INTRAOCULAR BIODISTRIBUTION OF INTRAVITREAL INJECTED FLUORESCENT DEXAMETHASONE-CHITOSAN NANOPARTICLES IN RABBIT EYES

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INTRAOCULAR BIODISTRIBUTION OF INTRAVITREAL INJECTED FLUORESCENT DEXAMETHASONE-CHITOSAN NANOPARTICLES IN RABBIT EYES (Abstract): Aim: To evaluate intraocular biodistribution of fluorescent nanoparticles composed of dexamethasone bound to chitosan after intravitreal administration in rabbit eyes. Material and methods: The chitosan and gelatin based nanoparticles were synthetized using a reverse emulsion-double crosslinking technique (ionic and covalent) and then dexamethasone was bound. Two units of 1% suspension of these nanoparticles in saline solution were injected intravitreally into rabbit eyes. The histologic sections obtained at 72 hours were analyzed by confocal microscopy. Results: The chitosan-fluorescein conjugate bound to dexamethasone was present in all ocular tissues at 72 hours. The nanoparticles were present in the retina and lens in a larger amount than in the other ocular tissues. Conclusions: The reverse emulsion-double crosslinking technique was efficient in synthesizing a biocompatible polymeric nanosystem. The in vivo study of intraocular biodistribution of fluorescein-marked nanoparticles capable of binding dexamethasone revealed their affinity for the retina and lens after intravitreal administration. Keywords: BIODISTRIBUTION, INTRAVITREAL, NANOPARTICLES, DRUG DELIVERY, CHITOSAN, DEXAMETHASONE

Nanoparticles are nanometer sized molecules that behave as a whole unit with respect to their properties and transport behavior. These molecules are beginning to be more frequently studied and used in the therapy of many diseases as safe and efficient drug carrier systems. Recent studies have demonstrated the ability of nanoparticles to overcome the eye barriers (cornea, conjunctiva, sclera and the blood-retinal barrier), thus being able to deliver therapeutic agents to the different ocular tissues. (1-5).

Current research trends in ophthalmology aim at developing intraocular drug delivery systems able of exerting their pharmacological effect over a longer period of time at a lower administration rate and with
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a lower dose related toxicity rate.

The most recent therapies of choroidal and retinal neovascularization target the main pathogenic processes of the disease: inflammation and overexpression of pro-angiogenic factors. In addition to the anti-angiogenic and anti-inflammatory agents approved for use (ranibizumab, aflibercept, steroids) or widely used in "off-label" manner (bevacizumab) there are numerous ongoing researches on other potential pathogenic factors (platelet-derived growth factor, complement modulators).

The aim of this experimental study is to assess the intravitreal biodistribution of fluorescent chitosan nanoparticles loaded with dexamethasone and injected in rabbit eyes. These nanoparticle-dexamethasone systems are considered potential therapeutic agents for ocular neovascularization and its complications.

MATERIAL AND METHODS

1. Preparation of dexamethasone fluorescent nanoparticle. The polymers used for the preparation of nanoparticles were chitosan and gelatin. These polymers were chosen because they combine the benefits of proteins and those of polysaccharides, in order to obtain a good bioavailability and drug release capacity to target ocular tissues.

Chitosan is a linear polysaccharide obtained from crustacean shells. It is composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Gelatin is a polypeptide prepared by thermal, chemical and physical denaturation of collagen. In the preparation process a series of emulsifiers and surfactants were also used. A 10% fluorescein solution was used to mark the chitosan-gelatin nanoparticles.

Chitosan and gelatin nanoparticles were synthesized using the double crosslinking in the droplet method. This method was selected due to the low toxicity required by ophthalmic preparations.

The heterogeneous reaction system involved two emulsions. The aqueous phase of the first emulsion was represented by the solution of the two natural polymers, gelatin and chitosan (polymer concentration 0.5 %). The organic phase of the first emulsion, consisting of toluene and the specific amount of surfactant Span 80 was added to the aqueous solution and stirred at 15 000 rpm for 10 minutes. The thus obtained primary emulsion was dripped by syringe into a 600-ml Berzelius beaker, stirred ultra-fast for 10 minutes in order to obtain the secondary emulsion in the organic phase, represented by a volume of 260 ml toluene and the appropriate surfactant, Span 80, 2%.

After the emulsification of the two emulsions, ionic crosslinking was carried out using sodium phosphate (5%), while stirring (5000 rpm/10 min). Glutaraldehyde was dripped into the reaction vessel while stirring in order to complete the covalent crosslinking.

