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Original Article

The effect of all trans retinoid nanostructured lipid carriers on cytokines and chemokines releasing induced by zymosan

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Abstract: *Objective:* The inflammatory process of infectious fungus is relative to macrophages, helper T cells, neutrophils, dendritic cells, and Treg cells, and is characterized by proinflammatory, chemotactic and regulatory cytokines. Zymosan was component of the yeast cell wall which stimulated macrophages producing proinflammatory molecular. All trans retinoid (ATRA) was well known for its inflammation suppressing effect. We sought to investigate the role of ATRA nanostructured lipid carriers (NLC) in the zymosan induced cytokines (IL-1β, TNF-α, IL-6, IL-8) and to investigate the approach of ATRA incorporated in the nanoparticles. *Methods:* ATRA-NLC was prepared by the method of emulsification process from scratch and the light fastness research was performed by IPA-ATRA detection. Then the particle size and concentration of ATRA-NLC was analyzed. Four to seven passages in monolayer cultured rabbit corneal fibroblasts(RCFs) were prepared for experiments. The concentrations of IL-1, TNF-α, IL-6, IL-8 induced by zymosan in culture supernatants were also determined with the use of ELISA kits. The ability of cells proliferation was assayed using CCK-8 according to the instructions. *Results:* The calculated ATRA-NLC solution concentration is 17.9 μg/mL. The value of Peak area was detected by HPLC. ATRA-NCL showed good light resistance. ATRA-NLC average particle size was detected by the particle size analyzer. The mean diameter was 200 nm. RFCs were incubated in the absence or presence of ATRA-NLC in the additional presence of zymosan for 24 h. ATRA-NCL can inhibit the release of IL-1ß, TNF-α, IL-8, IL-6 in a dose manner. Different concentrations of ATRA-NCL at different time points showed no inhibition effect on the ability of cells proliferation. ATRA-NCL at different time points showed no inhibition effect on the ability of cell viability. ATRA-NCL showed mild stimulation of cell viability. ATRA-NCL showed no effect on cell proliferation. *Conclusions:* ATRA-NLC can inhibit the cytokines including RFCs IL-1ß, TNF-α, IL-8, IL-6 releasing induced by zymosan.

Keywords: Nanostructured lipid carriers, zymosan

Introduction

Fungal keratitis (FK) is a worldwide visual impairment disease in lower income countries, particularly in agricultural areas [1]. Contact lens wear was the main risk factor in the USA and other industrialized countries, and traumatic injury was the main risk factor in developing countries [2]. The most effective treatments of opportunistic infections, especially less common fungi remain unknown. Portunistic fungi such as Fusarium spp. or Scedosporium sp have been showed limitations of the antifungal drugs. New and effective therapies should be explored [3]. This infectious fungus initiates the innate immune and the adaptive immune response. The inflammatory process is related to several immune cells, and is associated with proinflammatory, chemotactic and regulatory cytokines [4]. Zymosan is a ligand found on the surface of fungi, like yeast. Zymosan is prepared from yeast cell wall. In macrophages, zymosan induced the responsing of proinflammatory cytokines [5]. The levels of these cytokines and chemokines of MIP-1α, MIP-1β, interleukin (IL)-1α, IL-1β, and tumor necrosis factor (TNF)-α by neutrophils were induced by zymosan [6]. Zymosan-induced arthritis and febrile

