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MANUSCRIPT**SIX MONTH DELIVERY OF GDNF FROM PLGA/VITAMIN E BIODEGRADABLE
MICROSPHERES AFTER INTRAVITREAL INJECTION IN RABBITS**

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ABSTRACT

Local long-term delivery of glial cell line derived neurotrophic factor (GDNF) from vitamin E/poly-lactic-co-glycolic acid microspheres (MSs) protects retinal ganglion cells in an animal model of glaucoma for up to 11 weeks. However, the pharmacokinetics of GDNF after intravitreal injection of MSs is not known. We evaluated the GDNF levels after a single intravitreal injection of GDNF/VitE MSs. Biodegradable MSs were prepared by the solid-oil-in-water emulsion-solvent evaporation technique and characterized. Rabbits received a single intravitreal injection (50 μ L) of GDNF/VitE MSs (4%w/v; 24 right eyes; 74.85 ng GDNF), blank MSs (4%w/v; 24 left eyes), and balanced salt solution (4 eyes). Two controls eyes received no injections. At 24 hours, 1, 4, 6, 8, 12, 18, and 24 weeks after injection, the eyes were enucleated, and the intravitreal GDNF levels were quantified. Pharmacokinetic data were analysed according to non-compartmental model. Intraocular GDNF levels of 717.1 ± 145.1 pg/mL were observed at 24 hours for GDNF-loaded MSs, followed by a plateau (745.3 ± 25.5 pg/mL) until day 28. After that, a second plateau (17.4 ± 3.7 pg/mL) occurred from 8 to 24 weeks post-injection, significantly higher than the basal levels. Eyes injected with GDNF/vitE and Blank-MSs did not show any abnormalities during the six-months follow up after administration. The single injection of GDNF/VitE MSs provided a sustained controlled release of the neurotrophic factor in a controlled fashion for up to six months.

Keywords: Poly (lactic-co-glycolic acid) microspheres, protein delivery, vitamin E, glial cell line-derived neurotrophic factor (GDNF), pharmacokinetics, intravitreal injection.

1. Introduction

Glaucoma is a group of neurodegenerative diseases in which the optic nerve is affected (Almasieh et al., 2012). Although high intraocular pressure (IOP) is the main risk factor, some patients have glaucoma even with normal tension values (Sommer, 1989). Neuronal damage can occur by different pathways, including excitotoxicity, protein misfolding, mitochondrial dysfunction, oxidative stress, inflammation, and neurotrophin deprivation among others. All of these mechanisms ultimately lead to programmed cell death with loss of retinal ganglion cells (RGCs) (Qu et al., 2010).

Treatments directed to avoid irreversible damage of the neuronal cells are of great interest (Baltmr et al., 2010). Taking into account that glaucoma is a multifactorial disease, the most feasible pharmacological approach is directed toward the combination of several active substances with additive or synergistic effects. Based on this, the combination of a neurotrophic factor and an antioxidant agent would seem to be an optimal therapeutic strategy.

Among the neurotrophic factors, the glial cell line-derived neurotrophic factor (GDNF) enhances the survival of dopaminergic and motor neurons in neurodegenerative diseases such as Parkinson and Alzheimer (Allen et al., 2013; Chen et al., 2000; Ghiso et al., 2013; Siegel and Chauhan, 2000). At the ocular level, exogenous, intravitreally injected GDNF has neuroprotective effect for RGCs, promoting the survival of axotomized cells (Cuenca et al., 2014; Klocker et al., 1997).

Antioxidants are also beneficial in neurodegenerative diseases in which neuronal death is associated with the damage of healthy neurons located in the proximity of massive releases of oxidative molecules (Engin et al., 2010; McManus et al., 2014; Mozaffarieh et al., 2008). Among the antioxidants, the free radical

scavenger vitamin E (α -tocopherol, VitE) produces results of great interest (Engin, 2009). Furthermore, it has been proposed that VitE has antiproliferative properties that might reduce some of the side effects typically linked to repeated intravitreal injections such as proliferative vitreoretinopathy and retinal detachments (Larrosa et al., 1997).

Due to the chronic nature of glaucoma, the applicability of GDNF and VitE in the neuroprotective therapy is totally linked to the development of effective administration devices that are able to supply therapeutic concentrations at the retina for extended periods of time (Checa-Casalengua et al., 2011). During the last two decades, the development of intraocular drug delivery systems (IODDS) is one of the main research areas for the treatment of chronic diseases affecting the back of the eye (Achouri et al., 2013; Goyal et al., 2014; Kompella et al., 2013). Depending on the size, IODDS are classified as nanoparticles (1-1000 nm), microparticles (1-1000 μ m), and implants (>1 mm). Microparticles and implants have received a lot of interest because they can provide long-term delivery of the active substance. Microparticles have the advantage of easy administration because injection can be performed through conventional 25-34 gauge needles and thus avoiding surgery.

Two structurally distinct types of microparticles are typically used to deliver the active substance: microcapsules and microspheres (MSs). Microcapsules are composed of a polymeric material that creates a reservoir in which the active substance is included. In contrast, MSs are composed of a polymer network that produces a matrix microsystem within which the active substance is dispersed (Herrero-Vanrell et al., 2014). Biodegradable MSs are an emerging therapeutic tool in the treatment of chronic intraocular pathologies because they are suitable for

administration close to the target site by periocular or intraocular routes. Additionally, they disappear from the site of administration after releasing the therapeutic molecule (Andres-Guerrero et al., 2015; Herrero-Vanrell and Refojo, 2001; Herrero Vanrell and Refojo, 2001). Among the biodegradable polymers, poly-lactic-co-glycolic acid (PLGA) has been the most employed, with one device already used in clinical practice (Ozurdex[®]). IODDS made of PLGA are gradually converted into CO₂ and water *in vivo* and eliminated by ocular tissues (Makadia and Siegel, 2011).

The potential inflammatory reactions and damage to retinas and vision function caused by intravitreally injected PLGA microspheres and their degradation products have been studied by several authors. They indicated that no inflammation nor cell toxicity in the retinas was triggered by the microspheres, the degraded products, or the changed microenvironments in the eyes. Ocular tissues show great tolerability and biocompatibility after the intravitreal administration of PLGA material (Abrego et al., 2015; Giordano et al., 1995; Rong et al., 2014; Veloso et al., 1997). However, an initial foreign body response after intravitreal injection can occur under normal physiological conditions with no damage to the retina (Giordano et al., 1995). These same authors did not observe inflammatory signs 4 days after administration of microspheres and thereafter.

PLGA-based GDNF-loaded MSs have been developed by different authors to provide slow drug release and long-lasting therapeutic effects (Andrieu-Soler et al., 2005; Jiang et al., 2007; Ward et al., 2007). In a previous study, we developed GDNF/VitE-loaded MSs capable of providing *in vitro* release of the bioactive protein for up to 19 weeks. This formulation was intravitreally injected into rats with

elevated IOP, an animal model of glaucoma. It effectively protected the RGCs for at least 11 weeks (Checa-Casalengua et al., 2011).

