·Basic Research·

Experimental study on the photodynamic treatment of choroidal neovasculization with nanophthaloc - yanine photosensitizer

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Abstract

• AIM: To investigate the therapeutic effects of nanophthalocyanine photosensitizers on an experimental rat choroidal neovescularization (CNV) model, as well as to evaluate the cytotoxicity of which on human retinal pigment epithelia (HRPE) and human retinal endothelial cells (HRECs).

• METHODS: Two types of photosensitizers, G_1-ZnPc$(COOH)_8$ and G_1-ZnPc $(COOH)_8$/m respectively, were administrated for photodynamic therapy (PDT) after a successful establishment of CNV model on Brown-Norway (BN) rats via fundus photocoagulation. The therapeutic effects of the two drugs were assessed through optical coherence tomography (OCT), fluorescein fundus angiography (FFA) and transmission electron microscopy (TEM). For cytotoxicity tests, cell counting kit-8 (CCK-8) assays and changes of mitochondrial transmembrane potential ($\triangle \Psi m$) were conducted on HRPE and HRECs after initial uptake of the two drugs.

• RESULTS: Both photosensitizers demonstrated an improvement of vascular leakage and closure of CNV 1 week after PDT as confirmed by fundus image, OCT, FFA and TEM. Two weeks after PDT, G_1-ZnPc $(COOH)_8$/m showed a better CNV closure effect versus G_1-ZnPc$(COOH)_8$ ($P<0.05$). A significant difference ($P< 0.01$) was found in uptake of the two drugs in HRPE and HRECs, with no difference between the drugs($P > 0.05$). Both photosensitizers showed cytotoxicity on HRPE, but G_1-ZnPc$(COOH)_8$/m induced a lower cell viability.

• CONCLUSION: G_1-ZnPc $(COOH)_8$/m mediated PDT is better than G_1-ZnPc $(COOH)_8$ in CNV closure and also have the advantage of fast metabolism leading to less side effect.

• KEYWORDS: phthalocyanine; carrier; nanoparticles; photodynamic treatment; retina pigment epithelial cells; choroidal neovascularization
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INTRODUCTION

Choroidal neovascularization (CNV) is an important pathological factor for age-related macular degeneration (AMD), pathological myopia, and severely diminishing visual acuity [1-3]. The CNV treatment methods currently include laser photocoagulation, photodynamic treatment (PDT), transpupillary thermotherapy (TTT), and the injection of anti-neovascularization drugs into the vitreous cavity [4-11]. PDT can act specifically on CNV through the targeting function of photosensitizers and two-fold irradiation of lesion regions with laser of specific wavelengths; this type of treatment presents nearly no damage on surrounding normal tissues. Hence, PDT is a mature, safe, and effective method for CNV treatment, and is particularly suitable for CNV at fovea centralis [12-13]. Phthalocyanine is a type of photosensitizer that has been extensively studied in recent years. It offers the advantages of good compatibility with body tissues, light phototoxicity on skin, as well as high stability and photodynamic activity.

In this study, a free phthalocyanine complex was compared with a photosensitizer. A new nanophthalocyanine photosensitizer was synthesized by introducing amphiphilic block copolymers as the drug-transport carrier. The nanophthalocyanine photosensitizer was used as the photosensitizer for the experiment, in which it was irradiated with laser of a specific wavelength. The effects of PDT mediated by the two types of photosensitizer on the CNV of BN rats and in vitro culture of HRPE cells were

observed. In addition, the structure activity relationship of the nanocarriers was evaluated. The results serve as experimental bases and theoretical foundation for the development of efficient, low-toxicity, and inexpensive photosensitizers for CNV treatment.

MATERIALS AND METHODS

Materials The nanophthalocyanine photosensitizer G_1-ZnPc $(COOH)_8$/m and corresponding free phthalocyanine complexes G_1-ZnPc $(COOH)_8$ were synthesized and characterized by Associate Professor Peng Yiru of the Chemical and Material College of Fujian Normal University. The photosensitizers were then stored at 4℃ away from light exposure. Subsequently, they were diluted with phosphate buffered saline(PBS) to a series of concentrations (ready for use) and filtered using a 0.22μm Millipore membrane before use.

HRPE cell strain D407 and human retinal capillary endothelial cell(HREC) strain H6530 were purchased from Shanghai Ruicong Laboratory Equipment Co., Ltd.

