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RESEARCH ARTICLE

Evaluation of the Performance of an Ophthalmic Thermosensitive Hydrogel Containing Combination of Suramin and Bevacizumab

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Abstract: Suramab (SUM) is a new pharmaceutical combination made up of suramine (SUR) and bevacizumab (BVM), which showed a high synergistic effect when administered jointly. As the pharmaceutical vehicle, poloxamer aqueous dispersions were used since this system is able to maintain their fluidity at low temperatures (<15ºC) but which become gel in the corporal environment (>35ºC). In the present study we aimed at evaluating the effect of Poloxamer to prolong the effect of SUM.

These formulations were characterized using rheological, biopharmaceutical (drug release) and morphological (SEM) technique. Corneal NV was induced in Sprague Dawley rats Corneal. At 15 days of follow up animals were sacrificed and perfused with black drawing ink. Digital photographs were taken and the area of neovascularisation (ANV) was calculated using the image programmed. The rheological behavior was influenced by the addition of drugs, resulting in a decrease in the gelation temperature ($T_{sol/gel}$). Both drugs were released from poloxamer gels by means of an anomalous mechanism. However, BVM was released faster than SUR, with their combination (SUM) to appearing to reduce delivery, probably due to interactions between the drugs or with the polymeric matrix. The *in vivo* studies showed that SUM-poloxamer gel was able to increase the corneal antiangiogenic effect compared to the SUM solution and BVM alone at 15 days of follow-up. Furthermore no injurious effects were observed in the histological tissue examination after drug administration. The presence of Poloxamer, known to modulate control release of biological agents, seems to have a favorable effect on SUM subconjunctival administered.

Keywords: Thermosensitive hydrogels, poloxamer, suramab, bevacizumab, neovascularization, controlled release, suramine.

INTRODUCTION

The cornea is localized in the anterior part of the eye, with the corneal tissue being avascular and its transparency being crucial to obtaining a good vision. Neovascularization (NV) is characterized by the formation of new vascular structures in areas that were previously avascular. The mechanisms that may be involved in NV regulation are vasculogenesis (the formation of new blood vessels from angioblasts, mainly during embryogenesis) and angiogenesis (the formation of new vessels from preexisting vascular structures). Corneal angiogenesis is associated with a wide variety of diseases, most of them associated with inflammation, trauma and/or limbal barrier disfunction [1].

The use of antiangiogenic drugs in ophthalmic diseases has dramatically increased during recent years [2–4]. BVM is a recombinant humanized monoclonal antibody (IgG1) that binds to soluble vascular endothelial growth factor (VEGF) and prevents the binding of this growth factor to VEGF receptors (Flt-1 and KDR), thereby inhibiting neovessel formation. This drug is also currently being used as an important neoadjuvant for colorectal and breast cancer. In addition, the off-label use of intravitreal injection of BVM is effective in neovascular inhibition and in improving vision of diabetic retinopathy and exudative age-related macular degeneration. However, the short half-live of this monoclonal antibody requires frequent injections in order to maintain its therapeutic effect with some recent.

Studies [5,6] having demonstrated that when the frequency of injection is decreased, the visual improvement derived from this treatment can be lost. This necessity for frequent injections is not only inconvenient but implies a psychological burden and an increased risk of infectious endophthalmitis, which is devastating to the vision [7].

The new pharmaceutical combination SUM, is made up of SUR and BVM. Its antiangiogenic and antitumoral effects were evaluated in a rat model of corneal angiogenesis and in a mouse model of colo-rectal cancer, respectively, with the results being quite promising. These preliminary studies were carried out using aqueous solutions of SUM and for these experimental conditions a higher and longer antiangiogenic effect was shown compared to almost double-fold monodoses of BVM and SUR [8] administered separately. Related to this, it is well known that a rapid clearance from an aqueous environment can be expected for aqueous solutions of drugs. Although the pharmacokinetics of this kind of compound are not know in detail, the administration of SUM vehiculised in a pharmaceutical carrier able to modulate drug release and prolong the residence time of the formulation may improve the therapeutic efficiency of this drug. Therefore, we proposed the formulation of these compounds in a novel pharmaceutical system.

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In this context, we focused our studies on the development of a thermosensitive gel based on the particular properties of Poloxamer, a copolymer of poly(oxyethylene)–poly(oxypropylene)–poly(oxyethylene), as the potential utility of this polymer in a viscous injectable dosage form has been widely described [9–11]. This material is able to load sufficient drug amounts, and possesses good water solubility, tolerability, biodegradability, non-toxicity, and more importantly, its increase in viscosity when the temperature is raised allows the controlled release of drugs[12]. In this way, poloxamer solutions are known to exhibit the phenomenon of reverse thermal gelation by remaining as solutions at low temperatures and forming gel when the temperature is high.

