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RESEARCH PAPER

Overcoming Glucocorticoid Resistances and Improving Antitumor Therapies: Lipid and Polymers Carriers

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ABSTRACT

Purpose To improve chemotherapy protocols of lymphoid malignancies, by using polymeric and lipid microparticles as controlled delivery systems of dexamethasone, part of all combined chemotherapy protocols for its strong-inducing effect on malignant lymphoblasts.

Methods Polymeric microparticles were prepared by the oil-in-water-emulsion cosolvent evaporation method, andlipid microparticles by spray drying. Their cytotoxic effects on GC-sensitive PC12 cells and GC-resistant PC3 cells were characterized by cell proliferation and apoptosis assays.

Results Both elaboration methods rendered optimal-sized microparticles for parenteral administration with high drug loading. *In vitro* assays showed sustained dexamethasone release from polymeric microparticles over a month, whereas 100% dexamethasone release from lipid microparticles was achieved within 24 h. Similar PC12 cell death to that obtained with dexamethasone solution administered every 48 h was achieved with dexamethasone polymeric microparticles in 26-days assays. Dexamethasone solution and loaded polymeric microparticles induced apoptosis around 15.8 and 19.9%, respectively, after 2 days of incubation. Lipid microparticles increased further apoptosis induction in PC12 cells and, unlike dexamethasone solution and polymeric microparticles, showed antiproliferative effects on PC3 cells.

Conclusions Dexamethasone polymeric microparticles constitute an alternative to current dexamethasone administration systems in combined chemotherapy, whereas dexamethasone lipid microparticles represent a potential tool to revert glucocorticoid resistance.

KEY WORDS apoptosis · cytotoxicity · dexamethasone · lipid microparticles · polymeric microparticles

ABBREVIATIONS

ABC	ATP-binding cassette
ALL	Acute lymphoblastic leukemia
ATCC	American type culture collection
DCM	Dichloromethane
Dex	Dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxidel
DSC	Differential scanning calorimetry
FACS	Fluorescence-activated cell sorting
GCs	Glucocorticoids
GR	Glucocorticoids receptor
HPLC	High performance liquid chromatography
MM	Multiple myeloma

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mTOR	Mammalian target of the rapamicin
MTT	Bromide (3-[4, 5-dimethyltiazol-2- yl]-2, 5-diphenyl)
OD	Optical density
PAO	Phenylarsine oxide
PBS	Phosphate buffer solution
PI	Polydispersity index
PpI	Propidium iodide
PLGA	Poly(lactic and glycolic) acid
PVA	Polyvinyl alcohol
RPMI	Oswell park memorial institute medium
SD_{mean}	Standard deviation of the mean diameter values
SEM	Scanning electron microscopy

INTRODUCTION

Glucocorticoids (GCs) are used in therapeutics as anti-inflammatory and immunosuppressive agents. Thanks to their ability to induce cell cycle arrest and cell death, these agents have a central role in the treatment of lymphoid malignancies, particularly acute lymphoblastic leukemia (ALL) and multiple myeloma (MM). Indeed, dexamethasone (dex), a potent synthetic glucocorticoid, is a part of all combined chemotherapy protocols, for its strong apoptosis-inducing effect on malignant lymphoblasts (1), with an important synergic effect in combination with drugs such as bortezomib, rituximab or lenalidomide. In addition to their cytotoxic effect on hematologic malignancies, preclinical studies have shown that GCs also act on cell differentiation, proliferation and apoptosis of osteosarcoma cells (2), hepatoma cells, mammary tumor cells, glioma cells, melanoma cells and thyroid cancer cells (3). In spite of the high effectiveness of dex treatment in ALL, mainly in children, GC resistance occurs in 10–30% of untreated patients, being more frequent in T-lineage than B-precursor acute lymphoblastic leukemia. Furthermore, systemic administration of high doses of dex is required for inducing tumor cell apoptosis but causes severe side effects such as osteoporosis, Cushing's syndrome or an increased risk of infections (4). Even though most combined chemotherapy protocols currently used in clinics include dex at high doses, recent clinical investigations on the efficacy of dex in refractory multiple myeloma have shown synergic effects of low doses of this GC combined with pomalidomide or lenalidomide (5,6).

The apoptotic effect of GCs is cell type-specific and time- and concentration-dependent (7). GCs mediate most of their effects via their receptor (GR), a ligand-activated transcription factor. The GR is expressed in almost every cell in the body and regulates genes controlling development, metabolism, and the immune response. The unbound receptor is complexed with a variety of proteins in the cytoplasm of cells. Upon GC binding, proteins are released and the GR migrates to the nucleus where it is involved in the regulation of gene transcription by two main mechanisms: trans-activation and trans-repression (8). Trans-activation occurs by direct binding of GR dimmers to Glucocorticoid Response Elements around the target genes and generally results in transcription enhancement . Activated GR can also complex with other transcription factors preventing them from binding their target genes and, hence, repressing the expression of genes that are normally up-regulated. This indirect mechanism of action is referred to as trans-repression.

It seems that GC-induced apoptosis critically depends on sufficient levels of GR and a subsequent alteration in gene expression. However, the precise nature of the GC-regulated genes responsible for the apoptotic effects remains elusive (9), although the changes induced in mitochondrial membrane properties seem to also play an important role in GC induced apoptosis (10,11) Mechanisms underlying the development of GC resistance are also poorly understood and likely vary with disease type, treatment regimen and genetic background of the patient. Resistance to GC therapy may occur if inactive GR isoforms are present, if the members of the ABC-transporter family are over-expressed or if the apoptotic pathway is inhibited (12). An increasing numbers of reports indicate that activation of the mammalian target of the rapamicin (mTOR) signaling pathway may contribute to GC resistance in hematological malignancies (1).

In conclusion, the complex pattern of GC-induced cell death warrants further investigation, and more efforts must be made to improve therapy protocols and overcome GC resistance.

The aim of this work was to improve chemotherapy protocols with dex by developing controlled drug delivery systems, and to evaluate the influence of these systems on dex effectiveness as an apoptotic drug and their ability to overcome GC resistance for chemotherapy purposes. Thus, we have developed two types of microparticles, polymeric and lipid, for parenteral administration of dex.

Microparticles could improve the dosage of dex, reducing the number of administrations and improving patient compliance. But more importantly, microparticles could also improve treatment effectiveness and reduce GC side effects by providing a suitable pharmacokinetic drug release profile (13). Indeed, these systems could allow an extended drug release (for weeks or months after an unique administration) while degrading (14), providing controlled constant blood levels of drug and avoiding the pharmacokinetic peak trough fluctuations characteristic of conventional formulations. With this in mind, polymeric and lipid microparticles were elaborated. These microparticles were designed thinking on their subcutaneous, intramuscular or even intratumoral administration; and thus biocompatible and biodegradable materials were used for their elaboration. The microparticles present the advantage, with regard to other controlled release parenteral systems, of their easy administration by a conventional needle.

The most of the parenteral microparticles approved to date are elaborated from polymers and provide a prolonged (but not sustained) release of the drug encapsulated enough to maintain its pharmacological effect during months. Nevertheless, in the last years the investigations on lipid carriesrs have increased, because of their high biocompatibility and the no use of solvents highly toxic in their elaboration. In fact, even though some polymer materials, such as polyglycolic acid, poly(lactic-co-glycolic) acid (PLGA), and polyalkylcyanoacrylate, are approved for parenteral use and considered safe, cytotoxicity studies have evidenced lower toxicity of phospholipids and other lipid materials used in the development of micro and nanoparticles por parenteral administration (15).

Polymeric and lipid microparticles were characterized in terms of size, dex loading and encapsulation efficacy, drug disposition into microparticles and drug-excipient compatibility. *In vitro* dex release from both types of carriers was determined and compared. Next, the cytotoxic effects and apoptotic activity of dex released from both types of microparticles were tested using a GC-sensitive cell line, comparing the results obtained after a single administration of the microparticles with those obtained with dex solution administration every 48 h. Finally, the potential of both types of carriers to overcome GC cell resistance was also investigated.

MATERIALS AND METHODS

Materials

Dexamethasone 21-phosphate disodium, polyvinyl alcohol (PVA, Mw = 30,000–70,000), albumin from chicken egg, Thiazolyl Blue Tetrazolium Bromide and propidium iodide were obtained from Sigma- Aldrich (Madrid, Spain). PLGA Resomer RG502 (Mw = 12,000) and RG504H (Mw = 48,000) were provided by Boehringer Ingelheim (Barcelona, Spain), Dichloromethane (DCM) (HPLC grade), acetonitrile, potassium dihydrogen phosphate, disodium hydrogen phosphate dehydrate, methanol, acetic glacial acid, ethanol, sodium chloride, lactose, mannitol and trehalose were provided by Panreac (Barcelona, Spain). Lipoid E80 (egg phospholipids with 80% phosphatidylcholine) was a generous gift from Lipoid GmbH (Switzerland). Distilled demineralised Milli-Q® water (Millipore, Madrid, Spain) was used. PC12 and PC3 cell lines obtained from American Type Culture Collection (ATCC). PC12 cell line was maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal bovine serum, 7.5% horse serum, 1% L-glutamine and 0.1% penicillin-streptomycin; and PC3 cell line was maintained as a monolayer in Roswell Park Memorial Institute medium (RPMI) 1649 supplemented with 10% fetal bovine serum and penicillin-streptomycin-amphotericin B 25 mg/mL. Both media were provided by Life Technologies (Madrid, Spain). Cells were incubated at 37°C in a humidified chamber in an atmosphere containing 5% CO_2.