response in rats depends on the centrally acting pyrogenic cytokines TNF-α, IL-1β, and IL-6 [7, 8]. Proinflammatory cytokines as IL-6 and PGE_2 occurred with vestibular strains after C albicans, C glabrata, and zymosan challenges, resulting in the ability to predict IL-6 and PGE_2 production by genital tract location [9]. IL-1β and chemotactic cytokines, such as MIP-2, recruit PMN in response to bacterial infection. IL-1β and TNF-α are elevated in the early stage of aspergillus and fusarium corneal infections. The Th1 cytokines have an immunity protection effect in the early stage of infection. Immunity initiation is essential in the early stage of infection, and immune intervention should be underlied in the late stage for the severe host response induced by cytokines in the late stage of infection thus leads to corneal destruction and perforation. Th1/Th2 cytokines play key roles in immune responses to Leishmania major by controlling macrophage activation for NO production and parasite killing [10]. Because the inflammatory lesions of stromal keratitis are primarily orchestrated by Th1 cells, and to a lesser extent by Th17 cells, inhibiting their activity represents a useful form of therapy [11]. IL-4, IL-10 cytokines may be protective during Gram-positive corneal infection and therefore may be useful for adjunct therapies in the treatment of this disease [12]. Agents with immunosuppressive effects may be a good candidate for treatment of keratitis [13]. Nu-clear receptors are ligand-activated transcription factors linking lipid signaling to the expression of the genome. These receptors and their activating lipid ligands in immunopathologies combined with complementary and focused translational and clinical research will be crucial for the development of new therapies to target nuclear receptors [14]. ATRA and 9-cis-retinoic acid (9-cis RA) promote IL-4, IL-5 and IL-13 synthesis, while decreasing IFN-gamma and TNF-α expression by activated human T cells. These results strongly support a role for RAR-alpha engagement in the regulation of genes and proteins involved with human T cell activation and type 2 cytokine production [15]. Th1 cells respond to corneal destruction, while Th2 cells respond less to corneal destruction. ATRA decreases the destruction of inflamed tissue by regulating the ratio of Th1/Th2 cytokines. Decreasing of Th1 cytokines and increasing of Th2 cytokines is potent for the control of fungi keretitis [16]. Although the conventional causes are categorized into a variety of pathogens, confusion in the etiological diagnosis superadded with the problem of increasing resistance to the older anti-microbials often leads to a delay in appropriate therapy [17]. Different formulation strategies like suspension, ointment, gels, nanoparticles, implants, dendrimers and liposomes have been employed in order to improve drug permeation and retention by evading rate limiting factors at the site of absorption [18]. Ophthalmic formulations for the treatment of ocular pathologies are for topical administration, this lead to low bioavailability, a reduced therapeutic effect. The use of polymeric and lipid nanoparticles has demonstrated to promoting the increase in the precorneal residence time and the ability to penetrate through the ocular tissues, enhancing the therapeutic efficacy of ophthalmic formulations [19]. NLCs are a potential controlled release formulation for lercanidipine hydrochloride and may be a promising drug delivery system [20]. It is essential to explore appropriate drug. ATRA-NLC was prepared in our experiment and was investigated its effect on cytokines producing of RFCs induced by zymosan.

Materials and methods

ATRA-NLC preparation

Emulsification process from scratch method was used to prepare the ATRA-NLC. Oil phase: weight 1.5 mg, whales wax palm 150 mg, 3.6 mg, lecithin BHT 0.1 mg, oleic acid 37.5 mg, and 112.5 mg soybean oil accurately, then mixed and preheat to 70°C. Water phase: 8 ml poly yamanashi resin 80 and 92 ml distilled water preheat to 70°C. The water phase mixture was mixed into the oil phase mixture with magnetic stirrer under the speed of 1400 RPM stir for 1 minute. In order to control the droplet size in the nanometer range, the mixture of the ultrasonic cell crusher under 25°C, 40% in 10 seconds interval by the probe type ultrasonic generator ultrasonic treatment for 5 minutes. With 1 N NaOH to adjust the pH value of 8 ± 0.1 preparations, then through a 0.22 micron filter membrane filtration sterilization. The preparation under 4°C was stored in dark sealed containers.

ATRA-NLC light fastness research

To study the photodegradation of ATRA-NLC, samples were placed in a 1 ml glass tube, and use the ZF-type I ultraviolet analyzer (wavelength is 320-400 nm) under 50 centimeters