Poloxamer 407 is a triblock copolymer with a central hydrophobic chain of polyoxypropylene (PPO) and two identical lateral hydrophilic chains of polyoxyethylene (PEO), with a molecular weight of 12500 Da and a PEO/PPO ratio of 2:1. It is biocompatible and is used in pharmaceutical preparations, due to its stability. In addition, its low toxicity and weak immunogenic properties make it suitable as a vehicle for drug delivery. A Poloxamer 407 aqueous solution is an injectable liquid when cold (5–10°C) and forms gels in situ at physiological temperatures[13]. However, for practical use, the most convenient gelation temperature of poloxamer solutions has to be experimentally determined in order to optimize the rheological effect of the temperature, which may be achieved by mixing different types (different molecular weight) of poloxamer and/or by adding appropriate auxiliary excipients. Such thermosensitive hydrogels have been used as controlled release drug delivery systems for injectable [13–17], ocular [18,19] and rectal administration [20–22].

In recent years, thermosensitive copolymers have attracted increasing interest and different applications such as biosensors, protein adsorption, drug delivery, and tissue engineering have been proposed. Thermo responsive injectable gels, which undergo a sol–gel transition in the physiological temperature range, have shown very interesting properties for the design of pharmaceutical systems based on the in situ formation of very viscous gels. Such a system could comprise a liquid with optimal syringe ability at ambient temperature, but once injected it would rapidly gel under physiological conditions with minimal syneresis. Post-injection gel formation has a number of advantages, such as i) this injectable matrix can be implanted in the human body with minimal surgical wounds, and ii) bioactive molecules or cells can be incorporated by mixing before injection. After gelation, these matrices become drug delivery reservoirs or cell-growth depots for tissue regeneration.

In the present work, we report on results concerning the efficacy of SUM -poloxamer injectable thermosensitive hydrogel with regard to the inhibition of induced corneal neovascularization in rats. In addition, an exhaustive characterization of the systems using rheological, biopharmaceutical (drug release) and morphological (SEM) techniques was addressed.

MATERIAL AND METHODS

1. Materials

Poloxamer 407 (Pluronic F-127®) was purchased from BASF and Suramin sodium salt was obtained from Biomol International (LP, USA). Bovine serum albumin (BSA) was purchased from Sigma Chemical (St. Louis, USA) and BVM (Avastin®) from Roche (Buenos Aires, Argentina). Avastin was provided as a concentrate for a solution for infusion in a single-use vial, which contained a nominal amount of either 100 mg of BVM in 4 ml or 400 mg of BVM in 16 ml (concentration of 25 mg/ml). α,α-trehalose dihydrate and polysorbate 20 were obtained from Anedra (Buenos Aires, Argentina) with the inorganic salts (NaCl, NaH_2PO_4, Na_2HPO_4) were purchased from Parafarm (Buenos Aires, Argentina). Phosphate buffer solution (PBS) was prepared according to Zimmer et al, 1995 [23]. Recombinant human $VEGF_{165}$ was purchased from R&D Systems (Minneapolis, USA) and the biotin mouse anti-human IgG, Streptavidin-Horseradish Peroxidase and TMB substrate set were obtained from BD Biosciences (San Diego, USA). Midazolam (Dormicum®) provided by Roche (Buenos Aires, Argentina), Proparacaine 0.5%, (Anestalcon®) by Alcon (Sao Pablo, Brazil) and Ketamine (Cost®) by Fada Pharma (Buenos Aires-Argentina) were used for the assays.

2. Preparation of Gels

An exactly weighed amount of P407 (16% w/w) was added to water or to an aqueous solution of drug previously equilibrated at 4–8°C. BVM and SUR (6 mg/ml of SUR or/and 1.8 mg/ml BVM) were dissolved in a solution containing 51 mM sodium phosphate pH 6.2, 60 mg/ml α,α-trehalose dihydrate and 0.04% polysorbate 20 which was diluted with an exactly weighed amount of cold water containing poloxamer P407. Four formulations were prepared with the composition of the formulations being shown in Table **1**.

3. Osmolality and pH

An Osmomat 030-D Cryoscopic Osmometer Printer Ganatec apparatus (using NaCl solution as reference (0.303 Osmol/kg)) and a Hanna HI 112 instrument, were used to determine osmolarity and pH, respectively.

4. Dynamic Rheology Assays

- Measurement of Gelation Temperature

The solid–gel transition temperature (T sol/gel) was measured using a Rheoplus MCR301 Anton Paar with a stainless steel cone, plate geometry (50 mm diameter, 1° angle and a gap of 50µm between the cone and plate) and a temperature ramp step oscillation procedure. Samples were carefully located onto the plate of the rheometer, thus ensuring that formulation shearing was minimized, and left to equilibrate for at least 10 min prior to analysis.

