·Basic Research·

Sustained-release genistein from nanostructured lipid carrier suppresses human lens epithelial cell growth

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Abstract

• **AIM: To design and investigate the efficacy of a modified nanostructured lipid carrier loaded with genistein (Gen-NLC) to inhibit human lens epithelial cells (HLECs) proliferation.**

• **METHODS: Gen-NLC was made by melt emulsification method. The morphology, particle size (PS), zeta potentials (ZP), encapsulation efficiency (EE) and *in vitro* release were characterized. The inhibition effect of nanostructured lipid carrier (NLC), genistein (Gen) and Gen-NLC on HLECs proliferation was evaluated by cell counting kit-8 (CCK-8) assay, gene and protein expression of the proliferation marker Ki67 were evaluated with real-time quantitative polymerase chain reaction (RT-qPCR) and immunofluorescence analyses.**

• **RESULTS: The mean PS of Gen-NLC was 80.12±1.55 nm with a mean polydispersity index of 0.11±0.02. The mean ZP was -7.14 ±0.38 mV and the EE of Gen in the nanoparticles was 92.3% ±0.73%. Transmission electron microscopy showed that Gen-NLC displayed spherical-shaped particles covered by an outer-layer structure. *In vitro* release experiments demonstrated a prolonged drug release for 72h. The CCK-8 assay results showed the NLC had no inhibitory effect on HLECs and Gen-NLC displayed a much more prominent inhibitory effect on cellular growth compared to Gen of the same concentration. The mRNA and protein expression of Ki67 in LECs decreased significantly in Gen-NLC group.**

• **CONCLUSION: Sustained drug release by Gen-NLCs may impede HLEC growth.**

• **KEYWORDS:** posterior capsular opacification; genistein; nanostructured lipid carrier; human lens epithelial cells

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INTRODUCTION

Cataract is one of the most common diseases that affect the elderly people and cataract surgery is the most frequently performed ocular procedure in the world. Despite a high success rate, displacement of intraocular lens (IOL), posterior capsule opacification (PCO) and anterior capsule contraction are highly likely to reduce the visual quality and even result in second vision loss [1]. During the first few months after cataract surgery, the residual lens epithelial cells (LECs) begin to migrate, proliferate, and undergo epithelial-to-mesenchymal transition (EMT) by which results in collagen deposition and fibrosis of the lens capsule. This process can not only cause contraction and narrowing of the anterior capsule opening, but also results in PCO which leads to poor visual acuity[1]. Although Nd:YAG laser capsulotomy is the most common method to treat PCO, its relative complications and increased financial burden to the health care system are the main concerns [2]. Other methods have been employed to prevent PCO such as new IOL materials, square-edge IOL design, and improved surgical procedures[3-5], *etc*. However, the rate of PCO was not reduced as presumption.

Theoretically, the drugs which could prevent LECs from proliferation and migration can be used to prevent PCO[6-8]. Pharmacological therapies are another way brought about recently and some anti-inflammation and anti-metabolic agents have been proved to be safe and effective prophylactic strategies. Genistein (Gen), (4',5,7-trihydroxyisoflavone), a potent tyrosine kinase inhibitor, is a phytoestrogen with a wide variety biological functions such as anti-oxidant [9], phyto-oestrogenic and tyrosine kinase inhibitor activities [10] and has been shown to be useful against breast and prostate cancers [11], cardiovascular diseases and post-menopausal

ailments. Gen has been reported to protect against lens opacity in human lens epithelial cells (HLECs) and in rat eyes [12] and its safety also has been proved as an intravitreal drug in the rabbit model[13].

Although Gen has been proved to be effective, the short residence time in ocular, especially in anterior chamber, hinders its performance in clinics. Due to the multiple constraints imposed by the eye against the penetration of drugs, the ocular delivery and targeting are particularly problematic. The major challenge in ocular drug therapeutic treatment was poor intraocular penetration and rapid ocular elimination [14]. One of the promising approaches to improve ocular drug effectiveness is nanostructured lipid carrier (NLC). NLC, as the new generation of lipid nanoparticle drug carrier system, have many advantages for enhancement of drug permeability, controlled release, targeting and so on[15-16].

