Chitosan/TPP-hyaluronic acid nanoparticles, a new vehicle for gene delivery to spinal cord

Min Ho Kong

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Do Heum Yoon

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This certifies that the Doctoral Dissertation of Min Ho Kong is approved.

Thesis Supervisor : Do Heum Yoon

Thesis Committee Member #1: Keung Nyun Kim

Thesis Committee Member #2: Ho Taek Song

Thesis Committee Member #3: Seung Woo Cho

Thesis Committee Member #4: Jin Woong Bok

The Graduate School
Yonsei University

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Chitosan/TPP-hyaluronic acid nanoparticles, a new vehicle for gene delivery to spinal cord

Min Ho Kong

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The Graduate School, Yonsei University

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Abstract

Gene delivery offers therapeutic promise for the treatment of neurological diseases and spinal cord injury. Several studies have offered viral vectors as vehicles to deliver therapeutic agents, yet their toxicity and immunogenicity, along with the cost of their large-scale formulation, limits their clinical use. As such, non-viral vectors are attractive in that they offer improved safety profiles compared to viruses. Polyethylenimine (PEI) is one of the most extensively studied non-viral vectors, but its clinical value is limited by its cytotoxicity. Recently, chitosan/DNA complex nanoparticles have been considered as a vector for gene delivery. Here, we demonstrate that DNA nanoparticles made of hyaluronic acid (HA) and chitosan have low cytotoxicity and induce high transgene expression in neural stem cells and organotypic spinal cord slice tissue. Chitosan-TPP/HA nanoparticles were significantly less cytotoxic than PEI at various concentrations. Additionally,

chitosan-TPP/HA nanoparticles with pDNA induced higher transgene expression *in vitro* for a longer duration than PEI in neural stem cells. These results suggest chitosan-TPP/HA nanoparticles may have the potential to serve as an option for gene delivery to the spinal cord.

Key words : chitosan, gene delivery, spinal cord

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I. INTRODUCTION

The delivery of therapeutic genes to neurons has the potential to treat injury to the central nervous system.[1,2] Viral vectors have been used successfully to deliver genes in animal models of injury,[3,4] yet safety concerns regarding their toxicity and immunogenicity remain, preventing its translation into the clinic.[5] As such, improvement of non-toxic polymeric vectors with high transfection efficiency has become a primary goal in non-viral gene carrier research.

Chitosan is a natural, non-toxic, biodegradable, polycationic polysaccharide, and has been extensively employed in the development of micro- and nano-carriers, with a specific focus on the use of complex actives such as nucleic acids, small-inhibitory RNA (siRNA), and proteins.[6,7] The percentage of de-acetylated primary amine groups along the chitosan chain determines the potential, while the positive charge density is responsible for the electrostatic interaction with negatively charged molecules. These

nanosystems have been shown, among other advantages, to have prolonged time at the target sites of absorption;[8-10] these results were mainly attributed to the capacity of the polymer to interact with the negatively charged cell surfaces.

Hyaluronic acid (HA) is a natural, anionic, non-toxic, biodegradable polysaccharide that is distributed widely throughout the human body, mainly in connective tissue and organs such as the eyes, neural tissues, and lungs. Many studies have reported that mammalian cells have a variety of HA receptors (CD44) on the cell surface.[11] CD44 mediates cell interaction with HA and is likely involved in the uptake, cell aggregation, migration, proliferation and activation of HA.[11] Additionally, HA is one of the most sought after materials when degradability and biocompatibility are necessary. It has been used as a viscous gel for cell scaffolding and drug, protein, and peptide delivery.[12] It is possibly adsorbed through polyelectrolyte complexation with polycations such as collagen or chitosan.[13-15]

In this study, we designed chitosan- tripolyphosphate/hyaluronic acid (chitosan-TPP/HA) nanoparticles using ionotropic gelation technique. To date, no study has compared the cytotoxicity and long-term transgene expression between PEI and chitosan-TPP/HA nanospheres. We evaluated the cytotoxicity and transgene expression efficacy of chitosan-TPP/HA specially designed for intracellular delivery of genes to neural stem cells and spinal cord slice tissue *in vitro* and gene transfection efficiency after direct injection into the injured spinal cord.

II. MATERIALS AND METHODS

2.1. Preparation of chitosan-tripolyphosphate/ HA nanoparticles encapsulating pDNA

CS-TPP/HA nanoparticles were prepared according to the ionotropic gelation technique.[10,16] Chitosan tripolyphosphate (TPP) and HA were separately dissolved in ultrapure water at different concentrations. Nanoparticles were loaded with pSV-Luc by incorporation of the required amount of the model plasmid in the HA/TPP phase. Nanoparticles were formed instantaneously upon the drop-wise addition of a fixed volume of TPP/HA solution to a fixed volume of chitosan solution under magnetic stirring.

2.2. Nanoparticle characterization and in vitro release of DNA

The size distribution and zeta potential of the chitosan-TPP/HA nanoparticles encapsulating pSV-Luc were evaluated using dynamic laser light scattering (Zetasizer 3000HS, Malvern, UK). To determine the amount of pSV-Luc encapsulated into the chitosan-TPP/HA, chitosan-TPP/HA nanoparticles encapsulating pSV-Luc were isolated through centrifugation (10min, 100,000rpm). The pDNA content in the supernatant solution was quantified using a UV spectrophotometer (Shimadzu 1204, Tokyo, Japan) at 260 nm by means of a standard curve of the pDNA in water.