After the double cross linked particles were obtained, separation was done by centrifuging and purification of the particles in several stages consisting of successive cycles of washing with double-distilled water/acetone/n-hexane to remove traces of toluene and centrifugation (5000 rpm). Drying of nanoparticles was carried out in n-hexane at room temperature.

The nanoparticles were marked with fluorescein, which was covalently bound to chitosan. Fluorescence was necessary to assess the biodistribution of nanoparticles within the eye.

Chitosan–fluorescein conjugate was synthesized using a method adapted from
De Campos et al. (2004) (6). The resulting product was dialyzed using a dialysis membrane in the form of tubes with a pore size of 12,000 Da. The last step was the lyophilization of the product. Chitosan fluorescein staining was highlighted by infrared spectroscopy.

Using double crosslinking in the double emulsion process, as described above, fluorescent nanoparticles were obtained by introducing in the reaction a fluorescein-labeled chitosan.

Afterwards, dexamethasone was bound to the fluorescent nanoparticles by immersing 100 mg nanoparticles in 9 ml solution of dexamethasone for 48 hours. Then, the suspension was centrifuged and the supernatant weighed.

2. Study of in vivo release of dexamethasone loaded fluorescent nanoparticles. The study was conducted in accordance with the Declaration of Helsinki, the current modifications (Somerset West Amendment). We used a group of 5 rabbits bred in laboratory under the same conditions and fed with standard food. Each rabbit received a 0.03 ml intravitreal injection of 1% solution of dexamethasone-loaded nanoparticles in the right eye. Before performing intravitreal injections the animals were anesthetized with intraperitoneal ketamine 25 mg / kg.

The animals used in the experiment were sacrificed 72 hours after the injection of fluorescent nanoparticles after prior anesthesia with Ketamine 25 mg/kg. The right eye was harvested, transferred to ice and sectioned within 15 minutes.

The tissue samples were then treated with mouse anti-fluorescein monoclonal antibodies, which were washed after 48 hours followed by a new treatment with secondary goat anti-mouse antibodies and labeled with R-phycoerythrin.

The sections were then transferred to microscope slides and coated with the fluorescence medium. To highlight the fluorescence, a confocal microscopy setup was used, including an inverted Nikon Eclipse TE-300 microscope. Images capture was done using red fluorescence (for R-phycoerythrin) and transmitted light in order to distinguish the morphological features.

**RESULTS**

**Analysis of the biodistribution of fluorescent dexamethasone loaded-nanoparticles within the eye.** Following the intravitreal injection and analysis of the obtained histological sections, the fluorescent dexamethasone-nanoparticles were found to be uniformly distributed and present in all ocular tissues. Red fluorescence was present in all ocular tissues of the experimental animals at 72 hours (fig.1 A, B; fig.2 A, B; fig.3 A, B; fig.4 A, B).

The intraocular biodistribution of dexamethasone nanoparticles recorded the highest values in the lens and retina, as seen at both the microscopic examination of histological sections and RGB diagram. A fluorescence value of 17.35 ± 1.85 UA inside the lens and 13.08 ± 3.75 UA in the retina was recorded, showing a better fixation of the drug loaded nanoparticles within the tissues surrounding the injection site.

In the structure of cornea and sclera a significant amount of dexamethasone loaded nanoparticles was detected, but lower compared with the retina and lens. Recorded values were 9.75 ± 3.24 UA for the cornea and 11.78 ± 2.94 UA for sclera, thereby highlighting the migration of nanoparticles in all ocular tissues at 72 hours after injection.
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**Fig. 1.** A. Analysis of red fluorescence from cornea section of the right eye at 72 hours (40x) (9.75±3.24 UA, n = 5). B. Cornea RGB at 72 hours. Red fluorescence is present at medium intensity.

**Fig. 2.** A. Analysis of red fluorescence from lens section of the right eye at 72 hours (40x) (17.35 ± 1.85 UA, n = 5). B. Lens RGB at 72 hours. Red fluorescence is present and intense.

**Fig. 3.** A. Analysis of red fluorescence from retina section of the right eye at 72 hours (40x) (13.08± 3.75 UA, n = 5). B. Retina RGB at 72 hours. Red fluorescence is present and intense.
DISCUSSION

Nanoparticles are currently studied in terms of efficiency as carriers of many drugs with application in ophthalmology and other medical specialties. Specific controlled release properties depend on the physicochemical properties of the drug, the chemicals used in the synthesis of the nanoparticles, as well as the methods of preparing such polymer systems. In general, when using optimal drug loaded nanoparticles the ocular bioavailability of many drugs significantly increases compared to conventional formulations. Increased ability for overcoming the blood-retinal barriers and releasing biologically active principles in a safe and effective manner that opens new opportunities in the treatment of many ophthalmic diseases.