The CNV model rats and BN rats were provided by Shanghai Super B&K Laboratory Animal Co., Ltd (Production License: SCXK (Hu) 2008-0016). CNV was induced by laser 532 (exposure interval: 100mS; power: 160-180mW). After successful modeling, 36 BN rats were randomly divided into six groups: the blank control, pure irradiation, pure G_1-ZnPc $(COOH)_8$ medication, pure G_1-ZnPc$(COOH)_8$/m medication, PDT1（G_1-ZnPc$(COOH)_8$-PDT), and PDT2（G_1-ZnPc$(COOH)_8$/m-PDT) groups.

Methods

Pharmacokinetics of intake of G_1-ZnPc $(COOH)_8$ and G_1-ZnPc$(COOH)_8$/m by HRPE and HREC cells HRPE and HREC cell strains were cultured to the third generation, to which G_1-ZnPc$(COOH)_8$ and G_1-ZnPc$(COOH)_8$/m (final concentration, 1.0×10^{-5}mol/L) were added, respectively. The same volume of PBS solution was added to the control group. Trition X-100 (2.0%) was added to split the cells. The products were centrifuged at a low temperature, after which the supernatant was collected. The quantity of ZnPc $(COOH)_8$ and G_1-ZnPc $(COOH)_8$/m in the cells was inspected by UV-vis spectroscopy.

Comparison of photodynamic activities of G_1-ZnPc $(COOH)_8$ and G_1-ZnPc $(COOH)_8$/m G_1-ZnPc$(COOH)_8$ and G_1-ZnPc $(COOH)_8$/m with a final concentration of 1.0×10^{-5}mol/L was added into the HRPE cell culture. The former was incubated for 3 hours and the latter for 2 hours. The following irradiation conditions were applied: laser emission (LD-670 semi-conductor laser machine) at 670nm with a power density of 60mW/cm^2 and energy density of 2.0 J/cm^2. No photosensitizer (the same volume of PBS was added instead, then irradiated after 3 hours of incubation) was added to the photo control group, while the control group contained 1.0×10^{-5}mol/L G_1-ZnPc $(COOH)_8$ and G_1-ZnPc $(COOH)_8$/m (incubated for 2-3 hours with only the sensitizer; no laser irradiation was applied).

Detection of the cell viability of HRPE cells by CCK-8 assay CCK-8 was added into the HRPE cells after photodynamic therapy (PDT) treatment. The OD values at a wavelength of 450 nm on were measured on a microplate reader, after which the inhibition rate was calculated as follows: Inhibition rate = (OD of untreated group -OD of experimental group)/OD of untreated group ×100%).

Analysis of the mitochondrial transmembrane potential of HRPE cells after PDT JC-1 dying liquid (1.0mL) was added, incubated for 20 min at 37℃, and washed twice with JC-1 dying buffer (1×). A cell culture solution (2.0mL) containing serum was added. Cells were taken from three seeded holes in each group, fixed with protein glycerin, and observed under laser scanning confocal microscope (LSCM).

Photodynamic treatment of CNV of BN rats The photosensitizer was slowly injected through the caudal vein; G_1-ZnPc $(COOH)_8$ was injected into the PDT1 group, and G_1-ZnPc $(COOH)_8$/m was injected into the PDT2 group (dosage: 1.0mL/kg). Thirty minutes after injection, the CNV region around the papilla was irradiated with a 689 nm laser with a light spot diameter of 3 500-4 000μm to cover all the CNV spots. Laser energy density and output power were fixed to 50J/cm^2 and 600mW/cm^2, respectively. The exposure time was 83s.

Fundus observation and OCT analysis At three time points of 1 day, 1 week, and 2 weeks after laser irradiation, the fundus of the BN rats was observed and photographed with an indirect ophthalmoscope. If the OCT inspection shows that subretinal fluid is reduced or disappears, and the strength of the CNV reflection region is reduced, the lesion is considered reduced. If the subretinal fluid is reduced and the strength of the CNV reflection region does not increase, the lesion is regarded as stable. If the subretinal fluid is increased and the strength of the CNV reflection area is increased, this phenomenon is regarded as CNVdevelopment.

FFA performance Into the abdominal cavity, 0.2mL (1.0mL/kg) of 10% fluorescein sodium was injected, and then photos of the blood vessels were taken under a fundus angiographer. The dynamic development and leakage of CNV were observed. The evaluation criteria used were as follows: CNV complete closure at the focus form; no changes in size and fluorescence intensity at early and late stages of CNV; incomplete CNV closure if there is a slight leakage of fluorescein at late stages; CNV expansion if there is fluorescein leakage and lesion expansion at late stages.