Table 1. Composition, pH and osmolarity of test formulations. Each value given in the table was calculated from n = 3, and is given by the mean ± standard deviation.

Formulations	Poloxamer P(407) (w/v)	Suramina SUR (mg/ml)	Bevacizumab BVM (mg/ml)	pH	Osmolarity (mOsmol/kg)
$P(407)_{16}$	16%	-	-	7.04± 0.12	269 ± 0.02
$P(407)_{16}$-Suramin	16%	6	-	6.88± 0.01	310 ± 0.12
$P(407)_{16}$-Bevacizumab	16%	-	1.8	6.79±0.10	300 ± 0.02
$P(407)_{16}$-Suramab	16%	6	1.8	6.53± 0.04	295 ± 0.03

For all oscillation experiments, the strain amplitude value was obtained from the linear viscoelastic region of the samples at 10 and 45°C. Following the application of a constant stress at a frequency of 10 Hz and a maximum shear strain of 0.07%, a temperature sweep analysis was performed over the temperature range of 10–45 °C, with the temperature being increased at 1.2 °C/min. The analyses were performed on at least three replicates of each formulation, with $T_{sol/gel}$ being considered to be the temperature at which the two moduli were of equal values (G' and G"crossover), as proposed in the literature [24,25]. The complex viscosity (Ƞ*), storage (G') and loss (G") modulus were used as quantitative indicators of the rheological behavior, and each sample was assayed in triplicate (n = 3).

5. *In Vitro* Drug Release

The polymeric solutions containing the drugs (1 ml) were allowed to remain in a water bath at 35ºC, until the solution became a gel. Then, 3.5 ml of buffer solution pH=6.8 was added with gentle magnetic stirring performed at 50 rev/min.

At appropriate intervals, 250 µL samples were taken and assayed for drug release, which were replaced by 250 µL of fresh buffer. The amount of SUR and BVM in the release medium was determined by high-performance liquid chromatography (HPLC, see section 6) and ELISA, respectively. Each sample was assayed in triplicate (n = 3), and in all cases the fitting process was performed using KaleidaGraph v4 software.

6. HPLC Determinations of SUR

The HPLC system consisted of an AGILENT 1100 HPLC pump and an AGILENT 1100 HPLC detector (Agilent Technologies) set at 283 nm. Samples were chromatographed on a reversed-phase Luna C18 column (150 x 4.6 mm, 5 mm, Phenomenex) and a 2 x 8 mm pre-column of the same material, with the mobile phase being at a 1.5 mL/min flow rate and consisting of 52.2 % MeOH, 4mM Tributil Ammonium Bromide, 10 mM monosodium phosphate and disodium phosphate, which was filtered and degassed before use. The column was thermostated at 40°C [26].

7. BVM Detection Assay

The 96-well flat-bottom plates (Greiner Bio One, Frickenhausen, Germany) were sensitized by incubating 25 µL $VEGF_{165}$ at a concentration of 0.4 µg/mL in the coating buffer (0.1M carbonate–bicarbonate buffer, pH 9.6) overnight at 4ºC. All plates were washed three times using 0.05 Tween® 20-PBS and the remaining protein-binding sites were blocked for one hour of incubation at room temperature with 100 µL blocking buffer (1% BSA-PBS). For each individual assay, a standard curve was included using Bevacizumab at known concentrations, ranging from 0.312 to $9.77x10^{-3}$ ng/mL. Plates were washed as described previously, and 50 µL of diluted standards, and 50 µL unknown samples were added. After incubating the plates for 1 hour at 37ºC and following a new washing step, 50 µL of anti-human Ig G antibody diluted in 1% BSA-PBS were added to each well. After 1 hour at room temperature followed by washing, 50 µL of Streptavidin-Horseradish Peroxidase diluted in 1% BSA-PBS were added to each well. Then 30 minutes after a new washing step, 50 µL of solution of TMB substrate were added to each well, and the reaction was allowed to develop at room temperature in the dark. The color reaction was stopped by adding 50µL of 1N sulfuric acid per well and reading were taken at 450 nm using an ELISA BIO-RAD (MODEL 680) microplate reader [27,28].

8. Hydrogel Morphology

SEM-Freeze-Dried Hydrogels: these formulations were gelled at 35 °C, frozen at -80 °C and lyophilized. Samples were fractured in liquid nitrogen, and sputter coated with palladium and gold. The resulting dried samples were examined using FEG-SEM Karl Zeizz equipment.

9. *In Vivo* Assay in Rats

Animals. Experiments were conducted using Sprague Dawley rats weighing approximately 600 g (CNEA, Buenos Aires, Argentina). Animals were anesthetized with 70 mg/kg intramuscular ketamine and 1 mg/kg IM Midazolam (Dormicum®), topical anesthesia was applied using 0.5 % Proparacaine (Anestalcon®).

