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PLGA Implants containing vancomycin and dexamethasone: development, characterization and bactericidal effects

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Post-operative endophthalmitis is an infection and an inflammation of the eye following a surgical procedure. Its treatment is based on drug injections into the eye. However, this treatment can lead to ocular complications. Intraocular implants could substitute the conventional therapy. Poly(lactic-co-glycolic acid) (PLGA) implants comprising on vancomycin and dexamethasone were evaluated as drug delivery system to treat endophthalmitis after cataract surgery. Implants were characterized by drug content uniformity, Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), Wide Angle X-ray Scattering (WAXS), Scanning Electron Microscopy (SEM) and *in vitro* drug release. The bactericidal effect of vancomycin, eluted from the implants, was demonstrated against *Staphylococcus aureus* and *Staphylococcus epidermidis*. The drugs were uniformly distributed in the polymer. The analytical techniques revealed the chemical integrity of the drugs incorporated into the polymer and the modification of dexamethasone semi-crystalline nature. Drugs were controlled released from implants; and the eluted vancomycin showed bactericidal effects. In conclusion, PLGA implants containing vancomycin and dexamethasone may represent a therapeutic alternative to treat post-operative endophthalmitis.

1. Introduction

Endophthalmitis is an ocular inflammation resulting from the introduction of an infectious agent into the posterior segment of the eye. During infection, damage to delicate photoreceptor cells frequently occurs, leading to irreversible loss of vision (Callegan et al. 2002). Post-operative endophthalmitis is a serious complication following intraocular surgeries, mainly the cataract surgery, which is the most performed type of ocular intervention (Anderson et al. 2009; Kresloff et al. 1998).

The inflammatory response triggered by intraocular pathogens can be acute or chronic. An acute inflammation is associated with more virulent bacteria strains (*Bacillus cereus*, *Enterococcus faecalis*, streptococci, *Staphylococcus aureus* and gram-negative organisms) and the visual prognosis is frequently poor. On the other hand, the chronic inflammation is associated with less virulent bacterial infections (*Proprionibacterium acnes* and *Staphylococcus epidermidis*) and a better visual outcome. However, in both case, the treatment of this infection needs to occur in the early stage to avoid vision loss (Callegan et al. 2007; Mandelbaum et al. 1996).

The treatment of bacterial post-operative endophthalmitis is based on topical, parenteral or intraocular administration of anti-microbial and anti-inflammatory drugs. In the early stage of the infection, the infectious agent is often unknown. As consequence, broad-spectrum antibiotics are generally used, including the combination of vancomycin and amikacin or ceftazidime (Callegan et al. 2007). Therefore, vancomycin is the drug of choice for gram-positive organisms including *Staphylococcus* and methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus* and *Bacillus* species (Han et al. 1996; Callegan et al 2007; Jampol et al. 1988).

The treatment of the inflammatory response caused by the intraocular bacterial infection is typically based on the administration of corticosteroids such as dexamethasone acetate and triamcinolone acetonide (Novosad et al. 2010). Intravitreal injections of these anti-inflammatory drugs allow a direct application of them into the posterior segment of the eye, thereby increasing the bioavailability. However, frequent administration of drugs via this route can lead to various complications, for instance retinal detachment, increased intraocular pressure and intravitreal hemorrhages.

To overcome the intravitreal injections drawbacks, novel implantable ocular drug delivery systems are required. These devices can be inserted into the vitreous cavity and control and prolong the delivery of the drug directly to the target tissue (De Souza et al. 2016). As a consequence, the frequency of intravitreal drug administration is reduced as well as the possibility of developing adverse effects. In this regard, the objective of this study was the design of an implantable intravitreal device, which could provide the controlled release of vancomycin and dexamethasone, capable of treating the bacterial endophthalmitis post-cataract removal.

2. Investigations and results

2.1. Preparation of implants and content of drugs

The implants were prepared by the dispersion of vancomycin and dissolution of dexamethasone acetate and PLGA in acetonitrile, followed by solvent evaporation. The blended powder was molded into cylindrical implants at approximately 70 °C. Before manufacturing the implantable devices, the thermal stability of the vancomycin and dexamethasone was evaluated. The DSC study showed no change on the thermal stability of the drugs at the temperature used to mold the implants.

The resultant monolithic implants showed 8.09±0.71 mg in average weight, 5.69±0.47 mm length, and 1.27±0.10 mm diameter ($n = 10$) (Fig. 1). The vancomycin and dexamethasone content into the PLGA implants was 100 ± 0.05% and 102.4 ± 0.09% (n = 10),

respectively. The obtained result demonstrated the uniform distribution of the drugs in the implants, since none of them was outside the specification (85.0-115.0%) (USP 35) of the pre-indicated amount of vancomycin (2 mg) and dexamethasone (1 mg).

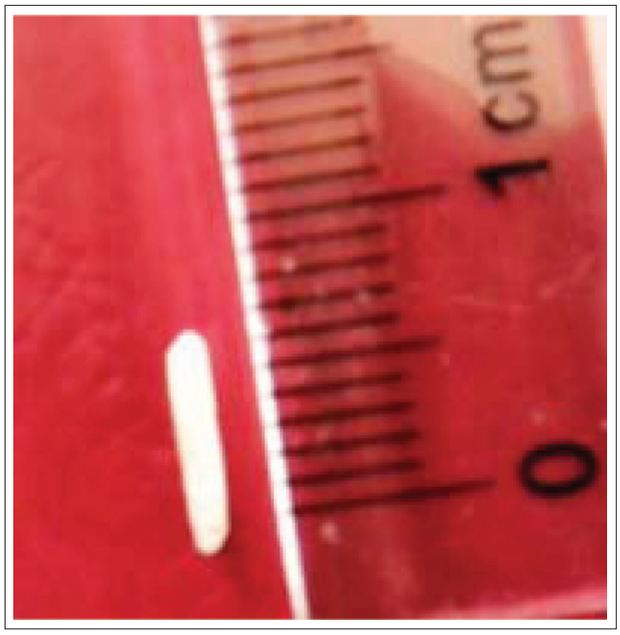

Fig. 1: Macroscopic picture of vancomycin and dexamethasone-loaded PLGA implant (average weight: 8.09±0.71 mg, length: 5.69±0.47 mm, diameter: 1.27±0.10 mm).