The injection frequency of the IODDS depends on the length of time that the active substance can be delivered after administration. For this reason, the evaluation of drug delivery systems designed to target retinal tissues or surrounding areas requires pharmacokinetic studies in the vitreous (Behar-Cohen et al., 2002; Fernandes-Cunha et al., 2014). Pharmacokinetic studies have been performed after injection of GDNF (bolus) in porcine eyes, and GDNF levels in the vitreous were quantified for seven days (Ejstrup et al., 2010). However, to the best of our knowledge, the study described here is the first to report the intravitreal pharmacokinetics of GDNF delivered from a microparticulate system over a long period (six months). The aim of the present study was to evaluate the GDNF levels after a single intravitreal injection of GDNF/VitE MSs. Pharmacokinetic study were done in rabbits, and data were analysed using non-compartmental treatment.

2. Materials and methods

2.1. Materials

GDNF and the enzyme-linked immunosorbant assay (ELISA) kit for GDNF quantification were supplied by R&D Systems (Minneapolis, MN, USA). PLGA ratio 50:50 (Resomer®503) was purchased from Boehringer Ingelheim Pharma GmbH & Co. (Ingelheim, Germany). Polyvinyl alcohol 72,000 g/mol (PVA) was obtained from Merck KGaA (Darmstadt, Germany). N-(tetramethylrhodamine-6-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (TRITC-DHPE) was purchased from Invitrogen (Carlsbad, CA, USA). VitE (α -tocopherol acetate),

bovine serum albumin (BSA), and human serum albumin fluorescein isothiocyanate (HSA-FITC) were obtained from Sigma-Aldrich (Schnelldorf, Germany).

2.2. Microsphere elaboration

GDNF/VitE-loaded PLGA MSs were elaborated using a solid-in-oil-in-water (S/O/W) emulsion solvent evaporation technique previously developed by our group (Checa-Casalengua et al., 2011). Briefly, 20 µg of GDNF was suspended in 20 µL VitE by gentle sonication at low temperature (0°C) (Sonicator XL, Head Systems, Farmingdale, New York, USA) for 30 sec. The suspension was then mixed with 1 mL of PLGA solution in methylene chloride (20% w/v). This organic phase was emulsified with 5 mL of PVA MilliQ® water solution (2% w/v) in a homogenizer (Polytron® RECO, Kinematica GmbH PT 3000, Lucerna, Switzerland) at 5,000 rpm for 1 min. The emulsion was subsequently poured onto 100 mL of an aqueous PVA solution (0.1%) and kept under constant stirring for 3 h to allow organic solvent evaporation. Once formed, the MSs were washed, filtered, freeze-dried, and kept at -20 °C under dry conditions until used.

2.3. Microsphere characterization

2.3.1. Production yield percentage (PY%)

The PY% of each formulation was calculated as the percentage of MSs weight divided by the total amount of the components (polymer, drug, and VitE) initially used in the formulation process.

$$\text{PY\%} = \frac{\text{weight of microspheres}}{\text{total amount of polymer, protein and VitE}} \times 100$$

2.3.2. Mean particle size and particle size distribution

The mean particle size and particle size distribution were measured by light scattering in a Microtrac[®] S3500 Series Particle Size Analyzer (Montgomeryville, PA, USA).

2.3.3. Morphological evaluation

The external morphology of freeze-dried MSs was evaluated by scanning electron microscopy (Jeol, JSM-6335F, Tokyo, Japan). Samples were gold sputter-coated prior to observation.

2.3.4. Protein and additive distribution in PLGA MSs

A model labelled-protein, HSA-FITC, was encapsulated in the same conditions described for GDNF (Checa-Casalengua et al., 2011). Combinations of the additive VitE with small proportions (6 µg) of the fluorescent lipid dye TRITC-DHPE (0.5 mg/mL in CH₂Cl₂) were also included in the Oil-phase. Samples of this formulation were placed in release media for 24 h (see *in vitro* release studies). Freshly prepared MSs samples (time 0 h) and samples taken 24 h after release were observed by confocal microscopy (Leica TCS SP2, Leica Microsystems Heidelberg GmbH, Germany).

2.3.5. Encapsulation efficiency

To determine the encapsulation efficiency, 5 mg of MSs were dissolved in 0.7 mL of methylene chloride. Then, the same volume of the reactive diluent composed of phosphate buffered saline, pH 7.4, and 1% BSA provided in the ELISA Kit (DY995, R&D System) was added. After vigorous mixing, the heterogeneous system was centrifuged (12,000 rpm, 15 min, 4°C) and the aqueous phase extracted. This liquid/liquid extraction was repeated four times to recover all of the encapsulated protein. Following the extraction, GDNF immunoassays were

performed in triplicate.

2.3.6. *In vitro* release studies

To determine the rate of GDNF release from the MSs, duplicate samples of GDNF/VitE-loaded MSs (5 mg) were suspended in a release medium composed of 1.5 mL of phosphate buffered saline (pH 7.4 isotonic with NaCl) containing 1% BSA and 0.02% sodium azide. LoBind Eppendorf® vials were used in all cases. Samples were maintained under a constant agitation speed of 100 rpm (Clifton Shaking Bath NE5, Nikel Electro Ltd, Avon, UK) at 37°C. At 1 h, 24 h, and once a week until the end of the assay (24 weeks), the MSs suspension were gently centrifuged (5,000 rpm for 5 min), and the supernatants were recovered and replaced by the same volume of fresh medium. GDNF in the supernatant aliquots were then assayed by ELISA as described above. When necessary, the aliquots were diluted with the reactive diluent.

2.4. *In vivo* assays

2.4.1. Animals

All of the animals were handled according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609/EEC. Animal assays were carried out in compliance with the ARVO Statement for the use of animals in experimental procedures and other scientific purposes. All methods were carried out according to the Project License PI12/02285 approved by the in-house Ethics Committee for Animal Experiments for the University of Zaragoza (Spain).

Investigations were performed in 27 adult female New Zealand albino rabbits (2 – 3.5 Kg, Servicio de Apoyo a la Experimentación Animal [SAEA], Zaragoza

University, Zaragoza, Spain). The animals were housed singly in standard cages, in a light-controlled room (12 h/12 h dark/light cycle) at $20 \pm 2^\circ \text{C}$ with a relative humidity of 40 - 70%. Diet and water were available *ad libitum* and clinical observations were performed daily.

