hydrophobic compound that has features similar to those of other drugs that have been formulated into albumin-nanoparticles for use in targeted drug delivery systems.(9, 38) Additionally, BRM can be entrapped in a protein structure and be cross-linked to glutaraldehyde.(23) More pertinently, there has been considerable interest in delivering BRM to the GCL in a sustained release manner due to its short elimination time. Shen et al. reported the pharmacokinetics of intravitreally injected BRM in various ocular compartments using rabbit eyes.(39) Their data demonstrated that the elimination half-life of intravitreal BRM in retina was 9.9 hours, which is relatively short compared to that of other intravitreal drugs (e.g. ranibizumab, aflibercept).(40, 41) Furthermore, the mean concentration of BRM rapidly decreased by approximately 90% after 12 hours. In consideration of much shorter time for maintaining effective concentration of topical BRM,(42) an effective drug delivery system assuring long-term release of BRM would certainly aid in sustaining its therapeutic effect.

In the present study, BRM, HSA-NP, and HSA-BRM-NP showed neuroprotective capabilities in the ONC model 5 days after ONC injury. Interestingly, HSA-NP itself showed significant improvement in RGC survival in the ONC model relative to the BSS group. Previous studies have reported the pathogenic role of Aβ aggregation in neurodegenerative disease(29, 43, 44) and glaucoma.(45) Abnormal Aß increase was associated with RGC apoptosis in an experimental model of glaucoma and intravitreal injection of Aß induced

significant RGC apoptosis in vivo.(45, 46) We speculated that accumulation of Aß could be associated with RGC apoptosis in an ONC injury model. Our immunohistochemistry results indicated positive Aβ expression in the GCL of the ONC model, which expression was decreased by the intravitreal injection of HSA-NP. Moreover, the RGC-5 cell viability test showed increased cell survival by the addition of HSA-NP to Aβ compared to that with Aβ. The capability of HSA-inhibiting Aβ plaque formation by regulation of Aβ fibril growth has been previously reported by Stanyon et al.(29) They reported that HSA binds to Aß molecules and traps them in a nonfibrillar form, thus inhibiting Aß fibrillization.(29) Some may argue the possibility of different mechanisms between HSA and HSA-NP for inhibiting Aß aggregation. Although HSA is assembled with nanoparticles to form HSA-NPs, we speculate that the fabricated nanoparticles still partially carry the surface property of HSA. Thus, the Aß monomers can accumulate on the HSA-NPs' surfaces as they did with HSA. In addition, the inhibitory mechanism of HSA-NP on protein (Hen Egg White Lysozyme) fibrillation has been reported that after HSA-NP helped protein monomers to unfold and expose their hydrophobic core, the monomers tended to accumulate on the HSA-NP's surfaces rather than folding with each other.(47) Direct comparison between studies may be difficult since different proteins (Hen Egg White Lysozyme vs Aß) were used, but their results showed the possible inhibitory effect of HSA-NP on protein fibrillation. Consistent with previous studies, the neuroprotective effect of HSA-NP on RGC survival was most likely to be via

regulation of Aβ aggregation. Further studies are needed to investigate the detailed mechanism of HSA-NP with intraocular Aβ (e.g. direct elimination of Aβ or inhibition of Aβ plaque formation) or other possible mechanisms.

Our study has several limitations. First, the ocular pharmacokinetic properties of intravitreal HSA-BRM-NP were not determined in the present study. Although in vitro release profile of BRM from HSA-BRM-NP and the RGC survival analysis have been provided in the present study, further experiments to investigate the pharmacokinetic properties of intravitreal HSA-BRM-NP in animal model is warranted for the confirmation of effective drug concentration and sustained drug release in vivo. Second, the neuroprotective effect of BRM was not investigated separately in the present study. ONC injury has been reported to induce RGC apoptosis associated with caspase-2, 3, 9, and Bax(48, 49) and BRM is known to potentially attenuate RGC loss by activating anti-apoptotic proteins, such as bcl-2 and bcl-xl.9 Because such neuroprotective mechanisms of BRM in the ONC model have already been reported, and also because a similar ONC model was used in this study, we assumed that BRM would exert its neuroprotective effect through the identified pathway. However, the possibility of BRM's different neuroprotective mechanism under the condition of its co-existence with HSA-NP remains. Therefore, further separate experiments with BRM, HSA-NP, and HSA-BRM-NP should be performed to investigate each agent's original mechanism of neuroprotection. Lastly, intravitreal injection was performed for the agent administration. Although the rate of severe complication in human subjects is low,

intravitreal injection can still cause pain, hemorrhage, and intraocular infection.(50) Thus, longer intraocular drug availability accompanying less number of injections should be achieved for HSA-NP based drug delivery system to be in practical use.