Table 1 HRPE and HREC cells with the aggregate amount of 5×10^6, intake quantity of G_1-ZnPc(COOH)$_8$ and G_1-ZnPc(COOH)$_8$/m (10^{-6}mmol)

Incubation period (Hr)	HRPE		HREC	
	G_1-ZnPc(COOH)$_8$	G_1-ZnPc(COOH)$_8$/m	G_1-ZnPc(COOH)$_8$	G_1-ZnPc(COOH)$_8$/m
1	2.16±0.03	3.09±0.02	0.38±0.01	1.47±0.02
2	4.63±0.05	5.26±0.01	1.41±0.03	2.34±0.01
3	5.63±0.03	4.46±0.02	2.15±0.03	1.85±0.03
4	2.52±0.04	2.32±0.03	1.65±0.03	0.78±0.03
5	1.44±0.03	1.88±0.01	0.73±0.05	0.25±0.01
6	0.45±0.01	0.75±0.03	0.55±0.04	0.28±0.01

Transmission electron microscopy After the above-mentioned FFA inspection, the rats were sacrificed. The eyeball was excised and then cut open along its equator 30 minutes after pre-fixation. The cornea, iris, lens, and vitreous body were removed. Then, 4℃ 3% glutaraldehyde and -1.5% paraformaldehyde were added into the reserved eye cup for full fixation. After laser spots were identified using an anatomic microscope, tissue lumps were cut and then subjected to post-fixation, lump dying, dehydration, embedding with epoxy resin 618, slicing, and dying with uranyl acetate and citric acid for 5 minutes. The ultra-micro structural changes of CNV and surrounding tissues were observed using a transmission electron microscope (HU-12A, Hitachi, Japan).

Statistical Analysis All statistical analyses were carried out with SPSS 13.0. For the metering data, the t test was adopted, while the χ^2 test was used to measure counting data. A difference of P<0.05 was regarded as statistically significant.

RESULTS

Pharmacokinetics of the intake of G_1-ZnPc (COOH)$_8$ and G_1-ZnPc (COOH)$_8$/m by HRPE and HREC cells Table 1 lists the intake quantity of G_1-ZnPc (COOH)$_8$ and G_1-ZnPc (COOH)$_8$/m by the HRPE and HREC cells after different periods of incubation. The factors that drove intake quantity in the four groups were analyzed. The two types of cells showed F=9.108 and P<0.001, hence the significant difference between the cells. The two types of drugs showed F=0.44 and P>0.05; thus, they were nonsignificantly different. The line graph is plotted in Figure 1, and it can be seen that 2-3 hours of incubation was the peak for photosensitizer intake. G_1-ZnPc (COOH)$_8$/m reached its intake peak one hour ahead of G_1-ZnPc(COOH)$_8$.

Survival rate of HRPE cells after PDT PDT mediated by G_1-ZnPc(COOH)$_8$ and G_1-ZnPc(COOH)$_8$/m in each group showed obvious killing and damaging effects on the HRPE cells, with significant difference compared with the control group (P<0.01), as detailed in Table 2.

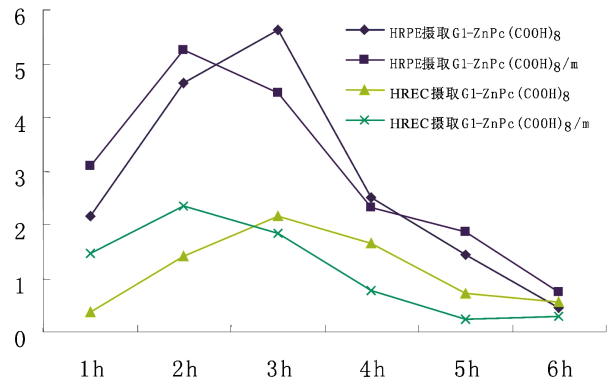

Figure 1 Intake quantity of G_1-ZnPc (COOH)$_8$ and G_1-ZnPc (COOH)$_8$/m by HRPE and HREC cells

Table 2 CCK-8 assay results for HRPE cells 24 hours after PDT (n=9)

Group	OD(mean±s)	Inhibition rate (%)
Blank control group	0.85±0.01	—
G_1-ZnPc(COOH)$_8$ group	0.74±0.02	12.94
G_1-ZnPc(COOH)$_8$/m group	0.68±0.02	20.00
Pure irradiation group	0.79±0.02	7.06
G_1-ZnPc(COOH)$_8$-PDTgroup	0.37±0.03★	56.47
G_1-ZnPc(COOH)$_8$/m-PDT group	0.17±0.02★▲	80.00

★Compared with the control group: P<0.05; comparison of ★▲ with ★: P<0.05.

