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Tolerance Assays Performed in Animal Models During the Evaluation of Nanoparticles for Ocular Drug Delivery

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Abstract. Nanotechnology is one of the most advanced technologies regarding the development of formulations for ocular drug delivery. Nanoparticles have shown great efficacy in the treatment of different ocular diseases, but the question arises as to whether nanoparticles are tolerable to the ocular surface of the animal and human eye, because that request needs to be fulfilled during the evaluation of their properties. The aim of this research is to provide a short overview of the tolerance assays performed in animal models during the evaluation of nanoparticles for ocular drug delivery. The following databases were searched: PubMed, Web of Science, Scopus, Science Direct, EBSCO. Both abstracts and full texts were included in the search and there were no exclusion criteria regarding the year when a certain article was published. Results showed that rabbits, rats and guinea pigs are typically used animal models for the evaluation of ocular tolerance of nanosystems, because of the precision of the animal-to-human extrapolation. The Draize test is the most commonly used test when assessing ocular tolerance in animal models. Other methods to evaluate ocular tolerance are the grading system of the macroscopic signs for the colloidal systems tested, the Nelson's classification for squamous metaplasia or the clinical recovery regarding ocular irritation. *In vivo* studies conducted in animals have shown that nanoparticles usually cause no irritation or inflammation, because of their small size. Still, further research needs to be carried out to get firmer confirmation about the safety of these nanoformulations in humans.

Keywords: Nanoparticles · Ocular drug delivery · Tolerance assays · Animal models

1 Introduction

Ocular drug application can be achieved using different dosage forms, such as eye drops or ointments. These conventional dosage forms are safe and noninvasive but are inconsistent concerning bioavailability, solubility and pericorneal drug retention. In the last

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few decades, nanoformulations have been intensively analysed regarding drug delivery to anterior and posterior parts of the eye. One of the most important steps in the evaluation of nanoparticles for ocular drug delivery is checking whether they cause irritation or inflammation because the tolerability of certain formulation impacts patients' adherence [1].

An animal model usually used in tolerance assays is rabbit. There are many benefits of using rabbits for this kind of analysis because they are very cheap and sensitive to irritating substances, have big eyes and appropriate anatomy and physiology for the animal-to-human extrapolation. Guinea pigs and rats are also commonly used [2].

Tolerability factors limit the use of many nanomaterials in ocular drug delivery [3]. For example, some polymers have both mucoadhesive and viscosity-modifying properties. If a drug solution becomes too viscous after the addition of a polymer, it will irritate the eye, consequently causing the tear production and increasing the drug clearance. The aim should be to enhance mucoadhesiveness without significantly changing the viscosity [4]. Chitosan (CS) is often used as a polymer in ophthalmic formulations. This is a cationic polysaccharide, so it reacts with the negatively charged mucus and conjunctiva of the eye. It is only soluble in acids, so it needs to be neutralized to be formulated into an ophthalmic formulation. Galactosylated chitosan (GC) and thiolated chitosan (TC), derivatives of CS, have better solubility properties for ocular drug delivery, so they are preferred in practice [5, 6]. Many polymers, such as poly(caprolactone) (PCL) and poly(lactic acid) (PLA), have also been thoroughly studied regarding the incorporation into nanotechnology-based ocular dosage forms [7]. Some of the surfactants used as stabilising agents caused ocular irritation when used above an optimal concentration. But, Kolliphor P188 showed no irritation signs even at the highest concentration, Tween 80 did not cause irritation signs up to the concentration of 0.05% and sodium dodecyl sulphate (SDS) caused severe inflammation. Therefore, once a nanosystem is prepared, the excess surfactant should be removed [8]. Timolol maleate-loaded polymeric nanoparticles containing flaxseed gum (FX) and CS were also evaluated. The pH value of the FX and CS solutions was 6.78 ± 0.02 and 6.42 ± 0.04, respectively. The near-neutral pH value implied that there would not be any irritation signs. Results of morphological studies showed that nanoparticles of size less than 100 nm and spherical or ovoid shape with no sharp edges did not cause any irritation signs in the cul-de-sac [9].

