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Prednisolone loaded-cationic nanoemulsion formulation for uveitis management

Mohamed A. Attia [a,*], Nermin E. Eleraky [a], Khaled Abdelazeem [b], Mohamed A. Safwat [c]

[a] Department of Pharmaceutics, Faculty of Pharmacy, Assiut University, Assiut, 71526, Egypt
[b] Department of Ophthalmology, Faculty of Medicine, Assiut University, Assiut, Egypt
[c] Department of Pharmaceutics, Faculty of Pharmacy, South Valley University, Qena, 83523, Egypt

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ABSTRACT

Uveitis is a sight-threatening complication that continues to be a major contributor to blindness. The etiology of uveitis mostly depends on inflammatory activities. The mainstay of uveitis treatment is the topical use of corticosteroids, although their therapeutic efficiency is constrained by poor corneal penetration and retention. Traditional eye drops are less potent when inflammation extends further into the eye. Nanoemulsions are efficient drug delivery systems for ocular applications owing to their many benefits, particularly sustaining drug action and their capability to penetrate the deepest parts of the ocular structure and the aqueous humor. Herein, a novel preparation of prednisolone-laden cationic nanoemulsion was designed to prolong the precorneal drug retention time, thereby improving the bioavailability of prednisolone for uveitis treatment. Pseudoternary-phase illustrations were created via a water titration approach. A cationic surfactant (cetalkonium chloride) was used to test the effectiveness of a cationic nanoemulsion in extending the precorneal retention of prednisolone. The developed nanoemulsion formulae were assessed for their physicochemical characteristics, morphology, in vitro release profile, and ex vivo permeation patterns. In addition, the clinical investigation and the safety of the proposed formulation in a uveitis-induced experimental animal model were assessed. The proposed nanoemulsion formulations displayed a spherical shape, a nanometer size range, a narrow size distribution, and negative surface charge. The incorporation of cetalkonium chloride decreased the droplet diameter and shifted the droplets' surface charge to positive. The developed cationic nanoemulsions exhibited a sustained in vitro drug release profile and enhanced flux through rabbits' corneas compared to the same formulations without adding cationic surfactant, and free prednisolone suspension (Pred forte® 1 %). Clinical studies showed that using cationic nanoemulsion formulations significantly reduced the severity of uveitis in rabbits' eyes throughout treatment period (three weeks) compared to drug suspension (Pred forte® 1 %). Prednisolone cationic nanoemulsion formulations did not cause an elevation in intraocular pressure (IOP) and any appreciable changes in the diameter of the rabbits' pupils in the investigated animal groups. Also, there were no adverse effects on the cornea, retina/choroid, or iris/ciliary body, demonstrating the safety of the suggested nanoemulsion formulations. Therefore, the developed prednisolone cationic nanoemulsion system may offer a potential vehicle for ophthalmic drug delivery and enhanced management of uveitis.

1. Introduction

Uveitis is an inflammatory illness that impacts the iris, ciliary body, and the choroidea. It can result in irreversible eye damage and vision loss. In the developed world, uveitis may account for up to 25 % of blindness, compared to 10 %–20 % in the US and Europe [1]. The exact etiopathogenesis is not known. However, interleukin (IL)-1, IL-6 and tumor necrosis factor-alpha (TNF-α) are among the pro-inflammatory cytokines that are recognized to have a substantial role in uveitis [2,3].

Mainly, corticosteroids and immunosuppressants have been used to treat uveitis. However, their application is restricted by the systemic and ocular side effects brought on by long-term, large-dose administration of particularly steroids [2–4]. Therefore, researchers have been looking for innovative ways to deliver corticosteroids to the eye. Topical ocular

* Corresponding author.
E-mail addresses: m.attia@aun.edu.eg (M.A. Attia), nermineleraky@pharm.aun.edu.eg (N.E. Eleraky), Abdelazeem.kh@aun.edu.eg (K. Abdelazeem), safwat_mohamad@svu.edu.eg (M.A. Safwat).

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Table 1
Composition of different nanoemulsion formulations.

Formulation Code	Surfactant/Co-surfactant mixture (1:1)	Composition (% w/w)		
		Water	Oil	Surfactant mixture
F1	$Tween_{80}$: Propylene Glycol	28.50	7.10	64.3
F2	Cremophor RH_{40}: Propylene Glycol	30.50	6.90	62.50
F3	Cremophor RH_{40}: Polyethylene Glycol 600	12.30	8.90	78.90
F4	$Tween_{80}$: Polyethylene Glycol 600	9.10	9.10	81.80
F5	Cremophor RH_{40}: Glycerin	9.10	9.10	81.80
F6	$Tween_{80}$: Glycerin	10.70	8.90	80.40
F1*	$Tween_{80}$: Propylene Glycol	28.50	7.10 + 0.01 % CKC	64.3
F2*	Cremophor RH_{40}: Propylene Glycol	30.50	6.90 + 0.01 % CKC	62.50

delivery is the most suitable approach for steroids administration. However, owing to the specific anatomical structure of the eye, topical medication instillation suffers from restricted drug penetration at the targeted location [5–7]. Additionally, the cornea can allow only 1 %–3 % of the administered total dose to pass through and reach the intraocular tissues [7,8]. All the obstacles mentioned above of eye constraints lead to poor penetration and reduced bioavailability of traditional ophthalmic formulations such as solutions and suspensions. Several novel ophthalmic drug delivery platforms have been explored to overcome the above mentioned challenges and to obtain improved therapeutic efficacy e.g. Liposomes [9], nanoparticles [6,10], microemulsions, nanoemulsions and lipid-polymeric hybrid nanoparticles [11,12].

Nanotechnology represents an intriguing strategy for delivering medications to the eye. Nanoemulsion formulations are thermodynamically stable and optically transparent fine dispersions [13]. They are two-phase systems comprising of oily phase, aqueous phase and a surfactant [14]. Nanoemulsions as ocular delivery system offer ease of application compared to other systems, in addition to better compliance of the patient resulting from decreasing the number of instillations, long contact time and extended drug action. O/W nanoemulsions are an ideal option for ophthalmic drug delivery because of the water existence as a continuous phase, which makes it easier for the drug to be diluted with tears, the potential to include water-insoluble medications in their oil core, and an improvement in drug penetration into ocular tissues is guaranteed by the improved residence duration. Reducing the contact angle between the cornea and the nanoemulsion droplets improves the spreadability and the wettability of administered nanoemulsions, thus enhancing their ocular residency. Moreover, ophthalmic nanoemulsions are mostly regarded as affordable and non-invasive formulations [15,16]. Cationic nanoemulsions are generally approved as prospective ophthalmic topical delivery vehicles for hydrophobic medications. In addition to facilitating the quick passage of nano-sized globules through the cornea's tight junctions, cationic nanoemulsions can also efficiently boost the bioavailability of ocular medications by extending the precorneal retention time through electrostatic attractions with the cornea's negatively charged mucin. Besides, cationic nanoemulsions provide enhanced physical stability over storage time by generating a repulsive force among the positively charged oil droplets [17–19]. Many therapeutics including antibiotics [4], antifungals [20], anti-inflammatory drugs [21], and immunosuppressants [22] have been formulated as nanoemulsions for the medical therapy of ocular diseases.

Prednisolone is a synthetic glucocorticoid used to reduce inflammation of the eye. Due to its potential anti-inflammatory and immunosuppression properties, prednisolone (PRD) is the most widely used corticosteroid in managing ocular inflammatory diseases [23]. The biopharmaceutical classification system assigns prednisolone to BCS class II, which is characterized by strong permeability and extremely low water solubility. Prednisolone is commercially available as a micronized ophthalmic suspension, and ointment. However, the use of suspension dosage form is restricted owing to aggregation, restricted corneal residency, poor dosage accuracy, and low effectiveness. Besides, stickness, low spreadability, and reduced stability are resulted from using the ointment formulation [24].

Accordingly, the main objective of this investigation was to design and assess a novel nanoemulsion preparation for local ocular administration of prednisolone (PRD) for uveitis management. A cationic nanoemulsion formulation of PRD was developed. Due to its compatibility with the ocular tissues, cetalkonium chloride (CKC) was chosen as the compound that confers positive charges to nanoemulsion droplets. This approach takes advantage of cationic nanoemulsion electrostatic attractions with the ocular surface to extend the precorneal retention period, which may boost prednisolone bioavailability, as well as the nanoscale droplet size of nanoemulsion dispersions to improve corneal penetration. Pseudoternary-phase diagrams were used to optimize the formulations of prednisolone incorporated nanoemulsions via water titration technique. The final formulations were assessed for droplet size, surface charge, polydispersity index, morphology, and in vitro drug release pattern before and after inclusion of cationic surfactant (cetalkonium chloride). Measurements of ex vivo corneal permeation across rabbits' corneas were conducted to examine the enhancement effect of applying prednisolone cationic nanoemulsion formulation compared to conventional eye drops (Pred Forte® 1 %). Finally, the in vivo clinical investigation of uveitis-induced experimental rabbits was performed to evaluate the therapeutic efficiency and safety of the proposed formulation.

2. Materials and methods

2.1. Materials

Prednisolone was a kind gift from El-Kahira Co. (Cairo, Egypt); Poly ethoxylated 40 hydrogenated castor oil (Cremophor® RH40) was obtained from BASF (Monheim, Germany). Tween 80 (ethoxylated sorbitan mono oleate), bovine serum albumin (BSA) and cetalkonium chloride (CKC) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Oleic acid was obtained from Alpha Chemicals Co. (Cairo, Egypt). Glycerin (spectrum 99.5 % USP) was provided from El-Nasr Chemical Co., Cairo, Egypt. Polyethylene glycol 600 was supplied from Merck KGaA (Darmstadt, Germany). El-Gomhouria Chemical Company (Cairo, Egypt) provided propylene glycol (99.5 % USP). Cellophane membrane MWCO 12.000–14.000 was obtained from Carl Roth GmbH Co. (Karlsruhe, Germany). All other ingredients were of analytical grade and used without further purification.