Changes in mitochondrial membrane potential after PDT In the G_1-ZnPc(COOH)$_8$-PDT and G_1-ZnPc(COOH)$_8$/m-PDT groups, the cells showed basically only strong green fluorescence given that the potential of the mitochondrial membrane dropped and red fluorescence was relatively weak when the two channels were open. The control group showed uniform orange fluorescence(Figure 2).

Fundus performance and OCT results The retinal fundus inspection of the rats in each group 1 day after PDT treatment showed that the retina had no fresh edema, bleeding, or exudation (Figure 3A). The OCT inspection demonstrated no observable change in CNV thickness (Figure 3B). The CNV region of the retina in groups PDT1 and PDT2 had slightly enhanced reflection 1 week after

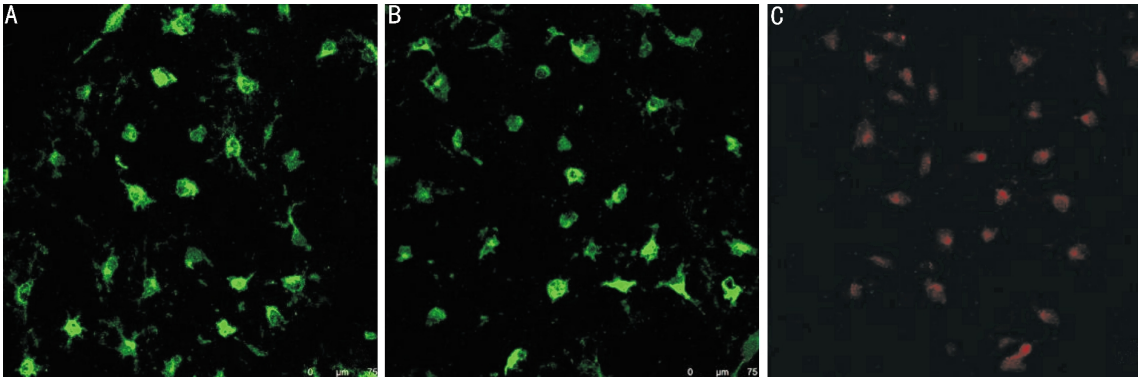

Figure 2 Changes in mitochondrial membrane potential in cells under LSCM A: G_1-ZnPc(COOH)$_8$-PDT group; B: G_1-ZnPc(COOH)$_8$/m-PDT; C: Control group

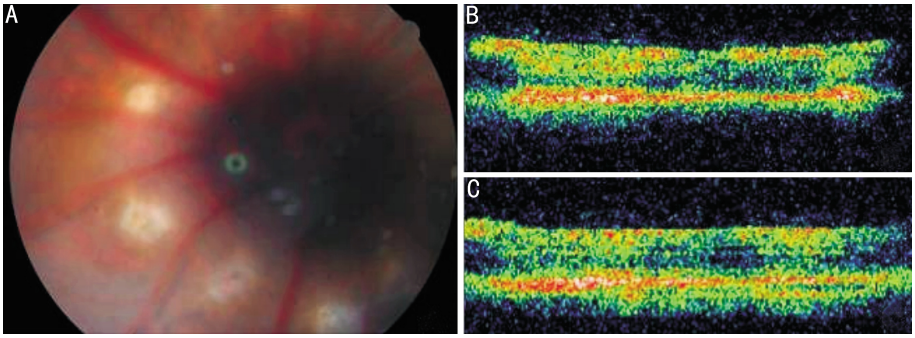

Figure 3 Fundus and OCT changes after PDT treatment A: 1 day, no fresh edema, bleeding, and exudation in the retina; B: 1 day, no apparent change in CNV thickness; C: 2 weeks, retina gets thinner to different extents