In this study, we designed and modified an innovative NLC for drug delivery of Gen based on our previous study to provide higher drug loading, sustained drug release and better biocompatibility. The effectiveness of inhibitory effect of nanostructured lipid carrier loaded with genistein (Gen-NLC) on HLECs growth *in vitro* was also evaluated.

MATERIALS AND METHODS

Gen was supplied by Huike Botanical Development Co., Ltd (Xi'an, China). Compritol 888 ATO was gifted by Gattefosse (Paris, France); Miglyol 812N was obtained from Sasol (Witten, Germany); Cremphor ® EL was provided by Ludwigshafen (Germany); egg phosphatidylcholine (EPC) was obtained from Shanghai Taiwei Pharmaceutical Co., Ltd. Human; Dulbecco's modified eagle's medium (DMEM; Gibco® Invitrogen, Carlsbad, USA) was supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, USA); cell counting kit-8 (CCK-8), trypan blue and penicillin-streptomycin was acquired from Beyotime Technology (Jiangsu Province, China); Trizol® Reagent was purchased from Invitrogen (NY, USA).

Preparation of Nanoparticle Gen-NLC was prepared by melt-emulsification method. Briefly, designed amount of Gen (0.14 wt%), Compritol 888 ATO (2.1 wt%) and Miglyol 812 N (0.9 wt%) were mixed and heated under moderate stirring at 87℃ to form the clear and uniform oil phase. EPC (0.88 wt%) and Cremphor EL (2.65 wt%) were dissolved in 10 mL double distilled water before being heated up to 87℃. The mixture were added into the oil phase and stirred at 800 rpm for 7min. The coarse emulsion was formed and homogenized by probe-ultrasonic cell disruptor (JY-92-II; Xinzhi, Ningbo, China) for 5min (active every 3s for 3s duration, 400 W). The nanoemulsion was immediately solidified in ice bath (0℃ -4℃) to form nanoparitcles.

Particle Sizes and Zeta Potentials The mean particle sizes (PS) and zeta potentials (ZP) were measured by photon correlation spectroscopy using a Zeta-sizer Nano (Malvern Instruments, Worcestershire, UK) at 25℃. All determinations were performed in triplicate.

Encapsulation Efficiency Entrapped Gen-NLC was separated from unentrapped drug by a Sephadex G-50 column. Sephadex G-50 was loaded into a 2.5 mL syringe followed by spinning at 2000 rpm for 2min to gain a dehydrated column. Afterwards 0.2 mL sample of Gen and Gen-NLC suspensions was added to the column and centrifuged (2000 rpm, 2min) again. After 3 times washed by distilled water and centrifuges, all the eluents were collected and a mix of solvent of dichloromethane and methanol (1:4, v/v) was added to destroy the lipid ingredient. The amount of encapsulated genistein was assessed by high-performance liquid chromatography HPLC (Hitachi, Tokyo, Japan) with the following conditions: a Diamasil® C18 column (200×4.6-mm^2, 5 μm, Dikma, China); a mobile phase with the mixture of methanol -0.05% phosphoric acid aqueous solution (60/40, v/v); a flow rate of 1.0 mL/min and a wavelength of 260 nm. Another 0.2 mL Gen-NLC suspension was added to the methanol and dichloromethane for destroying and determined by HPLC. Encapsulation efficiency (EE) was calculated using the following equations: EE (%)=(Gen encapsulated/total amount of Gen initially added)×100%.

Morphological Studies A drop of the nanoparticle suspension was placed on the copper grid with amorphous carbon film. The 1% phosphotungstic acid was used for megatively stainng. The morphologic character of the nanoparticles was ascertained by transmission electron microscopy (JH-7650, Hitachi, Tokyo, Japan).