In this paper, we aim to provide a short overview of the methodology of conducting tolerance assays during the evaluation of nanoparticles for ocular drug delivery, with an emphasis on *in vivo* animal models.

2 Materials and Methods

The following databases were searched: PubMed, Web of Science, Scopus, Science Direct, EBSCO. Search terms included: nanoparticles, ocular drug delivery, tolerance assays, animal models. Various combinations of Boolean terms (AND/OR/NOT) were used to capture all relevant articles but also to make sure our search is limited only to the papers that help answer our research question. Both abstracts and full texts were included in the search. There were no exclusion criteria regarding the year when a certain article was published.

3 Results

In Table 1. we present results of notable studies conducted in animal models, which investigated ocular tolerance of certain nanosystems.

Table 1. Results of notable studies conducted in animal models, which investigated ocular tolerance of certain nanosystems

Study	Animal model	Nanosystem	Notes
[10]	New Zealand white rabbits	Pranoprofen-loaded nanoparticles with poly(lactic-co-glycolic acid) (PLGA) as a polymer and Carbomer 934 as a hydrogel	Compared with the pranoprofen solution and eye drops, nanoparticles were preferred because they showed no signs of ocular irritation
[11]	New Zealand white rabbits	Silver nanoparticles	Conjunctival redness, discharge, and oedema were monitored and no signs of irritation to the cornea, iris, or conjunctiva were found 24, 48, and 72 h post-application
[12]	Rabbits	Fullerene nanoparticles	No signs of corneal opacity, iris anomaly, or chemosis were found after 24 h, but conjunctival redness and blood vessel hyperemia were observed one hour post-application. Thus, long-term tolerance studies need to be carried out
[13]	Rabbits	Multi-walled carbon nanotubes (MWCNTs) of two different sizes	Reversible conjunctival redness and discharge were found, indicating low ocular toxicity of MWCNTs

(*continued*)

Table 1. (*continued*)

Study	Animal model	Nanosystem	Notes
[14]	Rabbits	MWCNTs and single-walled carbon nanotubes (SWCNTs)	Only one of the MWCNTs showed a very weak and reversible acute ocular irritation potential, causing conjunctival redness and blood vessel hyperemia one hour post-application
[15]	Rabbits Sprague Dawley rats	Graphene oxide nanoparticles	Graphene oxide did not cause acute ocular irritation in rabbits, but short-term repeated exposure of Sprague Dawley rats to this substance caused reversible ocular damage because of the oxidative stress occurrence
[16]	Rabbits	Indomethacin-PCL nanoparticles	Optimal ocular tolerance was revealed, especially in the cornea, conjunctiva, and iris, with a possibility of the conjunctival hyperemia occurring later. Cationic polymer-coated nanocapsules as ocular drug carriers should generally be considered safe [17]
[18]	Rabbits	Poly(methylmethacrylate-co-sulphopropylmethacrylate) (PMS) nanoparticles	When formulated with arecaidine propargyl ester and aceclidine, no signs of ocular irritation were shown
[19]	Rabbits	Carteolol-PCL nanocapsules	No signs of ocular irritation were shown

(*continued*)

Table 1. (*continued*)

Study	Animal model	Nanosystem	Notes
[20]	Rabbits	Acyclovir-PLA and poly(ethylcyanoacrylate) (PECA) nanospheres	No signs of discomfort or inflammation were noted. Insignificant conjunctival hyperemia was noted 10 min post-application, but no signs of inflammation were observed six hours post-application. Both PLA and PECA nanospheres were thought to be safe in ocular drug delivery
[21–24]	Rabbits	Eudragit-based nanoparticles	Nanoparticles did not cause any ocular irritation, indicating their safe use in ocular drug delivery
[25]	Wistar rats	Ganciclovir-bovine serum albumin (BSA) nanoparticles	Results showed that prolonged ocular presence of these nanoparticles was well-tolerated

The evaluation criteria of the ocular irritation according to the Draize test are divided into four categories presented in Table 2.