Methods

Microparticle Preparation

Polymeric microparticles were prepared by the oil-in-water (O/W) emulsion-cosolvent evaporation method using PLGA 50:50 copolymers (16,17). Briefly, the organic phase consists of 500 mg polymer and 0.5 ml of dex solution in methanol (100 mg/ml), dissolved in 5 ml of a volatile organic solvent. The organic phase was emulsified by stirring in an aqueous phase with PVA and NaCl. Later, vigorous stirring was maintained at 500 rpm for 2.5 h in order to allow suitable evaporation of the organic solvent and hardening of the microparticles. The microparticles were collected by filtration (through a 5 μm Millipore® SMWP membrane filter) and washed with distilled water. Finally, the microparticles were freeze-dried at 200 mT (Flexi-Dry MP, FTS® Systems, NY, USA) and were stored at 4°C. Different experimental conditions were evaluated, and the details of each condition are summarized in Table I.

Lipid microparticles were obtained by spray drying (18,19). A spray drier (B-191, BüCHI) equipped with a 0.7 mm diameter fluid nozzle was used. The spray drying parameters were fixed at an air-flow rate of 800 L/h, feed-flow rate of 50%, inlet temperature of 110°C, outlet temperature of 50–55°C and aspiration at 100%. Lipoid® and dex were dissolved in ethanol, and ovoalbumin and lactose in water. Aqueous and ethanolic solutions were then mixed (30/70, v/v), and the mixture was maintained under moderate stirring while fed into the spray dryer. The total amount of solids used was 2 g/l, of which 10% were dex and 90% were excipients. The powder directly obtained from the cyclonic separator was stored at 4–8°C. Different batches of microparticles were prepared from 400 ml of solution (80 mg of dex and 720 mg of excipients). To optimize the process, different proportions of Lipoid® (from 40 to 80% of the total excipient) and different sugar types were employed, maintaining ovoalbumin and sugar at the same ratio.

The yield of the whole microencapsulation process was calculated as a percentage by dividing the amount of powder collected by the initial amount of solids in the solution.

Morphology and Size Characterization

The surface and shape of the microparticles were examined by scanning electron microscopy (SEM) (Jeol-JSM-6400

Table I Experimental Conditions Evaluated in the Optimisation of the Elaboration Procedure of Polymeric Microparticles

	Organic phase		Aqueous phase		Stirring rate
	Organic solvent	Polymer	Volume	Composition	
Protocol 1	4.5 ml DCM	RG502	100 ml	1% PVA, 1 N NaCl	800 rpm
Protocol 2	3 ml DCM	RG502	100 ml	1% PVA, 1 N NaCl	800 rpm
Protocol 3	3 ml Chloroform	RG502	100 ml	1% PVA, 1 N NaCl	800 rpm
Protocol 4	3 ml DCM	RG502	50 ml	1% PVA, 1 N NaCl	800 rpm
Protocol 5	3 ml DCM	RG502	100 ml	0.5% PVA, 0.5 N NaCl	800 rpm
Protocol 6	3 ml DCM	RG502	100 ml	0.25% PVA, 0.25 N NaCl	1,200 rpm
Protocol 7	3 ml DCM	RG504H	100 ml	0.25% PVA, 0.25 N NaCl	1,200 rpm

Electron Microscope Tokyo, Japan). The samples were deposited on aluminum tubs with a carbon tape and were coated with gold under vacuum (Emitech K550X, Eitech Ltd., UK). The particle size of the microparticles was measured by laser diffraction (Microtrac® SRA 150 Particle Size Analyzer, Leeds & Northrup Instruments, Ireland), after dispersing the particles in water. The volume mean diameter, the polydispersity index (PI) and the span were calculated. Each sample was measured in triplicate.

Drug Disposition Into Microparticles and Compatibility

The drug-excipients compatibility, as well as the physical state of the drug within the microparticle, was evaluated by differential scanning calorimetry (DSC). DSC scans were carried out using a Mettler-Toledo DSC820 with a Huber TC100 intracooler (Madrid, Spain) calibrated with indium. Samples were heated in pin-holed sealed aluminum pans under an inert nitrogen atmosphere at a flow rate of 70 mL/min. With polymeric microparticles, thermal events were measured in the second heating cycle of the following heat-cool-heat loop: the sample was heated from 20 to 80°C, held isothermally for 2 min, quenched from 80 to 20°C, held isothermally for 2 min and heated from 20 to 280°C. The rate of heating and quenching was 10°C/min. Lipid microparticle DSC runs were conducted from 0 to 280°C at a rate of 5°C/min. Samples of pure drug, raw excipients, drug: excipients physical mixtures, unloaded and loaded microparticles were analyzed in triplicate.

Drug Loading and Encapsulation Efficiency

High performance liquid chromatography (HPLC) was used for the quantification of dex inside the microparticles. The HPLC system consisted of a quaternary pump, an automatic injector, an ultraviolet/visible detector, a vacuum degasser and an oven, all from 1200 series Agilent Technologies (Madrid, Spain). The method, adapted from that of Thote *et al.* (20) was previously validated (21). The mobile phase was a mixture of PBS 0.05 M (pH 7.4):acetonitrile:glacial acetic acid (70:26:4 v/v, pH=4±0.2), and a flow rate of 1 mL/min was fixed. Samples of 20 µL were injected and detected at 244 nm. The analytical column was a Tracer Excel 120 ODSB 5 µm, 15×0.46 cm (Teknokroma, Barcelona, Spain). The retention time of dex was 5 min.

To determine the amount of dex encapsulated in the polymeric microspheres, 15 mg of microparticles were dissolved in 2 mL of DCM. The mixture was vigorously stirred until the complete breaking of the microparticles. Then, 18 ml of water at pH 4 (adjusted with acetic acid) were added. The partition coefficient DCM:water pH=4 was previously determined, obtaining a value of 0.201 (at pH 4 dex phosphate is speciated essentially as a monoanion). The sample was stirred in a vortex stirrer every 5 min for 35 min in order to completely extract the drug (21). Later, the aqueous phase was diluted and analyzed by HPLC. In the case of lipid microparticles, 5 mg of microparticles were dissolved in 2 ml of ethanol. After sample sonication for 1 min, it was centrifuged at 3,100 g for 5 min to eliminate the excipients that were not soluble in ethanol. Then, 500 µl of the supernatant was diluted in 4.5 ml of methanol (1:10) and analyzed by HPLC. The amount of dex was determined using a calibration curve constructed over the range of 5–50 µg/ml (r=0.99). All measurements were conducted in triplicate.

The dex content in the microparticles was expressed as mg of dex in 100 mg of microparticles. The entrapment efficiency (EE) was calculated using Eq. (1).

$$EE(\%) = \frac{actual\ Dex : Polymer\ ratio}{initia;\ Dex : Polymer\ ratio} \cdot 100$$

In Vitro Dex Release From Microparticles

In vitro release of dex from microparticles was carried out under sink conditions. For this, the solubility of dex phosphate disodium in PBS was previously determined by the shaking flask method, obtaining a value of 120 mg/mL. The same

protocol was used to evaluate dex *in vitro* release, from polymeric and lipid microparticles. 5 mg of microparticles were weighed, suspended in 10 ml of PBS pH 7.4 in closed vials, and immersed in a thermostatic shaking water bath (Clifton® NE5-28, United Kingdom) at 37°C with continuous agitation (50 strokes/min). At specific time intervals, 8 mL of the release medium were withdrawn, taking care to avoid the withdrawal of microparticles. The medium withdrawn was filtered through 0.45 μm nylon filters (Teknokroma®, Barcelona, Spain) and analyzed for dex content by HPLC (as described above). The amount of dex released was determined using a calibration curve constructed over the range of 0.25–10 μg/ml (r=0.99). The withdrawn volume was immediately replaced with an equal volume of fresh and prewarmed medium. The renovation of the release medium guaranteed the maintenance of sink conditions throughout the assay. The amount of drug withdrawn at each sampling time was taken into account to calculate drug release from microparticles. *Cell proliferation assay*GC-sensitive and GC-resistant cell lines were used.

