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RESEARCH ARTICLE

Chitosan-coated C-phycocyanin Liposome for Extending the Neuroprotective Time Window Against Ischemic Brain Stroke

Goo Yong Chung*, Kyou Hee Shim*, Hye Jin Kim, Seul Ki Min and Hwa Sung Shin**

Department of Biological Engineering, Inha University, Incheon, 22212, Republic of Korea

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Abstract: The time window for neuroprotection during ischemic brain stroke is short, and hence, development of neuroprotectants is critical to extend this time window. This study sought to verify if muco-adhesive chitosan coating improves the neuroprotective potential of the pre-proven C-Phycocyanin-pertaining liposome (C-Pc liposome). The use of chitosan-coated liposomes extended the neuroprotective time window by 6 h after occlusion, and further improved the neuroprotection efficiency of the C-Pc liposome in a rat middle cerebral artery occlusion (MCAO) model. Beneficial changes in mRNA expressions of antioxidants, inflammatory cytokines and glia scar proteoglycans were evident in the C-Pc liposomes. In addition, in the cultured astrocytes, the chitosan-coated C-Pc liposome expressed anti-oxidative activity without cytotoxicity.

Key words: Chitosan, C-phycocyanin, neuroprotection, liposome, ischemia and reperfusion, brain stroke.

1. INTRODUCTION

Stroke, the second leading cause of death in the world, is categorized as ischemic stroke and hemorrhagic stroke [1, 2]. The death rate from ischemic stroke is 5 times of death rate from hemorrhagic stroke. The most typical drug for recanalizing a blood vessel after an ischemic stroke is tissue plasminogen activator (t-PA), the only drug authorized by the United States Food and Drug Administration (US FDA). However, t-PA has many side effects, such as bleeding, hives, and rashes [3]. Hence, developing other treatments to surpass these obstacles is necessary. In particular, secondary damage caused by ischemia-reperfusion (IR) is the most critical problem. The abrupt reperfusion of oxygen creates large amount of reactive oxygen species (ROS), which can critically damage abnormal ischemic tissue [4]. For this reason, neuroprotection is crucial to minimize the secondary damages. Several neuroprotective drugs have been studied and evaluated in clinical trials [5, 6]. For example, C-Phycocyanin (C-Pc) from marine spirulina was demonstrated to be effective in neuroprotection after ischemic brain injury [7, 8], and nasally administered liposome-encapsulated C-Pc was shown to extend the neuroprotective time window [9]. Ischemic stroke has a short time within which a patient should be treated. Thus, extending the neuroprotective time window is one of the main objectives of ischemic stroke studies.

Nasal administration passes the drug through the olfactory bulb, which is a pathway to the brain. This route overcomes the difficulties of drug delivery through the blood-brain barrier (BBB) [10]. The strategy often utilizes the muco-adhesive potential of a delivery material or system, because the drug is absorbed by the nasal mucous membrane [11]. By binding with mucin, a muco-adhesive drug is delivered to olfactory bulb, and not the nasopharynx, and is effectively delivered to the brain. Traditionally, chitosan is used as an effective material for nasal administration [12]. Because positive amino groups of chitosan establish ionic interactions with negative residues in the mucus sialic acid, chitosan has the capacity to bind to mucus [13, 14]. In addition to muco-adhesiveness, chitosan is nontoxic, biocompatible and biodegradable, which makes it an effective material in a drug delivery system [15]. In this study, to increase biodegradability, a chitosan degradation method was used to make oligo-chitosan. Chitosan-coated C-Pc liposomes were evaluated for their ability to extend the neuroprotective time window.

2. MATERIALS AND METHODS

2.1. Fabrication of Chi/C-Pc/Chol/liposomes

C-Pc/Chol/liposomes were fabricated as previously described [9, 16]. L-α-phosphatidylcholine from egg yolk (PC; Sigma Aldrich, USA) and 33.3% cholesterol (Chol; Sigma Aldrich) dissolved in chloroform (Sigma Aldrich), were vacuumed for 3 h to make a film. The dried lipid film was hydrated in 1 ml PBS containing 500 μg/ml of C-Pc (Sigma Aldrich). Freeze at –80°C for 10 min using a deep-freezer and thawing 20°C for 10 min using a water bath method was repeated 10 times to prepare unilamellar liposomes. The solution was extruded to make nano-sized liposomes using an extruder (Avanti Polar Lipids, USA) equipped with a 100 nm-sized polycarbonate filter. The fabricated liposomes were coated with chitosan (Sigma Aldrich) dissolved in 1% acetic acid (0, 0.001, 0.01, 0.1, 0.5%). Adsorption of chitosan was determined using the drop-wise method for 1 h in 4°C.