2.2. Methods

2.2.1. Pseudoternary-phase diagram construction

The water titration approach was employed to develop a pseudoternary phase diagram with a 1: 1 mass ratio of surfactant to co-surfactant with the objective of establishing the concentration range of the nanoemulsion's constituents (water, oil, surfactant, and co-surfactant) [25,26]. A uniform mixture of oleic acid and surfactant (Cremophor® RH40 or tween 80)/Co-surfactant (PEG 600, propylene glycol, or glycerin) at weight ratios (1:9; 2:8; 3:7; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1) was gradually titrated with distilled water while stirring at 25 °C for sufficient period till equilibration. After equilibrium was attained the systems' transparency was visually examined. No attempts were made to explain the other zones of the pseudoternary phase diagram as only nanoemulsions were our target. The selected samples of nanoemulsions

whose composition is presented in Table 1 were subjected to further investigations.

2.2.2. Preparation of prednisolone (1 %) nanoemulsions

Prednisolone nanoemulsion was prepared by dissolving the specified quantity of the drug (1 % w/w) in the oil phase of each formulation (Table 1). The mixture of surfactant and co-surfactant was mixed with the oily phase under constant stirring utilizing a magnetic stirrer (1500 rpm), at ambient temperature (25 °C). The blend was titrated with distilled water according to the specified amount present in each formula. The whole mixture was emulsified by continuous stirring for 15 min. The nanoemulsion was further homogenized to minimize globule size with probe sonicator (Cole Parmer Inst., Illinois, USA) for 10 min, pulse 5 s, power 500-W, amplitude 60 %.

2.2.3. Preparation of prednisolone cationic nanoemulsions

A cationic surfactant (cetalkonium chloride) imparts positive charges to the oil nanodroplets. So, the cationic surfactant should be sufficiently lipophilic to be entrapped in the oil phase [27]. Cationic nanoemulsions were prepared by dissolving prednisolone in the oil phase then add the mixture of cationic surfactant (cetalkonium chloride 0.01 % w/w), and surfactant/co-surfactant mix to the oily phase, followed by addition of the determined quantity of water dropwise with continuous stirring at 1500 rpm at room temperature, utilizing a magnetic stirrer. The prepared cationic nanoemulsions were further homogenized to minimize globule size using probe sonicator (Cole Parmer Inst., Illinois, USA) for 10 min, pulse 5 s, at an amplitude of 60 %. The composition of cationic nanoemulsions of prednisolone (F1* and F2*) is listed in Table 1.

2.2.4. Physical stability of prednisolone nanoemulsion preparations

The physical stability of prednisolone loaded nanoemulsion preparations were assessed after subjecting to thermodynamic stability studies. Nanoemulsions were subjected to centrifugation test at 3500 rpm at 25 ± 2 °C for 30 min. The stable formulations should not exhibit any turbidity or separation of phases. Heating/cooling cycles between 4 °C and 40 °C for 48 h were applied and then the performance of nanoemulsions was evaluated. Freezing/thaw cyclings (3 cycles) between –21 °C and +25 °C (each cycle 24 h cooling and 24 h at 25 °C) were also investigated, the thermodynamically stable formulation should return to their original status within 2–3 min [28,29].

2.2.5. Characterization of prednisolone loaded nanoemulsions

The average size of droplets, surface charge, and polydispersity index of the selected nanoemulsion preparations, were evaluated by the dynamic laser light–scattering method employing the Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK), fitted with a 4-mW helium/neon laser functioning at λ = 633 nm.

The pH values of the produced nanoemulsions were measured utilizing an electronic pH meter (Mettler Toledo, Greifensee, Switzerland). The viscosity of the prepared nanoemulsions was checked employing a Brookfield digital DV-III viscometer (Brookfield Engineering Laboratories, INC, Stoughton, MA) with a UL-adaptor, spindle 00 at 10 rpm. All determinations were performed at 25 ± 2 °C. Measurements were made in triplicates and the outcomes are displayed as mean ± standard deviation (±SD).

2.2.6. Transmission electron microscopy (TEM)

Optimized nanoemulsion preparations were imaged using a transmission electron microscope (JEOL 2100, Tokyo, Japan). Aliquots (20 μl) were added to formvar®/carbon-coated 300 mesh grids. The specimen was placed on the grid for 1 min, and then any excess sample was filtered. The specimens were negatively stained using an aqueous uranyl acetate solution (20 μl of 2 % w/v) for a few seconds. The grids were left to dry overnight at ambient temperature. A high-resolution TEM instrument functioning at a 200 KV accelerating voltage was used to observe the samples.

2.2.7. In vitro prednisolone release investigation

The in vitro release of prednisolone from different nanoemulsion preparations (F1, F2, F1*, and F2*) in comparison to prednisolone suspension (Pred Forte® 1 %) was examined as represented previously [30,31]. A standard semi-permeable cellophane membrane (MWCO: 12,000–14,000) was used for the study. An elastic rubber band was used to tightly stretch the membrane over the end of a glass tube (2.4 cm internal diameter) that was opened on both ends. The examined formulation (0.3 g equivalent to 3 mg prednisolone) was introduced over the semi-permeable membrane in the release tube. The tube was submerged in 50 mL of phosphate buffer saline (PBS, PH 7.4). The test was conducted at a constant temperature in a water bath shaker (Gesellschaft für Labortechnik GmbH, Burgwedel, Germany) previously adjusted to 37 ± 0.5 °C and agitated at 50 rpm. Aliquots (5 mL) were drawn from the release medium at various time points up to 48 h and substituted with an equal volume of a freshly prepared PBS medium. The amount of prednisolone released was determined by measuring the absorbance at λ = 244 nm using a UV–visible spectrophotometer (Shimadzu Seisakusho, Ltd., Kyoto, Japan). Appropriate dilution of samples was done versus a blank, and the drug concentration was calculated using a previously constructed calibration curve ($R^2 > 0.999$). The cumulative amount of drug released over time was computed. The mean ± SD was represented (n = 3).

2.2.7.1. Drug release kinetics. Prednisolone in vitro release data was analyzed using various mathematical approaches (zero order, first order, Higuchi diffusion model, Baker-Lonsdale model, and Hixon-Crowell cube root law) to ascertain the mechanism of drug release from the produced nanoemulsion formulations. The proper model was chosen according to the highest correlation coefficient (R^2) value [32].

2.2.8. Ex vivo corneal permeability assessment

The permeation of prednisolone from the selected nanoemulsion formulations (F1, F2, F1*, and F2*) compared to prednisolone suspension (Pred Forte® 1 %) across the corneas of experimental rabbits, was tested using modified horizontal Franz diffusion cells (diffusion area 0.5 cm^2) [33]. Rabbits' corneas were removed immediately after the slaughter, put in phosphate buffer saline (PBS, PH 7.4), and kept at 4 °C. The excised rabbits' corneas were fixed between the donor and receptor compartments of the diffusion cells. The receptor cells were filled with 25 ml of PBS (pH 7.4) and kept at 37 ± 0.5 °C. Sodium azide (0.025 % w/v) was added during permeability experiment to avoid microbial growth. The agitation rate was adjusted at 50 rpm throughout the experiment. The selected nanoemulsion formulations and commercial suspension formulation (Pred Forte 1 %) (150 mg equivalent to 1.5 mg of prednisolone), were added to the donor cells. The aliquotes (3 ml) were removed from the receptor cell and substituted with an equal volume of fresh media every hour until 24 h. The quantity of prednisolone that permeated the cornea was determined spectrophotometrically at λ = 244 nm. Three runs were conducted for the permeation test.

2.2.8.1. Analysis of ex vivo corneal permeation data. The cumulative amount of prednisolone permeation was calculated and plotted as a function of time. The steady-state flux (J_{ss}, $\mu g/cm^2 \cdot h$) was estimated using the slope of the linear regression line. The permeability coefficient K_p (cm/h) of prednisolone through the cornea of rabbits was computed using the following equation [34]:

$$K_p = \frac{J_{ss}}{C_0}$$

Where C_0 = initial prednisolone concentration in the donor compartment. The effectiveness of nanoemulsions as ocular delivery systems for prednisolone is determined by enhancement factor percent calculated