Corneal alkali burns. Corneal neovascularization was induced by placing a Whatman filter paper disc (3-mm-diameter) soaked in 1M Na(OH) at the center of the cornea of the left eye for 30 seconds, after which, the eye was washed with saline solution [12,29].

Ocular therapy. Animals were treated immediately after injury. Group 1 (n=6) was treated with subconjunctival SUM solution (a blend of 3 mg/kg of BVM+10 mg\kg of SUR), Group 2 (n=6) was treated with subconjunctival $P(407)_{16}$-SUM (3mg/kg of BVM + 10 mg\kg of SUR), Group 3 (n=6) was treated with subconjunctival BVM (5 mg/kg of BVM) and Group 4 (n=5) received saline solution. Animals were kept in individual cages with free access to food and water, and were the reported experiments in accordance with the standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animals and were handled according to the ARVO Statement for the Use of Animals in Ophthalmic Research

10. Black-Ink Perfusion and Histological Examination

Black-ink perfusion. At 15 days of follow-up, animals were perfused with black drawing-ink and sacrificed. Their eyes were removed and the corneas (with limbal regions) were isolated, radial-cut and flat-mounted. Digital photographs were taken, and the area of neovascularisation (ANV) was calculated using the jimage program. The percentage of corneal neovascularization was compared among groups using the Mann Whitney test, with P values <0.05 considered statistically significant.

Histology. The corneas of each group of animals examined with black-ink were later stained with haematoxiline & eosin. Sections of 10-micron-thickness were analyzed with a Nikon Eclipse Microscope (Tokyo, Japan).

RESULTS

Poloxamer 407 (trade name, Pluronic F-127) is a non-toxic poly(oxyethylene/ oxypropylene/oxyethylene) (PEO/PPO/PEO) triblock copolymer with a weight-average molecular weight of 12.500 and contains 70% hydrophilic ethylene oxide units and 30% hydrophobic propylene oxide units.

A diluted aqueous solution (<20% w/w) of this compound is a clear liquid at low temperatures (<15ºC), which is able to form a gel on warming to room temperature as a consequence of a sol-gel transition and the process of reverse thermal gelation. The resulting gel has slow drug release characteristics.

In this study for 16 % (w/w) poloxamer solutions, a gel with desirable rheological properties (syringeability) was obtained and was thus chosen for further studies where the drugs were vehiculised.

Measurement of Gelation Temperature

The gelation temperature is the temperature at which the liquid phase makes its transition into a gel. This stage of the formulations was evaluated by monitoring the variation of the viscoelastic parameters with temperature. The mechanical properties of formulations were studied through small amplitude shear tests, at temperatures below 15°C and above 35°C the theoretical $T_{sol/gel}$. The elastic and viscous moduli of polymer-drug dispersions, at a frequency value of 10 Hz, being shown in figure **1**. The gelation temperature ($T_{sol/gel}$) was identified as the temperature at which the G' and G" curves intersected each other. At 15°C, the rheological behaviour of

the formulations was typical of a viscous fluid, with G" always being higher than G'. In contrast, at 35°C, the addition of SUM into the poloxamer formulations altered the rheological behaviour of the system, with there being two conformational changes (at 20.8 and 27.5 ºC) which appeared to be typical of a gel-like material, even though a significant increase in both viscoelastic moduli was apparent (Table **2**).

The observed $T_{sol/gel}$ for poloxamer aqueous dispersion was 29.6 ºC. Incorporation of BVM shifted the $T_{sol/gel}$ value to 22.8 ºC whereas SUR reduced this parameter by about 5.5 ºC to a value of 23.5 ºC. Finally, SUM produced a decrease in $T_{sol/gel}$ to 20.8ºC. This fact implies that this last formulation should be cooled to temperatures lower than 20 ºC at the moment of administration, in order to improve syringeability by decreasing the solution viscosity. Future , formulation studies should be conducted with aim of optimizing this issue, i.e. by the addition of electrolytes or other kinds of additives able to increase $T_{sol/gel}$, as already described in bibliography. However, such studies are beyond the scope the present article.

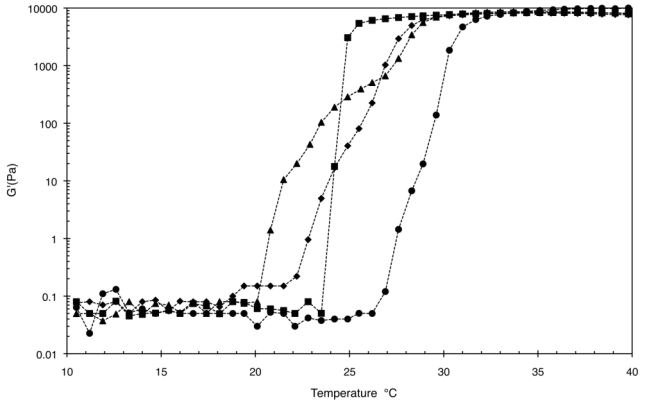

Fig. (1). Profiles of G' P (407)16 in water ● with Suramin ■, Bevacizumab ♦ and Suramab ▲.