2.2. Characterization of Liposomes

Transmission electron microscopy (TEM) using a CM200 microscope (Philips, USA) was used to ascertain if the chitosan was successfully adsorbed on the liposome surface. The sample was placed dropwise on a TEM grid (Electron microscopy Sciences, USA), stained with 1% uranyl acetate for 10 min, washed with distilled water (DW), and finally dried in vacuum condition. Zeta potential and size of the sample were observed with an ELS-Z zeta potential analyzer (Otsuka, Japan) equipped with dynamic light scattering (DLS).

**Address correspondence to this author at the Department of Biological Engineering, 2S113A, Inha University, Inha-ro 100, Nam-gu, Incheon, 22212, Republic of Korea; Tel: 82-32-860-9221; Fax: 82-32-872-4046;
E-mail: hsshin@inha.ac.kr
**Both authors contributed equally to this work.*

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2.3. Cytotoxicity of Chi/C-Pc/Chol/liposomes to Primary Astrocytes

Primary astrocytes were isolated and cultured following a previous procedure [8]. MTT assay (Sigma Aldrich) was performed to evaluate the cytotoxicity of Chi/C-Pc/Chol/liposomes to primary astrocytes. The cultured astrocytes in well of a 24-well plate were exposed to a 500 μM suspension of the liposomes and incubated for 24, 48 and 72 h. 1 ml of 500 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) diluted in culture media was added to each well and incubated for 1 h in 37°C, after which 500 mL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan that had formed. The absorbance of the formazan solution was measured at 540 nm using a VARIOSKAN LUX apparatus (Thermo Scientific, USA).

2.4. Antioxidative Potential of Chi/C-Pc/Chol/liposomes for Oxidized Primary Astrocytes

Primary astrocytes were seeded in a 24-well plate and cultured for 2 days. And then the cells were incubated with serum free media for 1 day. The oxidized astrocytes, which mimicked astrocytes exposed to a high level of reactive oxygen species (ROS) in brain IR, were produced by treatment with 200 μM hydrogen peroxide (H_2O_2). 24 h after H_2O_2 treatment, chitosan-coated or non-coated liposomes were treated for 24 h and then MTT assay was conducted.

2.5. Neuroprotective Potential of Chi/C-Pc/Chol/liposomes in a Rat Model of Middle Cerebral Artery Occlusion (MCAO)

A rat MCAO model was made following a previous procedure [9]. Six hours after occlusion, 8.34 µl of a liposome suspension was administered twice in both nasal passages. After 2 days, the modified neurological severity score (mNSS) was determined with the test lists (motor, sensory, reflex, beam balance), and the brain was sliced with a rat brain slicer (Zivic, USA) into 2 mm thick slices. The slices were stained with TTC (2,3,5-triphenyltetrazolium chloride) solution (Sigma Aldrich, USA) and the infarct size was measured.

2.6. Ethical Statement

All animal tests followed national guidelines for the care and use of laboratory animals and the ARRIVE guidelines (www.nc3rs.org.uk). The experiment procedure was approved by the Institutional animal care and use committee of Inha University (Korea, INHA 161012-447-1).

2.7. Quantitative Real-time PCR (qRT-PCR)

mRNA of liposome-treated rat brain was quantified using qRT-PCR as previously described [9]. Brain slices (3 mm) were homogenized and total RNA was purified using an RNeasy MiNi kit plus (Takara Bio, Japan). cDNA was synthesized using a QuantiFast Reverse Transcription kit (Qiagen, Germany). qRT-PCR was performed using the QuantiFast Reverse Transcription Kit (Qiagen, Germany) and Rotor-Gene Q PCR (Qiagen) was used to quantify the expression level of markers. The primer sequences are listed in Table **1**.

2.8. Statistical Analysis

All experiments were performed three times. T-test was done to verify statistical significance of data with p-values (* < 0.05, ** < 0.01, or *** < 0.001).