PC12 cells are derived from a pheochromocytoma of the rat adrenal medulla and can be differentiated into a neuronal phenotype by stimulation with nerve growth factor. Functional GR expression in PC12 cells has been reported (22,23). Because GCs up-regulate catecholamine-synthesizing enzymes and storage proteins (24,25), dex has been extensively used to improve catecholamine secretion in PC12 cells (26). The concentrations employed range from 1 to 5 μM (27,28) with different time exposures, but no cytotoxic effects were reported for dex. PC12 cells were used as GC-sensitive cell line.

On the contrary, PC3 cell lines were established from bone metastasis of grade IV of prostate cancer. These cells do not respond to androgens, glucocorticoids, or epidermal or fibroblast growth factors (29,30), and were selected as GC-resistant cell line.

PC12 or PC3 cells were seeded in 48-well plates and treated with different concentrations of dex in deionized water (3–1,000 μM) for 1, 2 and 3 days to determine the cytotoxic effect of the dex solution. The cytotoxic effect of dex released from the polymeric microspheres cannot be determined directly by adding dex-loaded microspheres to the cell culture since dex takes longer than cell cultures' maximum half-life (of approximately 6–7 days) to be completely released from polymeric microparticles (30 days). To solve this problem, parallel *in vitro* release assays were carried out, as previously described, employing sterile PBS pH 7.4 as the release medium, which was withdrawn and replaced by fresh medium at predetermined time intervals to maintain sink conditions during all of the assays. When target days were reached, the release medium was withdrawn and replaced with a fresh culture medium, adding this suspension of microparticles in culture medium to the cell culture. Once the microparticles were added, the culture was maintained for 6 days, and the resulting effects on cell proliferation were compared with that obtained with a daily dex solution administration during the same time interval.

As dex release from lipid microparticles occurred within 2–3 days, the cell proliferation assay was carried out directly by adding dex lipid microparticles to the cell culture and comparing the results obtained after 3 days of incubation with those obtained after daily administration of dex solution.

To assess the mitochondrial function of cells, bromide (3-[4, 5-dimethyltiazol-2- yl]-2, 5-diphenyl) (MTT) reagent was added to each well for 1 h at 37°C, and formazan crystals were formed; then, dimethylsulfoxide (DMSO) was added to dissolve the crystals. After resuspending the content of the wells, the optical density (OD) was read using an Elisa reader at 550 nm (Berthold Detection Systems, Sirius). Phenylarsine oxide (PAO) at 10 μM was used as a positive control for cell death. Cell viability, expressed as a percentage of the control, was calculated with the following equation: viability=(OD test/OD control) ×100. Each result described is based on 6 to 12 different determinations.

Apoptosis Assay

PC12 or PC3 cells were seeded in 6-well plates. After 24 h, cells were treated with 10 μM of dex, with the polymeric or lipid microparticles, or with 1 μM staurosporine as a positive apoptosis control for different time periods. Treated cells were fixed in 70% ethanol and DNA was stained with propidium iodide (PpI) at a final concentration of 20 mg/ml. The number of apoptotic cells (DNA content<2n) was determined by flow cytometry (FC500 MLP, Beckman Coulter). The percentage of the apoptotic cells in the sub-G1 fraction was determined using CXP analysis software. Each result described was based on 6 to 12 different determinations (31).

Statistical Analysis

Statistical analysis of data was made by Statgraphics® and GraphPad Prism v. 5.0 assuming sample homogeneity. Different formulations and different treatments were compared by ANOVA tests. Differences in p values below 0.05 were considered statistically significant. Mean values±standard deviations are shown.

RESULTS

Development of Polymeric Microparticles

Polymeric Microparticle Preparation and Characterization

The microencapsulation procedure developed was reproducible; with process yields for the different protocols tested above 82%, except in protocol 3, where the yield was lower (Table II). In most cases, the resulting microparticles were spherical, individualized, nonporous, and uniform, with a smooth surface without hollows or deformations (Fig. 1). The particle size was in the range from 60 to 140 μm, being the lowest mean diameter (59.69 μm) achieved when using protocol 6. As regards drug loading, low values were obtained when using protocol 3 (1.53 mg dex/100 mg MPs), whereas microparticles elaborated with protocol 6, showed the highest one (7.30 mg dex/100 mg MPs) (Table II).

Drug Disposition Into the Microparticles and Compatibility

DSC thermograms of pure dex, raw R502 polymer, a mixture of RG502:dex and microparticles are shown in Fig. 2. The DSC trace of pure dex showed two peaks near 100°C, which correspond to linked water molecules and free water molecules, respectively. A sharp and large exothermic peak was also observed at 228.05°C, which corresponds to the drug melting point with an enthalpy of fusion of $\Delta Hm = -77.8$ J/g. Raw polymer RG502 showed an endothermic peak at 46.17°C with heat of fusion of 4.32 J/g. The thermograms of the physical mixtures at actual proportions showed two peaks, which correspond to polymer RG502 and dex whereas the thermogram of dex microparticles, the peak corresponding to the melting of dex was not detected in DSC thermogram.

In Vitro Release of Dex From Polymeric Microparticles

As depicted in Fig. 3, dex release from polymeric microparticles showed a multi-stage release behaviour. A low burst effect in the first 24 h (with less than 10% of the drug released) was detected. Next, a rapid release stage was observed, showing 70% of the drug released by day 14. After that, a slower release stage was noticed, in such a way that after 28 days, 81% of the drug was released. The maximum drug release was reached after 90 days with a total of 92% of the drug released.

Development of Lipid Microparticles

Lipid Microparticle Preparation and Characterization

Microparticles were obtained directly as a powdered product. The microencapsulation procedure developed was reproducible, with microencapsulation yields around 40%.

Different lipid microparticle batches were elaborated using four different Lipoid® proportions (40, 60, 70 and 80%). With 40, 60 and 70% of Lipoid® (batches 1, 2 and 3), a powdered free-flow product was directly obtained from the spray drier, but when the Lipoid® proportion increased up to 80% the flowability of the obtained product decreased significantly, as deduced by its trend to stick on the walls of the cyclone and collector recipient of the equipment. When the size of batches 1, 2 and 3 were determined by laser diffraction, mean diameters ranging from 18 to 31 μm and narrow unimodal size distributions were obtained (Table III and Fig. 4e). These results do not correspond with the images obtained by SEM (Fig. 4a, b, c, d). Indeed, high aggregation was observed in all cases, although in most batches microparticles did not lose their spherical shape, and individual microparticles could be distinguished. In addition, an increase in Lipoid® proportion led to higher aggregation. No significant

Table II Microparticle Characteristics Obtained From the Different Protocols of Table I (SD_{mean}: Standard Deviation of the Mean Diameter Values; PI: Polydispersity Index)

	Yield (%)	Mean diameter ± SD_{Mean} (μm)	PI	Span	Drug loading (mg dex/ 100 mg MPs)	Encapsulation efficiency (%)
Protocol 1	90.06	66.06 ± 14.23	0.23	2.51	2.98 ± 0.20	30.62 ± 2.11
Protocol 2	92.12	73.31 ± 19.41	0.18	3.97	2.89 ± 0.18	29.79 ± 1.91
Protocol 3	63.23	83.59 ± 9.18	0.34	1.10	1.53 ± 0.10	15.48 ± 1.05
Protocol 4	81.88	81.34 ± 10.51	0.21	2.03	3.25 ± 0.24	33.48 ± 2.51
Protocol 5	95.68	92.81 ± 15.87	0.20	1.18	5.79 ± 0.29	61.51 ± 3.18
Protocol 6	97.08	59.69 ± 8.09	0.14	1.46	7.30 ± 0.26	78.84 ± 2.86
Protocol 7	93.05	135.26 ± 24.35	0.15	2.20	4.63 ± 0.29	48.19 ± 3.09