2.2. *Characterization*

Figure 2 shows the infrared spectra of the pure vancomycin (Fig. 2a), pure dexamethasone (Fig. 2b), PLGA implants (without drugs) (Fig. 2c) and PLGA implants containing the drugs (Fig. 2d). The infrared spectrum of the pure vancomycin (Fig. 2a) revealed absorption bands at ~3200 cm^{-1} corresponding to phenolic OH stretching; at 1600-1700 cm^{-1} related to aromatic C=O stretching; and at ~1500 cm^{-1} due to C=C stretching vibration. The described FTIR results for pure vancomycin were similar to those previously reported (Giandalia et al. 2001). The FTIR spectra of pure dexamethasone (Fig. 2b) demonstrated characteristic absorption bands at ~1750 cm^{-1} and 1650 cm^{-1} due to C=O vibration associated with aliphatic and ester, respectively; at ~900 cm^{-1} corresponding to axial deformation of C-F group. These absorption bands of dexamethasone were also previously described (Da Silva et al. 2009). Typical infrared absorptions bands observed in PLGA can be detected in spectra of Figs. 2c and 2d, such as at 1725-1750 cm^{-1} due to the C=O stretching vibration of ester groups; and at 1000-1250 cm^{-1} related to the stretching -CO group from esters (Parvin et al. 2013). The infrared spectrum of the PLGA implants containing drugs evidenced the contribution of the organic groups of the polymer and did not demonstrate the bands equivalent to the therapeutic agents. This was attributed to the superposition of the bands from all the implant components. For example, the bands in the range of 1600 and 1750 cm^{-1}, equivalent to the carbonyl group in PLGA, vancomycin and dexamethasone, could overlap. Additionally, the bands at 900 and 1250 cm^{-1} may be representative of the C-F group in dexamethasone and –CO of esters in PLGA. Finally, new bands for drug-loaded PLGA implants were not detected.

Figure 3 shows the WAXS results of pure vancomycin (Fig. 3a), pure dexamethasone (Fig. 3b), PLGA implants (without drugs) (Fig. 3c) and PLGA implants containing drugs (Fig. 3d). The WAXS patterns were characteristic to pure vancomycin (Fig. 3a) indicating the predominant amorphous nature of this drug (Giandalia et al. 2001).

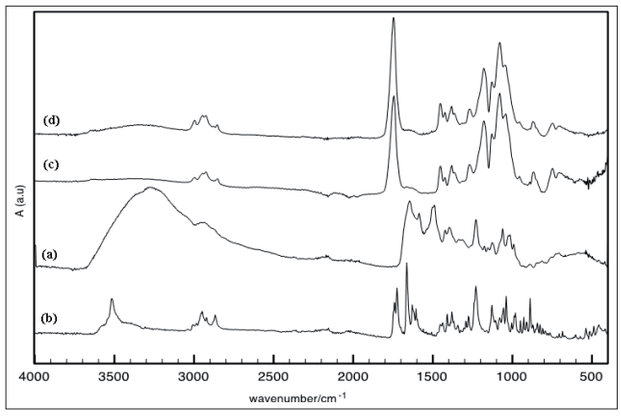

Fig. 2: FTIR spectroscopy of pure vancomycin (a), pure dexamethasone (b), PLGA implants (without drugs) (c) and PLGA implants containing drugs (d).

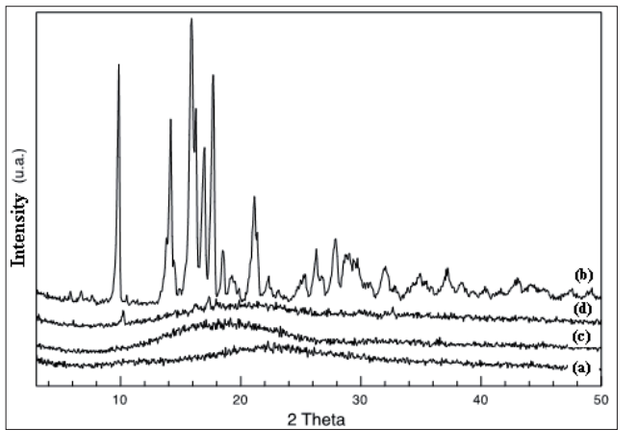

Fig. 3: WAXS patterns for pure vancomycin (a), pure dexamethasone (b), PLGA implants (without drugs) (c) and PLGA implants containing drugs (d).

The PLGA demonstrated also an amorphous structure (Figs. 3c and 3d). The pure dexamethasone showed sharp and the intense peaks (Fig. 3b) indicating its crystalline nature. However, the aforementioned peaks were not visualized in the WAXS pattern of the PLGA implants containing drugs (Fig. 3d).

Figure 4 depicts the DSC thermograms of the pure vancomycin (Fig. 4a), pure dexamethasone (Fig. 4b), PLGA implants (without

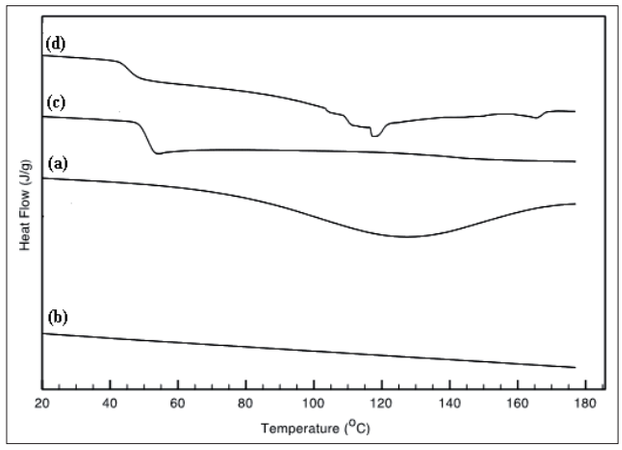

Fig. 4: DSC thermograms of pure vancomycin (a), pure dexamethasone (b), PLGA implants (without drugs) (c) and PLGA implants containing drugs (d).