The surface morphology of the particles was examined by atomic force microscopy (AFM, XE-100; PSIA Inc., Suwon, Korea). The AFM technique is an effective, widely applied method for obtaining surface morphological information.[17] The properties of the chitosan-TPP/HA nanoparticles were measured by tapping-mode AFM. The AFM sample was prepared by casting a

dilute solution on a solid silicon piece, which was then dried under vacuum. The image mode was set to tapping mode with a scanning speed of 1~5 Hz.
Plasmid DNA release was determined by incubating the chitosan-TPP/HA nanoparticles in pH 7.4 PBS at 37°C. At time intervals of 1 day, 2 days, and 1week, individual samples were isolated by centrifugation (10min, 100,000rpm). Supernatant samples were analyzed by agarose gel electrophoresis using un-encapsulated pDNA as the control sample.

2.3. Cytotoxicity assay

The cytotoxicity of chitosan-TPP/HA nanospheres and PEI (MW = 25,000 Da, Sigma, St. Louis, MO) was determined by measuring the mitochondrial metabolic activity of mouse neural stem cells (mNSCs) cultured in the presence of various concentrations of chitosan-TPP/HA nanospheres or PEI. mNSCs plated in 96-well plates at $2x10^4$ cells/well, and cultured for 24 hours. Various concentrations of chtiosan-TPP/HA nanoparticles or PEI were added to each well of the mNSC culture. After incubation for 4, 24, and 48 hours at 37°C, the MTT assay was performed as previously described[18]. The mitochondrial metabolic activity was reported as a percentage of the negative control (no polymer).

2.4. Cell uptake assay

To induce mNSCs to uptake chitosan nanoparticles, mNSC were plated at $1x10^{\wedge 4}$ cells/well and allowed to attach for 24 hours. The suspension 6-coumarin DMSO was mixed with TPP aqueous solution to obtain an alkaline environment, where the dye is chemically stable. Nanoparticles were formed instantaneously upon the drop-wise addition of a fixed volume of TPP/HA solution to a fixed volume of chitosan solution under magnetic stirring.

Chitosan nanoparticles containing a fluorescent dye were prepared and added to the cell cultures. After 24 hours incubation at 37°C, the medium was removed and the cells were washed with PBS. Cells transfected with the chitosan-TPP/HA nanospheres were observed with confocal laser scanning microscopy (Fluoview BX50, Olympus, Tokyo, Japan).

2.5. In vitro transfection

mNSCs were plated in 6-well plates at 2x10^5 cells/well and cultured for 24 hours. Chitosan-TPP/HA nanoparticles encapsulating pSV-Luc or PEI/pSV-Luc complexes (N/P ratio = 5/1) were suspended in DMEM/F12, added to each well at 10ug pDNA/well, and incubated for 24 hours at 37°C. PEI with a molecular weight of 25,000 Da was used as previously described.[19] Afterward, the medium was changed to DMEM/F12 supplement with 10% (v/v) FBS. The medium was changed every 2 days. 100µl lysis buffer (Cell Culture Lysis Reagent 5X, Promega, Madison, WI, USA) was added to each well and the lysates were cleared by centrifugation for 10 minutes at 16,000g using an ultracentrifuge. The transfection efficiency was measured using a luminometer (TD20/20, Turner Design, Sunnyvale, CA, USA) according to the Promega protocol. The total protein content in the supernatants was determined using a Bradford reagent (Sigma). The results are expressed as relative light units (RLU)/mg protein.

2.6. Organotypic slice cultures

Seven-day-old Sprague-Dawley rats were decapitated and the spinal cord was extracted and rapidly placed in ice-cold HBSS. The spinal cord was cut into 400µm thick slices using a tissue chopper (McIlwain, stoelting co, USA). Individual slices were placed into trans-well chambers (Becton Dickinson,

Franklin Lakes, NJ, USA) in 6-well plates with 50% OPTI-MEM supplemented with 25mM HEPES (Invitrogen) and 6mg/ml of D-glucose. Extracted slices were cultured for 24 hours at 37℃ and 5% CO_2, and the medium was refreshed every 2 days. Extracted slices were cultured in serum-free Opti-Mem media (Invitrogen, Carlsbad, CA, USA) under normal conditions at 37°C and 5% CO_2 for 24hours. Chitosan-TPP/HA nanoparticles or PEI/cDNA (N/P ratio 5) complexes were added to slice culture medium and incubated for 72hours at 37℃ in a 5% CO_2 incubator.

2.7. In vivo test

The spinal cord injury (SCI) animal model involved adult male Sprague-Dawley rats (250-300 g; OrientBio Gyeonggi-do, Korea). All protocols were approved by the Animal Care and Use Committee of Yonsei University College of Medicine. All experiments were performed according to international guidelines on the ethical use of animals, and the number of animals used was minimized. SD rats were anesthetized with sodium penthobarbital (20 mg/kg; Choongwae Pharma, Seoul, Korea). After anesthesia, laminectomy was performed at the T9 level. Chitosan-TPP/HA nanoparticles encapsulating pSV-Luc were immediately injected using an insulin syringe into the spinal cord. As a control, PEI/pSV-Luc complex or naked pSV-Luc was also injected into their spinal cords. After surgery, sepazolin (50mg/kg, Yuhan Corporation) was given to all animals for three days. Three days post-transplantation, all rats were sacrificed and perfused with saline (pH 7.4), with subsequent removal of the spinal cord including the injury epicenter. Lysis buffer (Promega) was added to each speciment and the lysates were cleared by centrifugation for 10 minutes at 16,000g using an ultracentrifuge. The transfection efficiency was measured using a luminometer and the total protein content in the supernatants was determined

using a Bradford reagent (Sigma).

2.8. Statistical analysis

Data is presented as mean values ± standard deviations (SD). The statistical significance was analyzed between groups by ANOVA (one-way analysis of variance). All data were analyzed using the Medcalc Program (Medcalc Software), where any p value less than 0.05 was counted as significant ($p<0.05$).

