

# Brinzolamide- and latanoprost-loaded nano lipid carrier prevents synergistic retinal damage in glaucoma

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**Glaucoma is a common eye disease and a major cause of blindness. We designed brinzolamide (Brla)- and latanoprost (Ltp)-loaded nano-lipoidal carriers (NLCs) for glaucoma treatment. Brla and Ltp-loaded NLCs were designed and characterized by assessing the zeta potential, polydispersity index, X-ray diffraction and scanning electron microscopy images, and particle size. Drug release was assessed by *in vitro* and *ex vivo* methods to determine transcorneal permeation, ocular irritation, and cell viability. Moreover, Brla- and Ltp-loaded NLCs were assessed in terms of the management of raised intraocular pressure (IOP). The size of Brla- and Ltp-loaded NLCs was <200 nm, the drug entrapment efficiency was 97.5%, and the zeta potential was 35.33 mv. The transcorneal permeation levels of Brla and Ltp after 8 h were 50.5% and 49.4%, respectively; after 24 h, they were 81.4% and 84.2%, respectively. However, NLCs are not cytotoxic. Moreover, Brla+Ltp-treated NLCs effectively reduced IOP in glaucoma patients. Therefore, Brla+Ltp-loaded NLCs showed promising effects against glaucoma.**

**Keywords:** nano-lipoidal carrier, glaucoma, brinzolamide, latanoprost, intraocular pressure

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**Abbreviations:** Brla, Brinzolamide; HPLC, high-performance liquid chromatography; IOP, intraocular pressure; Ltp, Latanoprost; NLCs, nano-lipoidal carriers; XRD, X-ray diffraction

## INTRODUCTION

Glaucoma is a neurodegenerative ophthalmic disease (Gupta & Yücel, 2007) characterized by raised intraocular pressure (IOP), which damages the optic nerve and causes vision loss (Khaw *et al.*, 2004). Approximately 80 million people worldwide had glaucoma in 2020, which is expected to increase to 111.1 million by 2040 (Allison *et al.*, 2020). Glaucoma is a major cause of vision loss worldwide (Parihar, 2016), and is managed by reducing the IOP using prostaglandin analogues, cholinergic drugs, carbonic anhydrase inhibitors,  $\beta$ -adrenergic antagonists, and/or  $\alpha$ -adrenergic agonists (Sambhara and Aref, 2014). These drugs are administered in the form of eye drop suspensions. However, there are several limitations to the use of eye drops in glaucoma patients, including limited ocular retention time, rapid tear turnover, and reduced corneal diffusion, which reduce drug bioavailability. The use of drug delivery systems that overcome these limitations may lead to poor patient compliance.

A combination of the aforementioned drugs is effective for glaucoma management and prevention of optic nerve damage (Nguyen, 2014). Brinzolamide (Brla) is a carbonic

anhydrase inhibitor derived from the class of heterocyclic sulphonamides (Menabuoni *et al.*, 1999). Brla reduces IOP and ocular hypertension (Iester, 2008) by decreasing bicarbonate ion and aqueous humour production by the ciliary body (Aslam & Gupta, 2021). Latanoprost (Ltp) is a prostaglandin analogue that enhances the outflow of aqueous humour, thereby reducing the IOP (Weinreb *et al.*, 2002). Ltp also prevents optic nerve injury in glaucoma patients (Ishii *et al.*, 2001). The combined use of Brla and Ltp increases their bioavailability in the ocular cavity.

Several recent studies have evaluated the use of nano-lipoidal carriers (NLCs) for ocular drug delivery (Bachu *et al.*, 2008). The use of NLCs enhances corneal drug bioavailability by increasing drug release and penetration, and maintaining the drug levels for a prolonged duration, in the ocular mucosa (Moiseev *et al.*, 2019). Encapsulated hydrophobic molecules achieve high drug loading with NLCs because of the abundance of lecithin in their cell membrane (Le *et al.*, 2019). Moreover, such drug molecules show enhanced penetration and reduced leakage during ocular delivery (Patel *et al.*, 2013). Drug uptake and residence time are enhanced due to increased ionic interaction of negatively charged mucus layer with positively charged NLCs and particle surfaces (Suk *et al.*, 2016). Therefore, many drugs are coated with a cationic moiety of cetyltrimethylammonium bromide, stearylamine, L-arginine, and chitosan (Baldim *et al.*, 2020). In the present study, we determined the bioavailability and effects of treatment with NLCs loaded with Brla and Ltp in a glaucoma model.