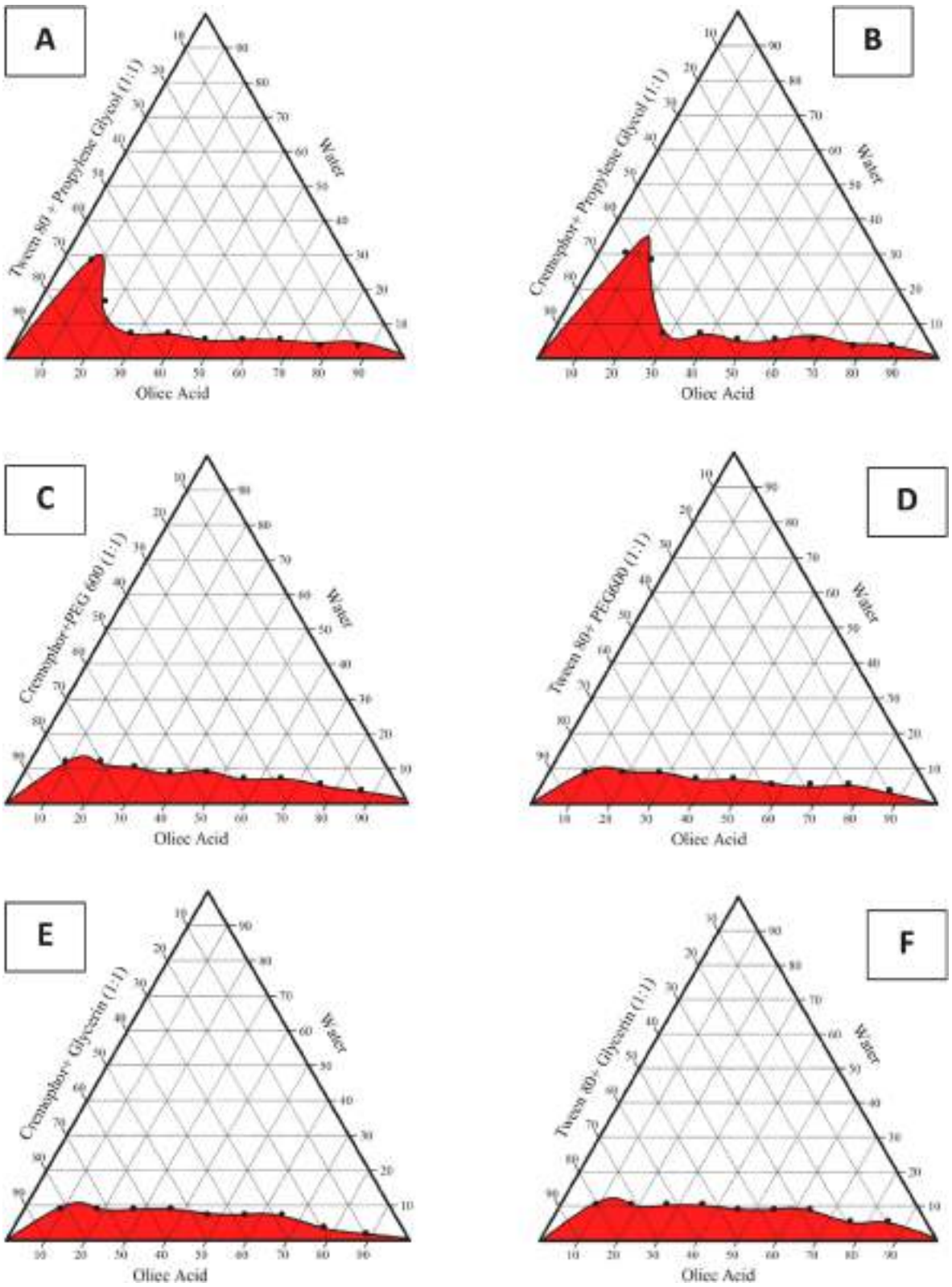

Fig. 1. Phase diagrams of: (Panel A): oleic acid, tween80: propylene glycol, water system. (Panel B): oleic acid, Cremophor® RH40: propylene glycol, water system. (Panel C): oleic acid, Cremophor® RH40: PEG 600, water system. (Panel D): oleic acid, tween 80: PEG 600, water system. (Panel E): of oleic acid, Cremophor®RH40: glycerin, water system. (Panel F): oleic acid, tween 80: glycerin, water system. The red area represents nanoemulsion region. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

according to the following equation:

$$EF\% = \frac{\text{Flux from nanoemulsion formation}}{\text{Flux from the commercial product}} X\ 100$$

2.2.9. Sterilization of the prepared formulations

The sterility of the selected nanoemulsion formulations was achieved by filtration through a sterile cap (0.22 μm GP Millipore Express® plus membrane). The preparations were then filled into propylene plastic bottles and subjected to a UV lamp for 20 min.

2.2.10. Evaluation of the efficacy and safety of prednisolone nanoemulsion formulations on uveitis-induced experimental rabbits' model

An experimental study was performed on 12 male albino New Zealand rabbits with approximate weights of 1.5–2 kg. The efficacy and safety of the proposed nanoemulsion preparations were assessed on rabbits after induction of uveitis using bovine serum albumin (100 mg/ml). The study was conducted consistent with the ARVO policy for using experimental animals in ophthalmic and vision investigations. The investigational study was authorized by the local ethics committee of the faculty of pharmacy, South Valley University, Qena, Egypt. (Approval No. A002/20). Rabbits (1.5–2 kg) were obtained from the University Animal Care Centre. Rabbits were kept in special cages with proper nutrition. Only the right eye per animal was investigated.

2.2.10.1. Experimental uveitis induction. A sterile bovine serum albumin (BSA) solution was prepared to obtain a 100 mg/ml solution. The proptosed globe was entered with a 30-gauge needle near the equator, and 0.1 ml (10 mg) of BSA solution was injected into the center of the vitreous body. The contralateral control eyes were injected with 0.1 ml of saline in the same manner. During injection, caution was taken to prevent damaging the lens and causing cataracts. One drop of 0.5 % ciprofloxacin eye drops (Epico, Egypt) was instilled to avoid eye contamination during uveitis induction.

The rabbits were divided into four groups:

- Group I: Prednisolone nanoemulsion formulation (F2*) two times daily.
- Group II: Prednisolone nanoemulsion formulation (F1*) two times daily.
- Group III: Commercial eye drop suspension (Pred Forte® 1 %, Allergan) administered two times daily.
- Group IV: Eye left untreated (negative control).

2.2.10.2. Clinical evaluation. After 7–10 days of uveitis induction, the rabbits' eyes were examined by slit lamp biomicroscope to determine the severity of iridocyclitis. Also, the safety of prednisolone nanoemulsion formulations was evaluated by measuring the IOP, pupil size, and histopathology of the tested eyes. Grading of the severity of iridocyclitis was done depending on the presence of signs of iritis (maximum score 9).

2.2.10.2.1. Determination of intraocular pressure (IOP). Intraocular pressure (IOP) was determined utilizing a Tono pen XL tonometer (Mentor, Norwell, MR) calibrated per the manufacturer's instructions. Before IOP measurement, a 10 μl of 4 % lidocaine solution was applied to the corneas to lessen any pain that might be experienced by the animal. Intraocular pressure was measured before the instillation of the formulation and at distinct time points after instillation. The right eye of the rabbit was administered 50 μl of the tested preparation, and the left eye served as a control.

2.2.10.2.2. Determination of pupil size. The pupil size of rabbits' eyes was measured before and after treatment using a ruler pupil gauge. A ruler pupil gauge was placed as close as possible alongside the pupil without tilting or bending the ruler. The outcomes were displayed as mean ± SD (n = 3).

2.2.10.3. Histopathological study. To test the safety of nanoemulsion formulations, a histopathological study was carried out using enucleated right eyes at the end of the clinical assessment of the induced uveitis. The animals were slaughtered, and the right eyeballs were removed for histological examination. Eyeballs were preserved in 10 % formalin and processed for typical histological sectioning. Preparation of paraffin wax was done. 5 μm parts of paraffin blocks were sliced with a microtome and stained with hematoxylin and eosin (HE) dyes. The stained blocks were visualized using a digital light microscope (Olympus CX31, Tokyo, Japan) equipped with a digital camera (Olympus, Camedia C-5060, Tokyo, Japan). Histopathological changes in the iris, ciliary body, choroidea, and retina were observed. Besides, the pathological alterations in the appearance of corneal epithelium were detected.

2.2.11. Statistical analysis

Each test was conducted in at least three separate runs. The results of each experiment were displayed as means ± standard deviations (SD). The statistically significant variations between the various groups were assessed using one-way analysis of variance (ANOVA) with Tukey Kramer multiple assessments or two-sided Student's t-test for pairwise comparison (GraphPad Prism 6.0, GraphPad, San Diego, CA).

3. Results & discussion

3.1. Impact of the type of co-surfactant on the composition of nanoemulsions

Preliminary experiments were performed to choose components for formulating prednisolone nanoemulsion. The choice of the vehicles is crucial because it ensures the drug will dissolve well, which is necessary to create a nanoemulsion [35]. Nonionic surface-active agents (Tween 80 and Cremophor® RH40) were selected due to their reported high safety profiles in ophthalmic formulations [11,36]. Besides, an HLB value > 10 is needed to create O/W nanoemulsions with good physical stability. This criterion was fulfilled by Tween 80 and Cremophor® RH40 surfactants, which have HLB values of 15 and 14–16, respectively [37]. The choice of oil and surfactant and the ratio of the oil to the surfactant/co-surfactant mix have an essential role in the production of nanoemulsions. The pseudoternary phase diagram was created to evaluate the influence of surfactant/co-surfactant type on the range of nanoemulsion existence. The transparent nanoemulsion zone is displayed in phase diagrams and highlighted in red (Fig. 1, Panels A, B, C, D, E, and F). According to ocular inspection, the remainder of the phase diagram reflects turbid and typical emulsions.

In this study, surfactant/co-surfactant at a 1:1 ratio was selected in all formulations because this ratio gives the most stable (after preparation) composition of the nanoemulsion. The illustrations demonstrated that nanoemulsions prepared using propylene glycol (PG) as a co-surfactant include higher amounts of water (28.5–30.5 %) compared to other nanoemulsion formulations containing PEG 600 or glycerin as co-surfactants. Consequently, the zone of nanoemulsion within the phase diagram was expanded upon using either Tween 80 or Cremophor® RH 40 surfactants in the presence of PG as a co-surfactant. The expansion of the nanoemulsion region upon the inclusion of PG could be due to the incorporation of PG into the surfactant layer at the interface, thus increasing the interfacial fluidity [38,39]. From a technological perspective regarding cosmetic and pharmaceutical applications, the most desirable nanoemulsion formulation should consist of a considerable quantity of water and oil phases with lower concentrations of surfactant and co-surfactant. In the current study, this composition was shown by nanoemulsion formulations in the ratio of oil to surfactant/co-surfactant mix of 1:9 w/w (F1 and F2), which incorporates a significant amount of water (28.5–30 %). The nanoemulsions' composition is summarized in Table 1.

Table 2
Physicochemical characteristics of prednisolone nanoemulsion formulations.

Formula code	Droplet size (nm) (±SD)	Zeta potential (mv) (±SD)	PDI (±SD)
F1	89.7 ± 0.4	−12.4 ± 0.6	0.443 ± 0.003
F1*	62.1 ± 0.3	18.1 ± 0.6	0.386 ± 0.021
F2	211.2 ± 1.0	−11.7 ± 0.6	0.421 ± 0.009
F2*	154.8 ± 2.9	17.4 ± 0.9	0.372 ± 0.011

Table 3
Appearance, pH and viscosity of prednisolone nanoemulsion preparations.

Formula code	Appearance	pH (±SD)	Viscosity (mPas) (±SD) at 10 rpm
F1	Transparent	6.1 ± 0.2	200.2 ± 3.6
F1*	Transparent	5.6 ± 0.2	202.6 ± 1.5
F2	Transparent	6.2 ± 0.1	304.7 ± 5.1
F2*	Transparent	5.8 ± 0.1	305 ± 3.6

3.2. Physical stability of prednisolone nanoemulsion formulations

The prepared nanoemulsion formulations (F1 to F6) were subjected to thermodynamic stress evaluations (centrifugation for 30 min at 3500 rpm, heating/cooling cyclings, and freeze/thaw rounds). All the nanoemulsion formulae passed the centrifugation test (no phase separation). However, concerning the data obtained from heating/cooling and freeze/thaw cyclings, most preparations failed the tests except formula F1 and F2 with or without cetalkonium chloride (CKC). Compared to conventional emulsions, which have kinetic stability and will ultimately experience phase separation, nanoemulsion formulations have a longer shelf life due to their thermodynamic stability [40]. Thermodynamically stable preparations (F1, F1*, F2, and F2*) were chosen for further evaluations.