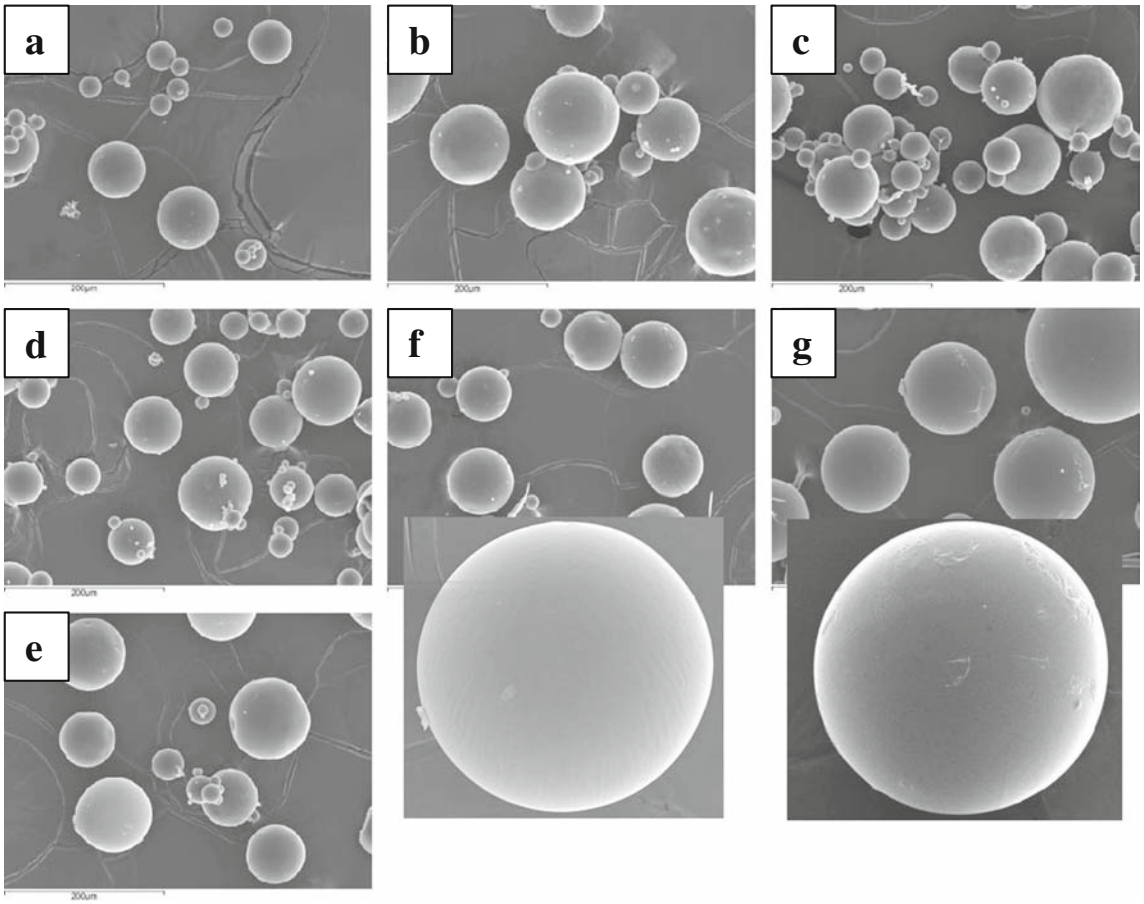

Fig. 1 SEM images of polymeric microparticles obtained under different experimental conditions (*scale bar* = 200 μm). Microparticles obtained in protocol 1 (**a**); protocol 2 (**b**); protocol 3 (**c**); protocol 4 (**d**); protocol 5 (**e**); protocol 6 (**f**); and protocol 7 (**g**). Representative microphotographs of the seven types of polymeric microparticles are shown.

differences in particle size (p=0.097), drug loading (p= 0.061 and encapsulation efficiency (p=0.062) were observed when lactose was replaced by mannitol or trehalose. Microparticles with different aggregation states, but maintaining their morphology and individual shape, were

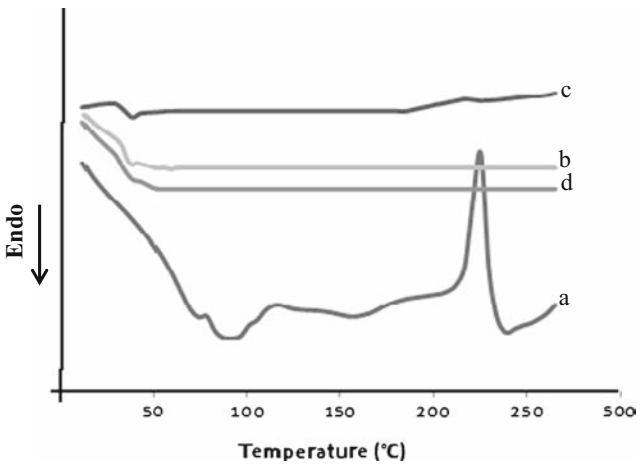

Fig. 2 DSC thermograms obtained upon heating from 20 to 280°C at 10°C/min of pure dex (**a**), pure polymer RG502 (**b**), physical mixture (**c**) and protocol 6 microparticles (**d**).

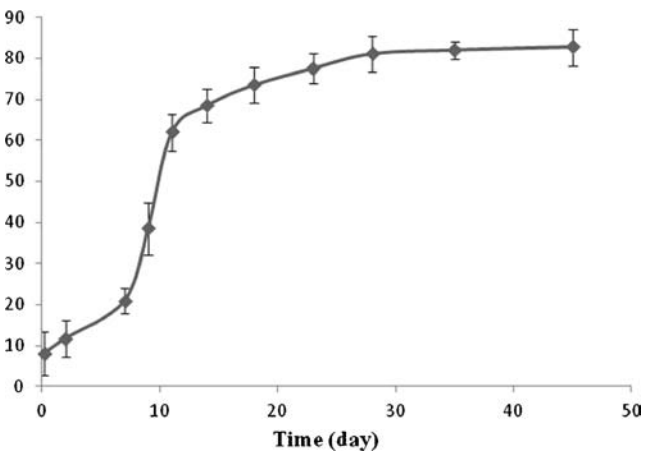

Fig. 3 Release profile of dex from polymeric microparticles obtained from protocol 6. For the *in vitro* release studies, microspheres were incubated in PBS (pH 7.4) and maintained in a shaking incubator at 37°C. At predetermined time intervals, supernatant was withdrawn and the medium was replaced. The concentration of dex in the release medium was quantified by HPLC. Data correspond to the cumulative amount of drug released at the indicated time points, and are expressed as mean percentage of dex released relative to the total amount of dex in microparticles (n = 3).

Table III Characteristics of the Different Batches of Lipid Microparticles (SD_{mean}: Standard Deviation of the Mean Diameter Values; PI: Polydispersity Index)

	% Lipoid	Sugar	Mean diameter ± SD_{mean} (μm)	IP	Span	Drug loading (mg dex/100 mg MP)	Encapsulation efficiency (%)
Batch 1	40	Lactose	29.8 ± 6.87	0.13	0.98	8.13 ± 0.22	79.63 ± 2.21
Batch 2	60	Lactose	27.93 ± 4.42	0.12	1.39	9.5 ± 0.41	94.32 ± 4.11
Batch 3	70	Lactose	19.48 ± 4.44	0.16	1.58	8.74 ± 0.21	86.27 ± 2.06
Batch 4	80	Lactose	30.42 ± 8.68	0.38	2.10	9.21 ± 0.37	91.34 ± 3.69
Batch 5	70	Mannitol	23.15 ± 2.74	0.23	1.52	9.06 ± 0.20	87.79 ± 1.96
Batch 6	70	Trehalose	25.36 ± 3.36	0.23	1.46	9.23 ± 0.49	89.61 ± 3.79

always observed. The lowest aggregation was observed when trehalose was used. For this reason it was batch 6, with 70% lipoid and trehalose, the one selected for the following studies.

The microencapsulation yield was in all cases around 40%, decreasing significantly in batch 4 where microsphere aggregation was higher.

Drug Disposition Into the Lipid Microparticles and Compatibility

DSC analysis of lipid microparticles was complex due to the high number of excipients that were used in the production procedure. Thermograms for all the pure excipients, all the physical mixtures (1:1) of all the components, and all the microparticles were analysed (data not shown). Lipoid DSC exhibited a single endothermic event at $T_{onset} = 38.5°C$ with an enthalpy (ΔH) of 18.99 J/g, Incompatibilities between components in the physical mixtures were not detected. In DSC thermograms of the microparticles, it is difficult to characterise the individual excipient peaks due to their low concentration and to the overlap of different peaks at the same temperatures.

In Vitro Release of Dex From Microparticles

As shown in Fig. 5A and B, dex release profiles obtained from the lipid microparticles were significantly different from the polymeric ones, because the release was much faster, reaching 100% of drug released after 24 h of assay. Figure 5A shows the different release profiles at different Lipoid® proportions. These profiles show that the higher the Lipoid® proportion the better the control of drug release. The drug was completely released in an immediate way from 40 to 60% Lipoid® microspheres, however, drug release was slightly slower with the 70% lipoid microparticles (although complete drug release was achieved within 24 h). Comparing the different 70% Lipoid® microparticle batches made with different sugars (Fig. 5B), differences in kinetic profiles were not detected.