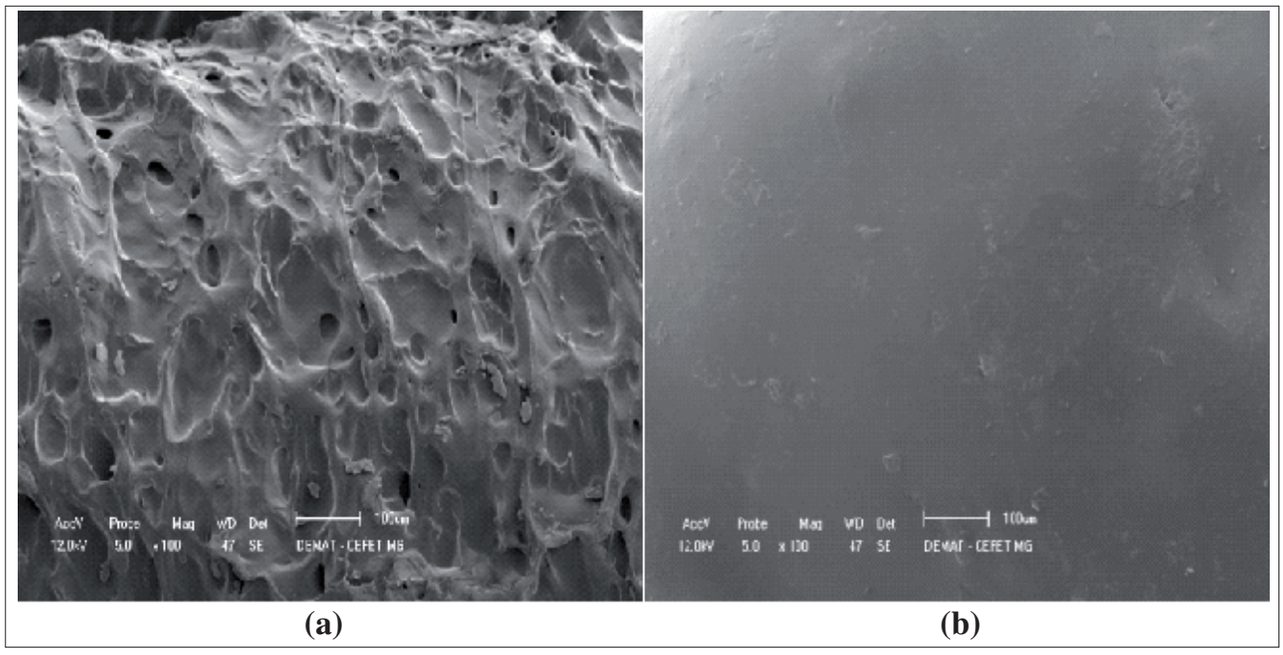

Fig. 5: SEM of the surface of PLGA implants (without drugs). (b) SEM of the surface of PLGA implants containing vancomycin and dexamethasone. ×100 magnification.).

drugs) (Fig. 4c) and PLGA implants containing drugs (Fig. 4d). The DSC thermogram corresponding to the pure vancomycin (Fig. 4a) exhibited only a broad endotherm in the temperature range between 110 and 130 °C due to the drug decomposition. This endothermic event was related to the drug decomposition (DeXia et al. 2013). The DSC curve of the pure dexamethasone (Fig. 4b), shows the absence of endothermic events, once the melting point for this anti-inflammatory drug occurs at about 265 °C (Meyres et al. 2000). The DSC curve for the PLGA implants (without drug) revealed the polymer glass transition temperature from 51 to 60 °C (Fig. 4c). The same endothermic events for pure vancomycin and for PLGA implants (without drug) are presented in the thermogram of PLGA implants containing drugs (Fig. 4d).
Figure 5 shows the SEM images of the surface of the PLGA implants (without drugs) (Fig. 5a) and PLGA implants containing vancomycin and dexamethasone (Fig. 5b). The surface of the polymeric implants without drugs revealed the presence of a porous structure (Fig. 5a). However, the incorporation of drugs into the polymeric matrix induced the formation of a smooth and homogeneous surface, with no evidence of pores or channels (Fig. 5b).

2.3. In vitro release of the drugs from the implants

Figure 6 demonstrates the cumulative release of vancomycin and dexamethasone from the PLGA implants over a period of 90 days. The release profiles of both drugs were similar, and showed a biphasic pattern. The first stage occurred for 15 days for dexamethasone and vancomycin, and approximately 41 % and 30 % of the drugs, respectively, were leached from the polymeric implants. In the second stage, which occurred between 15 and 90 days, the cumulative release of dexamethasone and vancomycin was approximately 88 % and 86 %, respectively.
To investigate the kinetics of drug release from the PLGA implant, two theoretical models describing drug release from polymeric systems were considered: Higuchi and Korsmeyer-Peppas models. To calculate the kinetic parameters of both models, the first 60% drug release data were used, since only this portion of the release curve should be used to determine the parameter *n* of the Korsmeyer-Peppas model (Costa and Lobo 2001; Liu et al. 2009). The determination coefficient (r^2) and kinetic parameters of each model are listed in the Table. The best fit for the vancomycin and dexamethasone release profiles of the loaded implant was obtained using the Korsmeyer-Peppas model. This model showed a higher coefficient of determination (r^2) compared to Higuchi. The magnitude of the release exponent *n* in the Korsmeyer-Peppas model indicated that the mechanism in which the drugs were released was an anomalous transport, indicated bey the *n* value (0.45-0.81) (Siepmann et al. 2008; Liu et al. 2009).