III. RESULTS

3.1. Nanoparticle characterization

Size and zeta-potential of the nanoparticles were dependent on their composition. The size of the chitosan-TPP/HA nanoparticles ranged from 300-500 nm (Fig.1A). The average diameter of the nanoparticles was 255.7 ± 42.5 nm. Nanoparticles containing the highest amount of HA underwent a positive-to-negative inversion of their zeta potential. The average zeta potential of chitosan-TPP/HA particles in this study was 45± 7.5 mv (Fig. 1B). AFM examination showed that chitosan-TPP/HA nanoparticles with pSV-Luc were discrete spherical particles without aggregation and that they were smooth when examined for surface morphology (Fig. 1C). The efficiency of pSV-Luc loading in chitosan –TPP/HA nanoparticles was 62.5 ± 2.0% (n=10**).**

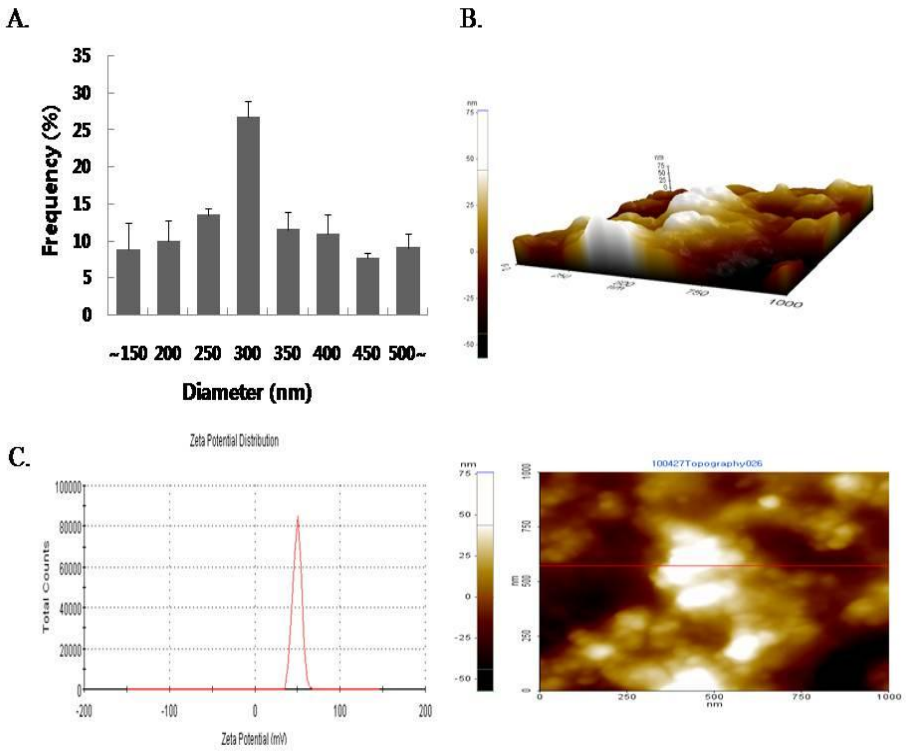

Figure 1. Characterization of chitosan/TPP-hyaluronic acid nanoparticles.

A : Size distribution.

B : Zeta potential (mV).

C : Atomic force microscopy.

3.2. In vitro DNA release from chitosan-TPP/HA nanoparticles

The binding capacity of chitosan with pDNA prepared at various N/P ratios was evaluated using the gel retardation assay (Fig.2). The chitosan-DNA complex with 1 N/P ratio was physically stable. When complexes were applied in an agarose gel and submitted to an electric field, no free DNA was detected for all formulations tested (Fig.2A). To formulate chitosan-TPP/HA with pDNA, chitosan was mixed with pSV-Luc at a 10 N/P ratio, because chitosan-TPP/HA particles showed the highest transfection efficiency at this N/P ratio in the *in vitro* study explained below. In order to study the physical stability and the pDNA release properties of chitosan-TPP/HA nanoparticles, they were incubated in PBS and assayed in agarose gel retardation assays. As shown in Fig. 2B, no DNA release was detected following incubation of pDNA-loaded nanoparticles for up to 1 week, irrespective of the nature of the incubation medium. These results indicate that pDNA is very tightly associated to chitosan-TPP/HA nanoparticles.

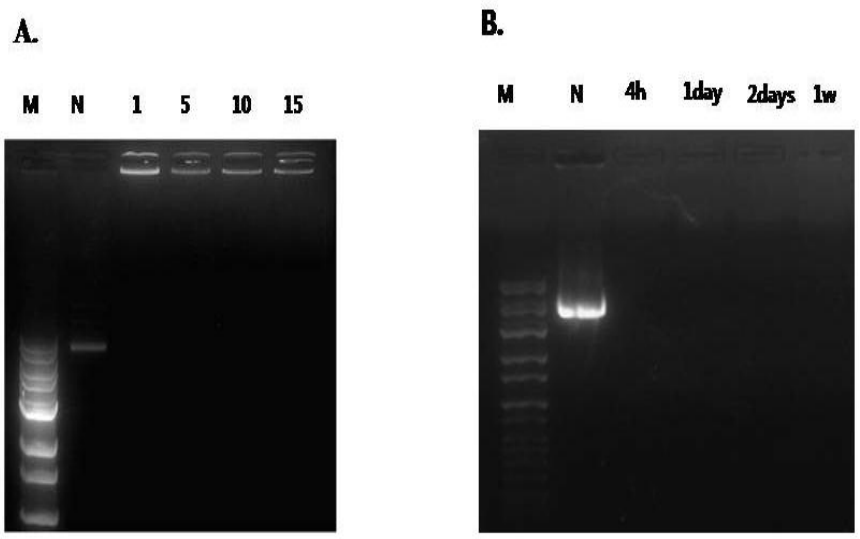

Figure 2. Agarose gel retardation analyses.