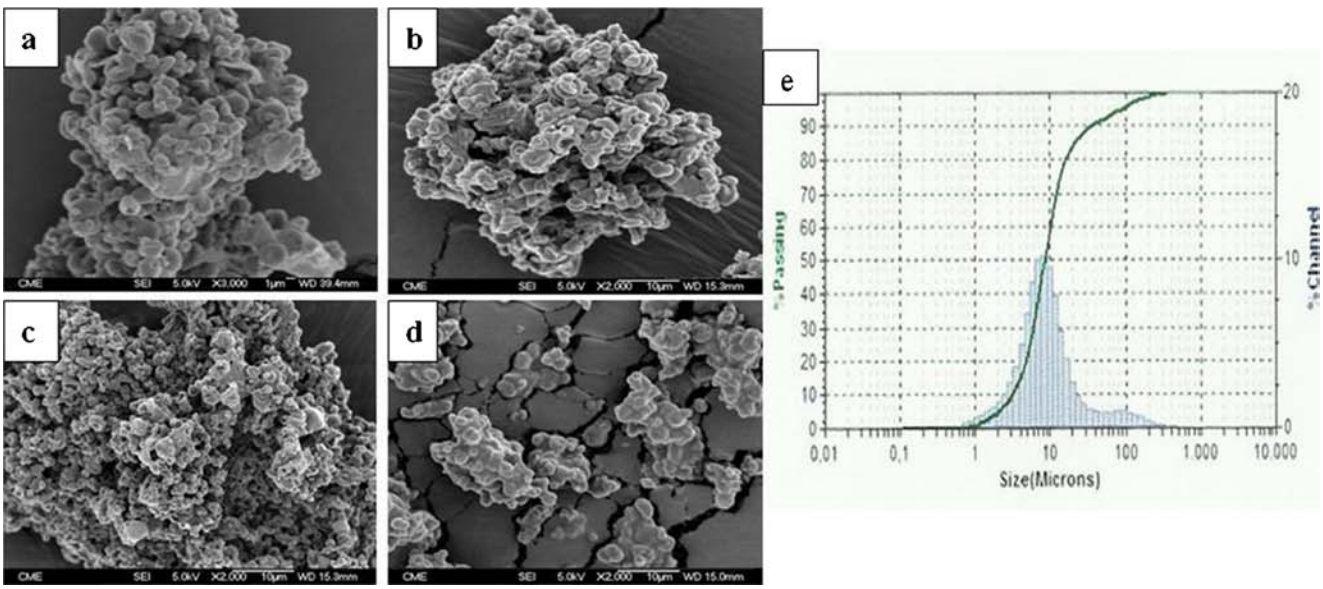

Fig. 4 SEM images of lipid microparticles with different lipoid proportions: (**a**) 40% lipoid, (**b**) 60% lipoid, (**c**) 70% lipoid and (**d**) 80% lipoid. Representative microphotographs of the four types of microparticles are shown. (**e**) shows the particle size distribution of batch 6 microparticles. Results correspond to percentage volume diameter distribution.

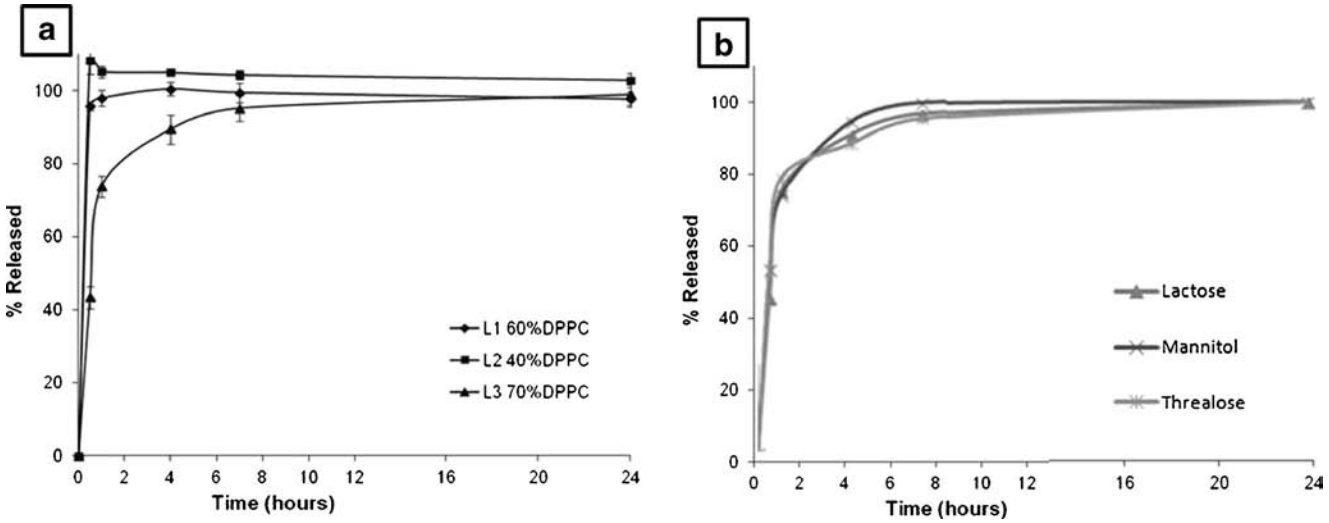

Fig. 5 (**a**) Release profiles of dex from lipid microparticles with different lipoid proportions. (**b**) Release profiles of dex from lipid microparticles obtained with different sugars. For the *in vitro* release studies, microspheres were incubated in PBS (pH 7.4) and maintained in a shaking incubator at 37°C. At predetermined time intervals, supernatant was withdrawn and the medium was replaced. The concentration of dex in the release medium was quantified by HPLC. Data correspond to the cumulative amount of drug released at the indicated time points, and are expressed as mean percentage of dex released relative to the total amount of dex in microparticles ($n = 3$).

Cell Proliferation Assay Dex Solutions

To establish the cytotoxic concentration of dex, a dose-response curve was performed by treating the cells with dex at 3, 10, 100 and 1,000 μM, using deionized water as solvent. In addition, cytotoxic time dependence was established by incubating for 1, 2 and 3 days. When PC3 cells were used, differences in cell viability between dex solutions and control were not detected. Nevertheless, PC12 cells were shown as GC- sensitives. As depicted in Fig. 6A, when 10 μM dex was used the viability was reduced by approximately 30% between days 1 and 3. However, after 3 days of incubation with 100

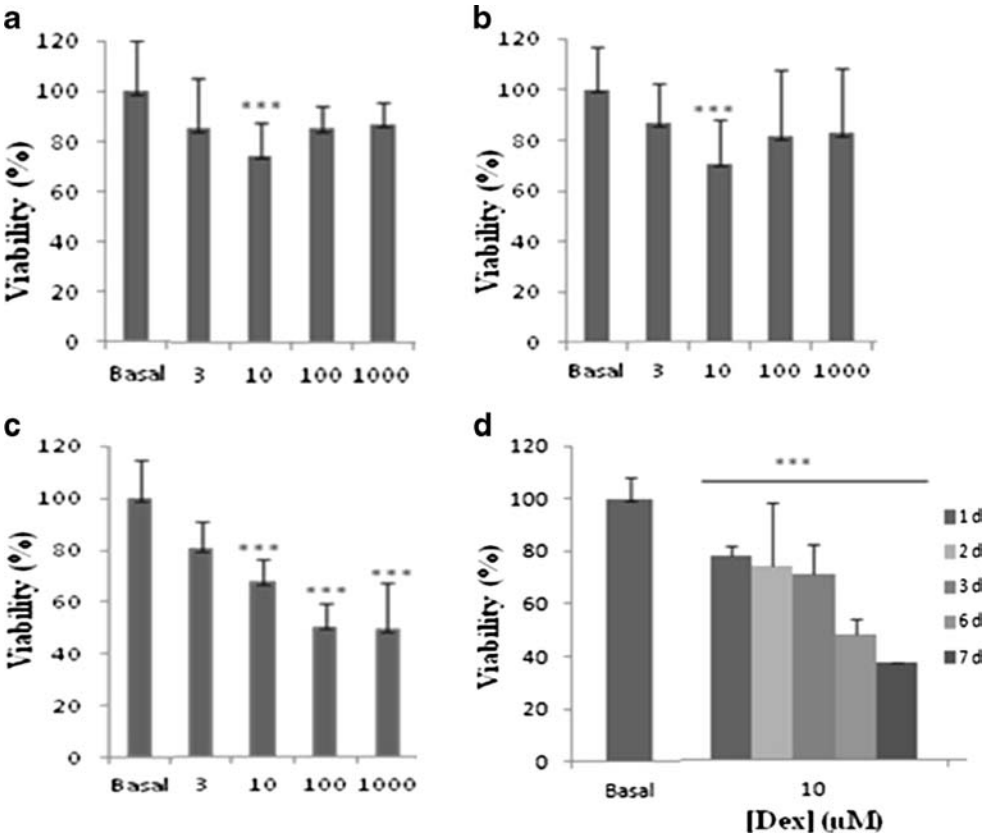

Fig. 6 Concentration and time dependence of the cytotoxic effects of Dex. Panel A, B and C shows the cytotoxic effect of increasing concentrations of Dex, measured as percentage cell viability, in cells exposed for 1 day (**a**), 2 days (**b**) and 3 days (**c**) to dex solution. Panel D shows the cytotoxic effect of 10 μM of Dex, measured as percentage cell viability, in cell exposed from 1 to 7 days. Data correspond to the means ± standard deviations of 6–12 wells for four different batches of cells. *** $P < 0.05$ with respect to control cell viability (ANOVA).

and 1,000 μM, approximately 50% of cell death was induced. In order to establish longer incubation periods, we used a concentration of dex at 10 μM since 50% of the cell death elicited by larger concentrations of dex could result in higher cytotoxic effects from longer exposure times.