Table: Fitting results of the experimental vancomycin and dexamethasone release data from PLGA implants to different kinetic equations

		Vancomycin	Dexamethasone
Higuchi	***K***	0.0795	0.0085
	r^2	0.9892	0.9251
Korsmeyer-Peppas	***K***	0.0352	0.0420
	r^2	0.9982	0.9916
	n	0.68	0.64

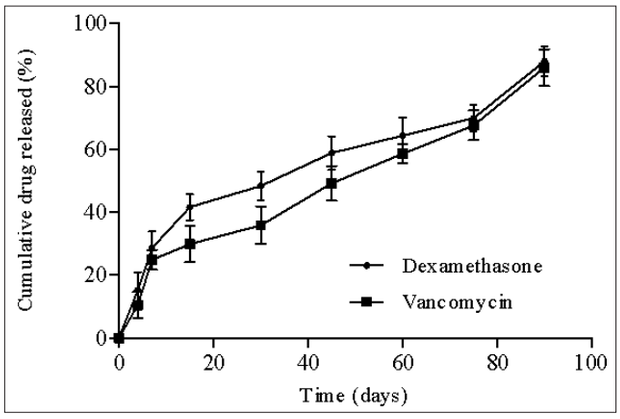

Fig. 6: In vitro cumulative release profiles of vancomycin and dexamethasone (%) from PLGA implants. Results represented mean ± standard deviation (n = 5).

2.4. *Determination of the bactericidal effect of the vancomycin released from PLGA implants*

The amount of vancomycin released from the PLGA implants was approximately 31 µg in 24 hours, which was capable of preventing the bacterial growth in the agar diffusion method (Fig. 7). The diameter of the inhibition zone of inhibition induced by the vancomycin leached from the implantable device and the pure drug was 21 mm and 18 mm, respectively, for *S. aureus*, and 26 mm and 25 mm, respectively, for *S. epidermidis*, after 24 hours of incubation.

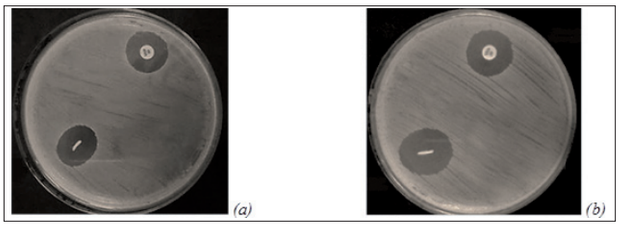

Fig. 7: Agar diffusion method – Zone of inhibition provided by the vancomycin leached from PLGA implants and discs containing 30 µg of the vancomycin: (a) S. aureus (b) S. epidermidis.

Figure 8 demonstrates the bactericidal effect of the vancomycin controlled released from the PLGA implants and pure vancomycin against *S. aureus* and *S. epidermidis* in liquid culture medium. The pure drug and the drug released from the polymeric implants reduced bacterial count when compared to the positive controls. The bactericidal effect of the vancomycin eluted from the PLGA implants was remarkable, since after 6 and 12 hours of incubation, growth was seduced by two and three log cycles, respectively, and after 24 hours, *S. aureus* was completely eliminated. The bactericidal effect of pure vancomycin against the bacteria was faster compared to the delivered drug. The statistical analysis (Student´s t test) showed that this difference was significant ($p < 0.05$).

The bactericidal effect of the pure vancomycin and the drug leached from the PLGA implants was also evaluated against *S. epidermidis*. It was verified that the released antibiotic reduced the bacterial count at two and three log cycles at 6 and 24 hours, respectively, and induced complete regression of the microorganisms after 36 hours of incubation. The bactericidal effect of the pure vancomycin against the *S. epidermidis* was faster than that of the drug delivered from the implantable system. The statistical analysis (Student´s t test) showed that this difference was significant ($p < 0.05$).