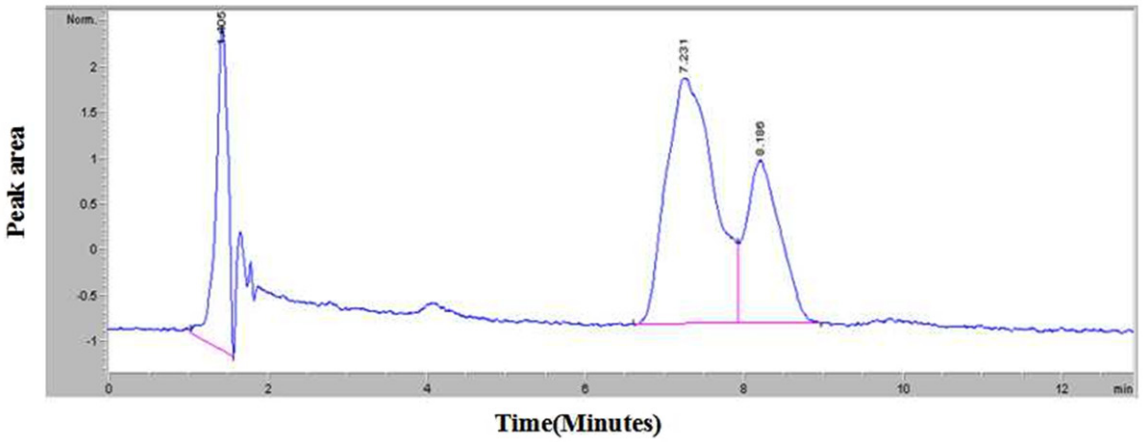

Figure 1. The value of peak area was detected by HPLC. The calculated ATRA-NLC solution concentration is 17.9 μg/mL. Quantitative determination of ATRA can get a good linear relationship between the range of 0.01~100 μg/mL. calibration curve Y = 61.82 + 20.33 X R^2 = 0.996.

for 4 hours. Diluted with Isopropyl (IPA) respectively, such as setting the time interval for (1, 2 and 4 hours) of eighty microliter sample. The quantity of ATRA by HPLC analysis. In order to compare, IPA-ATRA was detect under the condition of the same illumination as ATRA mixture samples for three times.

Cell isolation

New England white rabbits (bodyweight, 2.0 to 2.5 kg) were obtained from Animal department of Jilin University. This study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Animal Experimental Committee of Jilin University. Rabbit corneal fibroblasts (RCFs) were isolated and maintained as described previously [21]. The enucleated eye was washed with DPBS containing antibiotic-antimycotic mixture, the endothelial layers of the excised corneas were removed mechanically, the remaining corneal tissue was incubated with dispase (2 mg/ml, in MEM) for 1-2 h at 37°C. After mechanical removal of the epithelial sheet, the remaining tissue was treated with collagenase (2 mg/ml, in MEM) at 37°C until single-cell suspension of corneal fibroblasts was obtained. The isolated corneal fibroblasts were cultured under a humidified atmosphere of 5% CO_2 at 37 C in 60-mm culture dishes containing MEM supplemented with 10% FBS. Proliferating cells were harvested for experiments at the subconfluent stage after four to seven passages in monolayer culture.

Assay of cytokines and chemokines release

30000/ml cells cultured in 24-well plates until they achieved confluence were incubated first for 1 day with Serum-free MEM alone. For evaluation of the effects of ATRA-NLC on zymosan-induced cytokine or chemokine release, serum-deprived RFCs were incubated in the absence or presence of ATRA-NLC in the additional presence of Zymosan for 24 h. The culture supernatants were collected, centrifuged at 1500 r for 5 minutes to remove debris, and frozen at -80°C until subsequent measurement of cytokine and chemokine concentrations with the use of a cytokine assay system. Standard proteins were dissolved in MEM for producing of standard curves. The concentrations of cytokines and chemokines in culture supernatants were calculated. The concentrations of IL-1ß, TNF-α, IL-6, IL-8 in culture supernatants were also determined with the use of ELISA kits.

Cytotoxicity assay

The ability of cells proliferation was assayed using CCK-8 (Biyuntian Company, China) according to the instructions. RFCs were seeded at a density of 10000 cells/well in 96-well with 0.2 ml MEM with or without 0.001, 0.01, 0.1, 1 μmol/L ATRA. CCK8 was detected at 6, 12, 24, 36 and 48 h. the plate was read by a Microplate Reader.

Cell proliferation assay

Cells (10000 per well) seeded in a 96-well plate were incubated in MEM with or without ATRA-

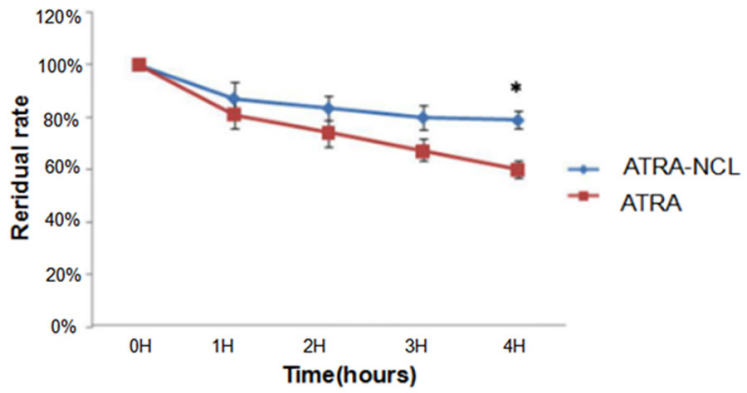

Figure 2. Residual rate = (CN/CI) × 100%. CN is the concentration of ATRA and ATRA-NLC measured in the sample; CI is the initial concentration of ATRA and ATRA-NLC in the sample. Under the same illumination condition, ATRA and the rate of the remaining ATRA-NLC were compared. *P < 0.05 versus ATRA residual rate (Dunnett's test).