Once 10 μM was set as the working concentration, cell proliferation assays were carried out with longer exposition periods. A time-response correlation was established in such a way that after 7 days of incubation, the cell viability was reduced by 35% (Fig. 6B). Dex solution was administered daily in fresh medium during all the assays. These experiments were carried out in parallel with phenylarsine oxide (PAO) as a positive control of cell death. PAO induced a cell death of 65% from day 1, which reached 90% after 7 days of treatment. PAO treatments also were administrated daily (data not shown).

Dex Released From Polymeric Microparticles

Polymeric microparticles were studied in terms of cell viability to determine if dex released from these systems has a similar effect as repeated dex solution administration. From the data of the *in vitro* release assay of dex from polymeric microparticles, a daily release rate of 2.89 μg/ml was established, which corresponds with the average rate of the release profile shown in Fig. 3. This value allowed us to calculate the amount of microparticles required to reach a daily dex release of 10 μM from a single microparticle administration.

Since dex takes longer to be completely released from polymeric microparticles than the maximum period of cell culture (6–7 days), direct incubation of microparticles in the cell culture medium only allowed us to evaluate the effect of dex released during the first 6–7 days. For this reason, microparticles with different degradation levels coming from a release test as described in the Methods section were added to the cell culture. Thanks to this strategy, proliferation assays were extended up to 26 days. To test the impact on cell viability of the unloaded polymeric microparticles, these microparticles were tested in parallel with loaded ones.

As with the dex solution, the effect on cell viability was only detected in PC12 cells. Unloaded polymeric microparticles (Fig. 7) were not cytotoxic for the time tested. However, loaded polymeric microparticles, after a single administration, afforded the same cytotoxic effect as dex solution administered every 48 h, during the entire assay period (26 days), showing statistically significant differences in comparison with control effect from day 1 and to unloaded microparticle effects from day 3.

The morphology of the cells during the viability assay was observed (Fig. 8). No differences in cell morphology were detected between control cells (Fig. 8A) and those incubated with dex in solution (Fig. 8B), unloaded polymeric microparticles (Fig. 8C, left panel) and loaded polymeric microparticles (Fig. 8C, right panel). A clear diminution of the number of cells was observed when cells incubated with dex loaded microparticles were compared to cells incubated with unloaded microparticles at all release times tested (Fig. 8C-1 to C-4). In addition, as the release time progressed, the microparticles showed a higher opacity and a loss in shape, with these changes more evident for loaded microparticles after 26 days of treatment (Fig. 8C-4).

To get a deeper insight on mechanisms underlying cell viability or toxicity, apoptosis assays were carried out in PC12 cells. To evaluate the cytotoxic effects of dex in solution and polymeric microparticle, FACS (Fluorescence-activated cell sorting) experiments were performed. Dex in solution and polymeric microparticles were incubated for short periods of 2–5 h. However, there was no effect on cell cycle progression or cell cycle arrest (data not shown). Cells were also incubated

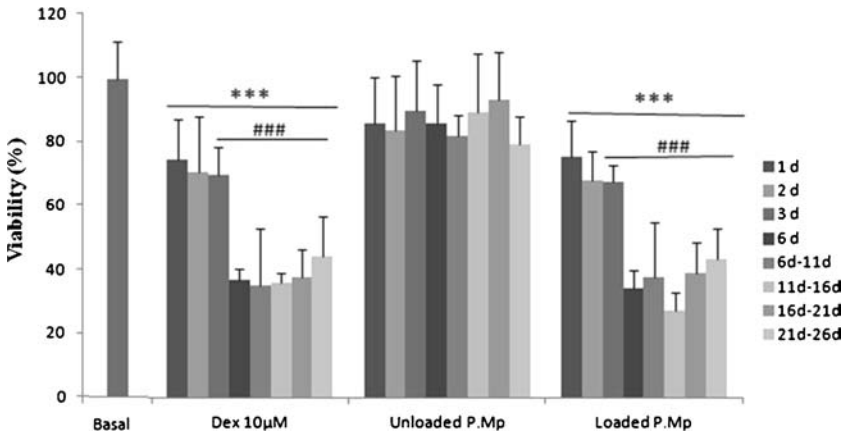

Fig. 7 Cytotoxicity of a 10 μM dex solution, unloaded and loaded polymeric microparticles from 1 to 26 days. Data correspond to the means ± standard deviations for 6–12 wells for four different batches of cells. 10 μM of dex was significantly cytotoxic after 3 days of exposure. Unloaded polymeric microparticles were not cytotoxic for the time exposure period tested. Loaded polymeric microparticles were significantly cytotoxic from 3 to 26 days of the incubation period. *** $P < 0.05$ with respect to control cell viability. ### $P < 0.05$ with respect to unloaded polymeric microparticles cell viability (ANOVA).

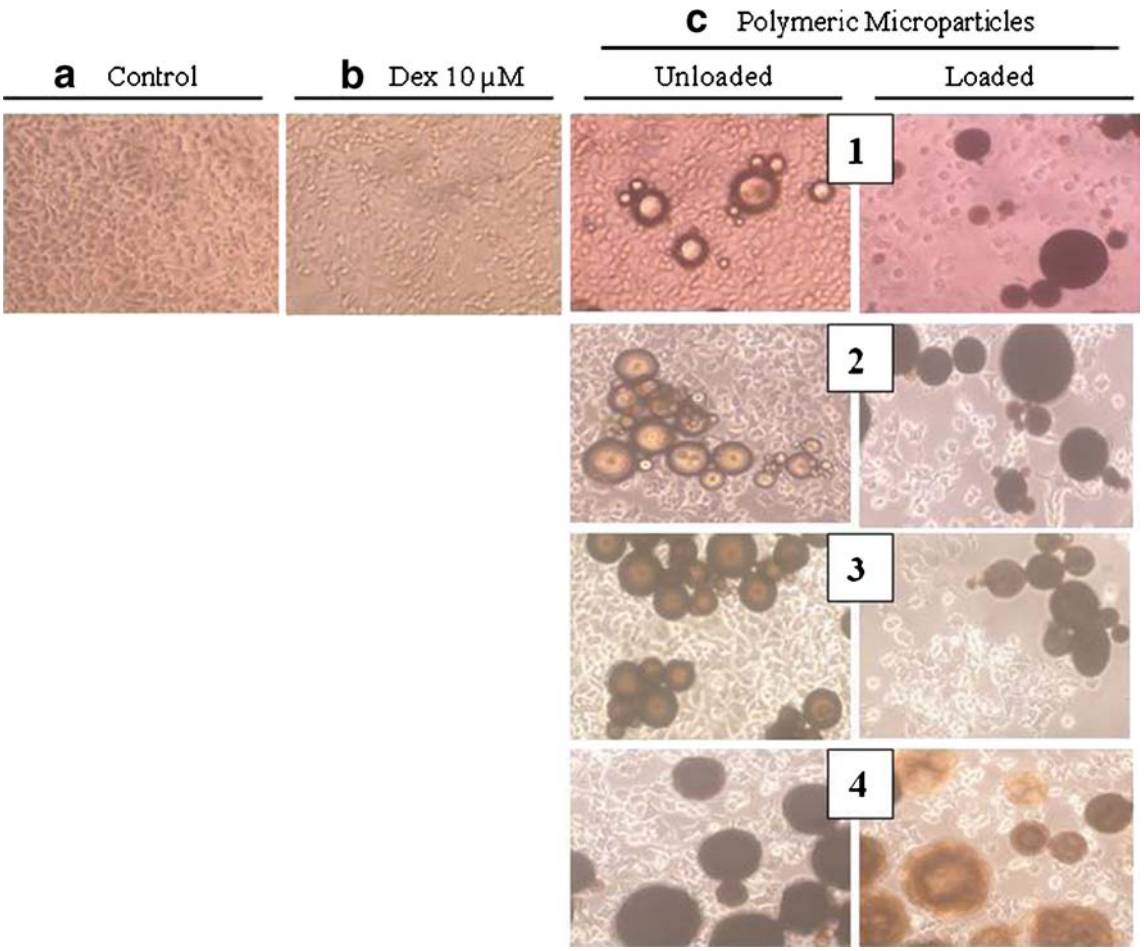

Fig. 8 PC12 cell morphology after polymeric microparticle treatment. (**a**) Microphotography of control cells, (**b**) After 1 day with dex solution, (**c**) Unloaded polymeric microparticles (*left panel*) and loaded polymeric microparticles (*right panel*). Optic microscope images were taken after 6 days of culture with polymeric microparticles from different release times; 1 day (1), 6 days (2), 16 days (3) and 26 days (4).

for longer exposure periods of 1–2 days. FACS analysis showed that the cell cycle was stopped with almost the disappearance of the G2 phase in cells treated with dex in solution and dex microparticles, but not in those treated with unloaded microparticles (Fig. 9A–B). Dex solution and loaded polymeric microparticles induced apoptosis around 15.8 and 19.9%, respectively, after 2 days of incubation. However, unloaded polymeric microparticles did not induce apoptosis during both times period tested (Fig. 9C). Staurosporine 1 µM was used as a positive control for apoptosis.