3.3. Characterization of prednisolone nanoemulsion formulations

Physicochemical parameters of the prepared prednisolone nanoemulsion formulations (F1, F1*, F2, and F2*) were evaluated and illustrated in Table 2. The selected nanoemulsion formulations exhibited a mean droplet diameter of 62.1 ± 0.3–211.2 ± 1.0 nm. This small droplet diameter was attributed to the co-surfactant molecules in the nanoemulsion system that reduce the fluidity and viscosity of the interfacial film, diminishing the radius of the nanoemulsion droplets and producing transparent formulations [41]. Due to their increased surface

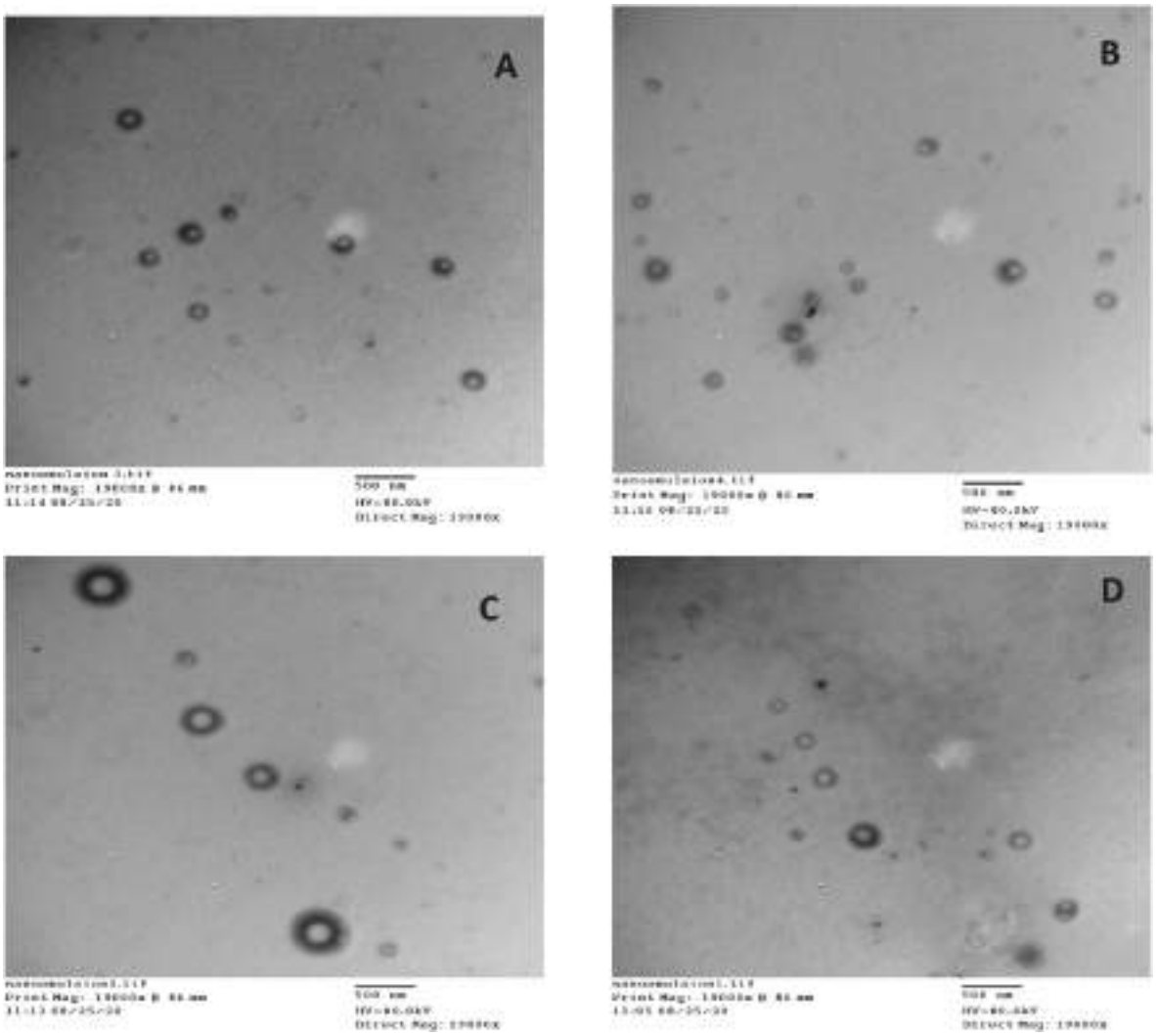

Fig. 2. Representative TEM photomicrographs of nanoemulsion formulation F1 (A), F1* (B), F2 (C), and F2* (D).

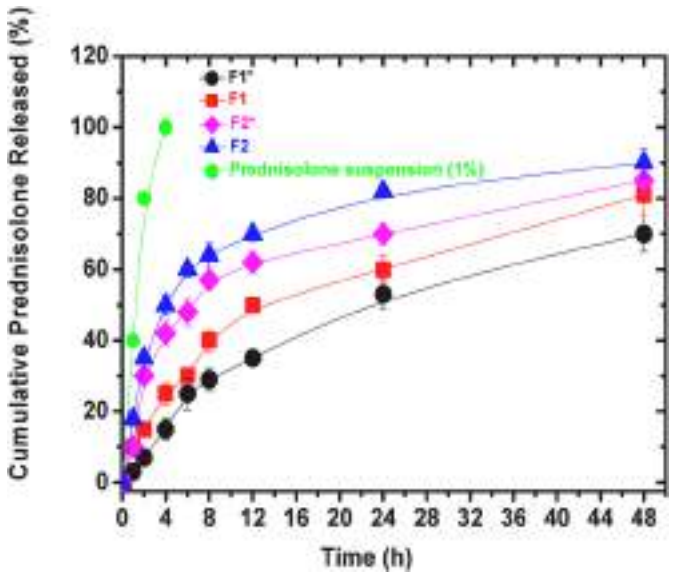

Fig. 3. Cumulative in vitro release patterns of prednisolone from various nanoemulsion preparations (F1, F1*, F2, and F2*) and Pred Forte® suspension (1 %) in phosphate buffer pH 7.4 at 37 °C.

area and enhanced capability to permeate ocular tissues, nanosized drops could provide higher doses of medication to the eye, lessening the frequency of dosing and the associated adverse effects [42]. Adding cationic surfactant (CKC) decreased the droplet diameter and polydispersity index and resulted in positive zeta potential values compared with the same formulations without CKC. The positively charged surface of oil droplets generates a repulsive electrostatic force, hindering the coalescence of nanoemulsion droplets during storage [43–45]. These findings agree with prior studies [18,46]. The polydispersity index values were less than 0.5. These outcomes demonstrate the uniformity and narrow distribution of droplet size.

3.4. Rheological properties of prednisolone nanoemulsion

Viscosity is an essential feature since it has a significant impact on the bioavailability of ophthalmic drugs and their corneal retention period. The prepared nanoemulsions (F1, F1*, F2, and F2*) have a viscosity range from 200.2 ± 3.6–305 ± 3.6 mPas, which makes them easily poured during dripping into the eye. The viscosity of the selected formulations (Table 3) did not change significantly with the incorporation of cetalkonium chloride (CKC) compared with the same formulations without CKC. The formulations containing Cremophor® RH40 surfactant (F2 and F2*) had high viscosity relative to those containing Tween 80 surfactant (F1 and F1*). These findings are in good agreement with the prior research data, which attributed the increase in viscosity of formulations containing Cremophor® RH40 surfactant to the difference in viscosity between Cremophor® RH40 (semisolid) and Tween 80 (liquid) at room temperature [11].

The pH of the tested prednisolone nanoemulsion preparations varied from 5.6 to 6.2 (Table 3). Coles and Jaros [47] conducted an investigation that demonstrated that a decrease in pH by two drops of Medriacyl (pH 4.8) and phenylephrine (pH 6.0) caused a prompt restoration to the initial pH within 20–40 min. The authors explained that based on the dilution effect of the reflux tears induced by irritation of the acidic pH. Thus, the prepared nanoemulsions comply with pharmacopeial requirements for ocular administrations [48,49].

According to the previously discussed experimental outcomes, the prednisolone-loaded nanoemulsion preparations (F1 and F2), either with or without the addition of cetalkonium chloride, are thermodynamically stable and remained isotropic and thus were selected for further evaluations.

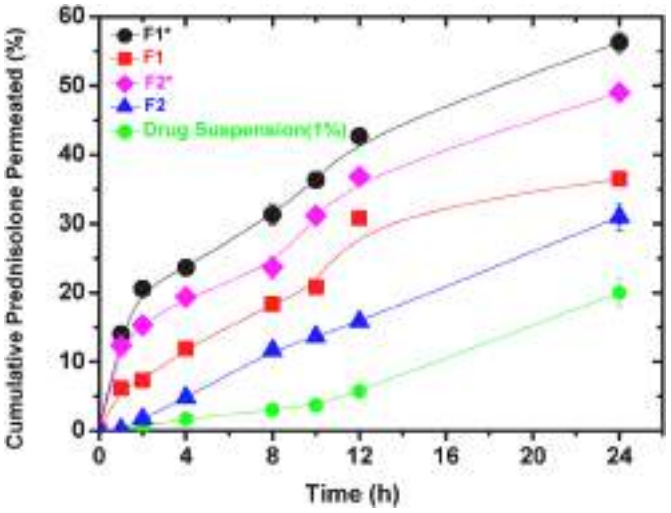

Fig. 4. Ex vivo permeation profiles of prednisolone from the developed nanoemulsion preparations (F1, F1*, F2, and F2*) in comparison with eye drop suspension (Pred Forte® 1 %).

Table 5
Permeation parameters of prednisolone through cornea of rabbit eye from different nanoemulsion formulations compared to drug suspension (Pred Forte® 1 %).