In conclusion, HSA-NPs showed a capacity to deliver ocular drugs to the GCL. Moreover, HSA-NPs loaded with BRM exerted a longer therapeutic effect and synergistically enhanced the neuroprotectivity. Therefore, HSA-BRM-NPs, having the potential to extend the release of BRM, can be a considered as a promising candidate drug delivery agent for treatment in glaucoma.

References

1. Weinreb RN, Aung T, Medeiros FA. The pathophysiology and treatment of glaucoma: a review. Jama. 2014;311(18):1901-11.

2. Heijl A, Leske MC, Bengtsson B, Hyman L, Bengtsson B, Hussein M, et al. Reduction of intraocular pressure and glaucoma progression: results from the Early Manifest Glaucoma Trial. Arch Ophthalmol. 2002;120(10):1268-79.

3. Kass MA, Heuer DK, Higginbotham EJ, Johnson CA, Keltner JL, Miller JP, et al. The Ocular Hypertension Treatment Study: a randomized trial determines that topical ocular hypotensive medication delays or prevents the onset of primary open-angle glaucoma. Arch Ophthalmol. 2002;120(6):701-13; discussion 829-30.

4. Schwartz GF, Quigley HA. Adherence and persistence with glaucoma therapy. Surv Ophthalmol. 2008;53 Suppl1:S57-68.

5. Spooner JJ, Bullano MF, Ikeda LI, Cockerham TR, Waugh WJ, Johnson T, et al. Rates of discontinuation and change of glaucoma therapy in a managed care setting. The American journal of managed care. 2002;8(10 Suppl):S262-70.

6. Koo H, Moon H, Han H, Na JH, Huh MS, Park JH, et al. The movement of self-assembled amphiphilic polymeric nanoparticles in the vitreous and retina after intravitreal injection. Biomaterials. 2012;33(12):3485-93.

7. Farjo KM, Ma JX. The potential of nanomedicine therapies to treat neovascular disease in the retina. Journal of angiogenesis research. 2010;2:21.

8. Kim H, Robinson SB, Csaky KG. Investigating the movement of intravitreal human serum albumin nanoparticles in the vitreous and retina. Pharm

Res. 2009;26(2):329-37.

9. Kratz F. Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. J Control Release. 2008;132(3):171-83.

10. Lafuente MP, Villegas-Perez MP, Sobrado-Calvo P, Garcia-Aviles A, Miralles de Imperial J, Vidal-Sanz M. Neuroprotective effects of alpha(2)-selective adrenergic agonists against ischemia-induced retinal ganglion cell death. Invest Ophthalmol Vis Sci. 2001;42(9):2074-84.

11. Lafuente Lopez-Herrera MP, Mayor-Torroglosa S, Miralles de Imperial J, Villegas-Perez MP, Vidal-Sanz M. Transient ischemia of the retina results in altered retrograde axoplasmic transport: neuroprotection with brimonidine. Exp Neurol. 2002;178(2):243-58.

12. Aviles-Trigueros M, Mayor-Torroglosa S, Garcia-Aviles A, Lafuente MP, Rodriguez ME, Miralles de Imperial J, et al. Transient ischemia of the retina results in massive degeneration of the retinotectal projection: long-term neuroprotection with brimonidine. Exp Neurol. 2003;184(2):767-77.

13. Mayor-Torroglosa S, De la Villa P, Rodriguez ME, Lopez-Herrera MP, Aviles-Trigueros M, Garcia-Aviles A, et al. Ischemia results 3 months later in altered ERG, degeneration of inner layers, and deafferented tectum: neuroprotection with brimonidine. Invest Ophthalmol Vis Sci. 2005;46(10):3825-35.

14. Ortin-Martinez A, Valiente-Soriano FJ, Garcia-Ayuso D, Alarcon-Martinez L, Jimenez-Lopez M, Bernal-Garro JM, et al. A novel in vivo model of

focal light emitting diode-induced cone-photoreceptor phototoxicity: neuroprotection afforded by brimonidine, BDNF, PEDF or bFGF. PLoS One. 2014;9(12):e113798.