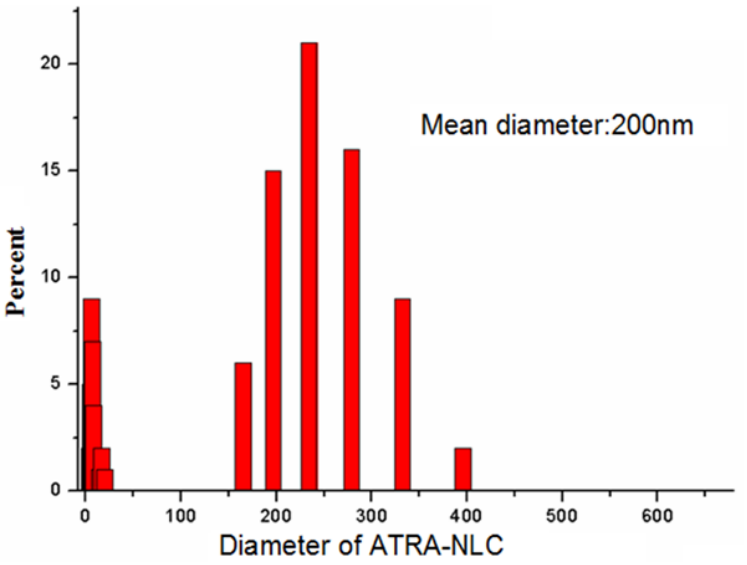

Figure 3. ATRA-NLC average particle size and the percent of ATRA-NLC were showed by Particle size analyzer. Datas are from an experiment that was repeated three times with similar results.

NCL for 24 h, incorporated with bromodeoxyuridine (BrdU) for the last 2 h. The cells were processed for colorimetric detection of incorporated BrdU by measurement of absorbance at 370 nm with a microplate reader.

Statistical analysis

Data are presented as mean values ± SD. All experiments were repeated at least three times. Statistical analysis using SPSS19.0 statistical software for statistical analysis. The single factor analysis of variance was used to compare the number of samples among the groups. Dunnett T3 analysis was used to investigate the differences in different groups. A *P* value of < 0.05 was considered statistically significant.

Results

The calculated ATRA-NLC solution concentration is 17.9 μg/mL

Determination of ATRA concentration curve equation is as follows: ATRA-NLC linear relation between concentration and Peak area: Quantitative determination of ATRA can get a good linear relationship between the range of 0.01~100 μg/mL calibration curve Y = 61.82 + 20.33 X R^2 = 0.996. The calculated ATRA-NLC solution concentration is 17.9 μg/mL. The value of Peak area was detected by HPLC. (**Figure 1**).

TRA-NCL showed good light resistance

Residual rate = (CN/CI) × 100%. Where CN is the concentration of ATRA and ATRA-NLC measured in the sample; CI is the initial concentration of ATRA and ATRA-NLC in the sample. Under the same illumination condition. ATRA and the rate of the remaining ATRA-NLC were compared. ATRA-NCL showed good light resistance (**Figure 2**).

Percent of ATRA-NLC nanoparticles were showed

ATRA-NLC average particle size was detected by the Particle size analyzer. The percent of ATRA-NLC nanoparticles were showed (**Figure 3**).

Concentrations of IL-1ß, TNF-α, IL-8, IL-6

30000/1 ml cells cultured in 24-well plates for 1 day with Serum-free MEM alone. Serum-deprived RFCs were incubated in the absence