In Vitro Drug Diffusion Kinetics

Different release behaviors were observed for BVM and SUR. used alone or vehiculised jointly (as SUM). In Fig. **2**, the drug release profiles of BVM, SUR and SUM are depicted.

It may be observed that BVM was released faster than SUR, although the difference in the initial concentrations of each drug was not taken into account (BVM= 6 mg/ml, SUR=1.8 mg/ml). However, the kinetic release for each drug alone was notably different compared to that of the SUM-poloxamer gel, with

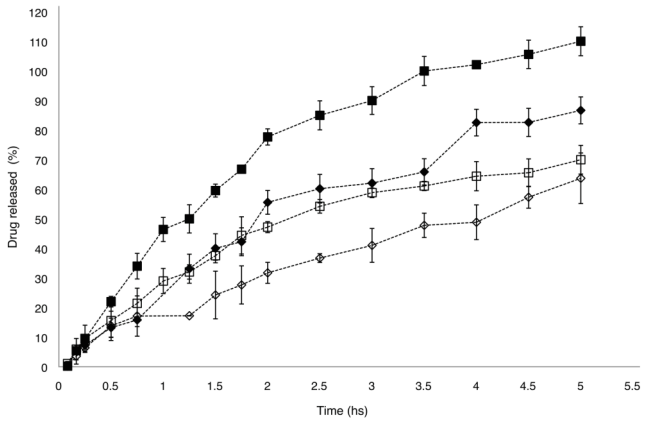

Fig. (2). Delivery of **Bevacizumab** from ■ $P(407)_{16}$ Bevacizumab and $P(407)_{16}$ Suramab hydrogel, and delivery of **Suramin** from □ $P(407)_{16}$Suramin and ◊$P(407)_{16}$ Suramab hydrogel.

both BVM and SUR being released faster than combined in SUM-poloxamer gel. Therefore, it is apparent that some kind of interaction took place between them and/or with the poloxamer resulting in a delay in the drug release.

Future studies concerning the evaluation of the mechanisms, involved in addition to permit the kinetics of drug release, would a better insight into the *in vitro* behavior of the systems regarding drug delivery. Although it is not usually useful to extrapolate in vivo conditions, this may provide valuable information related to the expected *in vivo* performance.

In this sense, since drug release kinetics reflect the different release mechanisms involved in drug delivery, we applied three kinetics models (zero-order, Higuchi and Korsmeyer-Peppas) to analyze the in vitro drug release data in order to find the best fitting equation (Table **3**).

In the case of the Korsmeyer-Peppas model, the following equation was used:

$$ft = \frac{M_t}{M_\infty} = a.t^n \quad (1)$$

Where ***ft*** is the ratio of absolute cumulative amount of the drug released at time ***t*** ($\boldsymbol{M_t}$) and at infinite time ($\boldsymbol{M_\infty}$), ***a*** is a constant incorporating structural and geometric characteristics of the carrier, and ***n*** is the release exponent, indicative of the drug release mechanism. If ***n*** = 0.5, the release is governed by Fickian diffusion, with ***n*** = 1, indicating that molecules are released by surface erosion, while both mechanisms play a role in the release for ***n*** values between 0.5 and 1.

Table 2. G', G'' modules, viscosity (at 15 and 35 °C) and sol-gel temperature of formulations.

Formulations	G'		G"		T sol-gel [°C]	Complex Viscosity [Pa.s]35ºC
	15°C [Pa]	35°C [Pa]	15°C [Pa]	35°C [Pa]		
$P(407)_{16}$	0.06	9070	0.16	1240	29.6	915
$P(407)_{16}$-Suramin	0.06	8340	0.42	780	23.5	921
$P(407)_{16}$-Bevacizumab	0.06	8180	0.37	760	22.8	822
$P(407)_{16}$-Suramab	0.00	8710	0.28	953	20.8	877

Table 3. Parameters calculated by Zero Order, Higuchi and Korsmeyer-Peppas models for Bevacizumab and Suramin *in vitro* release.