2.4.2. Intravitreal injections

All injections were performed under general anaesthesia and aseptic conditions. The animals were anaesthetized intramuscularly with a mixture of ketamine hydrochloride (25 mg/Kg; Ketolar 50[®], Pfizer, Madrid, Spain) and medetomidine (0.5 mg/Kg; Domtor[®], Esteve, Madrid, Spain). Topical anaesthesia was achieved by eyedrops with 1 mg/mL tetracaine chlorhydrate and 4 mg/mL oxibuprocaine chlorhydrate (Colircusí Anestésico Doble[®], Alcon Cusí SA, Barcelona, Spain). Povidone-iodine solution (5%) was applied for ocular surface antisepsis before and immediately after the intravitreal injection. All of the injections were performed by the same ophthalmologist. Before the injection, aqueous humour paracentesis of 0.03-0.05 mL approximately with a 30-gauge needle was performed to prevent backflow of MSs and an increase of IOP (Aref., 2012).

The animals were randomized into 9 groups (Groups 1 – 8, n = 3 each; Group 9, n = 2) and a single control rabbit (Table 1). During the anaesthesia, the right eyes of the 24 rabbits in Groups 1 – 8 were injected intravitreally with GDNF/VitE-loaded PLGA MSs. The left eyes of the same rabbits were injected with non loaded PLGA MSs (Blank-MSs). In Group 9, all four eyes were injected with 50 μL of balanced salt solution vehicle (BSS[®]; Alcon Laboratories, Inc.; Fort Worth, TX, USA). The control rabbit received no injection, and both eyes were enucleated to compare the basal concentration of GDNF with rabbits that received the intravitreal

injection. The bilateral injections were done to reduce the number of animals used for the study.

To ensure a homogeneous dispersion of MSs in the injection, 4% (w/v) suspensions of the MSs were prepared in BSS and briefly vortexed immediately before each injection. A 50 μ L-volume of suspension was then injected intravitreally with a 25-gauge needle placed in the superior-temporal quadrant of the eye. According to the encapsulation efficiency of GDNF in the MSs, the dose administered was 74.85 ng of GDNF. Under surgical microscope visualization, the needle tip was directed to the centre of the vitreous body, about 3-5 mm posterior to the limbus. At post-injection time points of 24 h and at 1, 4, 6, 8, 12, 18, and 24 weeks, the animals were humanely killed by rapid intravenous bolus injection of 30 mg/Kg sodium pentobarbital, and the eyes were enucleated and immediately dissected. The entire vitreous body was peeled from the everted globe and frozen at -20°C. The intravitreal concentration of GDNF was determined by ELISA as described below.

2.4.3. General health and clinical findings

IOP measurements for all animals were performed using a rebound tonometer (TonoVet[®] TV01, iCare Finland OY, Vantaa, Finland). Pressures were measured before and at 24 h and at 1, 4, 6, 8, 12, 18, and 24 weeks after the intravitreal injection. The ocular health status was observed over the study period by slit-lamp examination of the ocular anterior segments and by indirect fundus ophthalmoscopy through dilated pupils with a 20-diopter aspheric lens.

2.4.4. Quantification of vitreous samples by ELISA

All vitreous samples were sonicated (Sonicator XL) at a low temperature for 1 min. Afterwards, the samples were centrifuged at 12,000 rpm for 10 min. The GDNF content in the supernatant was determined by ELISA (DuoSet® ELISA Development System, R&D Systems Inc.). The assays were done according to the manufacturer's instructions on tissue culture treated 96-well plates (Costar® 3599, Corning Inc., Corning, NY, USA), and the absorption at 450 nm was read in a multi-detection microplate reader (BioTek® Synergy™ HT, BioTek Instruments, Inc., Friedrichshall, Germany). Experimental samples were tested in triplicate, and experimental GDNF concentrations were calculated using the average absorbance of the three readings plotted against the standard curve. Two anchoring points below the routine limit of 15.6 pg/mL (7.8 and 3.9 pg/mL) were used for GDNF quantification in vitreous (Smolec et al., 2005). The same restriction in the detection limit fixed for lower limit of quantification and upper limit of quantification (back calculated concentration within 25 % of nominal value) was also demanded for this anchoring point for acceptance (EMA guideline on bioanalytical method validation., 2012).

2.4.5. Pharmacokinetics and statistical analysis

All values were expressed as means \pm standard deviations except the mean concentration values from vitreous samples that were graphically represented by using the standard error of the mean. The data were analyzed by Statgraphics software (StatPoint Technologies, Inc., Warrenton, VA, USA). A p-value of less than 0.05 was considered to be statistically significant.

The mean intravitreal concentrations (C_{vitreous}) of samples collected after injection of GDNF/VitE MSs were used to perform the pharmacokinetic analysis.

2.4.5.1. Non-compartmental analysis

The primary pharmacokinetic parameter “apparent vitreous clearance” ($Cl_{app}(\text{vitreous})$) was calculated using the general equation:

$$Cl_{app}(\text{vitreous})/Fr = \frac{\text{dose}}{\text{AUC}}$$

where the experimental area under curve (AUC) was estimated by a linear-trapezoidal method with the data collected during the 24 weeks of the *in vivo* study. “Fr” is a correction factor that does not represent bioavailability (considered 100 %) in this case, but the fraction dose released during 24 weeks of the *in vitro* study.

The profile of the intravitreal concentrations showed two consecutive plateaus: the first one from 24 h to 4 weeks (Stage 1) and the second one from 8 to 24 weeks (Stage 2). Both exhibiting zero-order release kinetics. The steady state concentrations (Css_1 and Css_2) were calculated from the mean values of intravitreal concentrations corresponding to each stage.

The release rate constants at both stages (K_{01app} and K_{02app}), following the zero-order kinetics, were related to Css_1 and Css_2 according to the following equation:

$$K_{0app} = Cl_{app}(\text{vitreous})/Fr \times Css.$$

The K_0 data for both stages might be considered only as approximate values, according to the limitations offered by clearance calculations, that is why have named them “apparent” release constant.

The initial burst *in vivo* was estimated as follows:

Initial burst = Vitreal concentration at 24h x Anatomical volume of rabbit vitreous.

Where the anatomical volume of rabbit vitreous was taken as the value reported by Del Amo et al. for albino rabbits (Del Amo et al., 2015). It is necessary to point out that it is an inaccurate estimation, so there is no guarantee that the true peak concentration occurs at 24 h, the first sampling point, and the apparent volume of distribution might be higher than the anatomical volume.

3. Results

3.1. Microsphere elaboration and characterization

The production yield for microencapsulation of GDNF/VitE MSs was $79.1 \pm 7.8\%$ and the mean particle size was $19.1 \pm 9.4 \mu\text{m}$ (Fig. 1, inset). The particles were spherical and had a high number of small pores on the surface (Fig. 1).