3. RESULTS

3.1. Fabrication and Administration of C-Pc/33% Chol/PC Liposomes

Chitosan-coated liposomes containing C-Pc were evaluated concerning their drug delivery ability and degree of extension of the recovery time window. Liposomes were fabricated by freezing-thawing of lipid films consisting of PC and cholesterol. In the chitosan coating step, acetic acid mixed chitosan solution was used. The rat MCAO model representing ischemia and reperfusion was used for the *in vivo* assay. In Fig. **1**, Blocking blood flow for 1 h mimicked ischemia and physically unblocking the occluded blood vessel mimicked reperfusion, which caused secondary damage. Nasal administration of the drug proceeded 5 h after reperfusion for direct delivery to the brain. After 48 h MCAO, an extension of the neuroprotective time window was assessed by behavioral testing and tissue recovery.

3.2. Characterization of C-Pc/33% Chol/PC Liposome

Dried chitosan-coated liposomes and non-coated liposomes were observed by TEM. Non-coated liposomes had a diameter of 112.2 nm of diameter (Fig. **2a**) and 0.1% chitosan-coated liposomes had a larger diameter of 157.2 nm. The thickness of the chitosan layer was 19 nm (Fig. **2b**). Both liposomes were shown as spherical and 0.1% chitosan coated liposome was bigger than non-coated liposome. The size and zeta potential of liposomes were analyzed using a Z-analyzer (Table **2**). The diameter of non-coated and 0.1% chitosan-coated liposomes was 89.30 ± 2.23 nm and 105.37 ± 1.19 nm, respectively. Non-coated liposomes displayed a mean zeta potential of -41.55 ± 0.72 mV. Liposomes coated with 0.001, 0.01, 0.1, and 0.5% chitosan had a mean zeta potential of 18.59 ± 0.43 mV, 36.27 ± 0.26 mV, 52.49 ± 0.78 mV, and 53.96 ± 8.46 mV, respectively. Thus, the zeta potential showed increasing positive charge with increasing chitosan concentration.

3.3. *In vitro* Cytotoxicity of C-Pc/33% Chol/PC Liposomes to Primary Cultured Rat Astrocytes

Cell cytotoxicity and antioxidant effect were measured by the MTT assay by using rat astrocytes. On day 1, use of non-coated liposomes and all of the chitosan-coated liposomes decreased cell numbers (Fig. **3a**), compared with the non-treated cells. On days 2 and 3, the number of cells increased in the samples containing non-coated liposomes and all of the chitosan-coated liposomes. This indicated that C-Pc incorporated liposomes induce longer-term proliferation of astrocytes and that the chitosan coating is not cytotoxic. In the cell oxidation assay, both non-coated liposomes and chitosan-coated liposomes displayed antioxidation behavior in the presence of H_2O_2 (Fig. **3b**). Moreover, liposomes incorporating C-Pc showed better resistance to H_2O_2.

3.4. *In vivo* Tests of C-Pc/33% Chol/PC Liposome in the Rat MCAO Model

The rat MCAO model was used as the *in vivo* assay. By measuring the infarct volume and evaluating the behavior-related mNSS, the effect of the chitosan-coated liposomes was measured (Fig. **4**). The infarct volume of the PBS administered rats (Control) was 126.4 mm^3 on average, while the C-Pc liposome and the chitosan coated liposome treated groups displayed reduced infarct size by 112.1 mm^3 and 82.1 mm^3, respectively, compared to the control, especially in the chitosan coated liposome-treated rats (Fig. **4a** and **4b**). In the mNSS test for evaluating anomalous behavior, both of the liposome sample-treated groups showed statistically significant lower scores of 4.6 points and 5.8 points than control. Therefore, the 0.1% chi/C-Pc/liposomes can be considered to be a better neuroprotective effect than the C-Pc/liposomes.

3.5. Biochemical Analyses of the Effect of C-Pc/33% Chol/PC Liposomes in the Rat MCAO Model

Chito/C-Pc/liposomes displayed greater mRNA downregulation for an antioxidant enzyme (Fig. **5a**) and upregulation for the glia scar-associated proteoglycan such as phosphacan and neurocan than the C-Pc/liposomes (Fig. **5b**). Both C-Pc/liposomes and Chito/C-Pc/liposomes decreased the expressions of tumor necrosis factor-alpha (TNF-α) and no different expression of IL-6 (Fig. **5c**).

Table 1. Primer sequences for qRT-PCR analysis.