Table 2. The evaluation criteria of the ocular irritation according to the Draize test [26–28]

Score	Reaction
0–3.9	Nonirritant
4–8.9	Slightly irritant
9–12.9	Moderately irritant
13–16	Seriously irritant

In Table 3. we present results of notable studies in which the Draize test was used to evaluate ocular irritation of various nanosystems.

Table 3. Results of notable studies in which the Draize test was used to evaluate ocular irritation of various nanosystems

Study	Animal model	Nanosystem	Notes
[29]	Guinea pigs	Silver nanoparticles	An irritation score of 1 was noted for the conjunctiva after 24 h. It is also well-known that long-term exposure of eyes to silver could cause argyrosis [30, 31]
[32]	Rabbits	Ocular supersaturated self-nanoemulsifying drug delivery systems (SSNEDDS) of econazole nitrate with and without hydroxypropylmethylcellulose (HPMC) as a precipitation inhibitor	Rabbits were monitored for any ocular anomaly directly after the exposure and one hour post-application for three days. Both SSNEDDS-A and SSNEDDS-A2 produced a nonirritant effect to the eyes. They scored 0.111 ± 0.055 and 0.091 ± 0.087, respectively. Both of these formulations were well-tolerated and did not show any evidence of toxic or inflammatory effects on the ocular surface
[33]	New Zealand white rabbits	Naringenin-loaded sulphobutylether (SBE)-cyclodextrin (CD)-CS nanoparticles (Nag-SBE-CD-CS-NPs)	Both single-dose and long-term ocular irritation of Nag-SBE-CD-CS-NPs was studied. Before the test was conducted, both eyes were verified to be without inflammation. For the assessment of long-term ocular irritation, the right eye was treated with the formulation twice per day for seven days. This formulation was proven to be nonirritant to the eyes, because scores were 0 and 1.67 for single-dose and long-term ocular irritation, respectively. It can be concluded that Nag-SBE-CD-CS-NPs could be considered safe in ocular drug delivery
[34]	New Zealand white rabbits	Thermoresponsive gel with curcumin-loaded BSA nanoparticles (Cur-BSA-NPs)	Changes in the cornea, iris, and conjunctiva, secretion, and chemosis caused by this formulation were compared with the control. No lesion formation was observed during the study. The Cur-BSA-NPs gel did not irritate the eyes, because the irritation score was 0. This formulation could be considered safe for ocular use

(*continued*)

Table 3. (*continued*)

Study	Animal model	Nanosystem	Notes
[35]	Rabbits	Timolol maleate-loaded gelatin nanoparticles	Eight rabbits were grouped into three groups (three for each of two gelatin-nanoparticle formulations (F1: amino groups: glutaraldehyde (NH_2:GA) 1:2, F2: NH_2:GA 1:1) and two for eye drops). Time points were one hour, 24, 48, and 72 h, 7, 14, and 21 days post-application. Cornea, iris, conjunctiva, pupil, and anterior chamber were checked for any signs of ocular irritation. None of the gelatin-nanoparticle formulations irritated the ocular structures, whereas eye drops caused minor irritation in one rabbit's eye in the first hour. This minor irritation caused by timolol maleate eye drops was probably because of the preservatives used in this dosage form [36]

The evaluation criteria of the ocular irritation according to the grading system of the macroscopic signs for the colloidal systems tested are divided into three categories presented in Table 4.

Table 4. The evaluation criteria of the ocular irritation according to the grading system of the macroscopic signs for the colloidal systems tested [37]

Grade	Discomfort	Cornea	Conjunctiva	Discharge	Lids
0	No reaction	No alterations	No alterations	No discharge	No swelling
1	Blinking	Mild opacity	Mild hyperemia, mild oedema	Mild discharge without moistened hair	Mild swelling
2	Enhanced blinking, intense tearing, vocalisations	Intense opacity	Intense hyperemia, intense oedema, haemorrhage	Intense discharge with moistened hair	Obvious swelling

In Table 5. we present results of notable studies in which the grading system of the macroscopic signs for the colloidal systems tested was used to evaluate ocular irritation of various nanosystems.