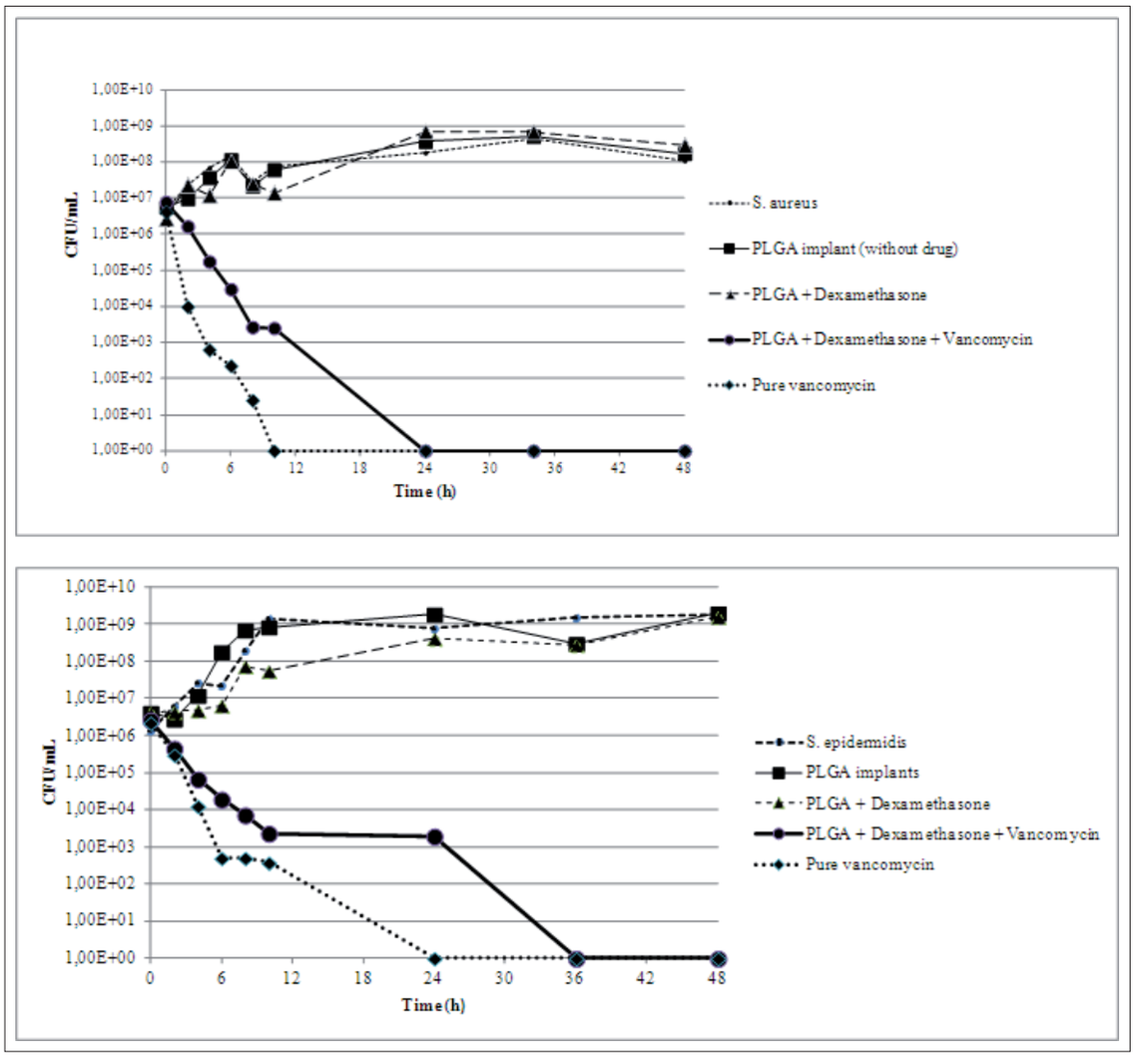

Fig. 8: Bacterial growth curves without vancomycin (positive control) (a); in contact with PLGA implants (without drugs) (b); in contact with PLGA implants containing dexamethasone (d); in contact with the vancomycin released from the PLGA implants (e); with pure vancomycin in solution. The first set of curves represents the bactericidal effect against S. aureus. The second set of curves represents the bactericidal effect against S. epidermidis. A mean of 3 experiments for each period of incubation was expressed in CFU mL-1. Standard errors were always below 0.35.

The Minimum Inhibitory Concentration (MIC) was 10 µg mL^{-1} and 0.6 µg mL^{-1} for *S. epidermidis* and *S. aureus*, respectively, which represented the lowest antibiotic concentration allowing no visible growth of bacteria.

3. Discussion

Severe endophthalmitis after cataract removal is a concern because it can result in significant vision loss and/or blindness. The treatment of bacterial endophthalmitis is based on topic and intraocular injections. The ocular injections need to be repeated every 3 days up to 2 weeks (Hachicha et al. 2007). This frequency can induced degeneration of the posterior ocular tissues.

In this study, a novel innovative intravitreal PLGA implants composed of vancomycin and dexamethasone were developed and evaluated as an alternative formulation to the conventional intraocular pharmaceutical dosage forms. This implantable device was developed by incorporating vancomycin into a solution of dexamethasone and polymer, followed by lyophilization method. The lyophilized sample was then hot molded and subsequent casting with appropriate heat, to obtain cylindrical implants. This method was able to provide systems with uniform drug content. PLGA was selected as polymeric matrix of the implantable device, since it is recognizably biocompatible and biodegradable, and its degradation products are natural metabolites. Additionally, this polymer is capable of entrapping the drugs into its polymeric chain, providing controlled and sustained release of them at therapeutic levels. Many implants composed on PLGA and a wide variety of drugs for treatment of ocular diseases have been investigated, considering the unique and attractive properties of this polymer (Thrimawithana et al. 2011).

During implant manufacture, the molding temperature was set at approximately 70 °C. This temperature was higher than the polymers' glass transition temperature; but low enough to avoid the degradation of the vancomycin and dexamethasone, as detected by DSC results. The fabrication process of the implants evolved the following steps (Hachicha et al. 2007): (1) the fusion of the polymeric particles began at their points of contact around the antibiotic and steroid particles, forming a three-dimensional network; (2) collapse of the networks in the voids spaces between the polymer and drugs, wich was induced by the progression of the fusion of the polymer. As the result of these manufacture phases, vancomycin and dexamethasone were incorporated into the PLGA matrix. The SEM images of the PLGA implants loaded with vancomycin and dexamethasone demonstrated clearly the filling of the polymer empty spaces by the added drugs, creating a monolith system.

The developed implantable devices were characterized using different analytical techniques to verify chemical-physical integrity of drugs in the implant. FTIR spectra and DSC thermograms indicated the absence of chemical interactions among drugs and polymer, thereby suggesting the preservation of their chemical structures. The maintenance of the chemical integrity of the vancomycin and dexamethasone is essential to ensure their therapeutic activities. The WAXS patterns demonstrated that the dexamethasone changed its crystalline state. This result was possibly caused by the dispersion of the drug into the polymeric matrix of the PLGA. Moreover, the random entanglement of polymeric chains with the drug as well as the lyophilization process prevented its re-crystallization (Elmostasem et al. 2008, Fernandes-Chunha et al. 2016).

The PLGA implants were capable of controlling the release of the incorporated drugs for a prolonged period. An initial burst release of the drugs was verified probably because of the diffusion of the drugs on the implant surface. This low portion of the drug particles did not permeate through the polymer during the manufacture process. In a second stage, the water penetrated into the polymeric chains, it was capable of diluting the vancomycin and dexamethasone incorporated into the polymeric matrix, and the solubilized drugs formed channels, which connected the interior to the surface of the implants. In a third stage, the solubilized drug mixture could also take up water, inducing the increase of the osmotic pressure through the PLGA. This promoted the breaking of the polymer and the fast drug delivery. Additionally, the polymer decreased its molecular weight, thereby increasing the release of the drugs. The drug release profile corroborated with those previously reported in the literature (Hachicha et al. 2007; Dunne et al. 1990). In these studies, it was demonstrated that the release mechanism of drugs from hydrophobic polylactide matrixes is controlled by channel diffusion, osmotic pressure and polymer degradation. Finally, the release of drugs from PLGA implants based on diffusion and erosion mechanisms complied with the Korsmeyer-Peppas model, considering the anomalous transport.