Dex Released From Lipid Microparticles

The cytotoxic effect, in PC12 cells, of dex incorporated into lipid microparticles in PC12 cells was compared to that produced by a 10 µM dex solution. As stated, lipid microparticles released *in vitro* 100% of microencapsulated dex within a day. Therefore, the data of microparticle drug loading were used to determine the amount of microparticles corresponding to 10 µM dex. This was the amount of lipid microparticles used, in a single administration, in a cell proliferation assay prolonged up to 3 days, whereas dex solution was added to the cell culture medium each 48 h.

As illustrated in Fig. 10, the cytotoxicity of unloaded lipid microparticles could be discarded because the cell viability after treatment with the microparticles during the incubation time tested did not show statistically significant differences with respect to the control. In addition, dex incorporation into these lipid carriers did not affect their activity, resulting in similar cytotoxic effects as the dex solutions. Cellular death observed after 1 day of treatment both with dex solution and with loaded lipid microparticles was statistically significant with respect to control cells. In the same way, cytotoxicity produced both by the dex solution and loaded lipid microparticles after 3 days of treatment also showed statistically significant differences with respect to unloaded microparticle treated cells (Fig. 10). Although the drug was released within a day, assays were carried out for 3 days, obtaining the same effects with a single administration of dex lipid microparticles as with dex solution administrated each 48 h.

Cell morphology was observed through an optical microscope. Control cells showed the classical polygonal PC12 cell

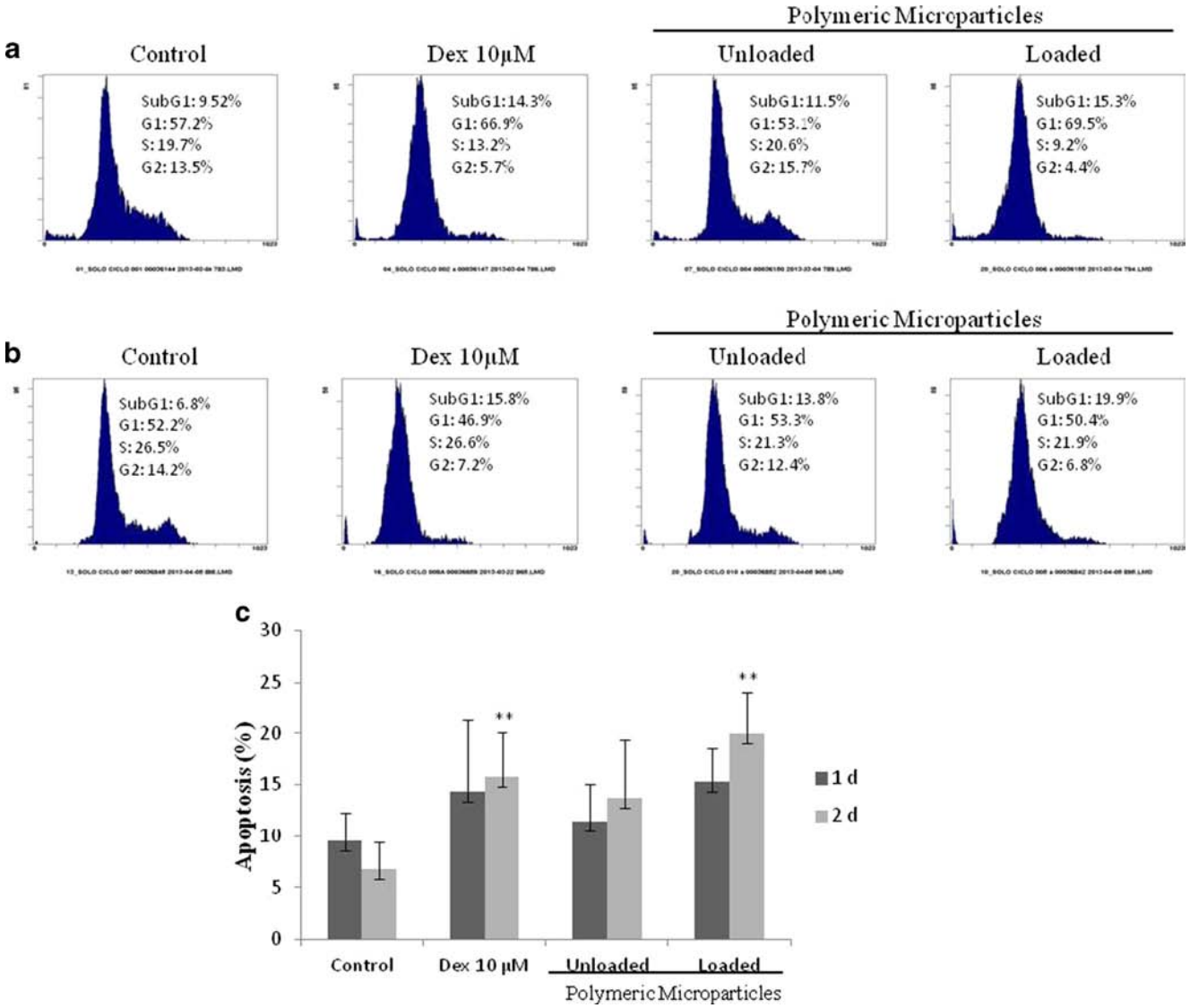

Fig. 9 Determination of apoptosis under dex and polymeric microparticle treatments. (**a**) and (**b**) show flow cytometry records obtained from control cells (Control) and from cells incubated with 10 μM dex solution and with unloaded and loaded microparticles for 1 day (**a**) and 2 days (**b**). **c** Averaged pooled results of all of the experiments, expressing the apoptotic effects (ordinate) of dex. Cells were also incubated with a positive reference compound (i.e., 1 μM staurosporine). Data are the means ± standard deviations of six experiments from at least six different cultures. Within each experiment, the measurement was performed at least in duplicate. ** $p < 0.001$ with respect to control.

morphology. This morphology was also observed when cells were treated with dex solution. However, when cells were treated with lipid microparticles (unloaded and loaded), they lost their classical morphology (Fig. 11).

The assay was repeated using Transwel, within 3 days of treatment, observing that cells did not show any morphological alteration after that time period. At the same time, cell viability results did not show any differences with those shown in Fig. 10.

Apoptosis assays were likewise conducted for lipid microparticles. As described in Fig. 9, 48 h was established as the optimal time to study apoptosis induction by dex solution or loaded polymeric microparticles. For this reason, we used the same incubation time for lipid microparticle analyses. Typical FACS analyses are shown in Fig. 12A, for the control, 10 μM dex solution, unloaded lipid microparticles and loaded lipid microparticles, respectively. However, the apoptosis induced by dex lipid microparticles was around of 17.9% higher (with statistical significance) than that obtained with Dex solution (Fig. 12B). Furthermore, cells treated with dex solution and loaded lipid microparticles, resulted in almost the disappearance of the G2 phase.

Such results motivated us to further evaluate the effect of dex lipid microparticles on PC3, a GC non-sensitive cell line, as long as in previous studies no cytotoxic effect was observed when PC3 cell cultures were incubated with different

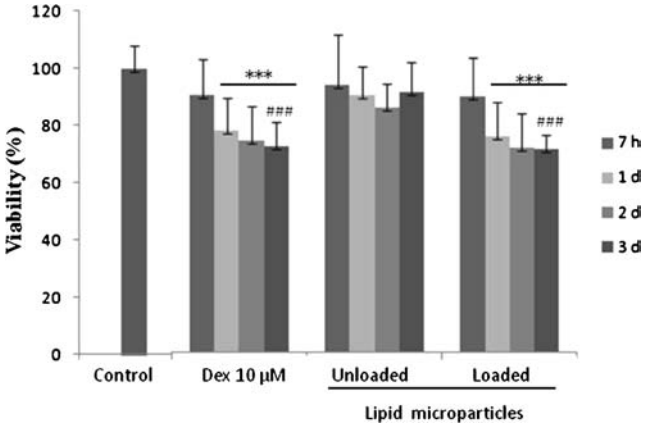

Fig. 10 Time dependence of the cytotoxic effects of lipid microparticles. The figure shows the cytotoxic effect of the 10 μM dex solution, unloaded and loaded lipid microparticles, measured as % cell viability, in cells exposed for 7 h and 1–3 days.

concentrations of dex solution. As depicted in Fig. 13, loaded lipid microparticles induced statistically significant cytotoxicity in these cells, whereas dex solution and unloaded lipid microparticles did not induce any significant effect after 48 h of incubation.