Formulation code	J_{ss} (μg. cm^{-2}. hr^{-1})	Kp x 10^{-3} (cm hr^{-1})	EF (%)
Drug suspension (1 %)	25.20 ± 2.44	2.52 ± 0.24	–
F1	41.77 ± 0.59	4.17 ± 0.05	165.75 ± 15.78
F2	40.16 ± 0.96	4.01 ± 0.09	159.36 ± 15.18
F1*	53.66 ± 1.92	5.36 ± 0.19	212.94 ± 26.42
F2*	48.49 ± 0.96	4.84 ± 0.09	192.42 ± 20.03

EF (%): Enhancement factor percent observed among nanoemulsion formulations compared to commercial drug suspension (Pred Forte® 1 %).
Kp: Permeability coefficient.

Table 4
Kinetic release mechanisms of prednisolone from different nanoemulsion formulations.

	Zero- order	First-order	Higuchi Diffusion	Hixon	Baker
Formulations	Correlation coefficient (R^2)	Correlation coefficient (R^2)	Correlation coefficient (R^2)	Correlation coefficient (R^2)	Correlation coefficient (R^2)
Drug suspension 1 %	0.923	0.997	0.954	0.996	0.998
F1	0.932	0.988	0.986	0.975	0.996
F1*	0.943	0.986	0.992	0.975	0.998
F2	0.804	0.948	0.911	0.908	0.957
F2*	0.833	0.954	0.927	0.921	0.979

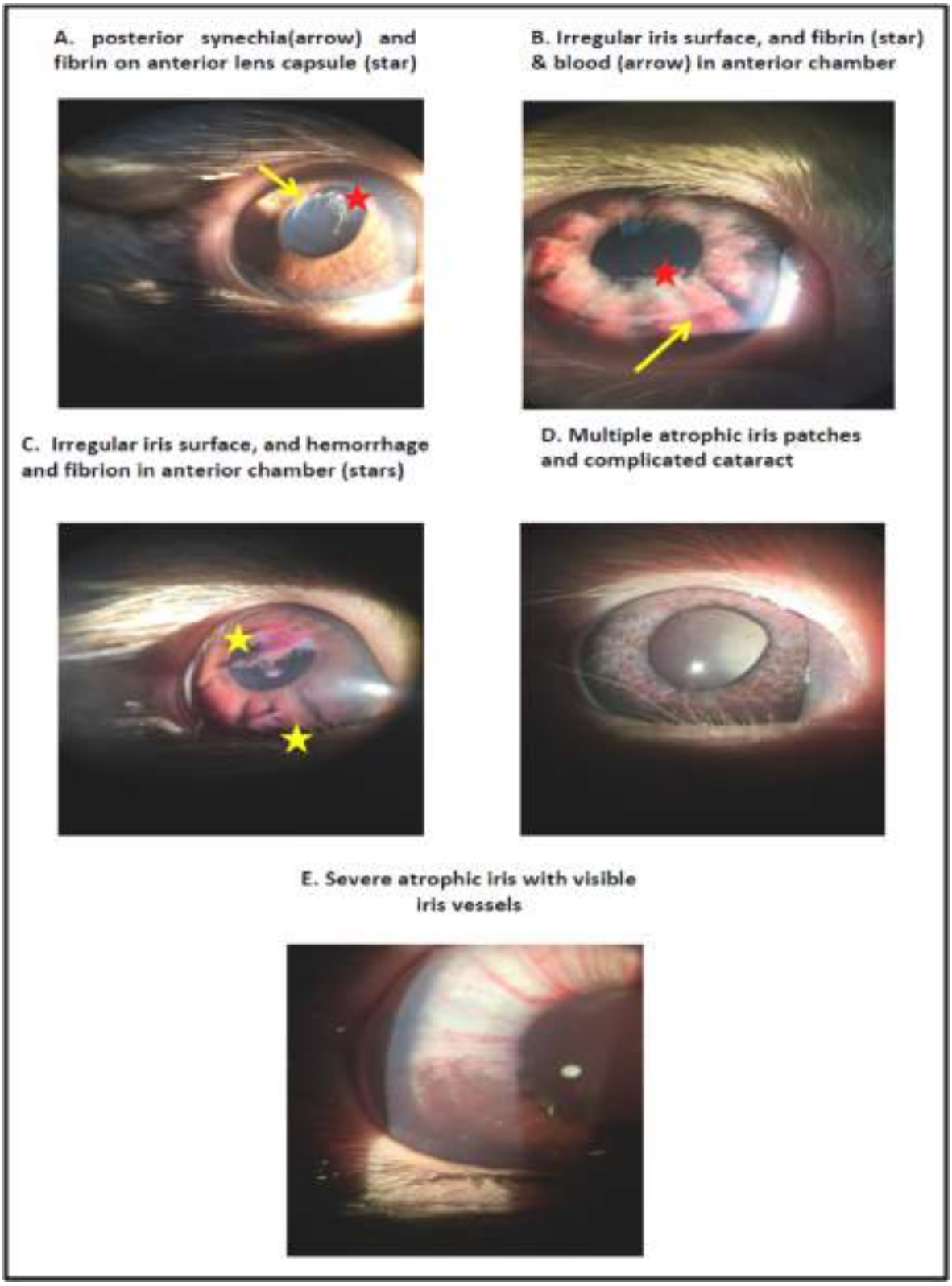

Fig. 5. Clinical signs of acute anterior uveitis after one week of interavitreal injection of BSA.

3.5. Transmission electron microscopy (TEM)

The morphology and surface properties of the optimum nanoemulsion formulations with and without the addition of cetalkonium chloride (F1, F1*, F2, and F2*) were investigated by TEM analysis and depicted in Fig. 2 (Panels A, B, C, and D). The TEM images displayed the spherical form and homogenous size of the nanoemulsion droplets. The droplets dispersed without agglomeration throughout the field. The effect of cetalkonium chloride addition on decreasing droplet size of the investigated formulations, was also demonstrated. The droplet diameter measured by TEM analysis agrees with that reported by DLS assessments (Table 2).

Table 6
Grading score of severity of iridocylclitis.

Type	Clinical observations	Score
Anterior chamber cells per field	None 0	0
	Faint <15	1
	Moderate 16-25	2
	Marked 26-50	3
	Severe 50+	4
Fibrin or synechiae	Absence	0
	Presence	1
Iris dilation	Normal	0
	Abnormal	1
Irregular iris surface, or iris bombe'	Absent	0
	Present	1
Hypopyon, or hyphema	Absent	0
	Present	1

3.6. In vitro prednisolone release

The cumulative prednisolone released from the developed nanoemulsion formulae (F1, F1*, F2, and F2*) was estimated in phosphate buffer saline (PBS, PH 7.4, at 37 ± 0.5 °C), utilizing the dialysis membrane diffusion method and compared to prednisolone suspension (Pred Forte® 1 %), Fig. 3. The prednisolone suspension exhibited a percentage drug release of 100 ± 2.5 % over 4 h. Prednisolone release was rapid from the Pred Forte® dispersion as it was controlled only by the rate of drug dissolution [50]. The prednisolone-loaded nanoemulsion formulation (F1) showed a drug diffusion of 50 ± 2 % in the first 12 h, then extended prednisolone release (81 ± 6 %) over 48 h, was subsequently followed. The prednisolone-loaded nanoemulsion formulation (F2) demonstrated a drug diffusion of 70 ± 2 % in the first 12 h, followed by extended prednisolone release (90 ± 4 %) up to 48 h. The higher in vitro release percentage of prednisolone from nanoemulsion formulation (F2) containing Cremophor® RH40 surfactant versus F1 formulation that contains Tween 80 surfactant may be ascribed to the increased solubilization of prednisolone in Cremophor® RH 40. Formulations F1* and F2*, which contain cationic surfactant (CKC, 0.01 % w/w), displayed more sustaining drug release profiles in comparison with the same formulations without CKC (F1 and F2). The efficiency of drug release from these nanoemulsion formulations is ordered as follows: F2 > F2* > F1 > F1*. The obtained release data revealed that prednisolone nanoemulsions exhibit a more sustained in vitro drug release in comparison with the aqueous drug suspension (Pred® forte 1 %). The sustained release pattern of prednisolone from nanoemulsion formulations may be due to the solubility of prednisolone in the oil drops, which act as a drug reservoir. Thus, prednisolone release is controlled due to the hydrophobicity of the drug that favors remaining in the oil phase [51]. This explanation can be potentiated by the results obtained from the kinetic release mechanism analysis of the drug from the optimized nanoemulsion preparations. Fitting of the drug release data with the Baker-lonsdale model was done, which explains the drug release from spherical particles (oil droplets) in which the drug dispersed through an inert diffusion matrix [52] (Table 4). This finding was in line with earlier investigations that outlined the same release profiles of drugs from nanoemulsion systems [53,54].