Confocal microscopy showed the initial distribution of HSA-FITC and VitE mixed with TRITC-DHPE in the MSs and the distribution after 24 h in the release medium (Fig. 2). Initially, the HSA-FITC was located mostly near the surface of the particles, and VitE (mixed with TRITC-DHPE during the microencapsulation procedure) was homogeneously distributed in the polymeric matrix. After 24 h in the release medium, the intensity and depth of the HSA-FITC fluorescent ring was diminished but still present. This fact can be related with an initial important release of the protein in the first 24 h, as occurs with GDNF. However, beanching phenomena cannot be discarded.

3.2. Encapsulation efficiency and *in vitro* release of GDNF

The GDNF/VitE formulation encapsulated $37.43 \pm 0.74 \text{ ng/mg}$ MSs of GDNF for an encapsulation efficiency of $42.74 \pm 1.66\%$. A sustained release of GDNF has been observed (Fig. 3). During the first 24 h, 49.3% of the GDNF incorporated in

the MSs was delivered from the GDNF/VitE formulation. After this initial GDNF burst, there was a sequence of fast and slow release steps (Fig. 3). First, there was a fast protein delivery for 7 days. After that, the release rate was 48 pg GDNF/mg MSs/day during the next 35 days (from 7th to 42nd day). After a fast delivery within 7 and 8 weeks (773 pg GDNF/mg MSs/day), the GDNF/VitE formulation released the GDNF in a sustained fashion for 42 days, from day 56 to day 98, with a release rate of 89 pg GDNF/mg MSs/day. From day 98 to 140 a release rate of 30 pg GDNF/mg MSs/day was denoted and finally, also a sustained release at a rate of 12 pg GDNF/mg MSs/day was observed until the end of the assay (day 168). At the end of the *in vitro* release study, the remaining GDNF content was more than 4% of the originally encapsulated agent.

3.3. *In vivo* assays

3.3.1. General health and clinical findings

IOP and clinical signs of ocular inflammation such as abnormalities in the cornea and inflammation in the anterior and posterior segments were evaluated before and after treatments. The eyes injected with the GDNF/VitE-loaded and blank MSs did not show any abnormalities during the six-month follow up after administration. In all animals, the corneas remained clear, and there were no observable anterior chamber reactions or iris abnormalities. Also, the posterior segments, particularly the retina and optic nerves, were free of any inflammatory response. Four eyes developed cataracts in the posterior lens capsule, probably due to trauma associated with the injection itself. Moreover, the IOPs were within normal limits over the study period, and no case exceeded 14 mmHg. There were no significant differences in IOP between eyes injected with GDNF/VitE or blank MSs (Fig. 4).

3.3.2. Ocular pharmacokinetics of GDNF after intravitreal administration

3.3.2.1. Vitreous levels of GDNF

The intravitreal GDNF concentrations after administration of 2 mg of GDNF/VitE and 2 mg of blank MSs are presented in Fig. 5. Consistent with the release burst observed *in vitro*, 24 h after injection of the GDNF-loaded particles, intravitreal GDNF concentrations reached 717.1 ± 145.1 pg/mL. At this initial point the injection of non-loaded MSs caused the basal levels of GDNF to increase to 10.2 ± 4.0 pg/mL. compared with the vitreous measured from non-treated animals (< 3.9 pg/mL, detection limit). During the following four weeks, GDNF levels were maintained at 745.3 ± 25.5 pg/mL (C_{ss1}) in eyes receiving GDNF-MSs, which was almost one hundred fold higher than the values in eyes receiving blank MSs, 7.8 ± 2.5 pg/mL. At six weeks post-GDNF MSs injection, the intravitreal GDNF concentration was 5.9 ± 0.6 pg/mL. After that, the GDNF concentration was maintained at 17.4 ± 3.7 pg/mL (C_{ss2}) from the 8th week to the end of the assay (24th week). GDNF quantification in animals receiving blank microspheres were over the detection limit during the first 4 weeks of the *in vivo* study.

3.3.2.2. Pharmacokinetic parameters

Based on the determinations of GDNF concentrations in the vitreous over the course of the study, the pharmacokinetic parameters for the non-compartmental analysis were determined (Table 2).

3.3.2.3. *In vivo* GDNF release from the MSs

Around 911 pg of the administered GDNF was initially released as a burst from the MSs during the first 24 hours. After that, GDNF was released during the following 4 weeks at a constant rate of 2 ng/day (Stage 1). The remaining neurotrophic factor was released at a constant rate of 46 pg/day until the end of the assay (Stage 2). These values were calculated considering that $Cl_{app}(\text{vitreous})/Fr$ describes the true vitreal clearance.

4. Discussion

Most neurodegenerative diseases induce irreversible loss of function in patients (Murakami et al., 2013). GDNF has a neuroprotective effect in delaying the degenerative process of retinal pathologies (Cuenca et al., 2014; Klocker et al., 1997). This effect occurs even at low concentrations if maintained for long periods of time. In fact, we have recently shown that 0.8 pg/day GDNF delivered by PLGA MSs resulted in RGC and axonal survival 11 weeks after injection in rats with induced glaucoma (Checa-Casalengua et al., 2011). These promising results indicated that biodegradable microparticulate systems can be considered as therapeutic tools in the treatment of chronic intraocular diseases in which the degeneration occurs slowly (Herrero-Vanrell et al., 2014). Furthermore, as the active substance is released close to the target site for long periods of time, the number of repeated applications can be significantly reduced (Herrero-Vanrell and Refojo, 2001).

In the present work, we prepared a MSs formulation with GDNF and VitE. We have selected PLGA as biocompatible and biodegradable polymer to control the release of the neurotrophic factor. This polymer undergoes homogeneous hydrolytic degradation that promotes the formation of pores and cavities (Wang et al., 2010) releasing the active compound from the PLGA matrix through diffusion

(Aubert-Pouessel et al., 2004). The particle size of about 19 μm and the spherical shape made this formulation suitable for being injected as a suspension through small needles (30 – 32 gauge) used for intravitreal administration (Herrero-Vanrell and Refojo, 2001). Scanning electron microscopy images showed the presence of pores in the MSs surfaces. This fact has been previously reported, and it is attributed to the rapid removal of organic solvent during the elaboration of the formulation (Al Haushey et al., 2007) and also to the presence of the oily additive VitE in the formulation (Checa-Casalengua et al., 2011).

One of the most remarkable technological aspect in the MSs formulation reported in this work is the presence of an oil (VitE) that allows the incorporation of the GDNF as a solid in the MSs. This fact helps to maintain the integrity of the neurotrophic factor (Checa-Casalengua et al., 2012) and extend the release of the active substance compared with the MSs without additive (data non shown). From a pharmacological point of view, the antioxidant properties of VitE presumably might help to reduce the oxidative stress associated with glaucomatous optic neuropathy (Mozaffarieh et al., 2008).