A : Chitosan-TPP/HA-pDNA complex nanoparticles prepared at different N/P ratios. The chitosan- DNA complex with 1 N/P ration was physically stable.

B : In vitro DNA release in PBS from chtiosan-TPP/HA. Samples were run on a 0.8% agarose gel and subsequently stained using ethidium bromide. No DNA release was detected following incubation of pDNA-loaded nanoparticles for 1 week.

3.3. Measurement of cytotoxicity and cellular uptake of nanoparticles

Excluding low concentrations (5ug/cm^2) and the initial exposure with mNSCs (4 hours), chitosan-TPP/HA nanoparticles were significantly less cytotoxic than PEI at various concentrations and incubation periods with mNSC (Fig. 3). The cytotoxicity of PEI dramatically increased as its concentration increased. The cellular uptake of chitosan-TPP/HA nanoparticles was confirmed with confocal microscopy. After mNSCs were treated for 24 hours with chitosan-TPP/HA nanoparticles labeled with a fluorescent dye and loaded with pDNA, an intense fluorescence was detected inside the cells (Fig. 4).

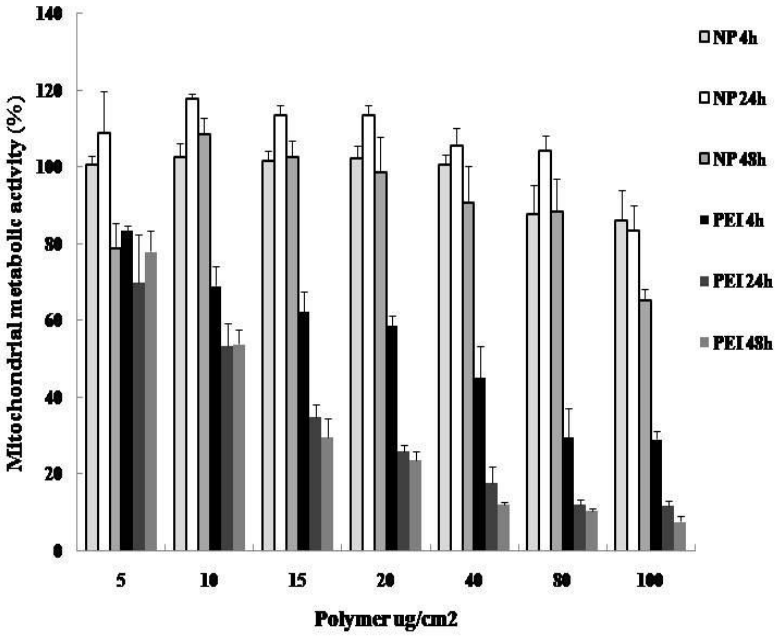

Figure 3. Cytotoxicity of chitosan-TPP/HA containing pDNA and PEI/pDNA complexes.

mNSCs were cultured for 4, 24, or 48 hours with indicated various polymer concentrations, and the mitochondrial metabolic activity of the mNSCs were measured with the MTT assay. The mitochondrial metabolic activities of the mNSCs were significantly higher in the chitosan-TPP/HA nanoparticles treated group (NP) than in the PEI group at various concentrations and incubation periods. $P<0.05$ between chitosan nanoparticles (NP) and PEI at any concentration and time point except at $5ug/cm^2$ and 48 h ($n = 4$)

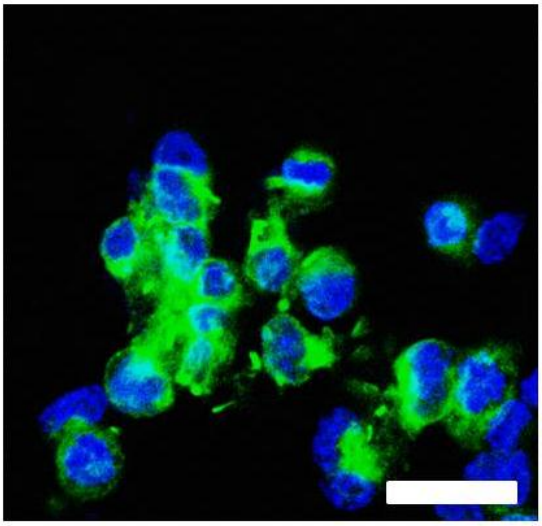

Figure 4. Cell uptake of chitosan-TPP/HA nanoparticles.

Confocal microscopic images examining the cellular uptake of chitosan-TPP/HA nanoparticles loaded with 6-coumarin (green) and pDNA into mNSCs after 24 h incubation. The nuclei were stained with DAPI (blue). Scale bar indicates 50 um.

3.4. In vitro transfection

The *in vitro* expression of transgenes delivered with PEI and chitosan-TPP/HA nanoparticles was compared. For the first 48 hours, transgene expression was higher in the PEI group than in the chitosan-TPP/HA nanoparticles group (Fig. 5). The transgene expression in the chitosan-TPP/HA nanoparticles group increased for 144 hours, whereas the expression in the PEI group decreased after 48 hours. The transgene expression in the chitosan-TPP/HA nanoparticles group was 5 fold higher than that of the PEI group at 72 hours.