15. Gao H, Qiao X, Cantor LB, WuDunn D. Up-regulation of brain-derived neurotrophic factor expression by brimonidine in rat retinal ganglion cells. Arch Ophthalmol. 2002;120(6):797-803.

16. Ahmed FA, Hegazy K, Chaudhary P, Sharma SC. Neuroprotective effect of alpha(2) agonist (brimonidine) on adult rat retinal ganglion cells after increased intraocular pressure. Brain Res. 2001;913(2):133-9.

17. Yoles E, Schwartz M. Elevation of intraocular glutamate levels in rats with partial lesion of the optic nerve. Arch Ophthalmol. 1998;116(7):906-10.

18. Lai RK, Chun T, Hasson D, Lee S, Mehrbod F, Wheeler L. Alpha-2 adrenoceptor agonist protects retinal function after acute retinal ischemic injury in the rat. Visual neuroscience. 2002;19(2):175-85.

19. Lonngren U, Napankangas U, Lafuente M, Mayor S, Lindqvist N, Vidal-Sanz M, et al. The growth factor response in ischemic rat retina and superior colliculus after brimonidine pre-treatment. Brain Res Bull. 2006;71(1-3):208-18.

20. Saylor M, McLoon LK, Harrison AR, Lee MS. Experimental and clinical evidence for brimonidine as an optic nerve and retinal neuroprotective agent: an evidence-based review. Arch Ophthalmol. 2009;127(4):402-6.

21. Wheeler L, WoldeMussie E, Lai R. Role of alpha-2 agonists in neuroprotection. Surv Ophthalmol. 2003;48 Suppl 1:S47-51.

22. Lee DA, Higginbotham EJ. Glaucoma and its treatment: a review. American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists. 2005;62(7):691-9.

23. Langer K, Balthasar S, Vogel V, Dinauer N, von Briesen H, Schubert D. Optimization of the preparation process for human serum albumin (HSA) nanoparticles. Int J Pharm. 2003;257(1-2):169-80.

24. Levkovitch-Verbin H, Harris-Cerruti C, Groner Y, Wheeler LA, Schwartz M, Yoles E. RGC death in mice after optic nerve crush injury: oxidative stress and neuroprotection. Invest Ophthalmol Vis Sci. 2000;41(13):4169-74.

25. Sarikcioglu L, Demir N, Demirtop A. A standardized method to create optic nerve crush: Yasargil aneurysm clip. Exp Eye Res. 2007;84(2):373-7.

26. Wadhwa S, Paliwal R, Paliwal SR, Vyas SP. Hyaluronic acid modified chitosan nanoparticles for effective management of glaucoma: development, characterization, and evaluation. J Drug Target. 2010;18(4):292-302.

27. Grove K, Dobish J, Harth E, Ingram MC, Galloway RL, Mawn LA. Trans-meningeal drug delivery to optic nerve ganglion cell axons using a nanoparticle drug delivery system. Exp Eye Res. 2014;118:42-5.

28. Prajapati KD, Sharma SS, Roy N. Current perspectives on potential role of albumin in neuroprotection. Rev Neurosci. 2011;22(3):355-63.

29. Stanyon HF, Viles JH. Human serum albumin can regulate amyloid-beta peptide fiber growth in the brain interstitium: implications for Alzheimer disease. J Biol Chem. 2012;287(33):28163-8.

30. Edelhauser HF, Rowe-Rendleman CL, Robinson MR, Dawson DG, Chader GJ, Grossniklaus HE, et al. Ophthalmic drug delivery systems for the treatment of retinal diseases: basic research to clinical applications. Invest Ophthalmol Vis Sci. 2010;51(11):5403-20.

31. Le Goff MM, Bishop PN. Adult vitreous structure and postnatal changes. Eye. 2008;22(10):1214-22.

32. Nishihara H. [Studies on the ultrastructure of the inner limiting membrane of the retina--distribution of anionic sites in the inner limiting membrane of the retina]. Nippon Ganka Gakkai zasshi. 1991;95(10):951-8.

33. Bunt-Milam AH, Saari JC, Klock IB, Garwin GG. Zonulae adherentes pore size in the external limiting membrane of the rabbit retina. Invest Ophthalmol Vis Sci. 1985;26(10):1377-80.