## MATERIAL AND METHODS

### Chemicals

Brla and Ltp were procured from Wuhan Wujing Medicine Co. Ltd. (Wuhan, China). Captex® 200P (propylene glycol dicaprate), Transcutol® P, and Capmul® MCM C10 (glyceryl monocaprate) were generously gifted by Abitec Corp. (Columbus, OH, USA). Soya lecithin was gifted by Lipoid GmbH (Ludwigshafen, Germany). Polysorbate 80 was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Stearylamine was purchased from Merck Co. (Kenilworth, NJ, USA). All other chemicals and reagents were of analytical grade, while the solvents were of high-performance liquid chromatography (HPLC) grade.

### NLC preparation

The hot microemulsion method was used to prepare the cationic Brla- and Ltp-loaded NLCs. A pseudo-ternary phase diagram was constructed to select the NLCs composition and drug solubility for various excipients. The lipid

phase contained Captex® 200 P (liquid lipid) and 1:1 ratio of soya lecithin (solid lipids) and Capmul® MCM C10. The liquid and solid lipids were added at a ratio of 25:75. The final content included 0.125% (50 mg) of oil and 0.5% (200 mg) of solid lipid. The surfactant mixture (100 mg) consisted of 1:1 ratio of polysorbate 80 and Transcutol® P; stearylamine was added to the surfactant mixture to provide a cationic charge. A total of 40 mg of drugs was added to the lipid mixture (100 mg/mL in dichloromethane); Captex® 200P was added to a small beaker at a 1:6 w/w ratio of the drug:lipid mixture. The mixture was added after heating the lipid mixture and drug to 60°C. A small quantity of hot water (200 mL; 50–60°C) was added after mixing stearylamine with the homogenous mixture for microemulsion. Drops of cold water (10–15°C) were added to the microemulsion. Thereafter, the mixture was kept at 16000 rpm for 10 min, for shear homogenisation. The mixture was stirred at 700 rpm for 3–4 h at room temperature before use.

### NLC characterization

#### Determination of zeta potential, polydispersity index, and particle size

The polydispersity index, particle size, and drug entrapment efficiency (%) were determined to characterize the NLCs. Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK) was used to estimate the polydispersity index and particle size. All experiments were performed three times.

#### X-Ray diffraction

X-ray diffraction (XRD) was performed on blank NLCs, pure drug samples, and Brla- and Ltp-loaded NLCs. An X-ray powder diffractometer using Cu Ka radiation (1.54 Å) was operated at 40 kV, with 40 mA passing through the nickel filter.

#### Field emission scanning electron microscopy

Field emission scanning electron microscopy (FE-SEM) was used to determine the NLC morphology. After placing a drop of NLC suspension, the carbon-coated stub was air-dried. An MC1000 ion sputter (Hitachi, Tokyo, Japan) was used to coat the sample with platinum; additional samples were photographed using the FE-SEM.

#### Determination of drug entrapment efficiency

Encapsulation efficiency was directly analysed. Drug-loaded NLCs were centrifuged at 4°C for 40 min at 20000 rpm. After washing the supernatant, distilled water was used to wash the pellets to remove the untrapped drug. A chloroform:dichloromethane (1:1) mixture was used to separate and lyse the pellet. The lysed samples were filtered and diluted with the mobile phase. HPLC was used to analyse the drug content in the sample. The samples were separated using chromatography (Hypersil C18 column; 250×4.6 mm; 5 µm; Thermo Fisher Scientific). The mobile phase consisted of 20 mM of acetonitrile and potassium dihydrogen phosphate buffer solution (50:50% v/v) at 4.2 pH; the absorbance was detected at 215 nm. The flow rate of the mobile phase was maintained at 1 mL/min and the injection volume was 20 µL.

$$\text{Entrapment efficiency (\%)} = \frac{\text{Amount of drug in NCL}}{\text{Total amount of drug added}} \times 100$$

#### Determination of *in vitro* drug release

The dialysis bag method was used to study *in vitro* drug release. The dialysis membrane contained an 80:20 mixture of simulated tear fluid (0.008% calcium chloride, 0.2% sodium bicarbonate, and 0.67% sodium chloride in water) and n-methyl pyrrolidine (30 mL) at 7.4 pH, for NLC dispersion. The dialysis bag was maintained at a constant temperature (37±0.5°C) and gently stirred at 400 rpm. Samples were withdrawn from the bag at regular intervals and replaced with fresh medium. Samples were analysed at a wavelength of 215 nm. HPLC analysis was performed according to the aforementioned method. The release profile of the formulation was fitted into different release kinetic models, such as zero order, first order, Hixon-Crowell, Higuchi, and Korsmeyer-Peppas, to evaluate the release behaviour using regression analysis.