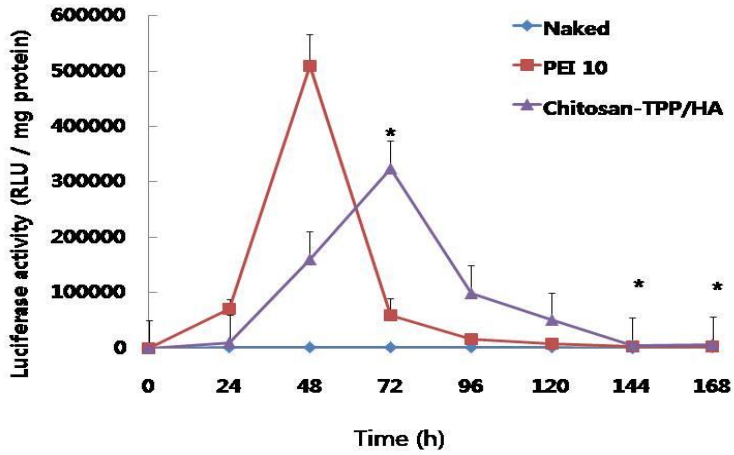

Figure 5. Transfection efficiencies of chitosan-TPP/HA in mNSC.
Luciferase gene expression in mNSC transfected with chitosan-TPP/HA nanoparticles encapsulating pSV-Luc or PEI/ pSV-Luc complexes. Transgene expression in the chitosan-TPP/HA nanoparticles group was maintained for 144 hours, whereas a decrease was observed in the PEI group after 48 hours. Gene expression was normalized to the total protein content. *: significant difference between each group ($P<0.05$). Data are shown as mean ± SD.

To assess the efficacy of luciferase gene delivery in organotypic spinal cord slice culture, chitosan-TPP/HA nanoparticles were transfected into spinal cord slice culture (Fig. 6A). At 72 hours after transfection, transgene expression was higher in the chitosan-TPP/HA nanoparticles group than in the PEI group. At 72 hours after transfection, luciferase mRNA expression was measured using RT-PCR (Fig. 6B). The chitosan-TPP/HA nanoparticles treated group showed a higher level of luciferase mRNA expression. Cytotoxicity in the organotypic spinal cord slice culture was determined using the apoptosis-related gene. RT-PCR analysis of the organotypic spinal cord slice culture showed that mRNA expression of *bax* and *p53*, both of which are apoptosis-promoting molecules, was more extensive in the PEI than in the chitosan-TPP/HA nanoparticles (Fig. 6C). The mRNA level of Bcl-2, an apoptosis-suppressive molecule, was significantly higher in the chitosan-TPP/HA nanoparticles than in the PEI.

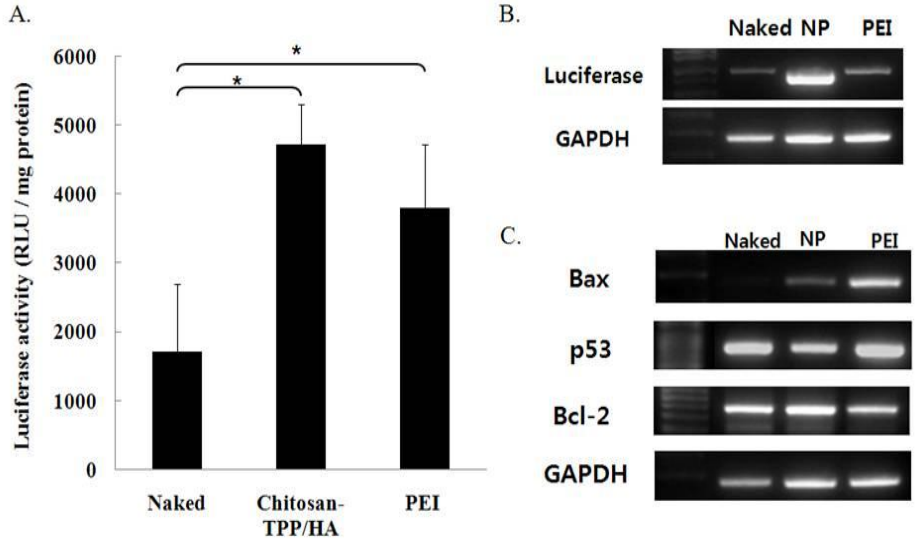

Figure 6. Gene expression in organotypic spinal cord slice tissue 3days after transfection.

A : Transfection efficiencies of chitosan-TPP/HA in spinal cord slice tissue. Gene expression was normalized to the total protein content.

B : mRNA expression of luciferase. Three days after transfection, the luciferase expression level increase in the chitosan-TPP/HA group (NP) compared to PEI or naked pDNA. B. mRNA expression of apoptosis–related genes was more extensive in the PEI group than in the chitosan-TPP/HA nanoparticle group. *: significant difference between each group ($P<0.05$). Data are shown as mean ± SD.

3.5. In vivo luciferase gene expression in the rat spinal cord

To evaluate the persistence of gene expression, chitosan-TPP/HA nanoparticles were injected into the rat spinal cord at a 10/1 N/P ratio. Naked pDNA was injected into spinal cord as a control. Luciferase activity was measured at three days after injection (Fig. 7). The chitosan-TPP/HA nanoparticle group showed higher transgene expression than naked pDNA.

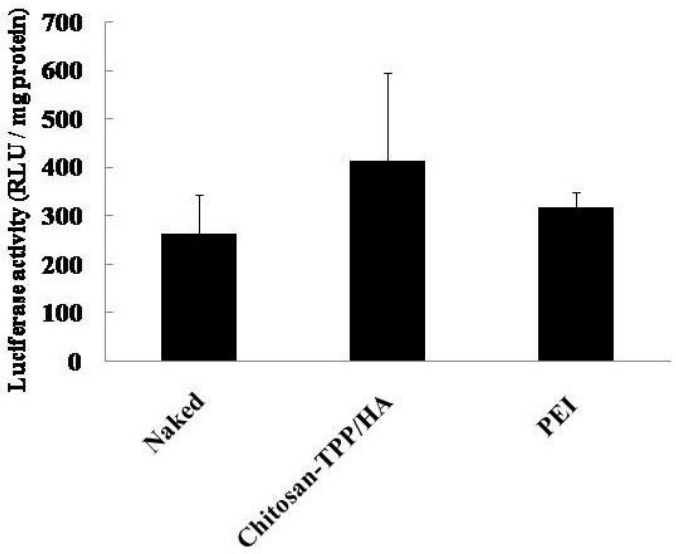

Figure 7. The luciferase activity of chitosan –TPP/HA containing pDNA in rat spinal cord at 3ays after injection.