Gene	Forward (5'→3')	Reverse (3'→5')
Catalase	GCGGATTCCTGAGAGAGTGG	TGTGGAGAATCGAACGGCAA
Phosphacan	TTGACAAGTGATGAAGAGAG	AATCAGCACATCTCGTTCTA
Neurocan	TTTCAGTCCACAGCGATCAG	AGGAGAGGGATACAGCAGCA
TNF-α	GCTTGGTGGTTTGCTACGAC	ATGGGCTCCCTCTCATCAGT
IL-6	ACAGCGATGATGCACTGTCA	ACGGAACTCCAGAAGACCAG
IL-1β	AAATGCCTCGTGCTGTCTGA	AGGCCACAGGGATTTTGTCG

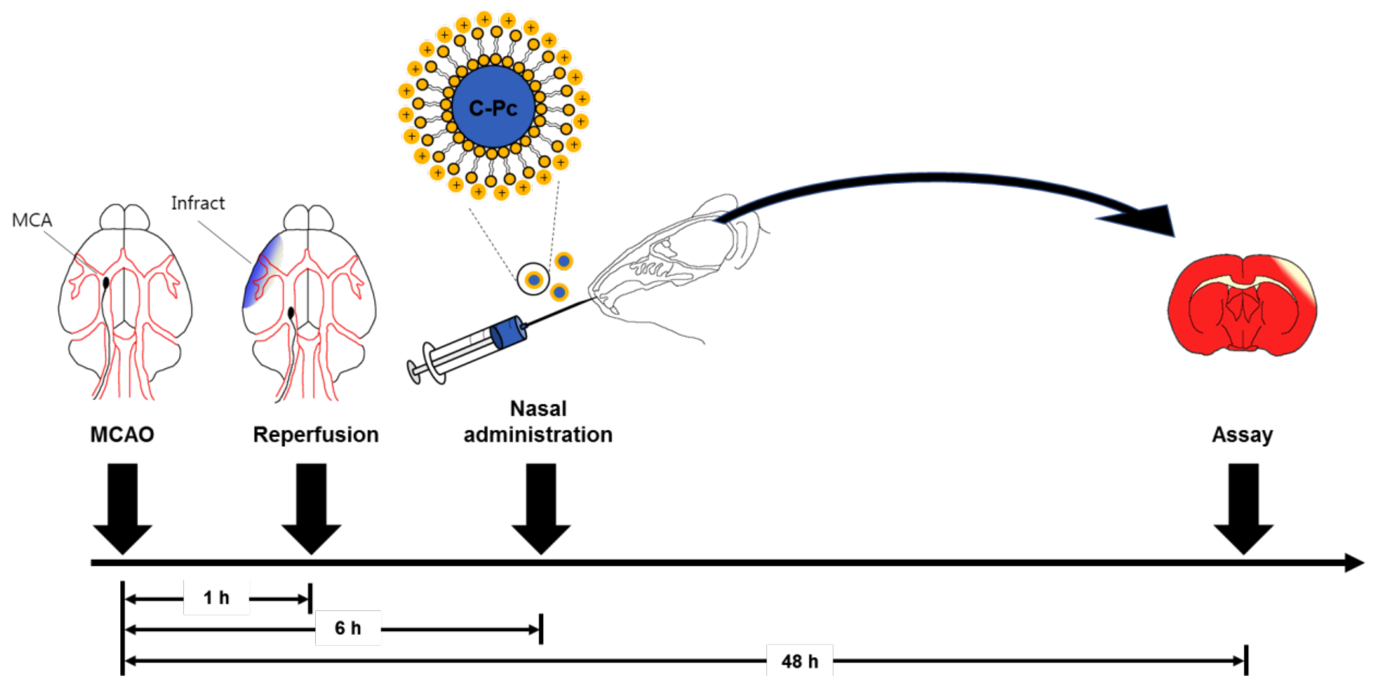

Fig. (1). Time-series procedure of *in vivo* administration of Chito/Lipo/C-Pc liposome.