For confocal studies, we used HSA-FITC to determine protein distribution in PLGA matrix instead of GDNF. Unfortunately to our knowledge, it does not exist GDNF fluorescently labelled commercially available. Although GDNF and HSA are two proteins with different molecular weight, it could be assumed that the handling procedures such as sonication in VitE and emulsification in homogenizer might have more influence in the final distribution of the protein in the polymeric matrix than its molecular weight, considering that the protein is always in its solid state (Checa-Casalengua et al., 2011). According to the confocal microscopy findings, the protein distribution among the polymeric matrix, seems to condition the release behaviour.

Although the main anatomical and physiological parameters in humans and rabbits' eyes show small differences, the pharmacokinetic parameters (clearance, volume of distribution and half-life) in the human and rabbit eye have good correlation and comparable absolute values. So, reliable rabbit-to-man translation of ITV pharmacokinetics should be feasible. For this reason, for pharmacokinetic studies, we selected rabbits (Del Amo et al., 2015; Los., 2008; Zahn et al., 2010; Bösze and Houdebine., 2006). We have intravitreally injected 50 μ L-volume of 4 % w/v homogeneous suspension of microspheres which lead to a concentration of microspheres in the rabbit vitreous of 1.6 mg/mL according to a theoretical rabbit vitreous volume of 1.27 mL (del Amo et al., 2015). In other works, higher amount of PLGA microspheres were evaluated. For example, Giordano et al used amounts of microspheres (2.5 mg, microspheres vitreous concentrations of 1.97 mg/mL) in order to study the biodegradation and the tissue reaction of microspheres intravitreally injected in rabbits. They conclude that these PLGA microspheres were not toxic to ocular tissues (Giordano et al., 1995). In this line, Rong et al, have explored the eye biocompatibility and the safety of PLGA/PLA microspheres through intravitreal injection in New Zealand rabbits. Intravitreal administration of different doses of PLGA/PLA microspheres were evaluated: low (2.5 mg, microspheres vitreous concentrations of 1.97 mg/mL), medium (5 mg, microspheres vitreous concentrations of 3.94 mg/mL), or high (10 mg, microspheres vitreous concentrations of 7.81 mg/mL). Examinations were performed by evaluating retinal histological and functional changes up to 12 weeks post administration. This study demonstrated that intravitreal injection of a PLGA/PLA microspheres drug delivery system resulted biocompatible and safe (Rong et al., 2014). In the present work a small amount of microspheres was used in an attempt to combine good tolerance at long term with the need of detectable

GDNF concentrations in the vitreous during 24-weeks study. However, this amount can be increased according to patient's needs

In order to reduce the number of animals used for the study and due to protocol ethical factors, bilateral injections were done. As microspheres are locally injected in the vitreous, the GDNF released from them might be confined to the treated eye. Although it is theoretically possible that part of the active substance could reach the blood stream by crossing the blood-retinal barrier, this phenomenon depends mainly on the compound molecular weight and also on their concentration. The access of the active compound to the contralateral eye in the present work was limited by the slow release of GDNF from the microspheres as well as by its molecular weight (23,000 g/mol). In fact, other authors such as Ward et al (2007) or Ejstrup et al., (2010), have also used the contralateral eye as negative control.

It has been postulated that the intravitreal injection itself is sufficient to cause substantial reactive changes in Müller cells and microglia throughout the entire retina at least for the first 24 hours (Seitz et al., 2014). Furthermore, attending to Igarashi et al, Müller cells and astrocytes express GDNF (Igarashi et al., 2000). It is then reasonable to hypothesize that, if activity of Müller cells and astrocytes increases, the expression of GDNF may also increase. To evaluate this point measurement of GDNF in vitreous injected only with BSS were performed. Although detectable, the analytical data resulted under the quantification limit (<3.9 pg/mL) and by hence not significant in this case. Furthermore, we also measured the GDNF vitreous concentration in control eyes injected with blank microspheres. In this sense we were able to observe an increment in this control group during the first 4 weeks of the study (values beyond 3.9 pg/mL). This low but detectable

increment in endogenous might be due to the presence of microspheres themselves in the vitreous.

MSs act like reservoirs in the vitreous, and the active substance delivered from the particles diffuses in the vitreous gel, as previously reported by Veloso et al. (Veloso et al., 1997). After that, the released molecules are distributed and eliminated. These events largely depend on the physicochemical properties of the substance and the tendency of GDNF to diffuse to the retina (Ejstrup, R., 2010). According to our results, GDNF/VitE formulation released the neurotrophic factor into the vitreous body at least for six months following intravitreal injection.

It has been reported that PLGA MSs tend to aggregate after intravitreal injection (Barcia et al., 2009) which surely might influence in the release profile of the protein. Although *in vitro*, also certain tendency of aggregation can be also observed after several weeks of incubation in release media (Checa-Casalengua et al., 2011), these two phenomena cannot easily compared. Furthermore, while in the *in vitro* study the released protein is maintained in the release media until quantification, *in vivo* occurs just the opposite, and the released protein can diffuse to the tissues (retina in this case) and/or is degraded in the vitreous once released. This could explain why the sequence of rapid then slow GDNF release steps *in vitro* was not correlated with the *in vivo* GDNF release behaviour postulated after pharmacokinetic data treatments. *In vivo*, the fast release of GDNF occurring at stage 1 can be explained for the diffusion of the active substance through the pores of the polymeric matrix, followed by a slow release due to polymeric matrix bulk erosion (stage 2). The *in vivo* assay was performed over a period of 168 days (24 weeks). At that time point, according to pharmacokinetic data, not all of the loaded GDNF had been released from MSs. It is possible that the remaining GDNF could

have resulted in an extended second stage, although this should be experimentally confirmed.

Because of the potency and mechanism of action, the concentrations of neurotrophic factors needed to provide neuroprotection are low (Brodski et al., 2002). *In vitro*, the EC₅₀ of GDNF that enhances dopaminergic neuron survival is 40 pg/mL (Lin et al., 1993). This estimated minimal concentration could be previously considered by other authors as the therapeutic concentration able to promote RGC rescue in a porcine elevated IOP glaucoma model (Ejstrup et al., 2010). These authors found that after the administration of 100 ng of GDNF in solution (bolus) into the porcine eye (vitreous volume 2.2 mL), values of the neurotrophic factor higher than the estimated minimal concentration were maintained for 15 days. However, other authors have observed neuroprotective effects for GDNF concentration in the vitreous that were lower than the estimated minimal value when it is continuously released from MSs. In fact, Ward et al. showed an increment in RGC survival in DBA/2J mice (vitreous volume 0.005 mL) after repeated injections of GDNF-loaded MSs with a total theoretical release of GDNF of 0.707 ng in two months (Ward et al., 2007). The same formulation of GDNF-particles was also neuroprotective in a rat glaucoma model (vitreous volume 0.05 mL) with chronically elevated IOP (Jiang et al., 2007). MSs (0.05 mg) containing GDNF were injected into the vitreous. After nine weeks, the theoretical total release of GDNF was 1.77 ng, which provided RGC protection. Kyhn et al. demonstrated also RGC protection for 6 weeks in a pig model of acute retinal ischemia after administration of 2 mg of particles that provided a total GDNF release of 70.8 ng (Kyhn et al., 2009). Finally, we previously demonstrated that even as little as 0.64 ng of GDNF administered by GDNF/VitE PLGA MSs in the chronic hypertension rat model of glaucoma protected RGCs for at least 11 weeks (Checa-Casalengua et

al., 2011). In fact, we postulated that *in vitro* release rates of GDNF as low as 0.8 pg/day could be neuroprotective (Checa-Casalengua et al., 2011).