***In Vitro* Release** The release of Gen from Gen-NLC was measured using a modified dialysis membrane diffusion technique[17]. Gen-NLC dispersions containing 0.2 mg Gen was transferred to a pre-soaked cellulose membrane (12 000 Da) and suspended in the dissolution flask containing 800 mL Ringer's solution. The cellulose membranes rotated horizontally with the blade in a speed of 50 rpm at 37℃. The release medium was withdrawn and the drug content was determined by HPLC at 270 nm at the predetermined time points. The release of Gen was conducted by adding equivalent amount of drug to NLC dispersions into 800 mL Ringer's solution, followed by the same procedure as Gen-NLC. All the results were the mean values of three runs.

Cell Culture SV40 T-antigen-transformed HLEC line (SRA01/04 cell) was kindly gifted by Dr. Yi-Sin Liu, Doheny Eye Institute, US. Cells were maintained in DMEM plus 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin at 37℃ in humidified 5% CO_2 condition.

Viability Assays of SRA01/04 Cells The viability assays were performed in HLEC lines (SRA01/04) using CCK-8 reagent. HLECs were prepared in 96-well plates at a cellular density of 10 000 cells/well with a confluent monolayer. The cells were treated with 100 μL of various concentrations of NLC, Gen or Gen-NLC at 37℃ for 24h after cultured in serum-free medium for 12h. The cells were washed three times with phosphate-buffered saline (PBS) and 100 μL 10% CCK-8 solution was added per well followed by incubating for another 2h at 37℃. Each sample was then measured spectrophotometrically at a wavelength of 450 nm using a micro-plate reader (Tristar LB 941; Berthold Technology, Hertfordshire, UK). Untreated cells were considered as a 100% cell viability control, and the media served as a background reference. The survival percentage was calculated in comparison with the control excluding the background reference. Cell viability (%)=[A (dose)-A (blank)]/[A (0 dose)-A (blank)]×100%.

Real-time Quantitative Polymerase Chain Reaction HLECs were treated with 0, 25 and 50 mg/L Gen-NLC for 24h after cultured in serum-free medium for 12h. Total RNA from cell lines was extracted using Trizol® Reagent (Invitrogen, NY, USA) according to kit instructions and the RNA quality was detected by a UV-Vis spectrophotometer UV-1800 (Shimadzu, Japan). RNA of Ki67 was reverse-transcribed with PrimerScript RT reagent kit (Takara, Dalian, China). The resulting cDNA was amplified with SYBR Premix Ex TaqTM II (Takara, Dalian, China) at 95℃ for 30s, followed by 40 cycles at 95℃ for 5s, and at 60℃ for 30s using designed primer on ABI 7500 (Applied Biosystems, USA). The primer sequences are listed in Table 1. In order to ensure product specificity, melting curve analysis was performed at the end of the cycles. The relative quantity of Ki67 normalized to β-actin, was calculated based on the equation $RQ=2^{-\Delta\Delta CT}$.

Immunofluorescence Assay After 12h incubation of serum-free medium, HLECs grown on coverslips were exposed to 0, 25 and 50 mg/L Gen-NLC for 24h. All the samples were fixed with pure methanol at -20℃ for 10min followed by 3 times of PBS washes. Cells were blocked with 5% goat serum (Ruite, Guangzhou, China) for 1h and then incubated with primary antibodies at 1:500 (Abcam, Cambridge, UK) overnight at 4℃. After 3 times rinses in PBS, cell were incubated with secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA) at 1:1000 for 2h, followed with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA, USA) staining for 5min. The cells were scanned with a fluorescence light microscope (BX51; Olympus, Inc., Tokyo, Japan).

Statistical Analysis All results are presented as the mean± SD and analyzed by SPSS16.0 statistical software. CCK-8 assay were assessed using repeated measure analysis of variance. One-way analysis of variance (ANOVA) with a post hoc test (Bonferroni test) for multiple comparisons was applied to evaluate the significant differences between groups. Values of $P<0.05$ were considered statistically significant.