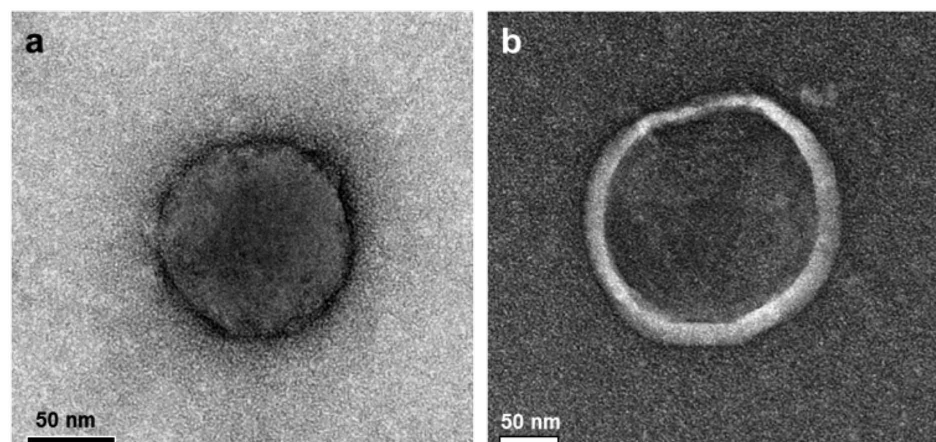

Fig. (2). Morphologies of A) chitosan non-coated liposome and B) 0.1% chitosan-coated liposome observed by Transmission electron microscopy. Scale bar = 50 nm.

Table 2. Zeta potential and size of Chito/Lipo/C-Pc.

Conc. of chitosan	0%	0.001%	0.01%	0.1%	0.5%
Zeta potential (mV)	-41.55 ± 0.72	18.59 ± 0.43	36.27 ± 0.26	52.49 ± 0.78	53.96 ± 8.46
Size (nm)	89.30 ± 2.23	88.53 ± 4.56	102.87 ± 30.77	105.37±1.19	122.13 ± 8.46

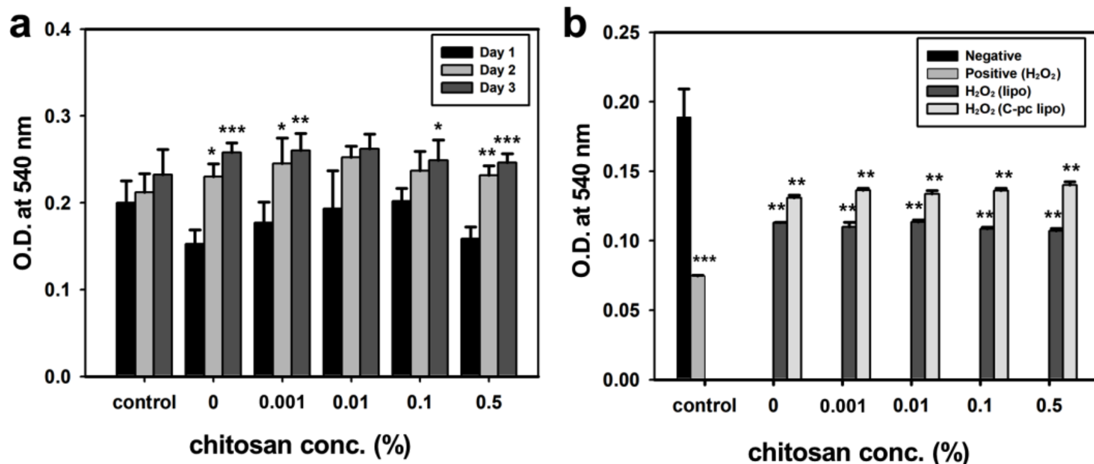

Fig. (3). (a) Cytotoxicity and (b) anti-oxidative potential of chitosan non-coated and coated liposomes on primary astrocytes under oxidative stress.