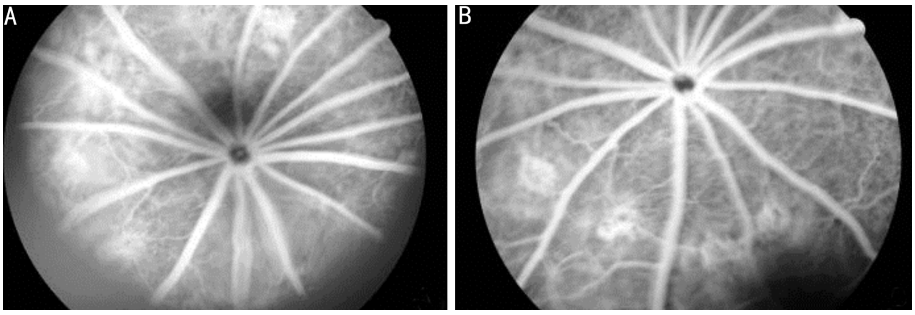

Figure 4 FFA changes after PDT treatment A: No apparent change in fluorescence leakage after 1 day; B: Weakened fluorescence leakage of CNV in the treatment group after 1-2 weeks

PDT treatment; the two groups exhibited similar performance. The OCT result showed that the retina became thinner to different extents; 2 weeks after PDT treatment, the two treatment groups had clearly observable enhanced reflection on the retina compared with the four control groups: disappearance of edema in the original laser-irradiated region, pigment disorder, cicatrix formation, and clear boundary were observed (Figure 3). No apparent changes were found in the fundus and OCT inspections of the blank control, pure medication, and two pure irradiation groups.

FFA performance FFA revealed that 1 day after PDT treatment, fluorescence leakage at the CNV region registered no apparent changes (Figure 4A). It also showed that 2 week after PDT treatment, CNV fluorescence leakage was rectified to different extents in the PDT1 group (Figure 4B). Fluorescence leakage at the CNV region of the four control groups exhibited no apparent change, and fluorescence leakage was reduced in a few occasions. The fluorescence leakage of the two experimental groups as detected by FFA inspection is shown in Table 3. The difference between groups PDT1 and PDT2 1 week after PDT treatment showed no statistical significance (P>0.05). The CNV closure rate in PDT2 was clearly higher than that in PDT1 2 weeks after treatment. The difference between these groups also showed no statistical significance (P<0.05).

Transmission electron microscopy Choroidal vascular endothelial cells, as well as the tubes and cavities enclosed by the cells, in the blank control, pure medication, and pure irradiation groups could still be clearly observed at different

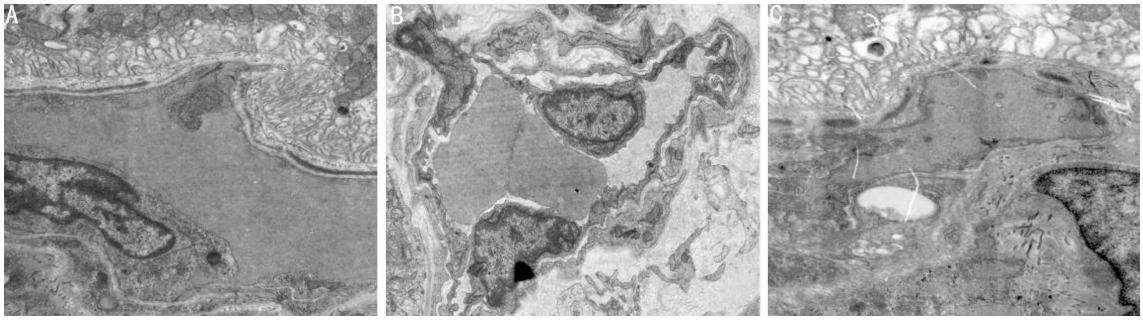

Figure 5 Choroidal vascular ultra-micro structure A: Blank control group (×8 000); B: PDT1 treatment group (×8 000); C: PDT1 treatment group (×10 000)

Table 3 Comparison of improvement rates (%) of CNV leakage in different PDT groups

	1 d after treatment		1 wk after treatment		2 wk after treatment	
	Improvement rate	Closure rate	Improvement rate	Closure rate	Improvement rate	Closure rate
G_1-ZnPc(COOH)$_8$-PDT	0	0	56.47±11.31	21.65±9.27	52.68±6.27	23.32±6.25
G_1-ZnPc(COOH)$_8$/m-PDT	0	0	61.38±9.25	24.33±8.36	51. 12±8.77	31.69±8.47

inspection times (Figure 5A). Choroidal blood vessel damages could be seen in groups PDT1 and PDT2 1 d after PDT: swelling of the vascular endothelial cells, as well as swelling of mitochondria cristae and vacuoles in some blood vessels, was observed. This phenomenon became apparent over time. The choroidal blood vessel cavity in the two treatment groups became narrower or obstructed 2 weeks after PDT; the thrombus was formed in some blood vessels and some choroidal vascular endothelial cells were shown as nuclearatypia (Figure 5B). Different quantities of fibroblast cells and collagen tissues were found in the choroidal tissues (Figure 5C).