Table 5. Results of notable studies in which the grading system of the macroscopic signs for the colloidal systems tested was used to evaluate ocular irritation of various nanosystems

Study	Animal model	Nanosystem	Notes
[38]	New Zealand white rabbits	Acyclovir-PLA nanospheres	These nanospheres caused no tissue alteration or ocular inflammation in the rabbits' eyes. Irritation scores of conjunctival congestion, swelling, and discharge, as well as iris hyperemia and corneal opacity, were 0
[39]	Rabbits	Tetramethyl rhodamine (TAMRA)-gelatin nanoparticles	A slight conjunctival redness was observed after four hours, but after 16 h, no difference was noted. Previous studies showed that the rabbits' eyes were more sensitive and needed more time for the epithelial repair compared with the human eyes [40], and in this study, the application of gelatin nanoparticles was safe and caused no irritation to the rabbits' eyes. It is reasonable to expect that positively charged gelatin nanoparticles would also be well-tolerated in the human eyes

Blinking test can also be used to evaluate ocular tolerance of nanosystems [41]. In Table 6. we present results of notable studies in which the blinking test was used to evaluate ocular irritation of various nanosystems.

Nelson's classification system, which evaluates the morphology of conjunctival ocular surface and the degree of squamous metaplasia, is presented in Table 7.

Table 6. Results of notable studies in which the blinking test was used to evaluate ocular irritation of various nanosystems

Study	Animal model	Nanosystem	Notes
[42]	Rabbits	Amikacin-loaded nanosuspension prepared with two bioadhesive, positively charged polymers, Eudragit RS 100 and Eudragit RL 100	Rabbits were forced to blink once to spread formulations (simulated tear fluid, amikacin eye drops, amikacin-loaded nanosuspension) uniformly on the cornea. Frequency of rabbits' blinking five minutes post-application was monitored. The normal blinking rate was 1–2 per minute. Results showed that the ocular irritation caused by these nanoparticles was almost the same compared with the control
[43]	New Zealand white rabbits	Cyclophosphamidepoly(butylcyanoacrylate) (PBCA) nanospheres	No irritation effects on the iris, cornea, and conjunctiva were shown, suggesting their good ocular tolerance. Blinking rate post-application revealed significant differences because cyclophosphamide solution was shown to be irritant to the cornea, whereas nanospheres did not cause a significant increase in blinking rate. Blinking rate caused by drug-loaded PBCA nanospheres was higher compared with the PBCA nanospheres, which could be associated with the amount of cyclophosphamide incorporated into nanospheres

Table 7. The evaluation criteria of the ocular irritation according to the Nelson's classification for squamous metaplasia

Grade	Feature
0	>500 goblet cells/mm^2; small, round epithelial cells with large nuclei
1	100–500 goblet cells/mm^2
2	
3	<100 goblet cells/mm^2; large, polygonal epithelial cells with small nuclei

In Table 8. we present results of notable studies in which the Nelson's classification for squamous metaplasia was used to evaluate ocular irritation of various nanosystems.

Table 8. Results of notable studies in which the Nelson's classification for squamous metaplasia was used to evaluate ocular irritation of various nanosystems