Formulations	$P(407)_{16}$-*Suramin*	$P(407)_{16}$-Suramab *(Suramin)*	$P(407)_{16}$-*Bevacizumab*	$P(407)_{16}$-Suramab *(Bevacizumab)*
Korsmeyer Peppas Model				
$k\ (h^{-n})$	28.331±0.92534	18.513±0.93052	42.844±1.2156	25.714±1.7059
n	0.66433±0.03575	0.7589±0.0387	0.8338±0.0848	0.9801±0.0951
R^2	0.9835	0.97908	0.97682	0.97205
Higuchi Model				
$k(h^{-1/2})$	31.833 ±1.1374	23.481±0.9091	44.053±2.632	33.057±2.6288
R^2	0.91417	0.90133	0.83798	0.78195
Zero-Order				
$K(h)$	22.678 ±0.99069	13.589±0.5163	41.735±1.4048	25.398±0.71471
R^2	0.87233	0.90494	0.94808	0.97179

As discussed above, BVM was released faster than SUR from drug-poloxamer gel as well as from SUM-poloxamer gel. However in the case of the former gels, both drugs showed an anomalous release mechanism (with the process being regulated by both diffusion and polymer chain/aggregate arrangements). The value of n was higher for BVM, which could be indicating that the ordering and alignment of the polymer chain/aggregates is the most relevant process for gels containing BVM, leading to a kinetic mechanism closer to zero order. This was especially noticeable in SUM-poloxamer gels, where $n \approx 1$ (0.98, Table **3**) for BVM release, allowing as to hypothesize that the BVM-SUR (SUM) interactions in poloxamer gel affected the kinetic release as a consequence of an increase in the gel viscosity, which is in agreement with rheology studies discussed above.

Hydrogel Morphology

The samples were analyzed by scanning electron microscopy (SEM) in order to evaluate the porous structure of the hydrogels and to elucidate any possible modifications of this porous morphology, as a consequence of the incorporation of the drugs within the hydrogel.

As shown in Fig. **3**, clear differences existed among the four tested formulations, which indicates that the presence of the drugs clearly exerted an influence on the micro-structure of the hydrogel, in agreement with the rheological and drug delivery behaviors. In fact, $P(407)_{16}$ (without drug) displayed a non-interconnecting porous structure, with two anisotropic axes located perpendicular to each other. This micro-organization contrasts with those observed for $P(407)_{16}$ SUR and $P(407)_{16}$ BVM gels, where only one anisotropic axis was observed. In both of these cases, the pore shape was notably more irregular. However, in the case of $P(407)_{16}$ SUM, no particular pore arrangement was observed. In addition, the pore sizes seemed to be larger.

Inhibition of Corneal Neovascularization in Rats

The methodology used for inducing corneal neovascularization was able to produce an increase in the ANV, as shown in figure 4 and Table **4**. The administration of SUM in solution as well as $P(407)_{16}$-SUM gel was more efficient in reducing ANV in comparison to BVM and control saline solution ($p<0.05$), at 14 days of follow-up. However, in the case of SUM-poloxamer gel, the measured ANV was almost four times smaller than that of the SUM solution treatment ($p=0.01$).

Figure **5** shows the main histopathological findings. Black-ink neovessels were observed in the corneal stroma of control animals, with this group showing evidence of having more infiltrating cells in the cornea and an irregular corneal epithelium. $P(407)_{16}$-SUM treated animals revealed a fairly normal corneal epithelium and a corneal stroma free of neovessels.

DISCUSSION

For thermosensitive hydrogels, it was shown that following a rise in temperature, the polymer chains experienced an increase in the number of interactions due to their increased proximity. This increase in the number of interactions can be interpreted in terms of a corresponding increase in the molecular weight, thus favoring gel formation [30]. Desolvation and a consequent increase in solution entropy appeared to be the driving forces for these phase transitions.

Evidently, the presence of drugs perturbed the polymer-water equilibrium thereby affecting the micellization process, which was reflected in the change of gel temperatures ($T_{sol/gel}$). The influence of additives [31] or drugs [32] on the aggregation behavior of poloxamers has been previously reported. In the present study, a cooperative effect was observed, probably due to the influence of SUM (BVM+SUR) being greater than effects provoked by each drug alone. It can therefore be hypothesized that the presence of these drugs hampers the hydrophilic interactions responsible for the strengthening of the polymeric aggregates. However, specific and more extensive studies should be carried out in order to investigate this issue.

Temperature is a critical issue for any formulations intended for ophthalmic administration. The poloxamer gel, which shows a very low viscosity at low temperatures (< 20º C), gets warmer when administered (i.e. by the effect of eye temperature) which may then suffer a phase transition leading to a viscous gel with increased retention properties. Thus, the addition of any compound (drug and/or additive) has to be taken into account when investigating any possible influences on its rheological behavior.