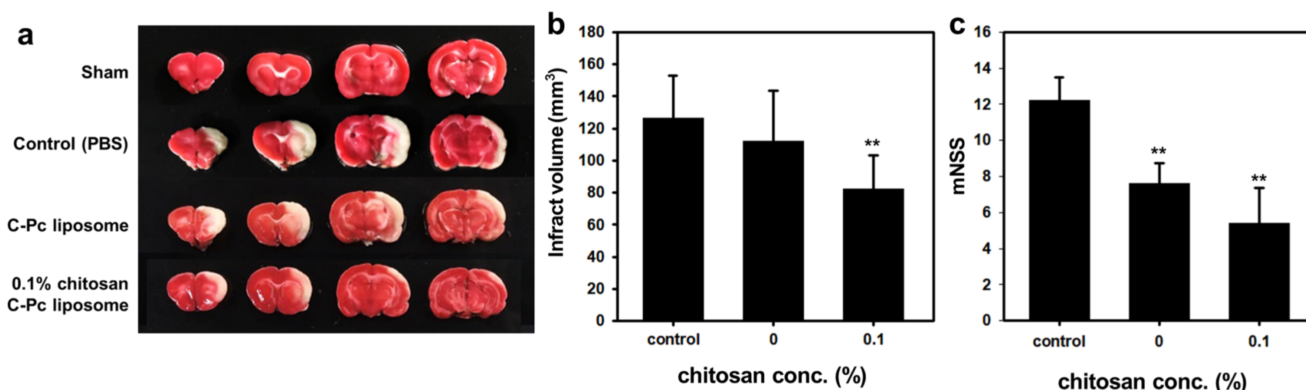

Fig. (4). Evaluation of (a,b) infarct volume and (c) neurological deficits after treating Chi/C-Pc liposomes.

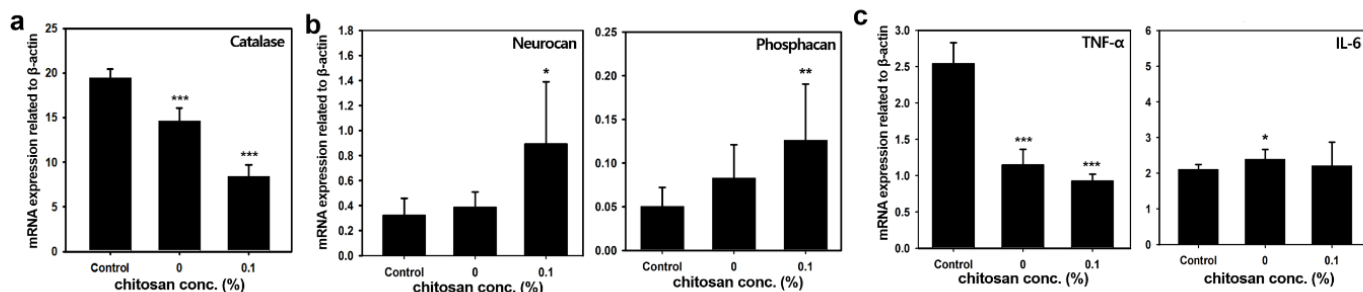

Fig. (5). mRNA analyses by qRT-PCR for (a) catalase, (b) glia scar factor phosphacan and neurocan and (c) pro-inflammatory factors TNF-α and IL-6.

4. DISCUSSION

Chitosan-coated C-Pc/liposomes were examined for their ability to extend the neuroprotection time window, over the pre-confirmed extension level of C-Pc/liposomes [9]. Previous studies demonstrated that C-Pc is neuroprotective in the ischemic brain, and that C-pc/Cholesterol/Phosphatidylcholine liposomes can extend the neuroprotection time window. The present study was focused on examining whether chitosan coating increases the neuroprotection effect, as well as the extension effect of time window of C-Pc/Chol/liposomes.

In order to verify this hypothesis, C-Pc/Cholesterol/PC liposomes were fabricated following the optimal formulation method [9]. In an isothermal condition, increasing chitosan concentrations produce increasingly thicker coating of the liposome surface until saturation. As seen in the zeta potential and DLS results, liposome size and charge increased with increasing chitosan concentration by 0.1%, but no significant differences were observed between 0.1% and 0.5% chitosan, which mean that the chitosan coating was saturated over the 0.1% concentration. In addition, cytotoxicity and antioxidant level did not differ significantly according to chitosan concentration. Based on these results, 0.1% chitosan was selected as the maximum coating concentration for C-Pc/liposomes.