Study	Animal model	Nanosystem	Notes
[45]	Rabbits	Fluoresceinamine-labelled hyaluronic acid-CS nanoparticles	Acute ocular tolerance was evaluated 24 h post-application and compared with the prior-application values. Five rabbits received fluorescent nanoparticles or fluorescent conjugate in the right eye, 12 applications total. Twenty-four hours post-application, evaluation of the ocular tolerance of these formulations was conducted and it included clinical signs, such as ocular discomfort, irritation, discharge, eyelid swelling, and presence of corneal and/or conjunctival alterations. Clinical signs in all rabbits at all time points scored grade 0 because there was no ocular discomfort, irritation, discharge, or eyelid swelling. After six hours, all of the nanoparticle-treated eyes scored grade 1 mucus-like discharge. No oedema, redness, or corneal vascularization were noted. Six days prior-application, conjunctival impression cytology (CIC) samples showed normal epithelial cells and abundant goblet cells, which were evenly distributed (grade 0). Scattered polymorphonuclear cells were present in all CIC samples. Twenty-four hours post-application, CIC samples from control eyes showed no difference when compared with the samples taken six days prior-application. The CIC samples for nanoparticle-exposed and conjugate-exposed eyes showed a normal distribution and morphology of epithelial cells, but there was an increased presence of polymorphonuclear cells. Rabbits showed no signs of ocular discomfort or irritation after exposure to these nanoparticles

(*continued*)

Table 8. (*continued*)

Study	Animal model	Nanosystem	Notes
[46]	Albino New Zealand rabbits	CS-BSA nanoparticles labelled with fluorescein isothiocyanate	The CS-BSA nanoparticles were applied to the right eye every 30 min for six hours, whereas the left eye was not treated and served as a control. The CIC samples were collected from both control and experimental eyes of all rabbits, six days before the beginning of the study and 24 h post-application. The CIC samples were analysed in a masked fashion according to the Nelson's classification. Grades 0 and 1 resemble normal morphology and grades 2 and 3 abnormal morphology. Grades were assigned for nuclear morphology, nuclear or cytoplasmic ratio, metachromasia, and goblet cell density. In CIC samples collected six days before the beginning of the study, animals showed no clinical signs and the ocular surface structures were normal, thus the score of mean clinical macroscopic signs was 0. For both control and experimental eyes, Nelson's grade was 0 to 1, because of the normal morphology of the conjunctival epithelia regarding goblet and nongoblet epithelial cells. Twenty-four hours post-application, CIC samples were obtained from both control and experimental eyes and between them, no difference was observed, thus Nelson's grade was 0 to 1, too. Ocular irritation was analysed using the grading system of the macroscopic signs for the colloidal systems tested, three, six and 24 h post-application. Rabbits showed no signs of discomfort 24 h post-application. Nanoparticle-treated eyes had a clinical macroscopic sign score of 1, because of the mucus-like discharge six hours post-application, but it was minimal and probably caused by the ability of CS-BSA nanoparticles to aggregate. It can be concluded that the ocular surface of rabbits showed no signs of inflammation and that these nanoparticles were well-tolerated

The evaluation of clinical recovery regarding ocular irritation is presented in Table 9.

Table 9. The evaluation of clinical recovery regarding ocular irritation [47]

Grade	Description
0	Absent
1	Mild
2	Moderate
3	Severe

Ocular irritation potential of clindamycin-PLGA nanoimplants was evaluated in rabbits by scoring the clinical parameters from 0 to 3. *In vivo*, PLGA degrades into lactic acid and glycolic acid that are utilized in the Krebs cycle and converted into water and carbon dioxide. These nanoimplants, with or without clindamycin, caused no inflammation and were biocompatible and well-tolerated [47].

4 Conclusion

Ocular tolerance of nanosystems must be evaluated, primarily in animal models and afterwards in humans. By conducting tolerance assays, one can conclude whether a nanosystem used for ocular drug delivery is safe or not for animals and humans. These nanoformulations usually show great ocular tolerance in animals, with no signs of irritation or inflammation, when ocular tolerance is assessed via the Draize test, or according to the grading system of the macroscopic signs for the colloidal systems tested, the Nelson's classification for squamous metaplasia or the clinical recovery regarding ocular irritation. Still, it is crucial to precisely extrapolate results from animals to humans, so these nanosystems could be used for the treatment of ocular diseases in humans. Further research needs to be carried out to get firmer confirmation about the safety and efficacy of these nanoformulations in humans.

Conflict of Interest. The authors have no conflicts of interest to disclose.

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