Table 1 Real-time quantitative polymerase chain reaction (RT-qPCR) primers (TakaLa, China)

Gene	Primer
Ki67 (F)	CCTGCTCGACCCTACAGAGTG
Ki67 (R)	GTTGCTCCTTCACTGGGGTCT
β-actin (F)	CATCCGTAAAGACCTCTATGCCAAC
β-actin (R)	ATGGAGCCACCGATCCACA

F: Forward; R: Reverse.

RESULTS

Physicochemical Characterization of Nanoparticle In our study, the average PS of Gen-NLC showed an effective particle diameter of 80.12±1.55 nm and polydispersity index of 0.11±0.02. The mean ZP was -7.14±0.38 mV and the mean EE of Gen in the nanoparticles was 92.3%±0.73%.

Morphological Study TEM pictures were taken to obtain more information about the morphology of the prepared NLCs. As displayed, Gen-NLC showed spherical-shape particles covered by an outer-layer structure (Figure 1). It can also be read from the picture that most particles are around 100 nm, similar to the size given by Malvern.

***In Vitro* Release** The cumulative release profiles of Gen-NLC were obtained by determining the percentage of Gen released relative to the amount of Gen originally loaded in the nanoparticles. The *in vitro* release of Gen-NLC illustrated a biphasic drug release pattern for about 3d. The release profile showed an initial release of about 20% drugs during the 2h, however, followed by sustained release for at least 72h. On the contrary, the release profile of Gen solution showed a prominent burst release of 80% drugs during the first 2h. The biphasic drug release is a characteristic for controlled drug delivery (Figure 2).

Inhibitory Effects of Nanoparticle on SRA01/04 Cells Proliferation After 24h exposed to different concentration of Gen-NLC, morphological and quantitative changes in SRA01/04 cells were found (Figure 3). From the figure, an obvious decreased tendency in cell numbers and changes of cell morphology could be seen when the concentration of Gen-NLC increased from 6.25 to 75 mg/L. The inhibitory effects of Gen, Gen-NLC and NLC on the proliferation of HLECs were evaluated by CCK-8 assay (Figure 4). The cell viabilities in NLC groups were all above 90% although there was a slight decline of viability with the increased NLC concentration. Both Gen and Gen-NLC showed inhibitory effects on HLECs. Incubated with Gen-NLC at different concentrations of 0, 6.25, 12.5, 25, 50, 75 mg/L for 24h, the cells viability were 98.68%±2.20%, 91.84%±3.92%, 71.81%±

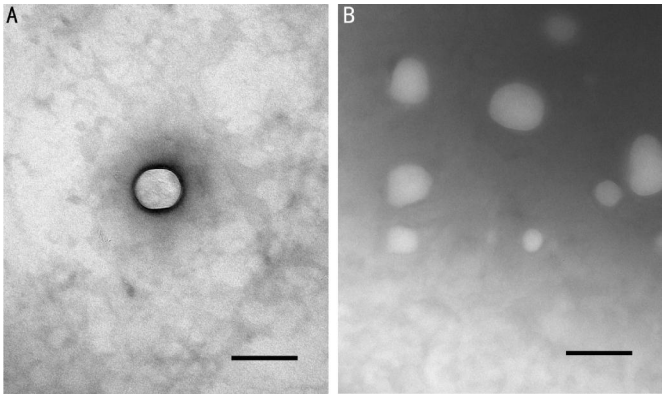

Figure 1 Transmission electron microphotographs of Gen–NLC in spherical shape A: Solo nanoparticle; B: Overview. Scale bar=100 nm.