#### Determination of *ex vivo* transcorneal permeation

Fresh goat eyes were procured from a butcher, and 2–4-mm-thick sclera and cornea were excised. The cornea was clamped between the receptor and donor cells of the Franz-type diffusion cell. Receptor cells were stirred at a constant temperature of 37±1°C using a magnetic stirrer in STF solution (20 mL). One mL of NLC was diluted in the STF solution and placed on the donor compartment cells. After a specific time interval, 2 mL of sample was withdrawn and replaced with fresh solution. The concentration of drug molecules was estimated at 215 nm using ultraviolet spectroscopy.

#### Determination of corneal distribution and penetration of dye-loaded drug

Nile red dye was used to determine the permeation and distribution of dye-loaded drug. NLCs were developed as described previously, except for the replacement of drug with dye. Goat cornea was isolated by biopsy and seeded in a 14-well plate using Hank's balanced salt solution. Then, 20-µL of dye-loaded formulation was added to the plate, followed by incubation for 1 h at 32°C. The cornea was fixed with 4% formaldehyde and a cryostat was used to cut the tissue. An AxioVision fluorescence microscope (Carl Zeiss, Jena, Germany) was used to observe corneal staining with DAPI.

#### Ocular irritability test

Albino rabbits (2–2.5 kg) were used in the ocular irritability test. NCL (50 µL) was administered to rabbit conjunctiva; the other eye of the same rabbit was considered as the control. The Draize scale was used to determine the severity of ocular injury (score range: 0–3, 0–4, and 0–3 for conjunctival discharge, swelling, and congestion, respectively).

#### Cell viability assay

MTT assay was used to determine cell viability, similar to previous studies. Cells (5,000 cells/well) were seeded into a 14-well plate and incubated for 24 h. Additional cells were separately treated with NLCs and Brla+Ltp-loaded NLCs (0.1, 0.2, 0.5, or 1 mg/mL; equivalent to the drug). The negative control group included untreated cells. The MTT solution was added to the cells and incubated at 37°C for 4 h. A microplate reader was used to determine absorbance at a wavelength of 540 nm.

#### Animals

Male 12-week-old Sprague-Dawley rats (weight: 125–180 g) were maintained under 12-h light/dark conditions.

**Table 1. Particle size, polydispersity index, and entrapment efficiency of nano-lipoidal carriers (NLCs) and drug-loaded NLCs**

| Sr. No. | Preparation          | Size (nm)   | Polydispersity index | Entrapment efficiency (% w/w) | Zeta potential |
|---------|----------------------|-------------|----------------------|-------------------------------|----------------|
| 1       | NLCs                 | 153.72±2.94 | 0.24±0.012           |                               | 32.46±0.42     |
| 2       | Brla+Ltp-loaded NLCs | 165.28±2.36 | 0.31±0.015           | 97.5±2.16                     | 35.33±0.37     |

### Induction of glaucoma

Glaucoma was induced using laser burns to increase the IOP; the laser burns were produced by exposing right eye episcleral veins and the trabecular meshwork to a diode laser (wavelength: 532 nm; duration: 0.5 S; power: 0.6 W); this was repeated after 1 week. The left eye of each rat was considered as the control. IOP was measured at 24 and 72 h after the second dose of laser. Rats with IOP>30 mmHg were diagnosed with glaucoma. The TonoPen XL tonometer (Medtronic Ophthalmics, Jacksonville, FL, USA) was used to determine the IOP according to the methods employed in previous studies. The rats were divided into a negative control group, in which the right eye was treated with saline solution, and a Brla + Ltp-loaded NLC group, in which the rats received Brla + Ltp-loaded NLCs for 1 week followed by IOP estimation. The left eye of all rats was untreated and considered as the control.