3.7. Ex vivo corneal penetration test

The cornea of rabbits was employed as an in vitro experiment to compare the ocular permeability features of prednisolone nanoemulsion systems (F1, F1*, F2, and F2*) versus free prednisolone suspension (Pred

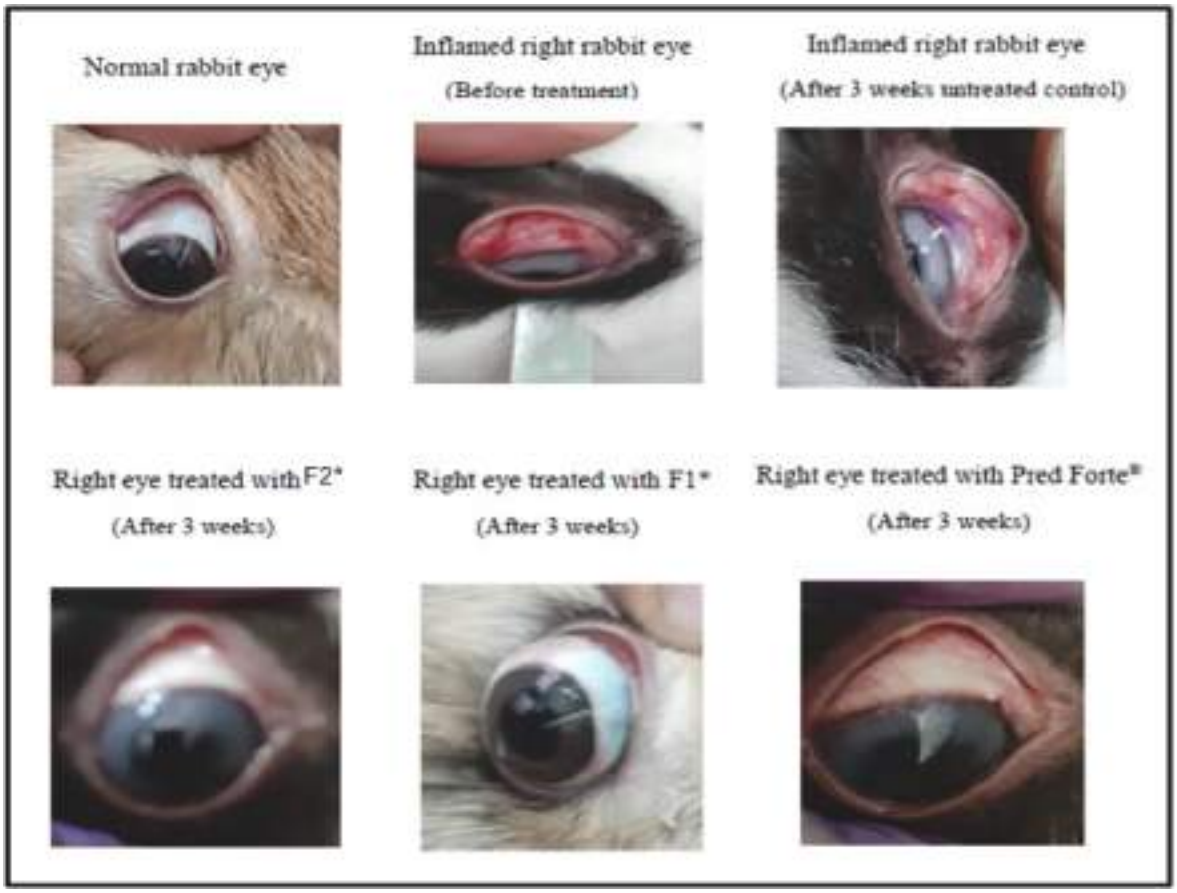

Fig. 6. Successive images of representative eyes of rabbits before and after 3-weeks treatment using nanoemulsion formulation (F1*, F2*) and Pred Forte® suspension.

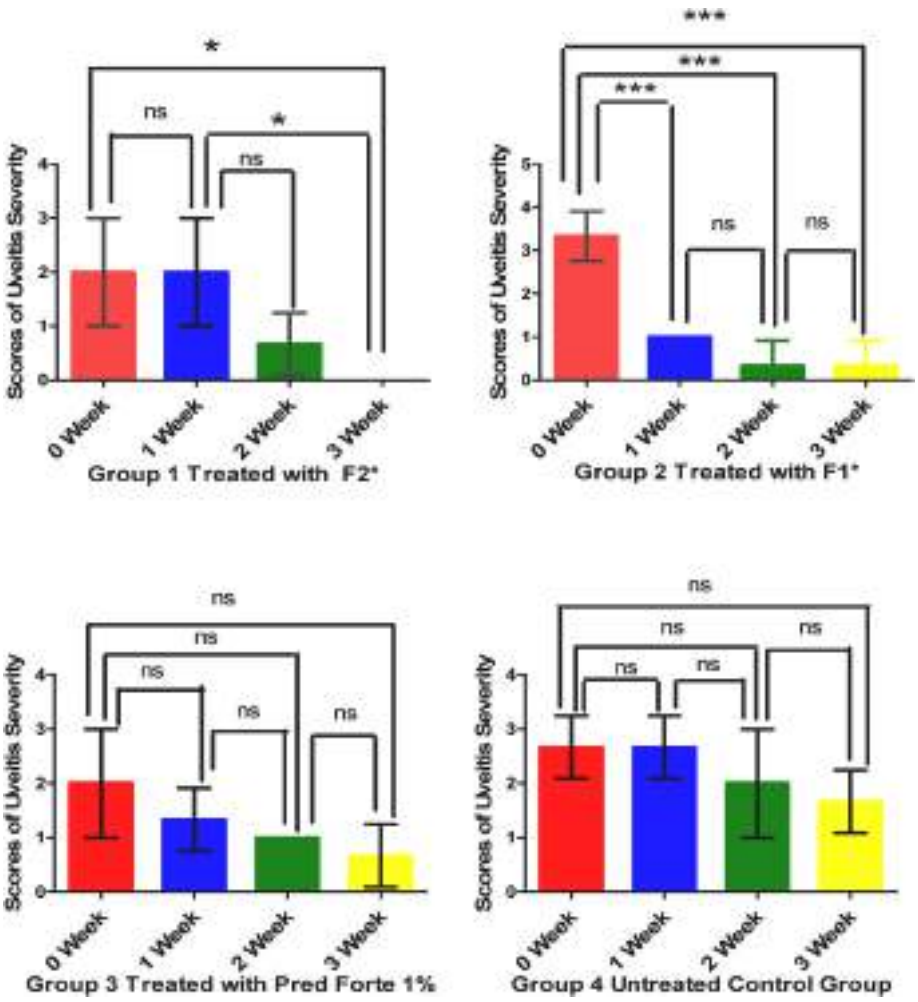

Fig. 7. Differences in uveitis scores within the period of treatment using prednisolone naoemulsion formulations (F1* and F2*), Pred® Forte 1 % suspension versus un-treated control group. ^{ns} Non-significant difference ($p > 0.05$). * Significant difference ($p < 0.05$). *** Extremely significant different ($p < 0.001$). Statistical significance was computed using Student's t-test.

Forte® 1 %) to gain knowledge on the in vivo ophthalmic bioavailability of the optimized prednisolone nanoemulsion formulations. The cumulative percentage of prednisolone permeated from the nanoemulsion formulations, as opposed to the drug suspension, and the respective permeability parameters are illustrated in Fig. 4 and Table 5. The prednisolone-loaded nanoemulsion formulations (F1 and F2) displayed an enhancement in the percent of cumulative drug permeation through the cornea in comparison with drug suspension, with 30.833 ± 0.76 % and 15.833 ± 0.76 % permeation of drug from F1 and F2, respectively, after 12 h, in comparison to only 5.733 ± 0.25 % prednisolone transport from the free suspension at the same period. The highest percentage of prednisolone diffused after 24 h from the selected nanoemulsion formulations (F1 and F2) was approximately 1.8–1.6 times, respectively, larger than that of the free drug suspension, Fig. 4. The incorporation of the cationic agent (CKC) results in a noteworthy increase in prednisolone percent permeability from F1* and F2* nanoemulsion formulations (56.333 ± 1.53 % and 49 ± 1 %, respectively) compared to only 20 ± 2 % percent permeation of drug dispersion at the end of 24 h experiment, Fig. 4. The drug permeability criteria also demonstrated that F1* and F2* nanoemulsions exhibited significantly higher ($p < 0.05$) ocular flux (J_{ss}) in comparison with prednisolone suspension **(Table 5)**. Moreover, the addition of CKC to formulations F1* and F2* resulted in a notable increase in the enhancement factor (EF%) relative to either the formulations without a cationic agent (F1 and F2) or the drug dispersion **(Table 5)**. This finding signifies the effectiveness of CKC as a potential enhancer in the studied system. The enhancing effect of CKC on the permeation of prednisolone through the cornea of rabbits may be attributed to the improved distribution of the cationic nanoemulsion eye drop formulation owing to the electrostatic attraction experienced between oil nanodroplets (positive charges) and the ocular surface (negative charges) [55]. Recently, nanosuspension preparation of prednisolone acetate was developed by Nandwani et al. [24]. Ex vivo goat corneal adhesion and penetration assessment revealed that a higher fraction of nanosuspension adhered to the cornea compared to a microsuspension formulation. However, penetration of the drug into the receptor compartment was not significant for both formulations. Moreover, prednisolone-loaded chitosan nanoparticles were previously prepared by the ionic gelation method following optimization via response surface methodology. The optimized formulation showed a maximum entrapment efficiency of 78.32 % and a controlled release behavior compared to the marketed drug formulations. However, no evaluations of the corneal permeation, the safety or therapeutic efficacy of the proposed preparation were done [56].

The current study evaluated the potential of nanoemulsion formulations comprising prednisolone and cetalkonium chloride to enhance the permeation parameters. The results indicated an approximate doubling of the permeation parameters in the presence of cetalkonium chloride. Through analysis of the existing literature about nanoemulsion formulations for *trans*-corneal drug delivery, it has been found that the majority of previous research highlights two primary factors that impact