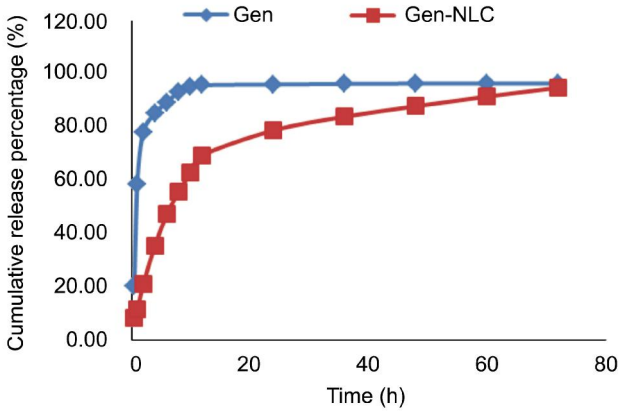

Figure 2 *In vitro* release profiles of the Gen and Gen–NLC.

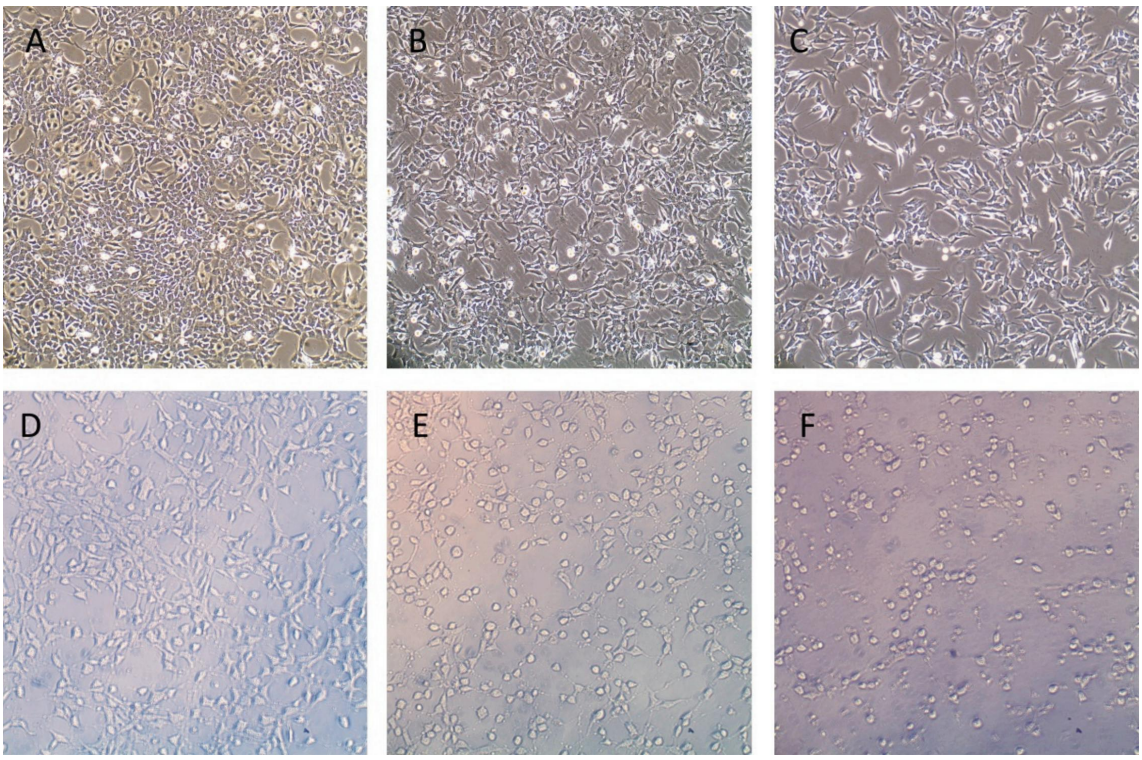

Figure 3 Inhibition of Gen–NLC on proliferation of SRA01/04 cells A: Untreated cells; B-F: Cells treated with different concentration of Gen-NLC for 6.25, 12.5, 25, 50 and 75 mg/L of Gen-NLC respectively (original magnification 100×).