DISCUSSION

PDT involves inducing photochemical reaction under light irradiation at relevant wavelengths to destroy lesions (slight damage is sustained by surrounding normal tissues) exploiting the capability of photosensitizers to accumulate and remain selectively in the tumor cells, CNV, and other actively proliferating tissues. Since its application in curing choroidal CNV eye diseases in 1995, an increasing number of reports that confirm the effect of PDT in curing CNV have been published. The effect of PDT depends mainly on three variables: the type of photosensitizer, its concentration in the target tissue, and laser irradiation time and laser energy. The greater the dosage of the photosensitizer, the higher the energy from laser irradiation, and the longer the irradiation time, the stronger the effect of PDT. If the laser energy and irradiation period are the same, the factors affecting the PDT effect will be the type of photosensitizer used and its concentration in the target tissue. In this study, the effects of two types of photosensitizers (free metallic phthalocyanine and polymer nanoparticle-carrying photos-ensitizers such phthalocyanine) on the PDT treatment of CNV were observed by establishing the same laser energy and irradiation period.

A phthalocyanine-type photosensitizer has a definite composition and clear molecular structure, and is regarded as a photosensitizer that presents considerable prospects for development and extensive application. The water solubility of a phthalocyanine-type photosensitizer is an important constraint on its clinical application; the ratio of hydrophilia and lipophilia must be modified by certain methods such as DDSs[14-15]. Third-generation nanophotosensitizers introduced in carrier takes us to a new direction for PDT study: changing the pharmacokinetics and *in vivo* distribution of drugs by changing the physico-chemical properties of nanocarriers (*e.g.*, particle diameter, charge, etc.) to achieve passive targeting. Nanocarriers present the following advantages [16]: 1) Lipophilic and amphiphilic molecules are difficult to administer through veins because of poor water solubility. Photosensitizers carrying hydrophilic nanocarriers make drug administration possible; 2) Lipophilic and amphiphilic molecules easily aggregate spontaneously in plasma and obstruct capillaries, and monomer photosensitizers exhibit the strongest photodynamic effect. Nanocarriers carry photosensitizer and reach the focus as monomers, hence, the drug effect is enhanced. 3) Nanocarriers have enhanced permeability and retention effect during *in vivo* circulation; 4) Nanocarriers cannot easily pass through the capillary walls of normal tissues, which reduces distribution in normal tissues and decreases the toxic and other side effects of drugs. PLL-PEG-PLL synthesized in this study contains a polymer nanocarrier to enable carrying of the aromatic oxide dendritic phthalocyanine and form nanoparticles with a diameter of 50nm; this changes the water solubility of the

phthalocyanine photosensitizer. The HRPE cells exhibited a more obviously enhanced intake rate of the two types of metallic phthalocyanine photosensitizers than did the HREC cells possibly because of the engulfing feature of the HRPE cells. As to the two types of photosensitizers (polymer nanoparticles carrying the aromatic oxide dendritic phthalocyanine and corresponding free phthalocyanine), they both demonstrated strong intake performance, with no significant difference in total intake quantity. The former reached its intake peak 1 hour ahead, indicating that cell intake speed increases after the introduction of nanocarriers. This result is consistent with those of international studies. The intake of G_1-ZnPc$(COOH)_8$ and G_1-ZnPc$(COOH)_8$/m by the HRPE cells increased as the incubation period increased, reaching its peak at 2-3 hours. After this, the concentration in the cells gradually decreased, indicating that the HRPE cells have the advantage of fast intake and discharge of G_1-ZnPc $(COOH)_8$/m. To observe the damage of photodynamics mediated by G_1-ZnPc$(COOH)_8$ and G_1-ZnPc $(COOH)_8$/m on the HRPE cells, irradiation after incubation for 2-3 hours was chosen and demonstrated by CCK-8 assay. PDT mediated by G_1-ZnPc $(COOH)_8$/m exhibited strong photodynamics, and its inhibition rate of HRPE cell proliferation was clearly higher than that of G_1-ZnPc $(COOH)_8$.