Another relevant aspect concerns the mechanisms and kinetics of drug release from poloxamer gels. As discussed above, the

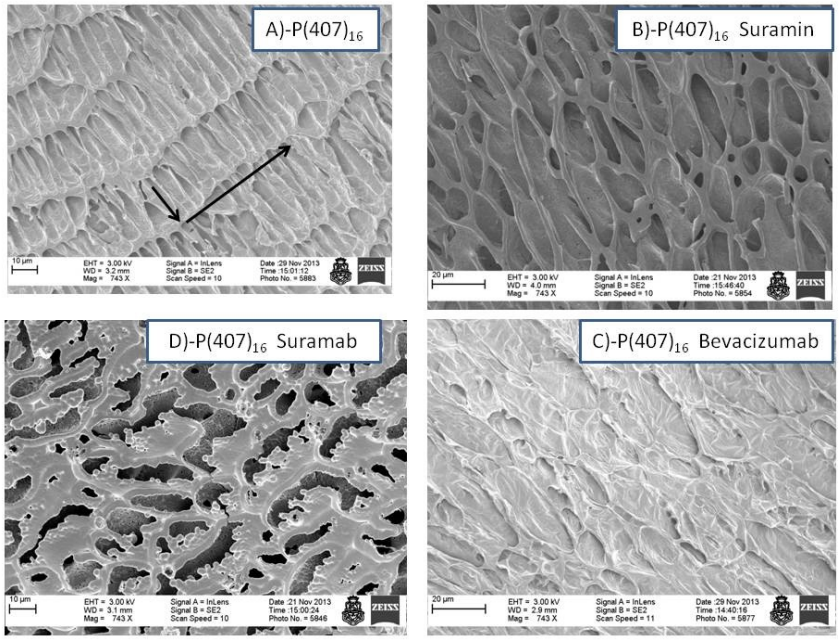

Fig. (3). SEM images showing the morphology of (A) $P(407)_{16}$, (B) $P(407)_{16}$ Suramin, (C) $P(407)_{16}$ Bevacizumab and (D) $P(407)_{16}$ Suramab hydrogel after incubation at 35ºC and freeze drying.

Table 4. The comparison of corneal neovascularization between groups of Suramab, BVM, P(407)16 Suramab and Control animals.

	CONTROL	SUM	$P(407)_{16}$-SUM	BVM
Number of values	5	6	6	6
Mean (pixeles)	14717	2948	760.2	5057
Std. Deviation	1669	1029	798.8	1163
Std. Error	746.4	420.2	326.1	474.7

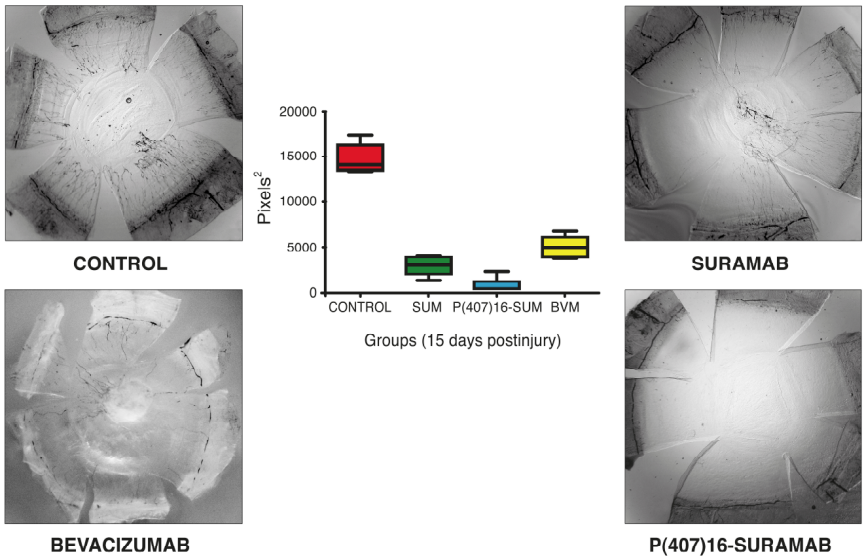

Fig. (4). Area of corneal neovascularization in animal groups.

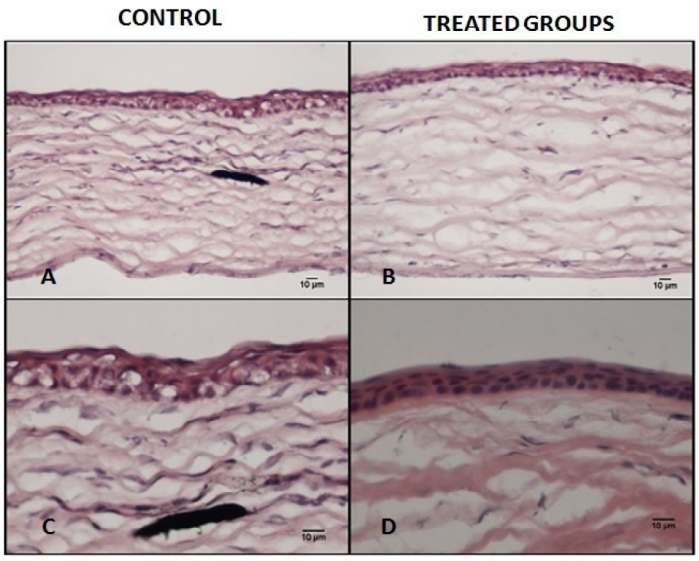

Fig. (5). Clinical evolution correlates with histopathological findings. A and C: control animal with a stromal vessel stained with black ink and irregular corneal epithelium. B and D: $P(407)_{16}$Suramab treated animals with corneal stroma free of neovessels, few infiltrating cells and a well stratified epithelium.