We have already proven that C-Pc/20~33% Cholesterol/PC liposomes are thermodynamically most stable, which helps to extend neuroprotective time window of C-Pc against ischemic brain stroke [9]. After chitosan coating, liposomes were being more stable because hydrophilic surface of chitosan hindered self-aggregation among liposomes, resulting in enhancing dispersion of liposomes [17, 18]. Chitosan coating can also improve adsorption ability of liposome on nasal mucous layer since chitosan is polysaccharide with positively charged ammonium residue and nasal layer is negatively charged [13, 14, 19]. In other studies, increased adsorption of bioactive materials on nasal mucopolysaccharide layer was reported to improve drug delivery [20, 21]. That is, with the same dosage, chitosan coated C-Pc liposome can efficiently deliver C-Pc more than non-coated C-Pc liposome because of higher stability and adsorption ability, which could increase the neuroprotective potential of C-Pc/20~33% Cholesterol/PC liposomes by improving the delivery efficiency of C-Pc to the injured brain tissue. Therefore 0.1% chitosan-coated C-Pc/33% Cholesterol/PC liposomes were examined in the rat MCAO model. By the TTC staining and mNSS test, both non-coated C-Pc/Chol/liposomes and 0.1% chitosan-coated C-Pc/33% Cholesterol/PC liposomes have reduced infract volume and behavioral deficit. Furthermore, chitosan coating on liposomes have shown improved efficacy on both assay.

C-Pc/liposomes increased the neuroprotective potential of C-Pc and maintained the extended time window of C-Pc/33% Cholesterol/PC liposomes. According to other studies, catalase gene expression is upregulated when the cells are exposed in oxidative stressful condition to decrease oxidants as ROS [22]. On the other hand, the downregulated activity of the antioxidative enzyme, catalase, means that the C-Pc/33% Cholesterol/PC liposomes sufficiently acted as an antioxidant, evading the cell catalase activity. The decreased inflammatory factor TNF-α indicates that the cells exposed to C-Pc/33% Cholesterol/PC liposomes become more resistant to the oxidative stressful environment and protect neuronal activity. The increased expression of glia scar mRNA coincides with the fact that these factors are upregulated to protect the penumbra from being oxidized [23]. As mentioned above, chitosan coating was proven in molecular-level studies to have better neuroprotection efficiency than non-coated C-Pc/33% Cholesterol/PC liposomes.

CONCLUSION

C-Pc/Chol/liposomes were previously proven to extend the neuroprotective time window by 6 h after occlusion in a rat MCAO model. In this work, muco-adhesive chitosan was coated onto C-Pc/Chol/liposomes and the neuroprotective potential was evaluated. Above all, the chitosan-coated C-Pc liposomes have shown no cytotoxicity but anti-oxidative potential in the cultured astrocytes. In the *in vivo* MCAO and reperfusion rat model, the chitosan coating also extended the neuroprotective time window by 6 h after occlusion, and additionally left a smaller infarct size and improved behavioral test results, compared with the non-coated C-Pc/Chol/liposomes in the rat MCAO model. C-Pc liposome mRNA expression of antioxidants, inflammatory cytokines and glia scar proteoglycans were positively affected. In conclusion, chitosan coating improves the neuroprotective potential of C-Pc/Chol/ liposomes in a rat brain model of IR.

LIST OF ABBREVIATIONS

C-Pc liposome	=	C-Phycocyanin-pertaining liposome
MCAO	=	Middle cerebral artery occlusion
t-PA	=	tissue plasminogen activator
USFDA	=	United State Food and Drug Administration
IR	=	Ischemia-reperfusion
ROS	=	Reactive oxygen species
C-Pc	=	C-Phycocyanin
BBB	=	Blood-brain barrier
Chi/C-Pc/Chol/liposomes	=	C-Pc incorporated and chitosan-coated cholesterol containing liposomes
PC	=	Phosphatidylcholine
Chol	=	Cholesterol
DLS	=	Dynamic light scattering
TEM	=	Transmission electron microscopy
DW	=	Distilled water
DMSO	=	Dimethyl sulfoxide
H_2O_2	=	Hydrogen peroxide
mNSS	=	Modified neurological severity score
TTC	=	2,3,5-triphenyltetrazolium chloride solution
qRT-PCR	=	Quantitative real-time PCR

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Bosch J, Yusuf S, Pogue J, *et al*. Use of ramipril in preventing stroke: double blind randomised trial. Bmj 2002; 324(7339): 699.

[2] Andersen KK, Olsen TS, Dehlendorff C, Kammersgaard LP. Hemorrhagic and ischemic strokes compared. Stroke 2009; 40(6): 2068-72.

[3] Benchenane K, López-Atalaya JP, Fernández-Monreal M, Touzani O, Vivien D. Equivocal roles of tissue-type plasminogen activator in stroke-induced injury. Trends Neurosci 2004; 27(3): 155-60.