The bactericidal effect of the vancomycin released from PLGA implants was demonstrated using the agar diffusion method and the time-kill curve studies. In the agar method, the drug inhibited the bacteria growth; and the diameter of the inhibition zone generated by the leached vancomycin from PLGA implants and pure drug was superior to 14 mm. According to the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2015), a diameter of the inhibition zone larger than 14 mm for *Staphylococcus* species demonstrates bacterial sensibility to the vancomycin. Moreover, the concentration of vancomycin eluted from the implants (approximately 31.2 µg) was greater than the MIC of *S. aureus* and *S. epidermidis*, since 100% of the microorganisms were eliminated, and they were maintained for the follow up period. The prolonged release of vancomycin in therapeutic concentrations, higher than MIC, represented a satisfactory experimental result. It was previously reported that sub-inhibitory intraocular concentrations of vancomycin, within a short period of just 4 h, could potentially enhance the biofilm matrix produced by coagulase-negative Staphylococci on the synthetic artificial intraocular lens surface. Once a bacterial biofilm has formed on the surface of the artificial lens, the staphylococci anchors firmly in this biomaterial, and it becomes protected from the antibiotic agents (Callegan et al. 2007; Dunne et al. 1990).

According to Jones et al. (2002), the bactericidal kinetics of the evaluated substance occurs when the initial *inoculum* are reduced to an equal or superior values of two log in a period equal or inferior to 24 hours of incubation. In this study, after just 6 h of incubation, the vancomycin leached from the systems reduced two log cycles of the microorganisms. The total elimination occurred at 24 h for *S. aureus* and at 36 h for *S. epidermidis*. Therefore, the vancomycin incorporated into implants had a bactericidal effect in a short period of time, which is essential to eliminate the infection in the posterior segment of the eye, and consequently, to reduce or avoid the irreversible damage to the retinal layers.

Despite the efficient bactericidal effect of the vancomycin leached from the PLGA implants, the time-kill curve studies demonstrated that the bactericidal effect of pure vancomycin was faster compared to the vancomycin released from implants. However, the experiment was performed in static conditions, which provided information of microorganism inhibition that were in direct contact with the fixed concentration of the antibiotic drug, for a pre-defined period, in a constant volume of medium. However, it is important to emphasize that the use of static bacterial time-kill curves offered detailed information about the antibacterial efficacy of the vancomycin inserted into the PLGA implants as a function of both time and antibiotic concentration (Liu et al. 2005).

On the other hand, under dynamic conditions, the time-kill curves would probably demonstrate that the greater bactericidal effect of the pure vancomycin would be lower than the effect generated by the vancomycin leached from implantable devices. Under these conditions, clearance of the drug occurs, and consequently, the initial concentration of the antibiotic would not be constant for the pre-defined period of experimentation (Liu et al. 2005). Therefore, for the pure vancomycin in contact with the microorganisms, the initial concentration would be lower through time, and this reduction could lead to an insufficient amount of the antibiotic drug, which would prevent the permanent elimination of *S. aureus* and *S. epidermidis*. By contrast, the PLGA implants provided the controlled and sustained release of vancomycin for 90 days, in concentrations capable of eliminating these microorganisms. The possibility of delivering the antibiotic drug for a prolonged period could guarantee

the bactericidal effect for several days, resulting in the complete remission of the infection.

In this study, it was not delay of action of the vancomycin due to the necessity of accumulation of effective drug concentrations released from the PLGA implantable devices. Experiments with microparticles based on vancomycin demonstrated also the effectiveness of these drug delivery systems against *S. epidermidis*; but with a delay of action of approximately 6 h, resulting from the initial rise time needed for vancomycin concentration to reach its minimum efficient value (Dunne et al. 1990). Considering the rapid progressive nature of bacterial endophthalmitis, effective therapy needs to be expediently applied to the patient due to the devastating consequences for the infected eye. Thus, the polymeric implants, developed in this study, could release vancomycin immediately after its intravitreal insertion, and within only 2 h of drug eluting, a significant bactericidal effect could be verified. These implantable devices may offer the advantages of providing the treatment of the bacterial endophthalmitis post-cataract removal during its early stage, and of maintaining the progressive release of the antibiotic and anti-inflammatory drugs, which would result in a longer and efficient therapy.

Finally, implantable devices have been designed to treat different intraocular diseases. Among them, Ozurdex®, a biodegradable implant containing dexamethasone, has been commercialized for the treatment of macular edema following branch retinal vein occlusion, central retinal vein occlusion or noninfectious uveitis affecting the posterior segment of the eye. Retisert®, a non-biodegradable implantable device based on fluocinolone acetonide was recently introduced in the market for the treatment of noninfectious uveitis (Bochot and Fattal 2012). Besides the existence of intravitreal implants to treat inflammatory auto-immune diseases, there are no drug delivery systems capable of treating post-operative bacterial endophthalmitis. Considering the necessity of arresting the endophthalmitis by providing rapid sterilization of the posterior segment of the eye and inhibition of the harmful inflammatory process, the polymeric implant containing antibiotic and anti-inflammatory drugs represents a promising alternative to successfully combat this blinding disease. As the PLGA implants composed on vancomycin and dexamethasone developed in this work presented satisfactory *in vitro* results; other experiments will be further performed to investigate not only the *in vivo* drug release and the toxicological profile and the efficacy in an animal endophthalmitis model. In this regard, many questions related to the performance of these implantable devices will be elucidated in order to consider their clinical application in the management of the post-operative endophthalmitis followed by the cataract removal, and to supplement their commercially absence.