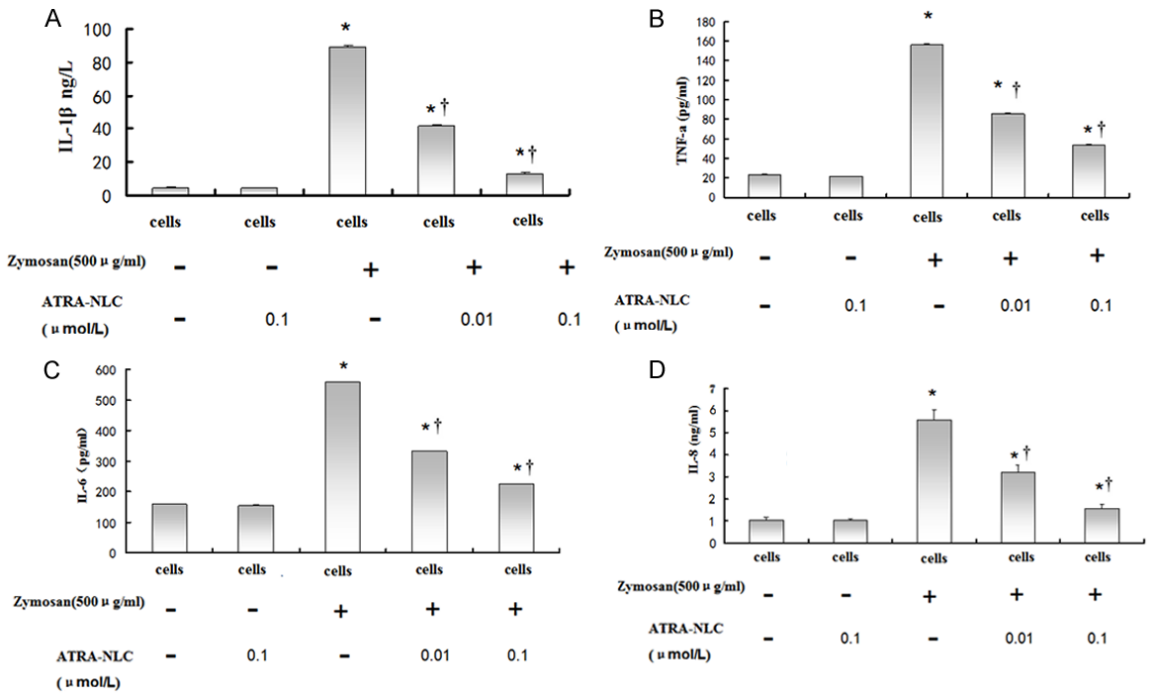

Figure 4. Serum-deprived RFCs were incubated in the absence or presence of ATRA-NLC in the additional presence of Zymosan for 24 h. IL-1ß, TNF-α, IL-6, IL-8 (A-D) concentrations were examined with the use of a cytokine assay system. Datas are mean values ± SD that was repeated a total of three times with similar results. †$P < 0.01$ versus the corresponding value for cells cultured with zymosan; *$P < 0.01$ versus the corresponding value for cells cultured alone (Dunnett T3).

or presence of ATRA-NLC in the additional presence of Zymosan for 24 h. IL-1ß, TNF-α, IL-8, IL-6 concentrations were examined with the use of a cytokine assay system (**Figure 4A-D**).

ATRA showed no effect on the proliferation of corneal fibroblasts

The ability of cells proliferation was assayed using CCK-8 (Biyuntian Company, China) according to the instructions. RFCs were seeded at a density of 10000 cells/well in 96-well with 0.2 ml MEM with or without 0.001, 0.01, 0.1, 1 μmol/L ATRA-NLC. RFCs were seeded at a density of 10000 cells/well in 96-well with 0.2 ml MEM. CCK8 was detected at 6, 12, 24, 36 h. The plate was read by a Microplate Reader. 0.01, 0.1 μmol/L ATRA-NLC were selected as the optimal concentrations (**Figure 5**). Datas are mean values ± SD that was repeated a total of three times with similar results. *$P < 0.05$ versus the corresponding value for cells cultured alone at different time points with different concentrations of ATRA-NCL (**Figure 6**).

Discussion

Fungi keratitis is a serious ocular infection leading to severe visual disability. The engagement of the receptors for fungal patterns induces the expression of cytokines, Zymosan induced the release of leukotriene (LT) B_4, LTE_4, 12-hydroxyeicosatetraenoic acid (12-HETE), and PA which is enough for optimal response of production of mediators. LTB_4, cysteinyl-LT, and PAF play a role in IL23A promoter trans-activation and the cytokine signature induced by fungal patterns [22]. The levels of these cytokines and chemokines of MIP-1α, MIP-1β, IL-1α, β, IL-6, TNF-α can be induced by zymosan [6-8]. CFs play key role in the immune and inflammatory responses to corneal infection in part by producing various cytokines and chemokines. Zymosan was represents for fungi [23]. IL-1β can promote inflammation and the evolute inflammatory states. IL-8 mediates recruit and activate circulating leukocytes. Higher levels of IL-8 and IL-6 in tears were detected in patients with fungal keratitis. Increased expression of mRNA to IL-8 and presence of PMN in the corneas from patients within Aspergillus flavus