Unfortunately, no GDNF quantification was performed in any of the *in vivo* studies. In the present study, we administered a total GDNF dose of 74.85 ng, which produced a high GDNF concentration, about 745 pg/mL. This was more than 18 fold higher than the estimated minimal threshold of 40 pg/mL that was detected in the rabbit vitreous for the first 28 days. According to our pharmacokinetic analysis, the *in vivo* release of the neurotrophic factor is consistent in a zero-order kinetic process that is followed by a predominant elimination step between weeks 4 and 6. After that, a second zero-order kinetic phase resulted in a low but sustained GDNF concentration, around 18 pg/mL and an estimated *in vivo* release rate of 50.2 pg/day, from day 56 until the end of the experiment.

Considering that there is no *in vivo* data concerning the therapeutic concentration of GDNF (Ward et al., 2007), it is difficult to estimate the optimal dosage, especially if it is released from MSs like those that were neuroprotective for 11 weeks in a glaucoma model (Checa-Casalengua et al., 2011). It is then rational to consider that our MSs might be neuroprotective not only for the first 28 days, with GDNF values *in vivo* > 40 pg/mL, but also for at least six months. Extended activity *in vivo* studies should be made to confirm this hypothesis.

5. Conclusions

The method described in this work allowed for the encapsulation and controlled *in vitro* release of GDNF for at least 24 weeks. After a single injection of the novel formulation GDNF/VitE into the rabbit vitreous, GDNF was released in a sustained fashion for at least six months.

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ABSTRACT

Local long-term delivery of glial cell line derived neurotrophic factor (GDNF) from vitamin E/poly-lactic-co-glycolic acid microspheres (MSs) protects retinal ganglion cells in an animal model of glaucoma for up to 11 weeks. However, the pharmacokinetics of GDNF after intravitreal injection of MSs is not known. We evaluated the GDNF levels after a single intravitreal injection of GDNF/VitE MSs. Biodegradable MSs were prepared by the solid-oil-in-water emulsion-solvent evaporation technique and characterized. Rabbits received a single intravitreal injection (50 μ L) of GDNF/VitE MSs (4%w/v; 24 right eyes; 74.85 ng GDNF), blank MSs (4%w/v; 24 left eyes), and balanced salt solution (4 eyes). Two controls eyes received no injections. At 24 hours, 1, 4, 6, 8, 12, 18, and 24 weeks after injection, the eyes were enucleated, and the intravitreal GDNF levels were quantified. Pharmacokinetic data were analysed according to non-compartmental model. Intraocular GDNF levels of 717.1 ± 145.1 pg/mL were observed at 24 hours for GDNF-loaded MSs, followed by a plateau (745.3 ± 25.5 pg/mL) until day 28. After that, a second plateau (17.4 ± 3.7 pg/mL) occurred from 8 to 24 weeks post-injection, significantly higher than the basal levels. Eyes injected with GDNF/vitE and Blank-MSs did not show any abnormalities during the six-months follow up after administration. The single injection of GDNF/VitE MSs provided a sustained controlled release of the neurotrophic factor in a controlled fashion for up to six months.