The blood-retinal barriers consist of an inner segment constituted of retinal endothelial cells, pericytes and astrocytes and an outer segment composed of the tight junctions between the retinal pigment epithelium cells. This barrier acts as a filter that restricts permeability of high molecular weight particles, including drugs, to the retina. Drug loaded nanoparticles of the appropriate size were proved to have favorable biological properties as prolonged residence time in the vitreal gel, higher availability of the drugs to the deeper layers of the retina and decreased toxicity (7-9). Also, these carriers can improve the availability of poorly water-soluble molecules to the retinal layers (10).

Nanopolimeric systems structured on chitosan and gelatin are used in the pharmaceutical composition of many hydrogels as drug carriers due to their property of being absorbed and metabolized without resulting toxic products. The blending of chitosan with gelatin increases the stability of the gel (11, 12).

Dexamethasone is a glucocorticosteroïd with anti-inflammatory and immunosuppressive properties. In ophthalmology, is widely used in the topical treatment of various inflammatory diseases. The intravitreal administration of dexamethasone is used in the treatment of macular edema secondary to vascular occlusions, diabetes, uveitis. Its effectiveness in the treatment of disorders of the posterior segment of the eye is due to a reduction in vascular permeability and leukocyte accumulation, by acting simultaneously on both VEGF and cytokines path (13).
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Particles stable in size and shape were obtained using the double crosslinking (covalent and ionic) in double emulsion technique. This method proved to be efficient due to covalent surface crosslinking. Also, the high swelling capacity of the nanoparticles is due to ionic crosslinking which enable a large diffusion capacity of drugs.

The histological samples were treated by indirect immunofluorescence to investigate the presence or absence of fluorescent drug-loaded nanoparticle in their structure. Tissue samples were then subjected to direct microscopy (by transmitted light) in order to highlight their morphological structure and confocal microscopy to assess the presence of fluorescently loaded nanoparticles inside ocular tissues. The presence of fluorescent dexamethasone nanoparticles was detected in the ocular tissues of experimental animals at 72 hours by marking red fluorescence.

Following intravitreal injection of fluorescently labeled polymer-drug nanosystems and analysis of the histological sections obtained from the rabbit eyeballs we found them to be uniformly distributed and present in all ocular tissues.

Comparing the results obtained at 72 hours after intravitreal administration of the fluorescent polymer-drug system with those achieved in our previous experimental study, in which only fluorescein loaded nanoparticles were injected in Wistar rat eyes, we concluded that the fluorescence values attained in the second experiment were higher (14). In both experiments the nanoparticles were predominantly fixed in the structure of retina and lens, but the fluorescence value was higher with a medium of 9 UA when dexamethasone was bound to the polymer. Although cornea and sclera presented a lower fluorescence than retina and lens in both experiments, higher values, in medium with 7 UA, were also registered when dexamethasone nanosystems were administered.

The higher values of fluorescence obtained in this experiment suggest that the dexamethasone carrier nanoparticles are stable and have the ability to persist in the tissues long enough to deliver the loaded drugs.

The results of this study are consistent with existing literature on the subject. Binding dexamethasone to polymeric nanoparticles systems has the effect of increasing the drug action period after intravitreal injection. Experimentally, it was demonstrated that dexamethasone-loaded nanoparticles can inhibit the development of subretinal neovascularization, the effect being dose dependent (15). The bioavailability of the therapeutic substance is increased by its binding to the polymer carrier, dexamethasone nanoparticles are gradually released over a period of up to 50 days, with maintenance of stable therapeutic doses at least 30 days (16).

CONCLUSIONS

In our study dexamethasone carrier nanoparticles injected intravitreally were found mainly in the lens and retina at 72 hours after administration. Smaller amounts were found in the cornea and sclera at the same interval of time after administration.

We demonstrated the ability of drug-polymer systems to penetrate tissues in the immediate vicinity of administration site and migrate, especially in the retinal and lens structure, at 72 hour interval. Their stability is higher after drug binding to the polymeric structure. Also higher levels of
fluorescence were recorded in this case compared to the values obtained when only fluorescein-loaded nanoparticles were administered.

REFERENCES