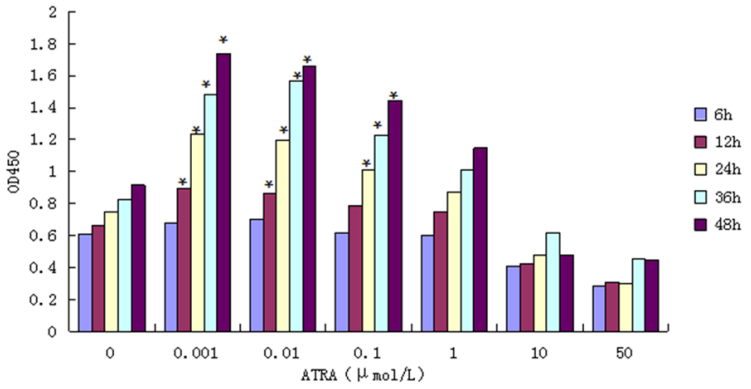

Figure 5. Different concentrations of ATRA-NCL at different time points showed no inhibition effect on the viability of cells proliferation. *$P < 0.05$ versus the corresponding value for cells cultured alone. (Dunnett's T3)

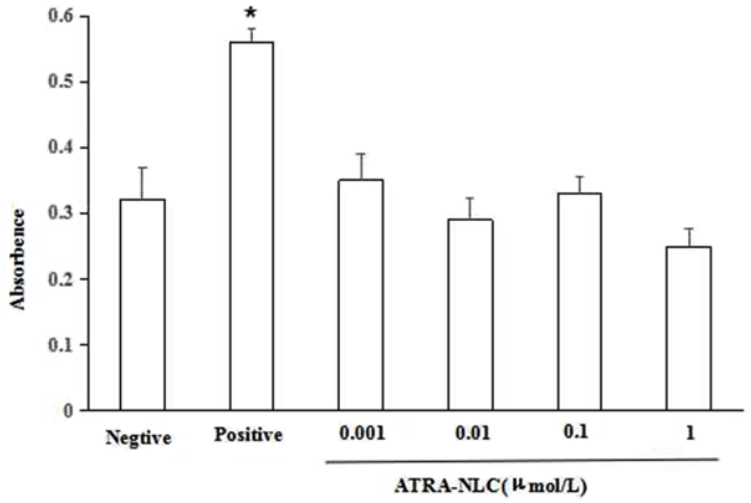

Figure 6. ATRA showed no effect on the proliferation of corneal fibroblasts. Cells were cultured for 24 h in MEM with or without ATRA-NCL. Cells cultured with 10% FBS in MEM was the positive control. Cell proliferation was evaluated by measurement of BrdU incorporation with an ELISA assay. Data are means ± SEM from an experiment that was repeated three times with similar results. *P < 0.05 versus negative control (Dunnett's T3).

and Fusarium solani infection [24-27]. This might explain the data observed in our study.

NLCs were useful for parenteral, dermal, pulmonary and topical delivery of drugs. These products have been developed to reduce toxic side effects of the incorporated drugs and increase the efficacy of the treatment. NLCs improve the stability, capacity loading and prevent the drug expulsion during storage [28, 29]. ATRA-NCL solution concentration is 17.9 μg/mL. ATRA-NLC average particle size was 200 nm. ATRA-NCL has the potent effect on ocular drug delivery. RCFs were cultured in our experiment, zymosan and ATRA-NCL were added to the culture system. From the datas above, pro-inflammatory factors IL-1ß, TNF-α, IL-8, IL-6 are elevated in zymosan induced RCFs. Our findings showed that ATRA-NCL can be used to decrease the cytokines induced by zymosan. Datas suggest that ATRA-NCL showed no inhibition effect on cells proliferation. It can also showed mild stimulation of cell proliferation. This can be proved by other cell lines [30]. ATRA-NCL was a potent drug for its capability of inflammation suppressing in fungi keratitis. Although we have performed the ATRA-NLC in our experiment and the nanoparticles showed no poisonous function on the RCFs. The number of products on the market is limited for limitations and difficulties related to them [28]. There were still some challenges of nanoparticles for drug delivery applications including the incompatibility of length-scales, weak interactions and solvation, the complexity of the thermochemical environment surrounding the nanoparticles, and the role of polydispersivity in determining properties and performance [31]. Therefore, further research is required to investigate the potential ability of ATRA-NCL in the therapy of fungi keratitis.

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Disclosure of conflict of interest

None.

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