In conclusion, vancomycin and dexamethasone-loaded PLGA implants were developed using the melting molding technique. The anti-inflammatory drug dispersed as an amorphous structure into the polymeric chains. Moreover, polymer and drugs apparently did not interact chemically. Drugs were controlled eluted from the PLGA implants for a prolonged period, demonstrating a biphasic pattern. The vancomycin, eluted from polymeric implants, had activity against *S. aureus* and *S. epidermidis*, which are important strains evolved in the endophthalmitis after cataract removal. Finally, the PLGA implants composed of vancomycin and dexamethasone could represent a promising therapeutic alternative to be applied into the intravitreal compartment to arrest infection and inflammation in cases of bacterial endophthalmitis after cataract surgery.

4. Experimental

4.1. Materials

Dexamethasone acetate reference standard was purchased from Sigma Aldrich (99% of purity). Vancomycin hydrochloride was kindly donated by ABL Antibióticos do Brasil (Brazil). Polymer poly(lactic-co-glycolic acid) 75:25 (PLGA 75:25; inherent viscosity (i.v.) = 0.50 - 0.70 dl g^{-1}; Resomer® RG 755 S) was obtained from Boehringer Ingelheim Pharma GmbH & Co; Ingelheim, Germany. Ultrapure water was produced by a Milli-Q® purification system (Millipore, USA). Acetonitrile and tetrahydrofuran HPLC grade were purchased from Merck® (Brazil). The other solvents and reagents used were of analytical grade. Filters of 0.22 μm diameter were purchased from Millipore (USA).

4.2. Preparation of the implants

Firstly, the vancomycin hydrochloride was dissolved in water at a concentration of 70 mg/mL followed by increasing pH to 8–8.2 (where vancomycin has no net charge and the lowest solubility) by adding an appropriate amount of sodium hydroxide (NaOH) solution (3 mol L^{-1}). After incubating for 60 min, the precipitated vancomycin free base was centrifuged, washed with10 mL of 70%(v/v) ethanol for 3 times and methanol for 2 times, separately, then dispersed in 10 mL of ultrapure water and lyophilized overnight (Li et al. 2010).

Dexamethasone and PLGA were dissolved in 2 mL of acetonitrile. Vancomycin free base was homogeneously dispersed to this solution. The solution was placed in a freezer under -80 ºC for 24 h. The mixture was lyophilized during 48 h. Then the mixture equivalent to one implant was weighed and was placed under a metallic mold, which was in direct contact with the heating plate. The blended power was heated at approximately 70 ºC for 5 min; and it was manually molded using a spatula into a cylindrical implant. Implants contained approximately 2 mg of vancomycin free base [25 %(w/w)], 1 mg of dexamethasone [12.5 %(w/w)]and 5 mg of PLGA were designed. Implants without drug were also prepared (PLGA implants).

4.3. Determination of the content of drugs incorporated into implants

The determination of vancomycin and dexamethasone into the PLGA implants was performed as follows: ten implants were selected and weighed. Each implant was dissolved in 5 mL of acetonitrile, since the polymer was soluble in this organic solvent. Additionally, the acetonitrile solubilized the dexamethasone. Afterwards, 10 mL of 0.1 mol L^{-1} hydrochloric acid (HCl) was added to dissolve the vancomycin. The resultant solution was quantitatively transferred to a volumetric flask (25 mL) and the volume was completed with 0.1 mol/L HCl. The solution was filtered through a 0.22 μm filter. The amount of vancomycin and dexamethasone present in each PLGA implant (mg) was determined by a HPLC method previously developed and validated. The method was carried out in isocratic mode using aC18 column (250 mm×4.6 mm) at 25 °C; acetonitrile and water (55:45, v/v) as mobile phase, 1 mL min^{-1} of flow rate, ultraviolet detection at 254 and at 280 nm for dexamethasone and vancomycin, respectively, 10 μL of injection volume. The method was linear ($r^2 > 0.99$), precise (RSD < 5%) and accurate (recovery ranged from 98.0 to 102.0%) for both drugs. The amount of vancomycin and dexamethasone in each implant was calculated and the results were expressed as the average percent of the pre-indicated value (2 mg for vancomycin and 1 mg for dexamethasone). The specification was 85.0 to 115.0% of the pre-indicated value, according to the American Pharmacopoeia (USP 35). The relative standard deviation was also calculated.

4.4. Characterization

4.4.1. Fourier Transform Infrared Spectroscopy

Infrared spectra were collected in a Fourier transform infrared spectrophotometer (FTIR; Perkin Elmer, model Spectrum 1000). Measurements were carried out using the attenuated total reflectance (ATR) technique. Each spectrum was a result of 32 scans with a resolution of 4 cm^{-1}.

4.4.2. Wide Angle X-ray Scattering

Wide angle x-ray scattering (WAXS) was performed in a Philips PW 3710 diffractometer with a copper target (λ= 1.54 Å) and Ni filters. Scans were performed from 2θ= 3.50 at rates of 0.01° min^{-1}.

4.4.3. Differential scanning calorimetry

Differential scanning calorimetric (DSC) thermograms were obtained on a Mettler Toledo DSC (Switzerland). Samples were put into aluminium pans. The calorimeter was calibrated for temperature and heat flow accuracy using pure indium melting (m.p. 156.6 °C and ΔH = 25.45 J g^{-1}). The temperature ranged from 20-180 °C with a heating rate of 20 °C min^{-1} under nitrogen atmosphere.

4.4.4. Scanning electron microscopy

Scanning electron microscopy (SEM) was performed using a JEOL microscope (model JSM – 6360LV) operating at 15 kV. The implants were fractured and mounted on aluminium stubs using double-sided adhesive tape. Prior to microscopical examination, all the samples were sputter-coated with a gold layer under argon atmosphere using a sputter apparatus (Balzers Union SCD 040 unit, Balzers, Germany). The implants surfaces were viewed at 100× magnification and the images were transferred to the computer by means of a Digital Image Transference Interface (DITI). The photomicrographs were adjusted using the software Adobe Photoshop 6.0 and Adobe Illustrator 9.01 (Adobe Systems Incorporated, 2000, USA).