34. Desai N, Trieu V, Yao Z, Louie L, Ci S, Yang A, et al. Increased antitumor activity, intratumor paclitaxel concentrations, and endothelial cell transport of cremophor-free, albumin-bound paclitaxel, ABI-007, compared with cremophor-based paclitaxel. Clin Cancer Res. 2006;12(4):1317-24.

35. Siddiqui SS, Siddiqui ZK, Malik AB. Albumin endocytosis in endothelial cells induces TGF-beta receptor II signaling. Am J Physiol Lung Cell Mol Physiol. 2004;286(5):L1016-26.

36. Ivens S, Kaufer D, Flores LP, Bechmann I, Zumsteg D, Tomkins O, et al. TGF-beta receptor-mediated albumin uptake into astrocytes is involved in neocortical epileptogenesis. Brain. 2007;130(Pt 2):535-47.

37. Diebold Y, Calonge M. Applications of nanoparticles in ophthalmology. Progress in retinal and eye research. 2010;29(6):596-609.

38. Elzoghby AO, Samy WM, Elgindy NA. Albumin-based nanoparticles as potential controlled release drug delivery systems. J Control Release. 2012;157(2):168-82.

39. Shen J, Durairaj C, Lin T, Liu Y, Burke J. Ocular pharmacokinetics of intravitreally administered brimonidine and dexamethasone in animal models with and without blood-retinal barrier breakdown. Invest Ophthalmol Vis Sci. 2014;55(2):1056-66.

40. Park SJ, Choi Y, Na YM, Hong HK, Park JY, Park KH, et al. Intraocular Pharmacokinetics of Intravitreal Aflibercept (Eylea) in a Rabbit Model. Invest Ophthalmol Vis Sci. 2016;57(6):2612-7.

41. Ahn SJ, Ahn J, Park S, Kim H, Hwang DJ, Park JH, et al. Intraocular pharmacokinetics of ranibizumab in vitrectomized versus nonvitrectomized eyes. Invest Ophthalmol Vis Sci. 2014;55(1):567-73.

42. Dong JQ, Babusis DM, Welty DF, Acheampong AA, Tang-Liu D, Whitcup SM. Effects of the preservative purite on the bioavailability of brimonidine in the aqueous humor of rabbits. Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics. 2004;20(4):285-92.

43. Bohrmann B, Tjernberg L, Kuner P, Poli S, Levet-Trafit B, Naslund J, et al. Endogenous proteins controlling amyloid beta-peptide polymerization. Possible

implications for beta-amyloid formation in the central nervous system and in peripheral tissues. J Biol Chem. 1999;274(23):15990-5.

44. Milojevic J, Melacini G. Stoichiometry and affinity of the human serum albumin-Alzheimer's Abeta peptide interactions. Biophys J. 2011;100(1):183-92.

45. Guo L, Salt TE, Luong V, Wood N, Cheung W, Maass A, et al. Targeting amyloid-beta in glaucoma treatment. Proc Natl Acad Sci U S A. 2007;104(33):13444-9.

46. Goldblum D, Kipfer-Kauer A, Sarra GM, Wolf S, Frueh BE. Distribution of amyloid precursor protein and amyloid-beta immunoreactivity in DBA/2J glaucomatous mouse retinas. Invest Ophthalmol Vis Sci. 2007;48(11):5085-90.

47. Mirzazadeh Dizaji N, Mohammad-Beigi H, Aliakbari F, Marvian AT, Shojaosadati SA, Morshedi D. Inhibition of lysozyme fibrillation by human serum albumin nanoparticles: Possible mechanism. International journal of biological macromolecules. 2016;93(Pt A):1328-36.

48. Ahmed Z, Kalinski H, Berry M, Almasieh M, Ashush H, Slager N, et al. Ocular neuroprotection by siRNA targeting caspase-2. Cell Death Dis. 2011;2:e173.

49. Magharious M, D'Onofrio PM, Hollander A, Zhu P, Chen J, Koeberle PD. Quantitative iTRAQ analysis of retinal ganglion cell degeneration after optic nerve crush. J Proteome Res. 2011;10(8):3344-62.

50. Jager RD, Aiello LP, Patel SC, Cunningham ET, Jr. Risks of intravitreous injection: a comprehensive review. Retina. 2004;24(5):676-98.

51. Kim KE, Jang I, Moon H, Kim YJ, Jeoung JW, Park KH, et al.

Neuroprotective effects of human serum albumin nanoparticles loaded with brimonidine on retinal ganglion cells in optic nerve crush model. Invest Ophthalmol Vis Sci. 2015;56(9):5641-9.