The expression of luciferase was measured after 3 days by luciferase assay. The chitosan-TPP/HA nanoparticle containing pDNA showed higher transgene expression than naked pDNA.

IV. DISCUSSION

This study showed that DNA nanoparticles made of hyaluronic acid and chitosan have low cytotoxicity and induce high transgene expression in neural stem cells and organotypic spinal cord slice tissue. Mouse neural stem cells treated with chitosan-TPP/HA nanoparticles had a significantly higher survival rate than those treated with PEI at various concentrations. Additionally, chitosan-TPP/HA nanoparticles containing pSV-Luc induced higher luciferase expression *in vitro* for a longer duration than PEI. The chitosan-TPP/HA nanoparticles-treated spinal cord slice tissue showed a higher level of luciferase expression and lower levels of apoptosis-related gene. In addition, luciferase expression in the spinal cord was higher in the chitosan-TPP/HA nanoparticle group than in the naked pDNA group.

The use of chitosan-TPP/HA nanoparticles promoted the rate cell viability of the mNSCs compared to PEI. A number of non-viral vectors have been developed for gene therapy because of their higher safety profile and lower cost. Liposomes and cationic polymers are major non-viral gene delivery carriers.[20,21] Although liposomes formed from cationic phospholipids offer several advantages over viral gene transfer, such as low immunogenicity and ease of preparation, the liposomal approach is limited due the serious cytotoxic side effects, low transfection efficiency, and instability in the serum.[22] Also, PEI is appeared to be the main toxicity due to their molecular weight.[23] This toxicity has severely limited its use as a tool for gene delivery *in vivo*. A non-toxic polymer, HA is biocompatible and biodegradable, and has been approved for use in humans. Perhaps most notably, it is used as injection filler for plastic surgery. In addition, chitosan has been extensively used in the development of carriers, with a specific focus on the use of complex actives such as nucleic acids, small-inhibitory RNA, and proteins.[6,7] Our results show that chitosan-TPP/HA nanoparticles indeed had a higher safety profile than

PEI.

Many studies have investigated the use of direct gene therapy to treat spinal cord injury, ranging from strategies for neuroprotection to the promotion of axonal growth promotion at the site of injury. Genes in viral vectors remain the leading therapeutic candidates for neuron-targeted therapy, as they have produced functional improvement in several animal models of neurodegenerative disease.[3,4] The few clinical trials that have been undertaken to treat neurodegenerative diseases have employed viral vectors almost exclusively. However, significant complications associated with viral vectors limit its widespread clinical use and lead to an inflammatory reaction.[24] The reduction in axonal growth has been attributed to this inflammatory reaction.[25] In contrast, with regard to gene delivery vehicles, chitosan-TPP/HA nanoparticles offer the advantage of being less toxic and not triggering an immune response. It has been reported that chitosan inhibits the production of pro-inflammatory cytokines in a human astrocytoma cell line[21] and that chitosan oligosaccharides possess anti-inflammatory and anti-oxidative properties.[26] Also, a previous study reported that chitosan nanoparticles reduced disruption of the cell membrane following traumatic spinal cord injury, secondary oxidative stress, and lipid peroxidation.[27] That study suggests that a chitosan-TPP/HA nanoparticle-based therapy to interfere with secondary injury is possible.

Chitosan-TPP/HA nanoparticles as a gene delivery vehicle for mNSCs provide the advantage of being able to induce expression of a transgene for 6 days *in vitro* (Fig. 5) due to high molecular chitosan. A previous study reported that chitosan of a lower molecular weight had an early release of the pDNA, and that chitosan of a higher molecular weight took longer to degrade and had longer gene expression compared with low molecular weight chitosan.[10,28] A previous study reported a sustained release of pDNA from chitosan-TPP/HA nanoparticles over 10 days *in vitro,*[16] in

contrast, our study shows a comparison with PEI.

In this study, the chitosan-TPP/HA nanoparticle group showed relatively higher transgene expression than naked pDNA *in vivo*. The delivery of a therapeutic gene that is beneficial for the injured spinal cord can be accomplished in many methods. Among the vectors that have been used to transduce neural tissue *in vivo* are naked DNA, cationic polymers, and herpes simplex viral, adeno-associated viral, adenoviral, and lentiviral vectors, each with their own merits and limitations.[24,29,30] A previous study has shown that viral vectors injection into central nervous system tissue may induce an inflammatory reaction.[24,31] The modified naked DNA or cationic polymer represents alternatives to viral vectors for gene therapy applications. However, these systems are typically less efficient than viruses.[29,30] In the present study, chitosan-TPP/HA nanoparticles showed relatively higher transgene expression than naked pDNA *in vivo*.

Chitosan-TPP/HA nanoparticles might be useful in the treatment of spinal cord injury as a tool for gene delivery. That study suggests that a chitosan nanoparticle-based therapy to interfere with secondary injury is possible. HA is bound by specific receptors, CD44 and RHAMM, depending on the cell type.[32] Also, highly hydrated HA has a role in the radial migration of spinal cord neurons. HA signaling through RHAMM and CD44 receptors mediates neuronal migration and angiogenesis.[33,34] RHAMM is expressed by normal neurons, where it regulates neurite migration and axon growth, and by oligodendrocytes and astrocytes, where it affects motility and proliferation.[35,36] The higher cell uptake in the chitosan-TPP/HA nanoparticle might be due to RHAMM receptor mediation in the neuronal cell or spinal cord.