### Statistical analysis

The data are presented as mean ± standard error mean (S.E.M.), and were analysed using one-way ANOVA, followed by Dunnett's post-hoc test (GraphPad Prism; version 6.1; GraphPad Software Inc., San Diego, CA, USA). *P*-values<0.05 were considered statistically significant.

## RESULTS

### Characterization of drug-loaded NLCs

Estimation of NLC particle size, polydispersity index, and entrapment efficiency

Table 1 summarizes the entrapment efficiency, polydispersity index, zeta potential, and particle size for the NLCs and drug-loaded NLCs. The drug-loaded NLCs had high entrapment efficiency (97.5±2.16% w/w). The zeta potentials of NLCs and drug-loaded NLCs were 32.46±0.42 and 35.33±0.37, respectively. The optimal formulations of NLCs and drug-loaded NLCs had aver-

age particle sizes of 153.72±2.94 and 165.28±2.36 nm, respectively. The polydispersity indices of NLC particles were 0.24±0.012 and 0.31±0.015 for NLCs and Brla+Ltp-loaded NLCs, respectively, suggesting homogeneous particle sizes.

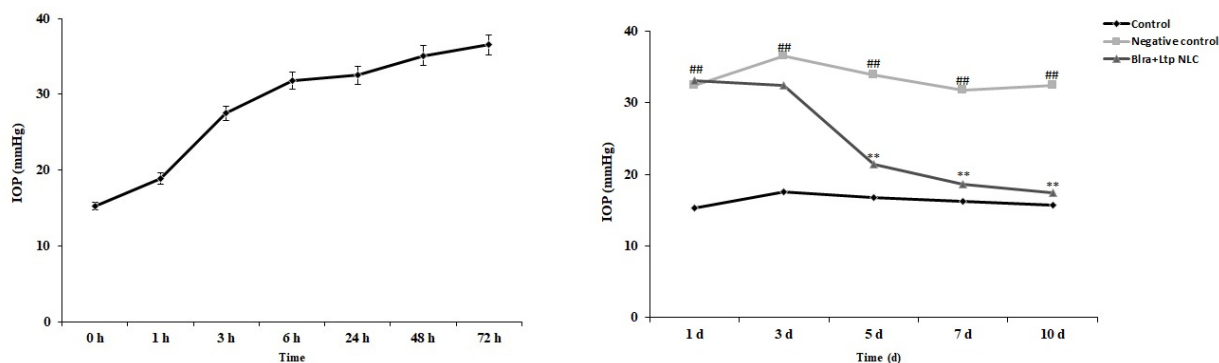
Estimation of the crystalline structure and surface morphology of NLCs

Figure 1A and B depicts the surface morphology and crystalline structure of drug-loaded NLCs. According to XRD, the crystalline peaks of Brla at 2θ positions were 9.87°, 17.18°, and 17.59°, while those for Ltp were 14.50°, 14.97°, 15.87°, 19.86°, and 24.68°. The characteristic peaks of NLCs at 2θ positions were 5.39°, 10.78°, 18.78°, 19.28°, 19.65°, 20.01°, 20.42°, 20.91°, and 23.65°. The XRD spectra of Brla+Ltp-loaded NLCs showed the reappearance of NLC peaks, suggesting complete entrapment of drug inside the nanoparticles. SEM showed spherical particles with round edges and smooth surfaces. The particles were uniformly sized (all <200 nm) and drug crystals were not observed on the surface of NLC particles. These data confirmed the absence of free drug molecules and proved that NLCs were non-irritants.

Estimation of transcorneal permeation and drug release profile of Brla+Ltp-loaded NLCs