Figures

Fig. 1

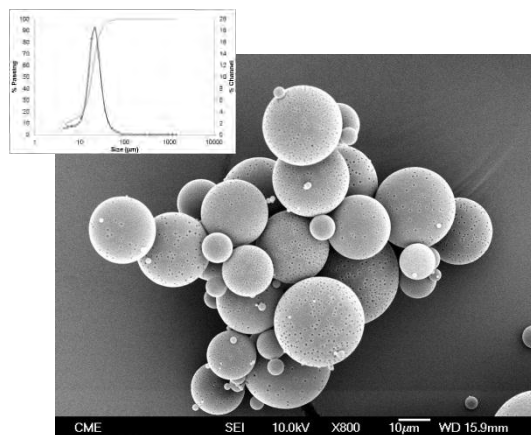


Fig. 2

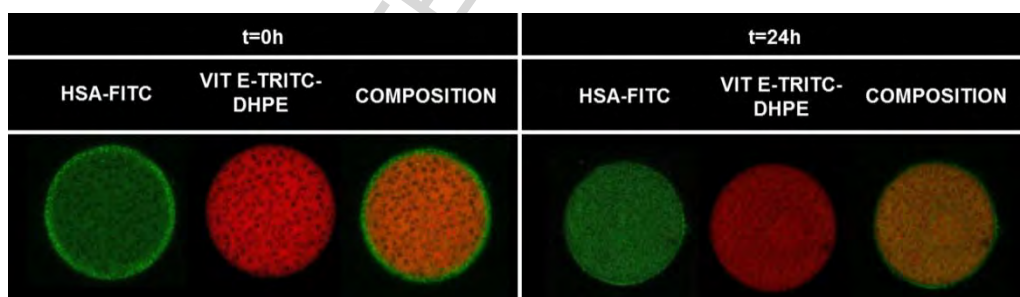


Fig. 3

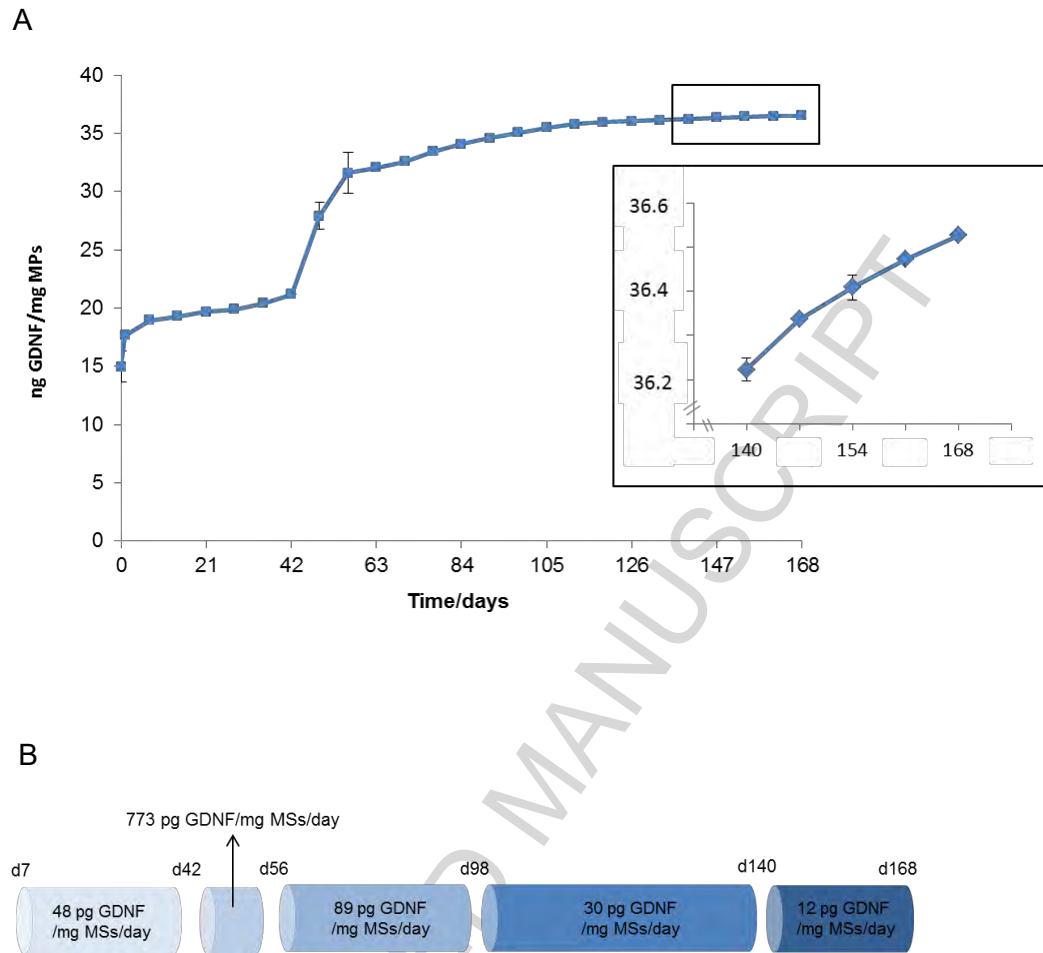


Fig. 4

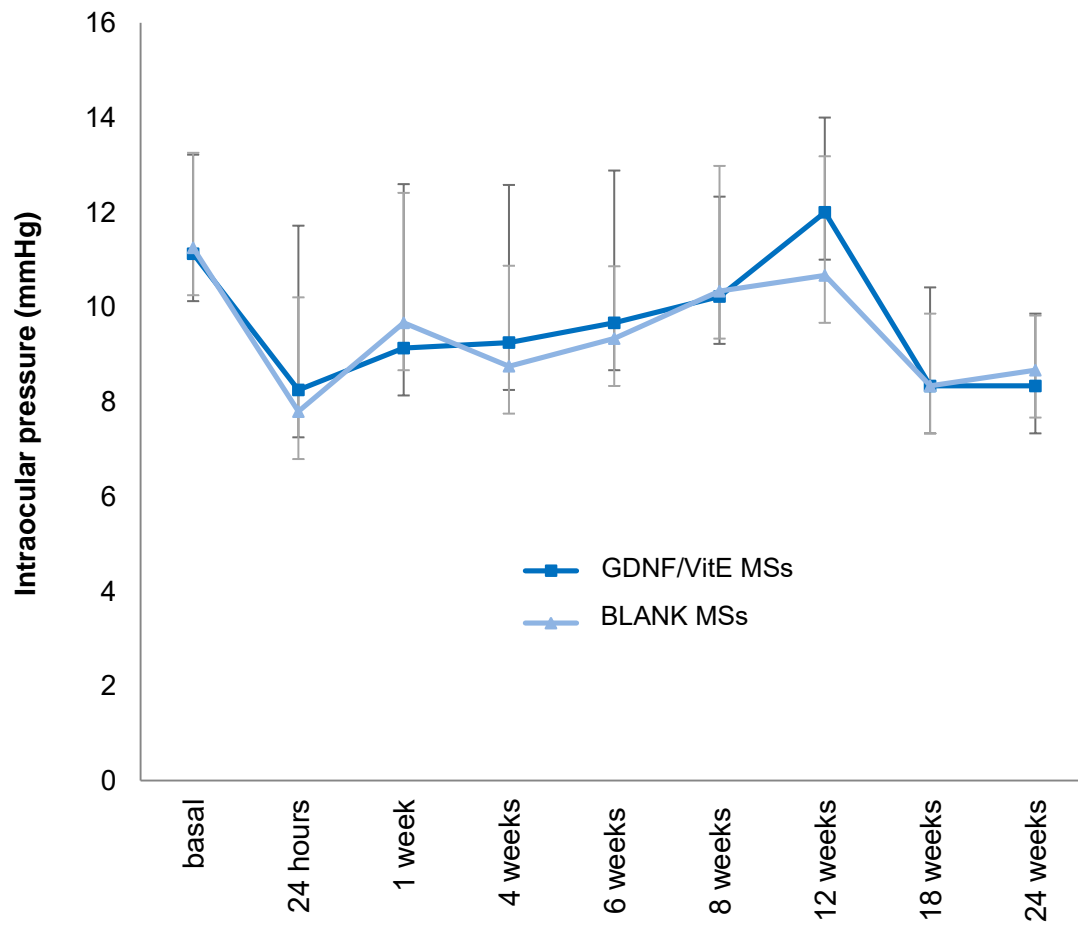
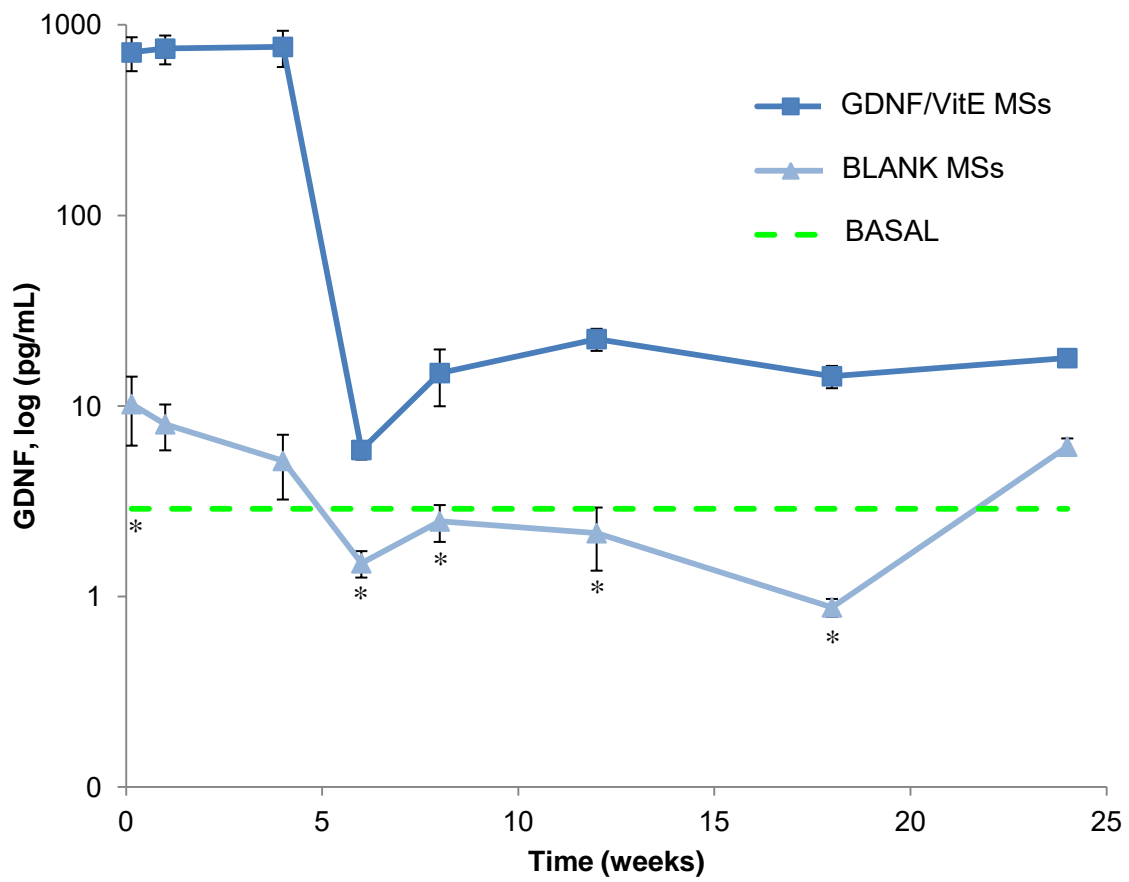