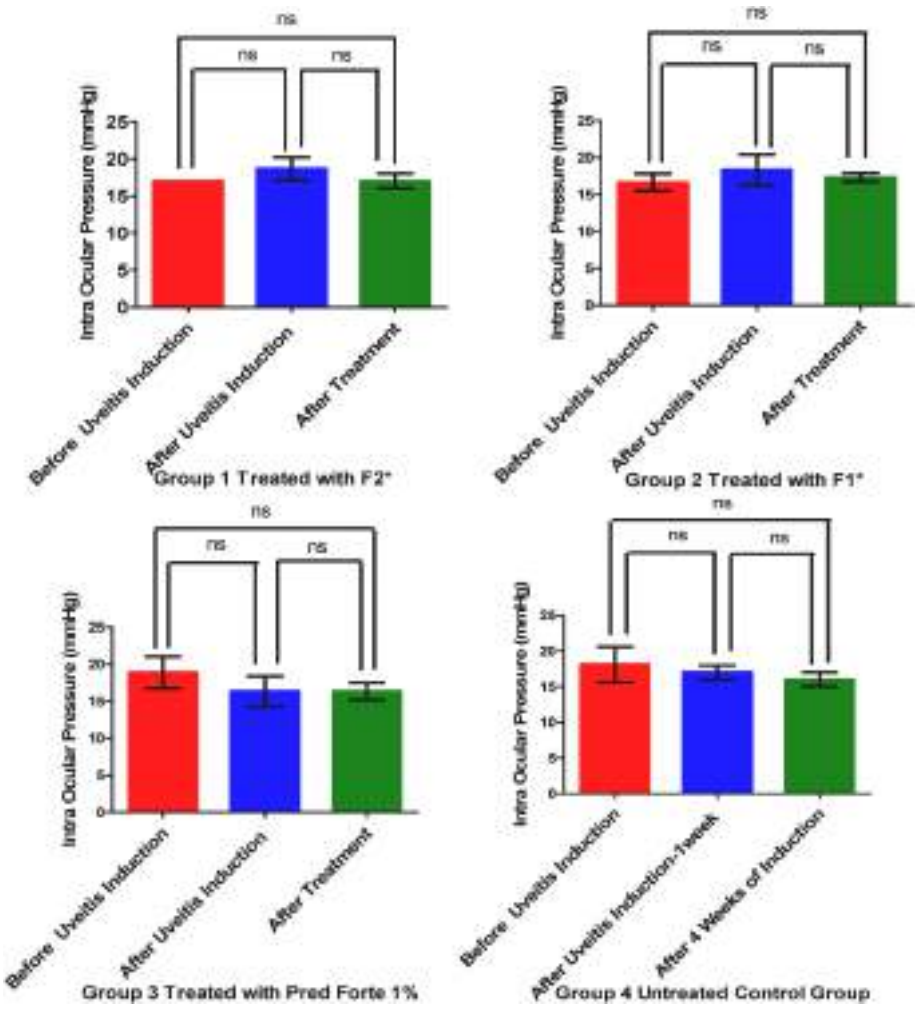

Fig. 8. Changes in IOP values within the period of treatment using different prednisolone nanoemulsion formulations (F1* and F2*), Pred® Forte 1 % suspension versus un-treated control group. [ns] Non-significant difference ($p > 0.05$). Statistical significance was computed using Student's t-test.

the efficacy of *trans*-mucosal or *trans*-dermal drug delivery: water content and oil contents [55,57,58]. In the current investigation, it was observed that additional variables exert an influence on the regulation of drug permeation, namely the nature of the surface-active agent employed and the proportion of surfactant to co-surfactant. Furthermore, the cationic nature of the dispersed phase (i.e., oil droplets) exhibits a significant influence on the drug permeability. According to the reported experimental outcomes, F1* and F2* were chosen for the in vivo clinical evaluation study to evaluate the therapeutic efficiency of the developed cationic nanoemulsions in uveitis management.

3.8. Evaluation of the efficacy of prednisolone loaded cationic nanoemulsion formulations

The anti-inflammatory potential of cationic prednisolone-loaded nanoemulsions (F1* and F2*) on the uveitis-induced animal model was evaluated and compared with prednisolone suspension (pred Forte®, 1 %). The first signs of uveitis appeared in the first week after the intravitreal injection of 10 mg BSA. The early signs developed as typical symptoms of ocular inflammation, while the onset of the second reaction occurred between 7 and 10 days following BSA injection (Fig. 5 A-E). The second inflammatory response was continued for several days with a gradual decrease in the severity of iridocyclitis during the period of treatment (3 weeks) by prednisolone nanoemulsions (F1* and F2*) in comparisons with prednisolone suspension 1 % (pred Forte®).

The first response of inflammation which lasts for not more than three days, may be attributed to a conterminal of protein preparation rather than to the trauma of injection itself [59]. This conclusion is potentiated by the fact that under experimental conditions, a pyrogen-free pharmaceutical solution of human serum albumin does not cause a similar initial episode of uveitis [59]. The clinical manifestations of acute anterior uveitis include blepharospasm, episcleral, conjunctival hyperemia, diffuse corneal edema, miosis, aqueous flare (indicative of protein and cells in the anterior chamber, fibrin in the anterior chamber, hypopyon and hyphema) [60].

The rabbit's eyes were examined using a slit lamp biomicroscope to determine the severity of iridocyclitis (a condition triggered by inflammation of the iris and ciliary body) after one week of intravitreal injection of BSA. The uveitis signs and scores are illustrated in Fig. 5 and Table 6. Grading of iridocyclitis severity was done depending on the presence of signs of iritis. Based on the scoring system outlined by McNeil Rod, the clinical symptoms of ocular inflammation were rated on a scale of 0–9 [61].

A three-week treatment regimen using prednisolone nanoemulsion formulations (F1*, F2*, and Pred Forte® suspension) was applied. The rabbits received treatment twice a day. The findings are depicted in Figs. 6 and 7. The study demonstrated that applying the prednisolone nanoemulsion formula (F1*) on rabbits' eyes resulted in an extremely significant outcome during 1, 2, and 3 weeks compared to the initial week (zero-week), $p < 0.001$. However, there was no significant variation in the clinical outcome of uveitis management during the third week when compared to either the first week or the second week and

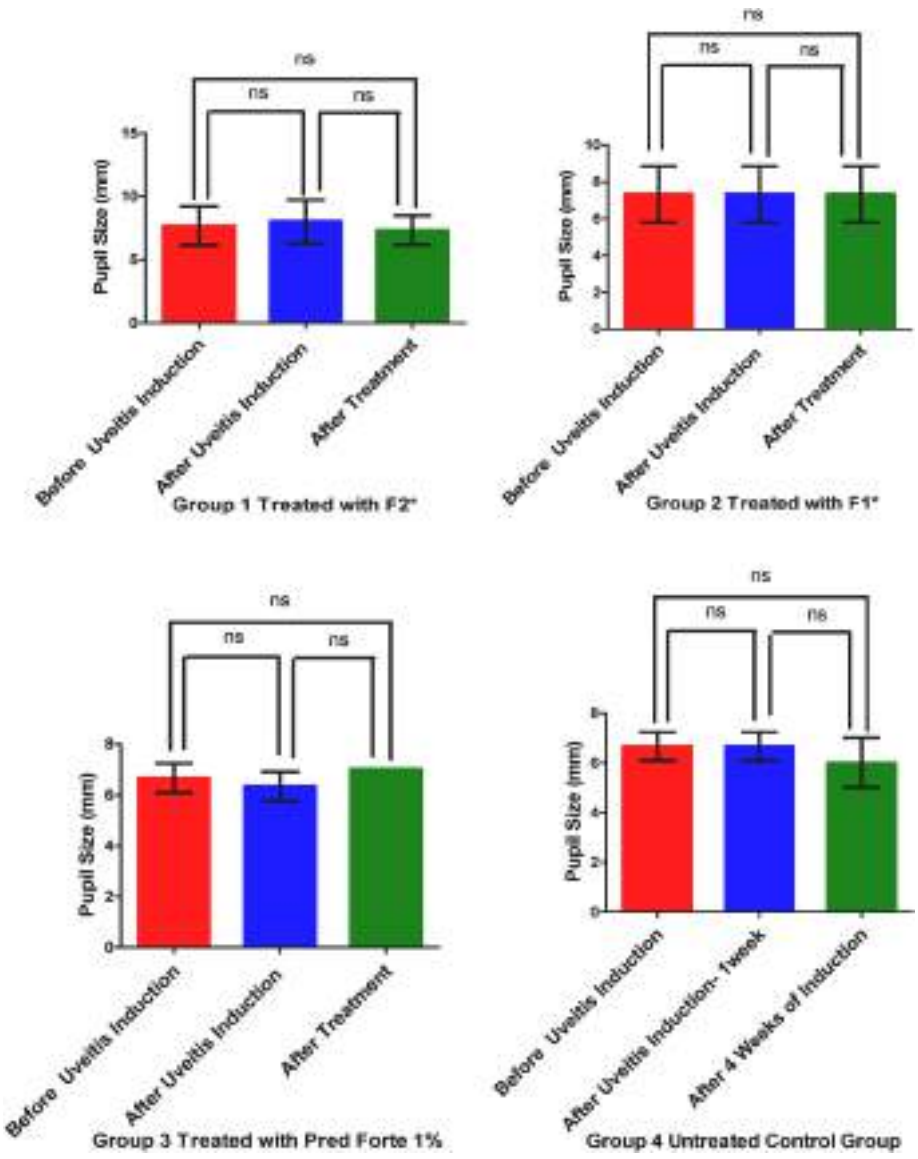

Fig. 9. Changes in right eye pupil size values within the period of treatment using different prednisolone nanoemulsion formulations (F1* and F2*), Pred® Forte 1 % suspension versus un-treated control group. ^{ns} Non-significant difference ($p > 0.05$). Statistical significance was computed using Student's t-test.

between the second week relative to the first week ($p > 0.05$). Moreover, during the treatment of uveitis with the prednisolone nanoemulsion preparation (F2*), there was a significant reduction in the intensity of uveitis within the periods of the third week in comparison with the first week and initial week (zero-week) ($p < 0.05$). There was no significant decrease in uveitis signs within the periods of the third week versus the second week, the second week versus the first week, the second week versus zero-week, and zero week versus the first week ($p > 0.05$), Fig. 7. Over three weeks, the application of prednisolone suspension on the eyes of rabbits (positive control group) did not show any significant impact on the reduction of the severity of uveitis (Fig. 7).

Recently, prednisolone acetate loaded chitosan-deoxycholate self-assembled nanoparticles were developed to increase the drug's ocular bioavailability, and efficacy in managing inflammatory disorders of the eye. The outcomes demonstrated a higher anti-inflammatory potential of prednisolone nanoparticles loaded gel compared to the drug loaded gel treatment. The findings were attributed to the nano-nature of drug in the nanoparticle preparation, the penetration enhancing efficiency of chitosan and deoxycholate, and the mucoadhesive properties of chitosan [62]. Katzer et al. [63], developed prednisolone-incorporated nanocapsules using a positively charged biocompatible, non-biodegradable polymer (Eudragit®RS100). No significant loss of viability of rabbit corneal epithelial cell line (SIRC) was recorded after the treatment with the developed nanocapsules formulations. Although no in vivo tests were conducted on an experimental animal model. Besides, prednisolone loaded polymeric nanoparticles were previously developed using poly lactide-co-glycolide (50:50) and poly DL-lactide polymers. The formulation variables were optimized using a 2^4-factorial layout. However, further in vitro and in vivo evaluations were required to assess the therapeutic efficacy of the produced formulation in managing inflammatory eye disorders [64]. Another investigation for improving ocular availability of prednisolone was reported by ElShaer et al. [65]. The authors developed prednisolone- PLGA nanoparticles loaded contact lenses. The outcomes showed that unloaded contact lenses had a better hydration level when compared to nanoparticle-loaded contact lenses. Further investigations are required to evaluate the developed formulation for maximum therapeutic efficiency and patient compliance.