Further studies are necessary to assess the clinical usage of this approach; namely, the need for chitosan-TPP/HA nanoparticles containing pDNA to be performed for a long term (more than 1 month) study assess its

efficacy in the injured spinal cord. Also, the pDNA in this study was performed in reporter DNA, and the same experiments need to be repeated in therapeutic DNA. Sustained therapeutic gene expression is important in the treatment of spinal cord injury; as evidenced by the use of genes encoding angiogenic growth factors, such as vascular endothelial growth factor and basic fibroblast growth factor, neovascularization can be induced in spinal cord injury.

V. CONCLUSION

In this report, we present a new type of nanoparticle consisting of chitosan and HA for spinal cord gene delivery. This nanoparticle exhibits low cytotoxicity, an ability to enter mNSCs through endocytic uptake, and the capacity to deliver pDNA into both the organotypic spinal cord slice and rat spinal cord, thus eliciting effective levels of protein expression. Therefore, these new NPs can be considered a promising means of spinal cord gene delivery.

References

1 Federici T, Boulis NM. Gene-based treatment of motor neuron diseases. Muscle Nerve 2006;33:302-23.

2 Blits B, Bunge MB. Direct gene therapy for repair of the spinal cord. J Neurotrauma 2006;23:508-20.

3 Mandel RJ, Spratt SK, Snyder RO, Leff SE. Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. Proc Natl Acad Sci U S A 1997;94:14083-8.

4 Frampton AR Jr, Goins WF, Nakano K, Burton EA, Glorioso JC. HSV trafficking and development of gene therapy vectors with applications in the nervous system. Gene Ther 2005;12:891-901.

5 Hsich G, Sena-Esteves M, Breakefield XO. Critical issues in gene therapy for neurologic disease. Hum Gene Ther 2002;13:579-604.

6 Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J Control Release 2004;100:5-28.

7 Katas H, Alpar HO. Development and characterisation of chitosan nanoparticles for siRNA delivery. J Control Release 2006;115:216-25.

8 Garcia-Fuentes M, Prego C, Torres D, Alonso MJ. A comparative study of the potential of solid triglyceride nanostructures coated with chitosan or poly(ethylene glycol) as carriers for oral calcitonin delivery. Eur J Pharm Sci 2005;25:133-43.

9 Koping-Hoggard M, Sanchez A, Alonso MJ. Nanoparticles as carriers for nasal vaccine delivery. Expert Rev Vaccines 2005;4:185-96.

10 De la Fuente M, Seijo B, Alonso MJ. Novel hyaluronic acid-chitosan nanoparticles for ocular gene therapy. Invest Ophthalmol Vis Sci

2008;49:2016-24.

11 Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 2007;23:435-61.

12 Gaffney J, Matou-Nasri S, Grau-Olivares M, Slevin M. Therapeutic applications of hyaluronan. Mol Biosyst 2010;6:437-43.

13 Van BM, Jones L, Sheardown H. Hyaluronic acid containing hydrogels for the reduction of protein adsorption. Biomaterials 2008;29:780-9.

14 Johansson JA, Halthur T, Herranen M, Soderberg L, Elofsson U, Hilborn J. Build-up of collagen and hyaluronic acid polyelectrolyte multilayers. Biomacromolecules 2005;6:1353-9.

15 Croll TI, O'Connor AJ, Stevens GW, Cooper-White JJ. A blank slate? Layer-by-layer deposition of hyaluronic acid and chitosan onto various surfaces. Biomacromolecules 2006;7:1610-22.

16 De la Fuente M, Seijo B, Alonso MJ. Bioadhesive hyaluronan-chitosan nanoparticles can transport genes across the ocular mucosa and transfect ocular tissue. Gene Ther 2008;15:668-76.

17 Chan JM, Zhang L, Yuet KP, Liao G, Rhee JW, Langer R, et al. PLGA-lecithin-PEG core-shell nanoparticles for controlled drug delivery. Biomaterials 2009;30:1627-34.

18 Gwak SJ, Choi D, Paik SS, Cho SW, Kim SS, Choi CY, et al. A method for the effective formation of hepatocyte spheroids using a biodegradable polymer nanosphere. J Biomed Mater Res A 2006;78:268-75.

19 Gao X, Kim KS, Liu D. Nonviral gene delivery: what we know and what is next. AAPS J 2007;9:E92-104.

20 Cullis PR, Chonn A. Recent advances in liposome technologies and their applications for systemic gene delivery. Adv Drug Deliv Rev 1998;30:73-83.

21 Kim MS, Sung MJ, Seo SB, Yoo SJ, Lim WK, Kim HM. Water-soluble chitosan inhibits the production of pro-inflammatory cytokine in human astrocytoma cells activated by amyloid beta peptide and interleukin-1beta. Neurosci Lett 2002;321:105-9.

22 Felgner JH, Kumar R, Sridhar CN, Wheeler CJ, Tsai YJ, Border R, et al. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J Biol Chem 1994;269:2550-61.

23 Kim YH, Park JH, Lee M, Park TG, Kim SW. Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. J Control Release 2005;103:209-19.

24 Dewey RA, Morrissey G, Cowsill CM, Stone D, Bolognani F, Dodd NJ, et al. Chronic brain inflammation and persistent herpes simplex virus 1 thymidine kinase expression in survivors of syngeneic glioma treated by adenovirus-mediated gene therapy: implications for clinical trials. Nat Med 1999;5:1256-63.