3.93%, 56.83%±6.63%, 43.84%±6.80% and 35.9%±5.23% respectively, indicating the inhibitory effect of Gen-NLC on HLECs was a concentration-dependent way. The same trend of inhibitory effects was also seen in Gen group. There were significant differences in cellular viability between NLC group and Gen or Gen-NLC groups at concentrations of 12.5 to 75 mg/L (P<0.05 for Gen, P<0.001 for Gen-NLC compared with NLC group). Gen-NLC displayed stronger inhibitory effect on cellular proliferation compared to Gen with statistically differences in the 25 to 75 mg/L concentration groups (P<0.05). The fifty percent inhibiting concentration of Gen-NLC on HLECs proliferation was 37.93 mg/L. Given the results, the following experiments were performed with 25 and 50 mg/L Gen-NLC for 24h to evaluate the protection effect of Gen-NLC from PCO.

Effect of Nanoparticle on Expression of Ki67 mRNA and Protein in SRA01/04 Cells RT-qPCR demonstrated that Gen-NLC altered the mRNA levels of Ki67 compared to control cells. A significant down-regulation of Ki67 mRNA in HLECs was shown in Gen-NLC treated groups (P< 0.001) compared with the control (Figure 5). The effect was dose-dependent.

The expression of Ki67 proteins in HLECs were further examined by immunofluorescence under fluorescence light microscopy (Figure 6). These Ki67 proteins were localized in nucleus. After treated with 25 and 50 mg/L Gen-NLC for 24h, the proliferation marker Ki67 proteins in HLECs became faint compared to the control group.

DISCUSSION

PCO is caused mainly by secondary pathological progression

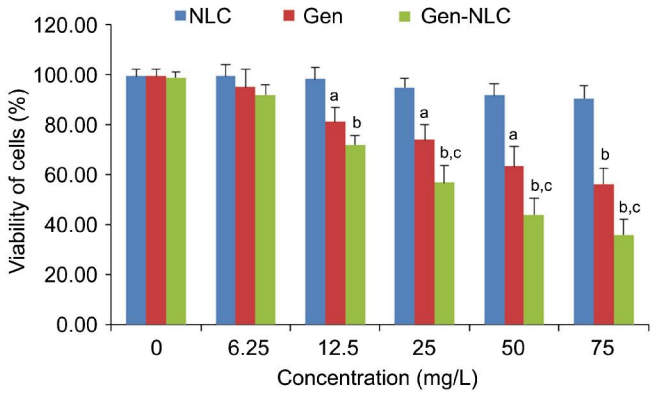

Figure 4 Inhibition of NLC, Gen and Gen-NLC on proliferation of SRA01/04 cells by CCK-8 assay [a]P<0.05, [b]P<0.001 compared with NLC group, [c]P<0.05 compared with Gen group.

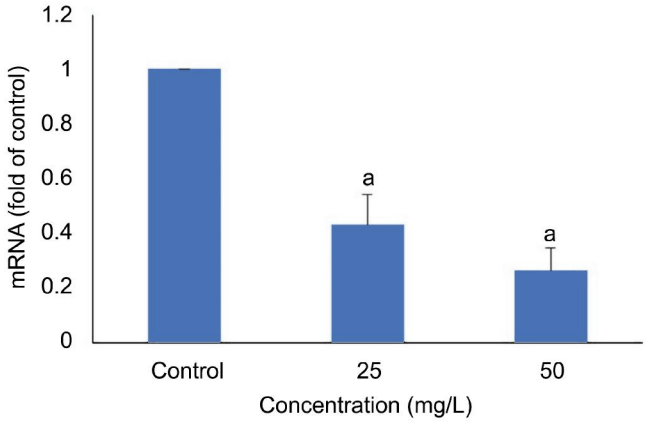

Figure 5 Inhibitory effect of Gen-NLC on expression of Ki67 in SRA01/04 cells [a]P<0.001, compared with the control cells.