[4] Pun PB, Lu JIA, Moochhala S. Involvement of ROS in BBB dysfunction. Free Radic Res 2009; 43(4): 348-64.

[5] Matsuda T, Arakawa N, Takuma K, *et al*. SEA0400, a novel and selective inhibitor of the Na^+-Ca^{2+} exchanger, attenuates reperfusion injury in the *in vitro* and *in vivo* cerebral ischemic models. J Pharmacol Exp Ther 2001; 298(1): 249-56.

[6] Green AR. Pharmacological approaches to acute ischaemic stroke: reperfusion certainly, neuroprotection possibly. Br J Pharmacol 2008; 153(Suppl 1): S325-S338.

[7] Pentón-Rol G, Marín-Prida J, Pardo-Andreu G, *et al*. C-Phycocyanin is neuroprotective against global cerebral ischemia/reperfusion injury in gerbils. Brain Res Bull 2011; 86(1): 42-52.

[8] Min SK, Park JS, Luo L, *et al*. Assessment of C-phycocyanin effect on astrocytes-mediated neuroprotection against oxidative brain injury using 2D and 3D astrocyte tissue model. Sci Rep 2015; 5: 14418.

[9] Min SK, Kwon YS, Cho MK, Shin HS. Nasal Delivery of Antioxidants by Cholesterol-incorporated Liposomes Extends the Neuroprotective Time Window in Cerebral Ischemia. Curr Pharm Des 2017; 23(40): 6223-30.

[10] Graff CL, Pollack GM. Nasal drug administration: potential for targeted central nervous system delivery. J Pharm Sci 2005; 94(6): 1187-95.

[11] Jain AK, Chalasani KB, Khar RK, Ahmed FJ, Diwan PV. Mucoadhesive multivesicular liposomes as an effective carrier for transmucosal insulin delivery. J Drug Target 2007; 15(6): 417-27.

[12] Mistry A, Stolnik S, Illum L. Nanoparticles for direct nose-to-brain delivery of drugs. Int J Pharm 2009; 379(1): 146-57.

[13] Luppi B, Bigucci F, Mercolini L, Musenga A, Sorrenti M, Catenacci L, Zecchi V. Novel mucoadhesive nasal inserts based on chitosan/hyaluronate polyelectrolyte complexes for peptide and protein delivery. J Pharm Pharmacol 2009; 61(2): 151-7.

[14] Alonso MJ, Sanchez A. The potential of chitosan in ocular drug delivery. J Pharm Pharmacol 2003; 55: 1451-63.

[15] Komi DEA, Hamblin MR. Chitin and chitosan: production and application of versatile biomedical nanomaterials. Int J Adv Res 2016; 4(3): 411-27.

[16] Akbarzadeh A, Rezaei-Sadabady R, Davaran S, *et al*. Liposome: classification, preparation, and applications. Nanoscale Res Lett 2013; 8(1): 102.

[17] Mertins O, Dimova R. Binding of chitosan to phospholipid vesicles studied with isothermal titration calorimetry. Langmuir 2011; *27*(9): 5506-15.

[18] Mady MM, Darwish MM. Effect of chitosan coating on the characteristics of DPPC liposomes. J Adv Res 2010; 1(3): 187-91.

[19] Law SL, Huang KJ, Chou VHY, Cherng JY. Enhancement of nasal absorption of calcitonin loaded in liposomes. J Liposome Res 2001; 11(2-3): 165-74.

[20] Borchard G, Lueβen HL, de Boer AG, Verhoef JC, Lehr CM, Junginger HE. The potential of mucoadhesive polymers in enhancing intestinal peptide drug absorption. III: Effects of chitosan-glutamate and carbomer on epithelial tight junctions *in vitro*. J Control Release 1996; 39(2-3): 131-38.

[21] Jiang L, Gao L, Wang X, Tang L, Ma J. The application of mucoadhesive polymers in nasal drug delivery. Drug Dev Ind Pharm 2010; 36(3): 323-36.

[22] Black AT, Gray JP, Shakarjian MP, Laskin DL, Heck DE, Laskin JD. Increased oxidative stress and antioxidant expression in mouse keratinocytes following exposure to paraquat. Toxicol Appl Pharmacol 2008; 231(3): 384-92.

[23] Rolls A, Shechter R, Schwartz M. The bright side of the glial scar in CNS repair. Nature Rev Neurosci 2009; 10(3): 235-41.