52. 장인석. [Multi-neuroprotective effects of human serum albumin nanoparticles loaded with brimonidine on retinal ganglion cells in optic nerve crush model]. 서강대학교:석사논문, 2015.

초 록

지속적 안구내 약물 방출을 위한
알부민 나노입자 기반 약물 전달 시스템 개발

김고은
의학과 안과학
서울대학교 대학원

목적: 현재 녹내장 치료는 안압하강용 점안제를 이용한 약물요법이 우선이다. 하지만 낮은 생체이용률로 인한 잦은 점안 횟수 및 부작용 때문에 환자들의 순응도가 저하될 수 있으며 이는 성공적인 녹내장 치료의 걸림돌로 작용할 수 있다. 이에 시신경 압박손상(optic nerve crush injury) 동물모델을 이용하여 알부민(human serum albumin)-나노입자(nanoparticle)를 기반으로 한 지속적 안구내 약물 방출 시스템을 개발하고자 하였다.

방법: 알부민(3.5%)-나노입자에 시신경보호 효능이 입증된 0.18% 브리모니딘(brimonidine)을 탑재하여 알부민-브리모니딘-나노입자 용액을 준비하였다. 나노입자에서 브리모니딘의 약물 방출 결과는 Spectra/por 투석막 및 고압액체크로마토그래피를 이용하여 분석하였다.

Sprague-Dawley 랫트를 이용하여 시신경 압박손상 모델 제작 및 유리체강내 나노입자 주사를 시행하였다. 랫트는 주입한 물질에 따라 정상군, 평형염류용액(balanced salt solution) 주입군, 알부민-나노입자주입군, 브리모니딘 주입군, 알부민-브리모니딘-나노입자 주입군으로 나누어 실험을 진행하였다. 정상군의 망막신경절세포 생존률을 100%으로 했을 때의 상대적 망막신경절세포 생존률을 각 군마다 측정한 후 시신경 압박손상 및 유리체강내 주사 시행 5 일 후 및 14 일 후에 각 군의 망막신경절세포의 생존률을 비교하여 약물의 효과를 확인하였다.

결과: 알부민-브리모니딘-나노입자에서 3 일간 약 95%의 브리모니딘이 지속적으로 방출되었고 5 일째까지 나머지 양이 서서히 방출됨을 확인하였다. 시신경 압박손상 5 일 후 알부민-나노입자 주입군(52.6 ± 3.3%), 브리모니딘 주입군(58.0 ± 4.2%), 알부민-브리모니딘-나노입자 주입군(63.5 ± 7.1%)의 생존률은 평형염류용액 주입군(29.2 ± 3.3%)에 비해 유의하게 높았다($P < 0.001$). 시신경 압박손상 5 일 후 알부민-브리모니딘-나노입자 주입군과 브리모니딘 주입군 사이에 유의한 생존률의 차이는 없었다($P = 0.014$). 하지만 시신경 압박손상 14 일 후에는 알부민-브리모니딘-나노입자 주입군(38.1 ± 3.6%)이 브리모니딘 주입군(18.6 ± 3.9%, $P = 0.006$)에 비해 유의하게 높은 생존률을 보였다.

결론: 알부민-나노입자는 브리모니딘과 결합시 장기간 약물의 효과가 지속될 가능성이 있음을 보여주었다. 본 연구를 통해 알부민-나노입자는 향후 지속적 약물 방출을 위한 약물 전달체로서의 가능성이 있음을 확인하였다.[1]

주요어: 알부민-나노입자; 브리모니딘; 지속적 약물 방출;
망막신경절세포; 시신경압박손상 모델

학 번: 2015-30584

[1] 본 박사학위 논문은 "*Investigative Ophthalmology & Visual Science*" 저널에 게재하였음.(51)위 논문의 공동제1저자인 서강대학교 장인석 학생이 시행한 나노입자 제작 및 특성 분석, 면역화학염색 및 세포 생존력 측정 실험 결과를 공유하였고,(52)본 학위 논문의 저자는 시신경압박손상모델 제작 및 유리체강내 주사, 안구 적출 및 망막 flat mounting과 관련된 모든 작업을 시행하였으며, 연구 결과 정리, 분석 및 집필을 담당하였음을 명시함.