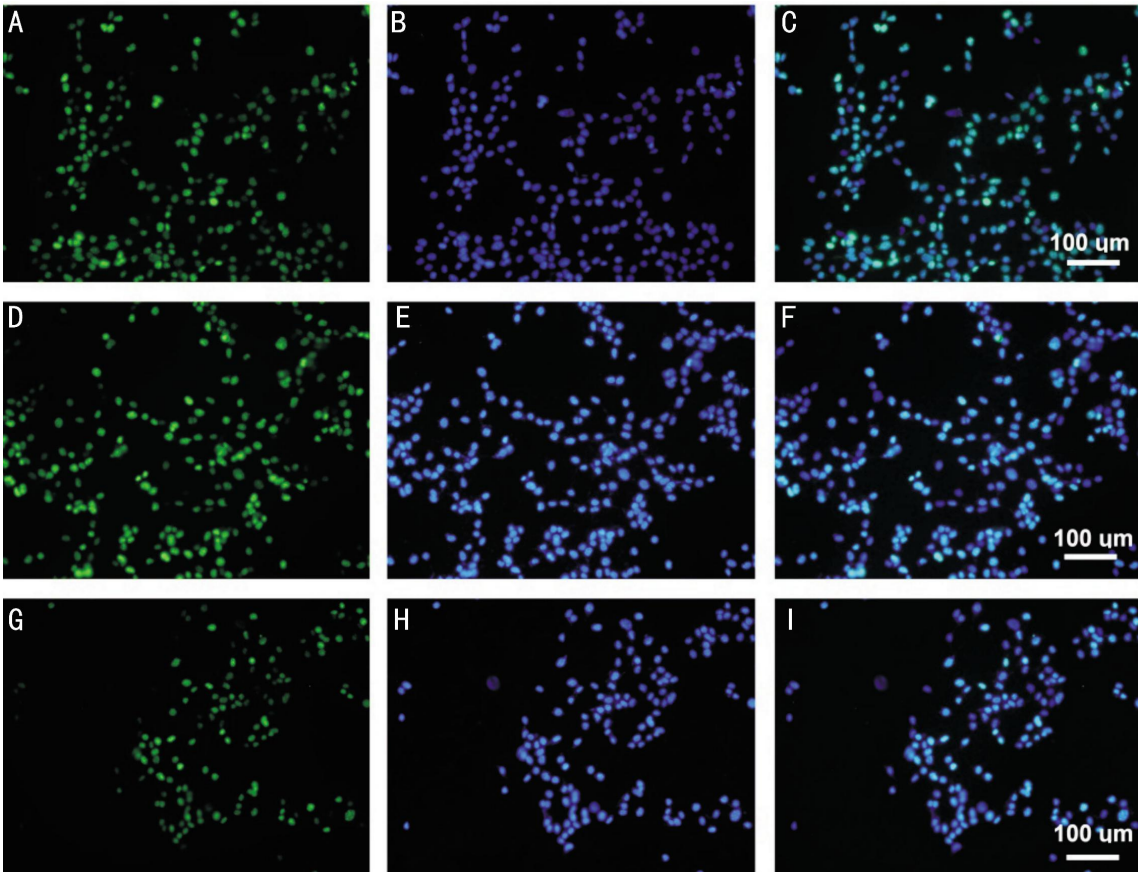

Figure 6 Inhibitory effect of Gen-NLC on expression of Ki67 protein in SRA01/04 cells A-C: Untreated cells; D-F: Cell treated with 25 mg/L Gen-NLC; G-I: Cells treated with 50 mg/L Gen-NLC. The nuclei stained by DAPI (blue).