Fig. 5



Captions

Fig. 1 Scanning electron microscopy images of GDNF/Vit E microspheres. Inset: Particle size distribution.

Fig. 2 Confocal microscopy images of HSA-FITC and VitE-TRITC-DHPE distribution in PLGA microspheres at $t = 0$ h and $t = 24$ h after incubation in the release medium. The combination images were created by merging the HSA-FITC images with the VITE-TRITC-DHPE images.

Fig. 3 (A) Cumulative *in vitro* release of GDNF from GDNF/Vit E microspheres (ng/mg MSs) over 168 days (six months) from the GDNF/VitE MSs. Release media: PBS (pH 7.4), 1% BSA and 0.02% Na azide. Inset: Cumulative release of GDNF from the GDNF/VitE formulation from day 140 to day 168. **(B)** Schematic representation of the release rate for formulation GDNF/VitE for 168 days.

Fig. 4 Intraocular pressure before and after the intravitreal administration of a MS suspensions of GDNF/VitE (■) and blank MSs (▲) in rabbits.

Fig. 5 Semilog GDNF time-dependent concentration profiles in rabbit vitreous humour after the administration of MS suspensions of the GDNF/VitE formulation (■), and blank MS suspensions (▲). The green dashed line represents the average basal concentration of intravitreal GDNF in two eyes of one rabbit. (*) GDNF values under the detection limit (< 3.9 pg/mL).

Table 1. Experimental groups.

Group	N° of rabbits	Injection	Time of enucleation	Treatment		N° of eyes
				RE	LE	
1	3	MSs	24 h	GDNF-MSs	Blank-MSs	6
2	3	MSs	1 week	GDNF-MSs	Blank-MSs	6
3	3	MSs	4 weeks	GDNF-MSs	Blank-MSs	6
4	3	MSs	6 weeks	GDNF-MSs	Blank-MSs	6
5	3	MSs	8 weeks	GDNF-MSs	Blank-MSs	6
6	3	MSs	12 weeks	GDNF-MSs	Blank-MSs	6
7	3	MSs	18 weeks	GDNF-MSs	Blank-MSs	6
8	3	MSs	24 weeks	GDNF-MSs	Blank-MSs	6
9	2	BSS	24 h	BSS	BSS	4
Control	1	-	-	-	-	2
TOTAL	27					54

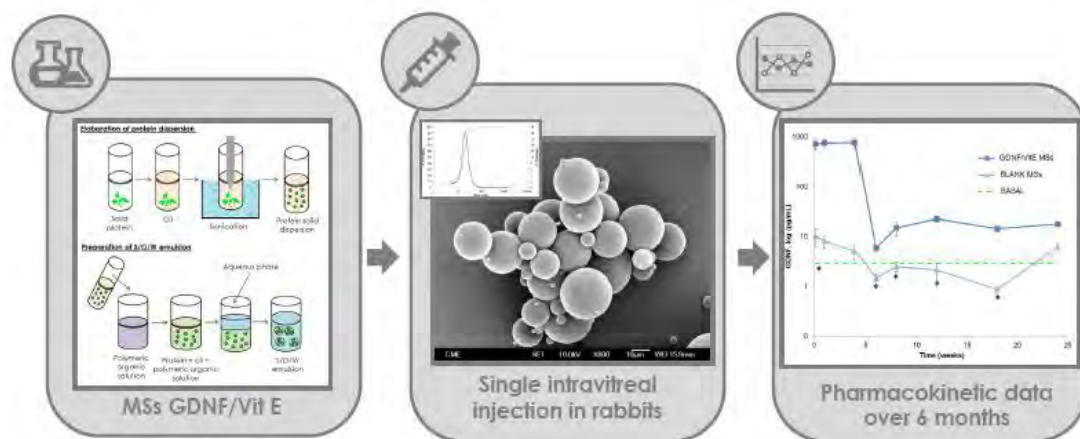
Table 2: Pharmacokinetic parameters of GDNF distribution in the vitreous

Pharmacokinetic parameter	Non-compartment analysis
$Cl_{app}(\text{vitreous})/Fr$ (mL/day)	2.65
AUC (pg·day/mL)	28238
K_{01app} (pg/day)	1973.4
K_{02app} (pg/day)	46.2
t_{02} (day)	> 42

$Cl_{app}(\text{vitreous})$, apparent vitreous clearance; AUC, area under the curve (0-168 days); K_{01} , zero-order release constant for 24 h to 4 weeks; K_{02} , zero-order release constant for 8 to 24 weeks; T_{02} , onset time of the stage 2.

Note: Calculation of AUC value from blank microspheres group resulted meaningless so part of the experimental data were under the detection limit (< 3.9 pg/mL)

Note: Fr does not represent bioavailability (considered 100%) but the fraction of dose released during the 24 weeks assay.



Graphical abstract