4.5. In vitro release of drugs from the implants

The United States Pharmacopeia states in the dissolution procedure: development and validation that ''sink conditions are defined as the volume of medium at least three times that required in order to forma saturated solution of drug substance. When sink conditions are present, it is more likely that dissolution results will reflect the properties of the dosage form'' (USP 35). The *in vitro* release study was carried out under sink conditions during 90 days. Assuming that the aqueous solubility of the vancomycin and dexamethasone acetate is about 0.1 mg/mL and 20 mg/mL, at 37 °C, respectively (Li et al. 2010; Einmahl et al. 2001), sink conditions are achieved with 30 mL at least for each implant. Five implants were immersed inside different tubes containing 30 mL of PBS. The tubes were placed inside a shaker incubator set at 37 °C

and 30 rpm. At predetermined intervals (0, 4, 7, 15, 30, 45, 60, 75 and 90 days), 30 mL of the medium was sampled and 30 mL of fresh medium was immediately added to each tube. The release profile was evaluated as the cumulative percentage of the drugs released in the medium. The amount of leached vancomycin and dexamethasone was measured using the HPLC method developed and validated, as previously described.

4.6. Mechanism of drug release

Various mathematical models to describe the mechanisms of drug release from polymeric systems have been reported in the literature (Costa and Lobo 2001; Siepmann et al. 2008). The release data were evaluated by model-dependent (curve fitting) and two theoretical models were used: Higuchi and Korsmeyer-Peppas models.

Higuchi describes drug release as a diffusion process based on Fick's law, according to Eq. (1).

$Mt / M\infty = KH\ t^{1/2}$ (1)

where $Mt/M\infty$ is the fractional drug release at time t and KH is Higuchi's dissolution constant. According to this model, a straight line is expected for the plot of $Mt / M\infty$ versus the square root of time if the drug release from the matrix is based on a diffusion mechanism.

The Korsmeyer-Peppas model considers that the drug release mechanism may deviate from Fick's law and follow an anomalous behavior, described by Eq. (2).

$Mt / M\infty = Ktn$ (2)

where $Mt / M\infty$ is the fractional drug release at time t, K is the kinetic constant, and n is the diffusional exponent characteristic of the release mechanism. A Fickian diffusion (Case I) is observed if $n \leq 0.45$. A non-Fickian diffusion (anomalous transport) is verified if $0.45 \leq n < 0.89$. If $n \geq 0.89$, a Case II transport drug release mechanism dominates (polymer swelling) (Saliba et al. 2012).

To calculate the kinetic parameters of both models, the first 60% drug release data were used since only this portion of the release curve should be used for the determination of parameter n of the Korsmeyer-Peppas model (Costa and Lobo 2001). The determination coefficient (r^2) was used to define the best fit between the two models. Thus, the model that provided r^2 closest to 1 was considered more adequate.

4.7. Preparation of the inoculum

Staphylococcus aureus ATCC 29213 and *Staphylococcus epidermidis* ATCC 12228 growth in agar nutrient at 37 °C for 24 h were collected and transferred to saline solution. The bacterial concentration was adjusted to 1.5 x 10^8 cells mL^{-1} using turbidity similar to 0.5 from the MacFarland scale.

4.8. Determination of the bactericidal effect of the vancomycin released from PLGA implants

4.8.1. Agar diffusion method

The implants were sterilized using ultraviolet light for 20 min on each side prior to cell culture. The microrganisms' solutions (1.5 x 10^8 cells mL^{-1}) were inoculated onto three plates containing Muller-Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2015). The plates of *S. aureus and S.epidermidis* received (1) the vancomycin-loaded PLGA implants and (2) the sterile discs incorporated into 30 µg of vancomycin. The plates were incubated for 24 h at 37 °C. Afterwards, the bacterial inhibition zones were measured and the analysis of the sensibility of the microorganisms was evaluated using the CLSI (CLSI, 2015).

4.8.2. Minimum Inhibitory Concentration (MIC)

The MIC was determined using the microdilution test according to The European Committee on Antimicrobial Susceptibility Test (EUCAST). The saline solution of 1.0 x 10^8 cells mL^{-1} was diluted in Muller-Hinton broth to yield a suspension of 5.0 x 10^6 cells mL^{-1}. The 96-well microtiter plate received (1) 225 µL of the *S. aureus* suspension and (2) 25 µL of pure vancomycin in the concentrations of 0.3, 0.6, 1.2 and 2.4 µg mL^{-1}. Additionally, the 96-well microtiter plate received (1) 225 µL of the *S. epidermidis* suspension and (2) 25 µL of pure vancomycin in the concentrations of 5, 10, 20 e 30 µg mL^{-1}. The microtiter plate were sealed and incubated for 24 h at 37 °C. The MIC was considered the lowest vancomycin concentration which prevented the turbidity.

4.8.3. Time-Kill curve studies

The diluted bacterial suspension (1 x 10^6 cells mL^{-1}) was inoculated in 10 mL of Mueller-Hinton broth. The bacteria were incubated for 48 h at 37 °C in direct contact with the following constituents: (1) vancomycin and dexamethasone-loaded PLGA implants; (2) solution of pure vancomycin at 20 µg mL^{-1}; (3) PLGA implants containing dexamethasone (positive control); (4) PLGA implants without drugs (positive control). The diluted bacterial suspension (1 x 10^6 cells mL^{-1}) was also inoculated as positive control, and the Muller Hilton medium alone was classified as sterile control. To obtain bacterial-killing curves, samples were collected at fixed intervals (0, 2, 4, 6, 8, 10, 24, 34 and 48 h) and plated in Mueller-Hinton medium. The plates were incubated at 37 °C for 24 h and colony counting was performed. This procedure was based upon three replicate determinations.

4.9. Statistical analysis

The standard formula for bacterial reduction assays was used to calculate the antibiotic effect. The formula consists of comparing the number of CFU mL^{-1} in the control with the number of CFUmL^{-1} obtained in the presence of an antibiotic [(control - antibiotic)/control x 100]. Data were tested for normality and investigated for statistical significance using the Student´s t test ($p < 0.05$).

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