of postoperative residual LECs, including proliferation, migration, EMT, and followed by wrinkling and fibrosis of the lens capsule. Pharmacological agent as a promising therapeutic tool might hinder the process and prevent the formation of PCO. Gen, as one of the most extensively studied isoflavones, has been found to have many properties such as weak extrogenic and antiestrogenic aspects, inhibitor of topoisomerase II, angiogenesis and protein tyrosine kinase, antioxidant effect and so on [18]. Although accumulating evidence has suggested that Gen has favorable beneficial effects on reducing formation of PCO *in vitro* [12], the therapeutic concentration may not be reached due to its poor water solubility and low concentration in aqueous humor[19]. It has been reported that prolonged ocular retention and sustained intraocular drug concentrations can be realized through delivering drug by *in situ* gel, microemulsion, microspheres, liposomes and slid lipid nanoparticles (SLN)[20]. NLC, a new generation of SLN, which is composed of a solid lipid matrix with certain content of liquid lipid, was put forward for its many advantages, *i.e.* controlled drug release, higher drug loading, drug targeting, good bioavailability and good biocompatibility due to the introduce of physiological and biodegradable lipids [21]. More and more studies [22-23] reported that NLC has been a good alternative as an ocular

drug delivery system for ibuprofen and flurbiprofen, which gave us an impetus to explore new method for treatment of other eye diseases.

The physicochemical properties of nanoparticles, including PS, ZP and surface characteristics have an essential impact on their biological performance. In our previous studies [24-26], Gelucire 44/14 and Solutol HS15 were used as emulsifier. Gelucire 44/14 is a semi-solid lipid with good drug permeability across skin, and these two emulsifiers are often used in the formulation of NLC. However, their toxicity to LECs could not be ignored especially in high concentration. As a result, in our experiment, Cremphor EL was added instead of ingredients mentioned above due to its slight cellular toxicity. The new formula Gen-NLC has mean PS of 80.12±1.55 nm. The tolerant PS for human eyes is less than 10 μm, yet smaller PS is more acceptable due to good translucency, less irritant sense and longer time residence. The polydispersity index was 0.11±0.02 suggested the Gen-NLC was homogeneous and the system was stabilized. The EE for Gen-NLC was high (92.3%±0.73%) which implied the drug loading was sufficient for this drug delivery system. From the results of *in vitro* release, despite a relative faster release in the first 2h, a sustained release of drug for the prolonged time afterwards were seen. About 70% drug was loaded in the outer shell of the nanoparticle, which accounted for the burst release, and 30% was cumulated in the inner part, which explained the sustained drug release pattern [27]. Compared to Gen, the biphasic drug release of Gen-NLC reflected an obvious controlled drug delivery, which qualified the success of our production of NLC.

The CCK-8 assay results that NLC hardly had any influence on cellular viability suggesting that the ingredients used as drug carrier were safe and the inhibitory effect was caused by Gen itself. Both Gen and Gen-NLC could inhibit the HLECs growth effectively in a dose-dependent way. However when the concentrations increased (from 25 to 75 mg/L), Gen-NLC revealed a much stronger inhibitory effect on cellular proliferation compared to Gen. The underlying reason was considered that Gen-NLCs were produced by covering Gen with different lipids by which improve the lipid solubility and biocompatibility of Gen. As a result, Gen-NLCs could penetrate the cell membrane more easily and had a higher drug concentration in cells. The expression of Ki67 mRNA and protein was significantly down-regulated after 24h treatment with 25 and 50 mg/L gen-NLC in SRA01/04 cells. All the results implied that Gen could inhibit the proliferation of HLECs and might have preventive impacts on the formation of PCO, since the proliferation of LECs was the first step for EMT. One of the tyrosine kinases families: Src family kinases have an essential role in the signaling pathways that regulate cell proliferation, migration, and EMT, and its inhibitor was investigated to inhibit the development of PCO in the chick lens capsular bag model[28].

In conclusion, tyrosine kinases signaling pathway was believed to be involved in the process of PCO and Gen-NLC could effectively inhibit the HLECs growth. Further studies are warranted to explore the *in vivo* drug release of Gen-NLC and its preventive effects and potential application on the formation of PCO.

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