Herin, the developed cationic nanoemulsion formulation of prednisolone, improved the residence time of the product in the eye via the electrostatic interaction with the opposite charges of the mucus layer of the eye. Thus, the developed formula showed enhanced corneal

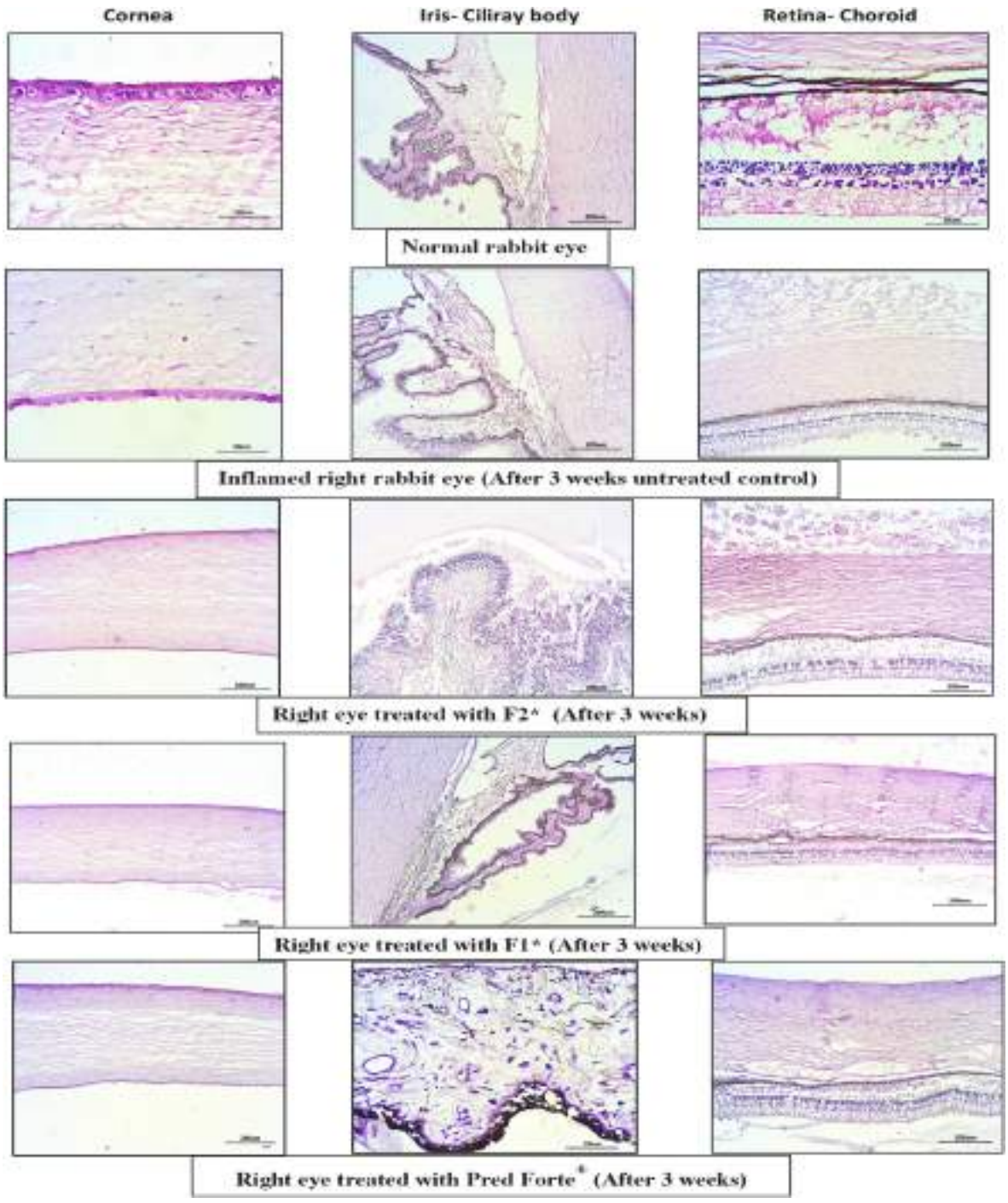

Fig. 10. Light microscopy images of representative cornea, iris, ciliray body, choroid and retina of rabbit after treatment using nanoemulsion formulations (F1*, F2*) and Pred® Forte 1 % suspension.

residence and permeation. Besides, improved anti-inflammatory efficacy was due to the increased retention time at the ocular surface after instillation. Moreover, the better stability of the cationic nanoemulsion formula during the preparation course and its better long-term stability via repulsive electrostatic forces between the positively charged oil nanodroplets were other merits. Additionally, cationic nanosystems have bio-adhesive characteristics that extend their corneal retention. Compared to cationic liposomes, cationic nanoemulsions have recently revealed better ocular tolerability and notable potential for drug delivery to the eye [17,66,67].

3.8.1. Intraocular pressure (IOP) determination

One of the essential measures for evaluating the safety of utilizing ophthalmic formulations involves assessing their impact on IOP. The occurrence of glaucoma induced by steroid treatment in the long term is a recognized complication concerning IOP. The present investigation confirmed that the developed prednisolone nanoemulsion preparations (F1* and F2*) did not trigger a statistically significant elevation in IOP in the rabbit model during the entirety of the three-week study period (Fig. 8). Although the addition of topical anesthetic (4 % lidocaine solution, 10 μl) may influence the measured IOP reading, any decrease in IOP due to the instilled local anesthetic will affect all the investigated animal groups (treated and untreated) equally. The present findings are

reinforced by the prior research conducted by Chennamaneni et al. [68], which demonstrated that the bio-erodible dexamethasone implant did not elicit a noteworthy elevation in intraocular pressure (IOP) in the rabbit model during the entire duration of the investigation (6 weeks).

3.8.2. Pupil size measurement

Measurement of pupil diameter of the rabbits' eyes after instillation of either prednisolone nanoemulsion formulations (F1* and F2*) or prednisolone suspension (1 %) revealed that there was no significant change in pupil diameter of all rabbits' groups ($P > 0.05$) (Fig. 9). According to the previous research conducted by Kahane and colleagues [69], it has been established that the use of prednisolone did not cause any changes in either the diameter of the pupil or the intraocular pressure within the eye. Also, a prior investigation demonstrated comparable outcomes in normal dogs, indicating that dexamethasone did not affect the diameter of their pupils [70].

3.8.3. Histopathological studies

In the previously mentioned data, it was demonstrated that the nanoemulsion formulae (F1* and F2*) exhibit a more prolonged drug release and an enhanced permeability of prednisolone compared to other tested nanoemulsion formulations. Therefore, these two formulations have been chosen to evaluate their impact on the ocular efficacy and safety of rabbits' eye tissues clinically and histopathologically. The histopathological examination of the rabbit's ocular tissues, comprising the cornea, ciliary body, iris, and retina, was carried out, Fig. 10. The results indicated that there were no pathological changes observed in the corneal epithelium, Bowman's layer, iris/ciliary body, or retina/choroid of the control rabbit eyes (Fig. 10).

On the other side, the eyes that were not treated demonstrated a thickened Descemet membrane and vacuoles in the cytoplasm of the cornea's endothelial cells, as well as edema and mild inflammation in the iris/ciliary body. Mild lymphatic infiltration and vascular congestion were also observed. Additionally, there was edema in the retina/choroid and a slight infiltration in the outer layers of the sclera. After examining the effects of the developed nanoemulsion formulations (F1* and F2*) and the prednisolone suspension (Pred® Forte 1 %) on rabbit eye tissue structure for three weeks, we discovered that F1* did not lead to any negative changes in the cornea, retina/choroid, or iris/ciliary body. Similar outcomes were observed using F2*, except for swelling in the iris and ciliary body and the presence of mild inflammatory cells. Regarding the effect of prednisolone suspension (1 %) on the corneal tissue, there was evidence of swelling and the presence of fluid within the endothelial cells, as well as the separation of layers within the retina/choroid accompanied by mild inflammation and a buildup of lymphocytes.

4. Conclusions

In the present study, cationic prednisolone nanoemulsions were efficiently formulated with a nanosized hydrodynamic diameter, low PDI values, and a positive surface charge. Moreover, cationic prednisolone nanoemulsions were capable of sustaining drug release more successfully, compared to the drug suspension (Pred Forte® 1 %) or the same formulation without cationic surfactant. Significant improvements in the permeability of prednisolone-incorporated cationic nanoemulsions through the rabbits' corneas compared to drug suspension (Pred Forte® 1 %) have been demonstrated. A notable reduction in the intensity of uveitis during the treatment course (three weeks) was recorded after the instillation of prednisolone cationic nanoemulsion formulations in the uveitis-induced rabbits' model as opposed to drug dispersion (Pred Forte® 1 %). It seems that the positive droplets' surface charge and the sustaining effect on drug release enhance the bioavailability of the nanoemulsion, besides the safety of the suggested formulae. Consequently, using prednisolone cationic nanoemulsion formulation for ocular drug delivery is considered a promising strategy for control of uveitis. Future perspectives will focus on the application of molecular and immunological investigations, particularly polymerase chain reaction (PCR) for the diagnosis of infectious uveitis. Furthermore, levels of cytokines (IL-1, IL-6 and TNF-α) and leukocytes will be determined via Enzyme-linked immunosorbent assay (ELISA), flow cytometry, and spectroscopic optical coherence tomography.

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CRediT authorship contribution statement

Mohamed A. Attia: Conceptualization, Data curation, Formal analysis, Investigation, Writing – review & editing. **Nermin E. Eleraky:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Khaled Abdelazeem:** Data curation, Investigation, Methodology, Writing – original draft. **Mohamed A. Safwat:** Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that have appeared to influence the work reported in this article.

Data availability

The data that has been used is confidential.

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