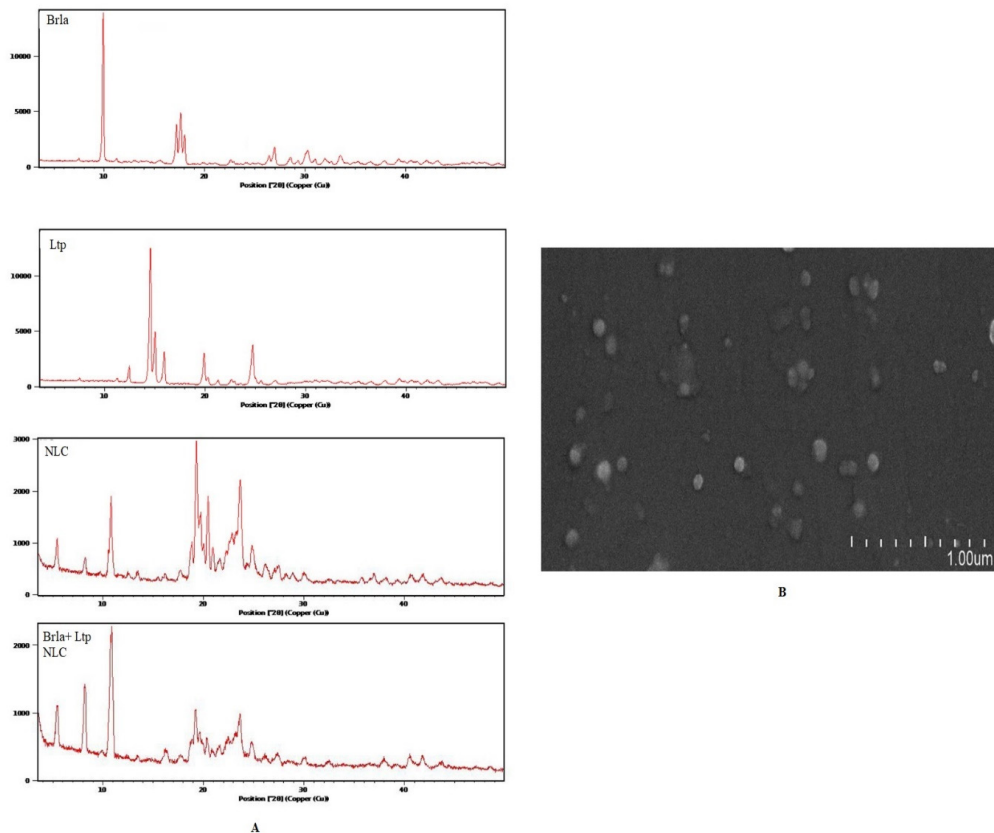
*Ex vivo* cumulative drug release and corneal permeation of Brla + Ltp-loaded NLCs was determined in goat eye (Fig. 2A–C). The percentage of cumulative Brla and Ltp release after 2 h was 40.33% and 43.8%, respectively; after 24 h, the values were 81.4% and 84.2%, respectively (Fig. 2A).

The results for transcorneal permeation of Brla + Ltp-loaded NLCs were similar to the drug release pattern. The drug permeation percentages of Brla and Ltp (Brla+Ltp-loaded NLCs) within 4 h were 42.8% and 40.6%, respectively. The total drug permeation percentages of Brla and Ltp after 8 h were 50.5% and 49.4%, respectively. Corneal permeation was lower for the Brla and Ltp suspensions compared to Brla+Ltp-loaded NLCs (Fig. 2B). Figure 2C depicts the corneal permeation for Brla+Ltp-loaded NLCs in goat



**Figure 1. Physical properties of brinzolamide (Brla)- +latanoprost (Ltp)-loaded nano-lipoidal carriers (NLCs).**

(A) Results of XRD of Brla, Ltp, blank NLCs, and Brla+Ltp NLCs; (B) Results of scanning electron microscopy of Brla+Ltp-loaded NLCs.



**Figure 2.** Determination of *ex vivo* cumulative drug release and corneal permeation from nano-lipoidal carriers (NLCs) in goat eyes. (A) Determination of cumulative drug release from Bra- and Ltp-loaded NLCs; (B) Determination of the percentage of cumulative drug permeation; (C) Determination of the corneal permeation of brinzolamide+latanoprost-loaded NLCs in goat eyes using Nile red dye.

**Table 2.** Drug release kinetics of nano-lipoidal carrier (NLCs) for different models

| Sr. No. | Formulation<br>(Bra + Ltp-loaded NLCs) | $r^2$ value release kinetic models |             |         |                  |               |
|---------|--|------------------------------------|-------------|---------|------------------|---------------|
|         |  | Zero order                         | First order | Higuchi | Korsmeyer-Peppas | Hixon-Crowell |
| 1       | Bra                                    | 0.7329                             | 0.5104      | 0.9279  | 0.9416           | 0.8768        |
| 2       | Ltp                                    | 0.7413                             | 0.5328      | 0.9433  | 0.9673           | 0.8917        |