25 Kim YT, Caldwell JM, Bellamkonda RV. Nanoparticle-mediated local delivery of Methylprednisolone after spinal cord injury. Biomaterials 2009;30:2582-90.

26 Qiao Y, Bai XF, Du YG. Chitosan oligosaccharides protect mice from LPS challenge by attenuation of inflammation and oxidative stress. Int Immunopharmacol 2011;11:121-7.

27 Cho Y, Shi R, Borgens RB. Chitosan produces potent neuroprotection and physiological recovery following traumatic spinal cord injury. J Exp Biol 2010;213:1513-20.

28 Csaba N, Koping-Hoggard M, Alonso MJ. Ionically crosslinked chitosan/tripolyphosphate nanoparticles for oligonucleotide and plasmid DNA delivery. Int J Pharm 2009;382:205-14.

29 Berry M, Barrett L, Seymour L, Baird A, Logan A. Gene therapy for central nervous system repair. Curr Opin Mol Ther 2001;3:338-49.

30 Choi BH, Ha Y, Ahn CH, Huang X, Kim JM, Park SR, et al. A hypoxia-inducible gene expression system using erythropoietin 3' untranslated region for the gene therapy of rat spinal cord injury. Neurosci Lett 2007;412:118-22.

31 Lawrence MS, Foellmer HG, Elsworth JD, Kim JH, Leranth C, Kozlowski DA, et al. Inflammatory responses and their impact on beta-galactosidase transgene expression following adenovirus vector delivery to the primate caudate nucleus. Gene Ther 1999;6:1368-79.

32 Baier C, Baader SL, Jankowski J, Gieselmann V, Schilling K, Rauch U, et al. Hyaluronan is organized into fiber-like structures along migratory pathways in the developing mouse cerebellum. Matrix Biol 2007;26:348-58.

33 Savani RC, Cao G, Pooler PM, Zaman A, Zhou Z, DeLisser HM. Differential involvement of the hyaluronan (HA) receptors CD44 and receptor for HA-mediated motility in endothelial cell function and angiogenesis. J Biol Chem 2001;276:36770-8.

34 Slevin M, Kumar S, Gaffney J. Angiogenic oligosaccharides of hyaluronan induce multiple signaling pathways affecting vascular endothelial cell mitogenic and wound healing responses. J Biol Chem 2002;277:41046-59.

35 Nagy JI, Hacking J, Frankenstein UN, Turley EA. Requirement of the hyaluronan receptor RHAMM in neurite extension and motility as demonstrated in primary neurons and neuronal cell lines. J Neurosci 1995;15:241-52.

36 Lynn BD, Turley EA, Nagy JI. Subcellular distribution, calmodulin interaction, and mitochondrial association of the hyaluronan-binding protein RHAMM in rat brain. J Neurosci Res 2001;65:6-16.

ABSTRACT (IN KOREAN)

신경 유전자 전달을 위한 키토산-하이알룬산-TPP나노입자 전달체

< 지도교수 : 윤 도 흠 >

연세대학교 대학원 의학과

공 민 호

의료기술의 발전에도 불구하고 중추 신경손상은 치료가 어렵고 회복률이 낮은 불치의 병이라고 인식되고 있다. 현재의 약물요법 및 수술, 재활치료는 과거에 비하여 개선이 되었음에도 아직까지 치료가 어려운 난치병이다. 신경 줄기세포를 이용한 유전자 치료는 현대의학으로는 완치가 어려운 신경계통의 질환들이 대상이 되므로 성공 시에 그 사회적 및 상업적 파급효과가 매우 큰 분야다. 신경 세포 및 신경 줄기세포에 외부 유전자를 도입하여 세포를 치료하는 방법은 신경손상 질환을 대상으로 진행되어 왔으며 레트로바이러스를 수송체로 이용하여 신경성장인자 유전자가 형질도입된 신경 줄기세포를 사용하여 생체 내에서 신경보호효과가 관찰된 바 있다. 그러나, 동물실험에서의 효과가 입증됨에도 불구하고 현재까지 연구되어온 신경손상의 유전자 치료는 임상적으로 획기적인 치료효과를 보여주지 못했다. 바이러스를 이용한 유전자 전달기술은 유전자 전달

효율이 비교적 높음에도 불구하고 바이러스 벡터의 안전성 문제로 인하여 임상적용이 매우 어려운 상태이다. 기존의 비바이러스 유전자 (Polyethylenimine, PEI) 치료법은 고분자를 통하여 유전자를 세포 내로 효과적으로 수송하는 전달 과정과 세포 내에서 유전자의 안정성 문제가 발생하여 임상에 적용하기 어렵다. 최근에 키토산과 유전자를 이용한 유전자 전달이 시도되고 있다.

본 연구저자들은 이온성겔화 방법을 사용하여 chitosan-hyaluronan/TPP (chitosan-HA/TPP)를 이용하여 유전자 전달체를 개발하였다. 신경 줄기세포 및 시험관 모델을 이용한 독성 검사를 한 결과 PEI 고분자보다 월등히 낮은 세포 독성을 보였다. 유전자 발현 효율을 측정하기 위해서 신경 줄기세포 및 시험관 모델에 적용해본 결과 PEI보다 장기간 유전자 발현이 확인되었다. In vivo 시험 결과 naked DNA와 비슷한 유전자 발현을 확인하였다.

본 연구에서 chitosan-TPP/HA를 이용한 비바이러스성 유전자 전달체는 유전자 치료에 이용될 수 있는 가능성을 보였으며 중추신경 질환 치료에서도 본 시스템이 적용 될 수 있을 것으로 기대한다.

핵심되는 말 : 키토산, 유전자 전달, 척수