Mitochondria play an important role in cell apoptosis induced by PDT, which is mainly related to the introduction of a large amount of photosensitizers in the mitochondria[17-18]. Free endogenous photosensitizers inside mitochondria or exogenous photosensitizers bonded onto the mitochondrial membrane can increase the permeability of the membrane under accurate action. The membrane potential disappears, and protein factors related to the induction of cell apoptosis in the abdominal cavity are released to cytoplasm and cause subsequent damage. JC-1 is a fluorescent probe used to detect the potential of mitochondrial membrane $\triangle\Psi m$ in cells [19-20], tissues, or purified mitochondria. When the mitochondrial membrane has high potential, JC-1 accumulates in the mitochondria matrix to form polymers, and emits red fluorescence under a fluorescent microscope. When the mitochondrial membrane has low potential, JC-1 exists as a monomer and cannot accumulate in the mitochondrial membrane; it emits green fluorescence. On the basis of these assumptions, the changing potential of the mitochondrial membrane can be detected by changing the fluorescence color, and the common index is the ratio of red and green fluorescence (to measure the ratio of depolarized mitochondria). A potential reduction in the mitochondrial membrane is a milestone in the early stages of cell apoptosis, and can be detected by the change in JC-1 fluorescence from red to green. The change in JC-1 fluorescence from red to green can also be regarded as an index for the early stage of cell apoptosis. This study employed LSCM to analyze the effect of PDT mediated by G_1-ZnPc$(COOH)_8$ and G_1-ZnPc$(COOH)_8$/m on the potential of mitochondrial membrane in the HRPE cells. The potential of the mitochondrial membrane in the cells was reduced or disappeared after PDT. That is, PDT mediated by G_1-ZnPc $(COOH)_8$ and G_1-ZnPc $(COOH)_8$/m possibly induces the apoptosis of HRPE cells through the mitochondria.

According to our experimental results, applying G_1-ZnPc $(COOH)_8$ and G_1-ZnPc $(COOH)_8$/m as the photosensitizers led to a higher CNV closure rate than did the control groups, further confirmed by fundoscopy, OCT inspection, and TSM inspection after PDT. As the duration was extended to 2 weeks, the CNV closure rate in the experimental group with nanophotosensitizers was higher than in the experimental group with free phthalocyanine, with significant difference. This result may be attributed to the following factors: 1) Hydrophilic nanocarriers can smoothly reach the target through veins while carrying drugs, hence, the enhanced medication effect. 2) The strong activity and engulfing capacity of CNV cells result in the high permeability of CNV blood vessels and incomplete development of the lymphatic drainage system. When injected through veins, nanoparticles cannot overflow from the endothelial tissue blood vessels with gaps in CNV and still remain in tumor cells. Thus, the period at which drugs remain in CNV is prolonged, i.e., enhanced permeability and retention effect, as well as improved targeting property of nanophotosensitizers on CNV, occurs [21-23]; 3) Nanocarriers are a new type of release control system. Their ultra-micro size enables direct action on cells and control over continuous and slow release of drugs. Therefore, nanocarriers effectively prolong the time of action, maintain effective concentrations of drugs, and improve the stability of drugs, which results in high local concentration.

We conclude that the introduction of nanocarriers improves the targeting property and photosensitive activity of photosensitizers on CNV in contrast to free phthalocyanine. However, an important criterion for screening of PDT photosensitizers is the singlet oxygen quantum yield rate of the photosensitizer [24],which characterizes sensitization capacity and competes with fluorescence intensity [25]. This study has confirmed that a nanophthalocyanine photosensitizer is characterized by low fluorescence intensity, and consequently, a high singlet oxygen quantum yield rate. We intend to examine the singlet oxygen quantum yield rate of *in vitro* and *in vivo* free

phthalocyanine complexes and nanoparticles with carriers to further quantify the structure activity relationship of nanocarriers.

In conclusion, PDT mediated by the phthalocyanine photosensitizer induced cell apoptosis through the mitochondria. The photosensitizer with nanocarriers presented fast intake and metabolism, strong photodynamic effect, and observably higher cytotoxic effect on the HRPE cells. The *in vivo* CNV closure rate induced by laser was visibly higher than that observed in the experimental group with free phthalocyanine.

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