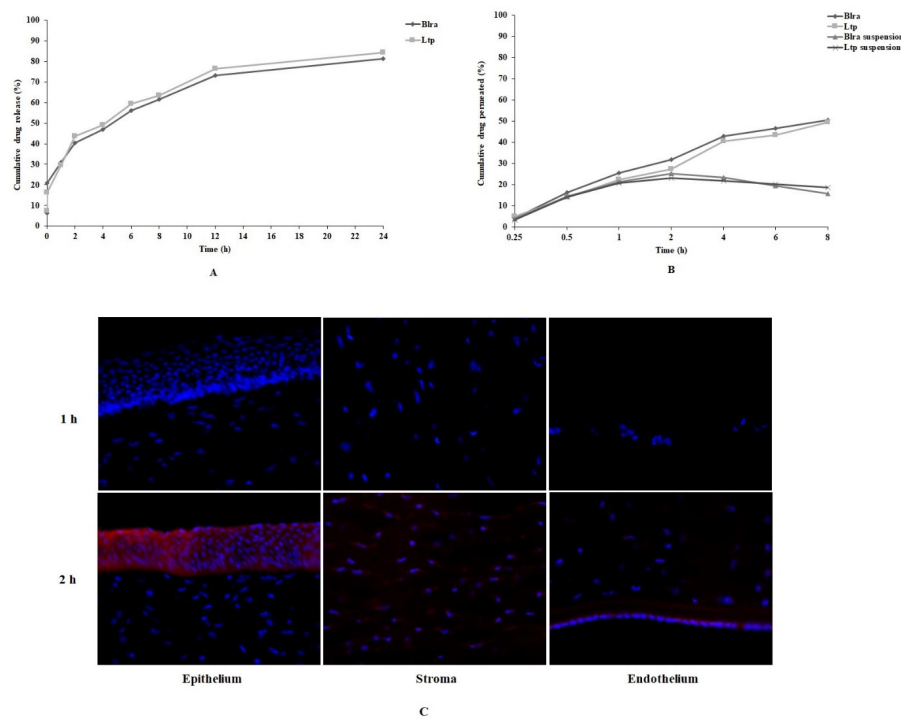
**Table 3.** Effects of drug-loaded nano-lipoidal carriers (NLCs) and NLCs on ocular irritation

| Sr. No. | Reaction        | Formulation  |              |                    |
|---------|-----------------|--------------|--------------|--------------------|
|         |                 | NLCs         | Bra+Ltp NLCs | Bra+Ltp suspension |
| 1       | Corneal opacity | 0.12 ± 0.013 | 0.14 ± 0.01  | 2.14 ± 0.19**      |
| 2       | Area of opacity | 0.15 ± 0.014 | 0.17 ± 0.015 | 2.33 ± 0.21**      |
| 3       | Iris            | 0.0 ± 0.0    | 0.0 ± 0.0    | 3.13 ± 0.25**      |
| 4       | Conjunctivae    | 0.0 ± 0.0    | 0.0 ± 0.0    | 1.23 ± 0.12**      |

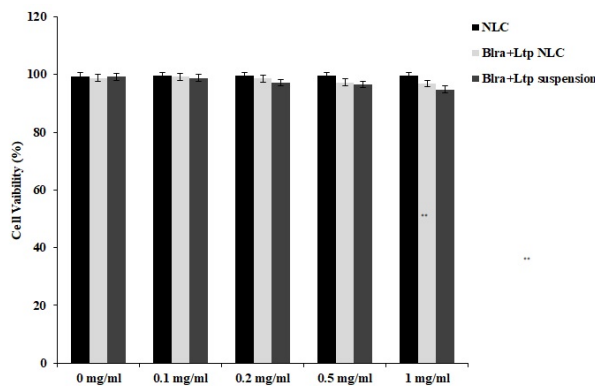
Mean ± S.E.M. (n=6), \*\* $P < 0.01$  compared to Bra+Ltp NLCs. The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: <http://www.textcheck.com/certificate/cepWjf>

eye based on Nile red staining. No fluorescence was observed after 1 h of drug administration. However, red fluorescence was observed, surrounded by blue

(DAPI)-stained nuclei, suggesting that the formulation requires 2 h to penetrate through the cornea.



**Figure 3.** Effect of brinzolamide+latanoprost-loaded nano-lipoidal carrier on cell viability determined by MTT assay. Mean  $\pm$  S.E.M. (n=6).