addition of BVM, SUR of SUM affected $T_{sol/gel}$ and the viscosity of the gels, and consequently the way in which the drugs were delivered.

By considering the chemical structures of these drugs, it may be possible to clarify, at least hypothetically, this behavior. BVM is a protein of high molecular weight (approximately 149 kilodaltons) [33] with it being well known, that this kind of protein is very prone to generate intra and intermolecular interactions which are able to define, in most cases, its primary, secondary and tertiary structures. Moreover, these compounds may interact with water molecules surrounding the surfactant aggregates, thus affecting the process of micellization. In contrast, SUR [34] possesses in its structure several strong acidic and amide chemical groups able to interact with the solvent, (water in the present case). In this way, it is highly predictable that these drugs could affect the sol-gel transition of poloxamer, and reciprocally, the drug release may also be influenced by the gel properties.

In vitro experiments showed that BVM and SUR were released faster when incorporated alone in the poloxamer gels than when they were combined as SUM. This reveals that some kinds of interactions exist between them and possibly with the gel structure. This inference is in agreement with microscopic observations, where a noticeable disorder of the gel structure was visualized as a consequence of drug addition.

In general alkali burns cause ingrowth of neovessels in the periphery of rat cornea at day 3 or 4 and they reach the central cornea at day 7 or 8 [12,29]. The entire cornea is usually vascularized at 2 weeks. For this reason most studies on rat corneal neovascularization last 2 weeks. Our investigation followed the same schedule.

With regard to the antiangiogenic effect of $P(407)_{16}$-SUM gels, we observed a significant increase in the efficiency in animal models, compared to the SUM solutions and BVM alone. Based on the properties of the poloxamer-gel analyzed in the earlier section, we attribute this improvement mainly to the ability of the system to maintain its integrity over an extended period of time (high viscosity), while the drugs are released in a sustained way. Furthermore, from a histological point of view, these gels seem to be harmless, according to the preliminary studies presented in this work.

It is interesting to note that $P(407)_{16}$-SUM was sixfold more effective than BVM alone at 2 weeks of follow-up. Although the route of administration was subconjuntival it is highly probable that the difference may persist if the drugs would have been intravitreally administered. The increased efficacy of $P(407)_{16}$-SUM makes it a possible option to lower the frequency of intravitreal injections needed to treat retinal diseases as exudative macular degeneration and diabetic retinopathy [5,6]. Results encourage us to further develop these potential useful systems

CONCLUSION

Thermosensitive poloxamer hydrogels were tested as innovative pharmaceutical dosage forms for the intraocular administration of SUM. The $P(407)_{16}$-SUM hydrogel exhibited a reversible sol−gel transition, with this behavior seemingly influenced by the presence of SUR, BVM and SUM, thereby affecting the $T_{sol/gel}$ values of the dispersions. Aqueous dispersions of poloxamer remained very fluid at low temperatures, which facilitated drug incorporation and syringeability. However, these were able to change into a gel form at a physiological temperature at which SUM might become entrapped inside the matrix of polymeric aggregates, thus exerting a sustained effect on drug release whose rate varied according to the drug. BVM was released faster than SUR, with their combination (SUM) seemingly reducing the delivery, probably to some kinds of interactions between them and/or with the polymeric matrix.

The *in vivo* studies revealed that this formulation strategy might be promising, since $P(407)_{16}$-SUM gel was able to increase the antiangiogenic effect compared to the SUM solution. Moreover, no injurious effects were observed in the histological tissue examination after drug administration. Consequently, this system seems to be an interesting candidate to be evaluated with the aim of reducing the frequency of injections, patient discomfort and the risk for intraocular infections.

LIST OF ABBREVIATIONS

SUR	=	Suramin
BVM	=	Bevacizumab
SUM	=	Suramab
NV	=	neovascularization
ANV	=	area of neovascularization
SEM	=	scanning electron microscopy
VEGF	=	vascular endothelial growth factor
PPO	=	polyoxypropylene

PEO = polyoxyethylene

$P(407)_{16}$ = Poloxamer 407, 16%(w/w)

CONFLICT OF INTEREST

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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