**Figure 4.** Effect of brinzolamide+latanoprost-loaded NLCs on the IOP in rat eyes with laser burn-induced glaucoma. Mean  $\pm$  S.E.M. (n=6); ## $P$ <0.01 compared to the control group; \*\* $P$ <0.01 compared to the negative control group.

The mechanism underlying drug release was assessed using the release kinetic model and based on the  $r^2$  value (Table 2). Drug release followed the Korsmeyer-Peppas ( $r^2=0.9416$  and  $0.9673$  for Brz and Ltp, respectively) and Higuchi ( $r^2=0.9279$  and  $0.9433$  for Brz and Ltp, respectively) models. These data suggest that drug release depended on the control of swelling and diffusion (Table 2).

#### Estimation of ocular irritability

The effects of drug-loaded NLCs and NLCs on ocular irritation were evaluated using the Draze irritation test (Table 3). Irritation in the opacified area, iris, conjunctivae, and corneal opacity were significantly greater for the Brz+Ltp suspension compared to NLCs and Brz+Ltp-loaded formulations ( $P$ <0.01).

#### Determination of the effect of Brz+Ltp-loaded NLCs on cell viability

The effect of Brz+Ltp-loaded NLCs on cell viability (%) was determined using the MTT assay (Fig. 3). The cells were treated with 0.1, 0.2, 0.5, and 1 mg/mL of Brz+Ltp-loaded NLCs. The MTT assay showed no cell toxicity.

#### Determination of effect of Brz+Ltp-loaded NLCs on IOP

The effect of Brz+Ltp-loaded NLCs on the IOP was determined in laser burn-induced glaucomatous eyes (Fig. 4). The IOP of eyes from the negative control was significantly higher compared to that for control eyes ( $P$ <0.01). However, treatment with Brz+Ltp-loaded NLCs significantly reduced the IOP in laser burn-induced glaucomatous eyes ( $P$ <0.01).

#### DISCUSSION

Glaucoma, a major cause of blindness, is associated with neurodegeneration (Artero-Castro *et al.*, 2020). Management of glaucoma is complicated by several issues, such as drug bioavailability, penetration, and the duration of residence on the cornea (Mobaraki *et al.*, 2020). Glaucoma has several underlying pathophysiological mechanisms that prevent adequate management. Therefore, we designed Brz+Ltp-loaded NLCs and evaluated the effects thereof on glaucoma.

NLCs are derived via a novel formulation, consisting of a mixture of liquid and solid lipids (particle size <100 nm) (Naseri *et al.*, 2015). The preparation of NLCs requires a distribution of small-sized particle emulsion droplets in the aqueous phase. NLCs are nanoparticles that improve drug loading and retention during storage (Ghasemiyeh and Mohammadi-Samani, 2018). Therefore, we designed a Brz+Ltp-loaded NLCs for glaucoma

management. The data related to particle size and drug entrapment suggest that NLCs have uniform sizes of <200 nm; the higher drug entrapment levels may be due to the lipophilic nature of drugs. Moreover, the round surface and complete drug entrapment of NLCs may explain the lack of irritation.

Bra reduces the IOP by decreasing aqueous humour production. Ltp reduces the IOP by improving the outflow of aqueous humour (Schmidl *et al.*, 2015). Moreover, Ltp also protects against optic nerve damage. In combination, these drugs may show synergistic effects and our data suggested that Bra+Ltp-loaded NLCs significantly reduced IOP in laser-induced glaucoma rats.

## CONCLUSION

We successfully designed Bra+Ltp-loaded NLCs with a particle size of  $165.28 \pm 2.36$  nm and entrapment efficiency of  $97.5 \pm 2.16\%$ . Moreover, SEM revealed spherical-shaped, rounded edges of NLCs. Bra+Ltp-loaded NLCs showed favourable results in the irritation test and adequate transcorneal permeation. Bra+Ltp-loaded NLCs are associated with significantly reduced IOP in the eyes of rats with laser-induced glaucoma.

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## Conflict of interest

None.

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## Data availability statement:

All data related to the current study are available in the manuscript.

## Authors' contributions

LC supervised and performed the research and edited the final version of the manuscript. RW analysed the data and wrote the first draft of the manuscript.

## Ethics approval

All animal experiments followed the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use and were approved by the Institutional Animal Ethics Committee (IAEC) of Putuo People's Hospital, Tongji University, China (IAEC/PPH/TU/2019/05).

## Consent to participate

All authors consent to the publication of this manuscript.

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