DISCUSSION

Two dex controlled-release systems were developed using different types of carriers: a synthetic lactic-glycolic copolymer was used to develop polymeric microparticles, and egg phospholipids with 80% phosphatidylcholine were used to obtain lipid microparticles. Polymeric microparticles were elaborated by an emulsion cosolvent evaporation method and lipid microparticles were elaborated by a spray drying method, which avoids the use of high toxicity organic solvents (18,19). Although both microencapsulation techniques are conceptually simple, the formulation and process variables that can affect the properties of the microparticles are numerous (17). Thus, for parenteral administration, it is important to take into account that microparticle size has to be less than 100 µm to be injected by means of a conventional needle, but larger than 10 µm to avoid macrophage phagocytosis (32). Optimization of the microencapsulation procedures was carried out in both cases to achieve an optimal size for parenteral administration, high encapsulation efficiency and, systems with high drug loading. Dex polymeric microparticles have been developed in the bibliography aiming to improve the anti-inflammatory effect of the drug. The three main elaboration methods described were O/W emulsion-solvent evaporation method, O/W emulsion-cosolvent evaporation method and W/O/W solvent-evaporation method; and dex was used as base, as well as phosphate or as acetate; trying to obtain higher drug loadings (16,17,20,33,34). To elaborate our dex PLGA microparticles we selected the O/W emulsion-cosolvent evaporation method using dex phosphate disodium, with which we obtained the best results. The organic solvent selected initially was DCM, whose volume was adjusted (Table I, protocols 1 and 2) to improve the extraction of such solvent during microsphere formation. Solvent extraction-evaporation leads to polymer precipitation encapsulating the drug. Therefore, the faster the solvent is evaporated, the faster the polymer precipitates and the higher the drug loading to be obtained (35). Nevertheless, in our study the reduction in solvent volume did not lead to any improvement in drug loading (Table II), nor did it result in any morphological alteration of the microparticles (Fig. 1). The smaller volume of DCM could be compensated by the higher viscosity of the internal phase in such a way that significant difference in solvent extraction rate was not produced. The presence of the cosolvent methanol, which is, because of the high hydrosolubility of dex phosphate, the main modulator of the drug diffusion to the aqueous external phase, could also contribute to cushion the effect of the extraction of the solvent.

DCM was substituted by chloroform in protocol 3, since many authors describe that the use of organic solvents with

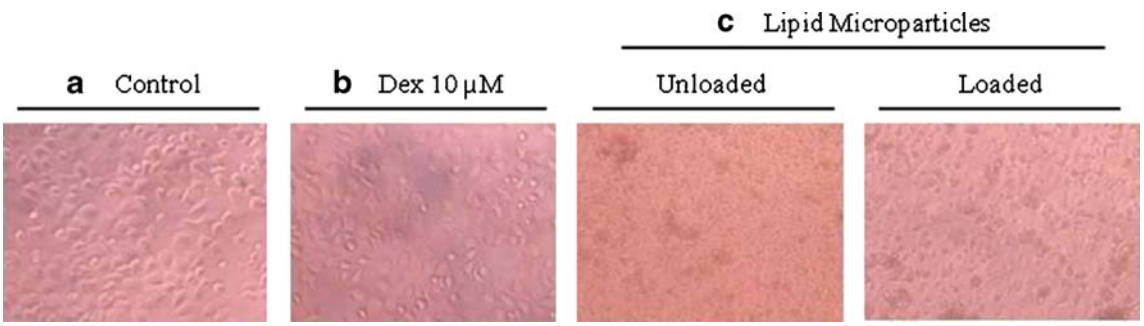

Fig. 11 PC12 morphology after 3 days of incubation with dex in solution, unloaded and loaded lipid microparticles.

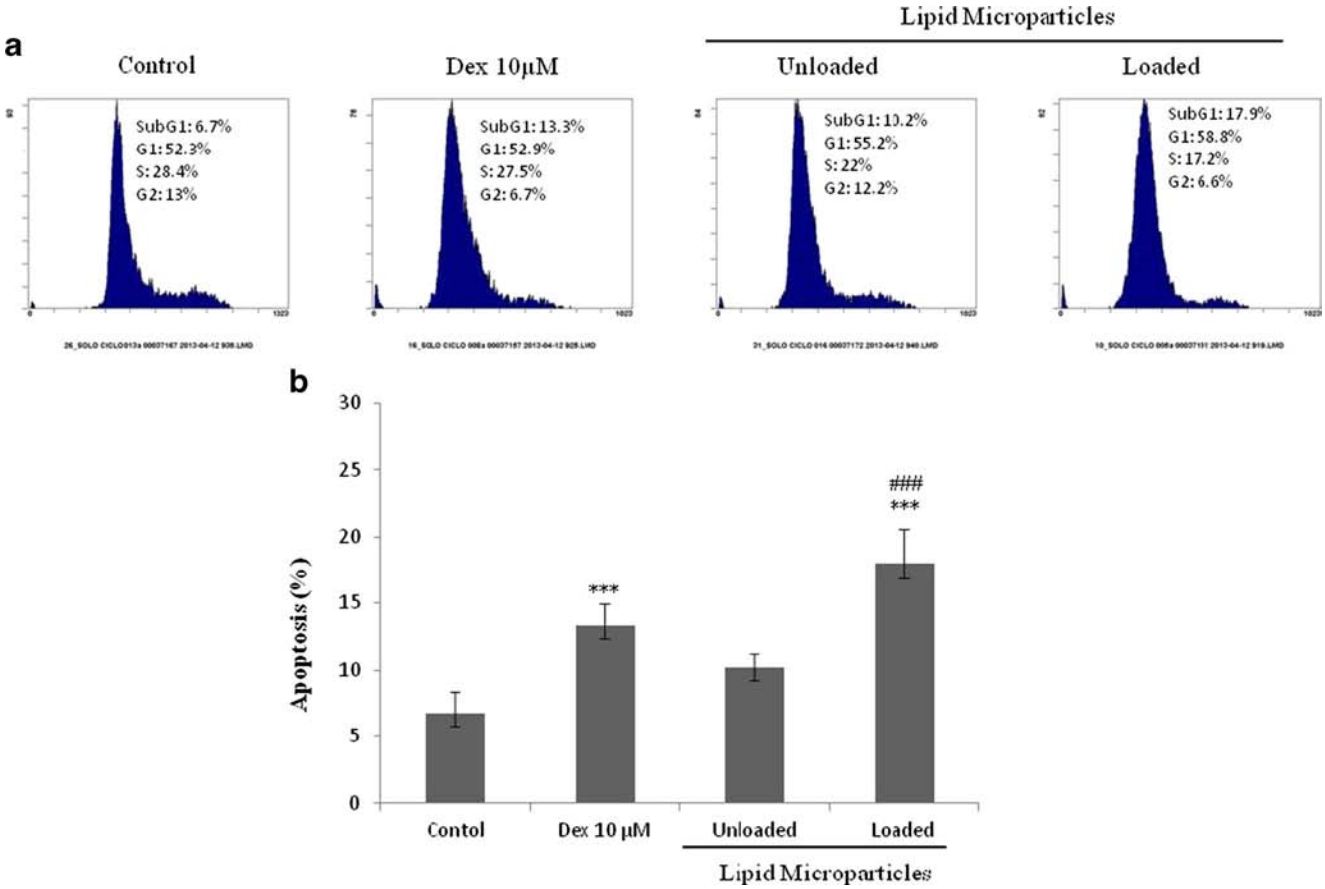

Fig. 12 Determination of apoptosis under dex and lipid microparticle treatments (**a**) Flow cytometry records obtained from control cells, cells treated with 10 μM dex solution and cells treated with unloaded and loaded lipid microparticles after 2 days of incubation. (**b**) Averaged pooled results of all of the experiments, showing the apoptotic effects (ordinate) of 10 μM dex solution, unloaded lipid microparticles and loaded lipid microparticles.

good miscibility in water (as it is the case of chloroform), improves microparticle drug loading (32,36,37). However, in our study, this change resulted in microparticles with a more irregular surface (Fig. 1), increasing microparticle aggregation and lowering microparticle drug loading (Table II). As the volume of both solvents was the same, the viscosity of the internal phase was not significantly changed, but the vapour pressure of chloroform (180 mmHg at 20°C) is lower than of DCM (375 mmHg at 20°C). In this situation chloroform was slower evaporated, which led to higher drug diffusion to external aqueous phase. In order to increase drug loading, the volume of the aqueous phase was reduced (protocols 2 and 4) so as to limit drug diffusion to the external aqueous phase during solvent removal. Nevertheless, the improvement in drug loading obtained was not significant (Table II), probably because the slower diffusion of the drug was compensated by the slower solvent diffusion and therefore the slower PLGA precipitationThe decrease of PVA concentration in the external aqueous phase (from 1% PVA in protocol 2 to 0.5% PVA in protocol 5) resulted in an increase in drug loading but also led to an increase of microsphere size (Table II). On the one hand, PVA acts as a stabilizer, reducing the surface tension between the aqueous and organic phases and, thus, facilitating internal organic phase dispersion in smaller droplets (36). On the other hand high concentration of PVA favors dex diffusion to the aqueous phase, thus, decreasing drug loading and encapsulation efficiency. To increase drug loading while maintaining a microsphere size of less than 100 μm, the

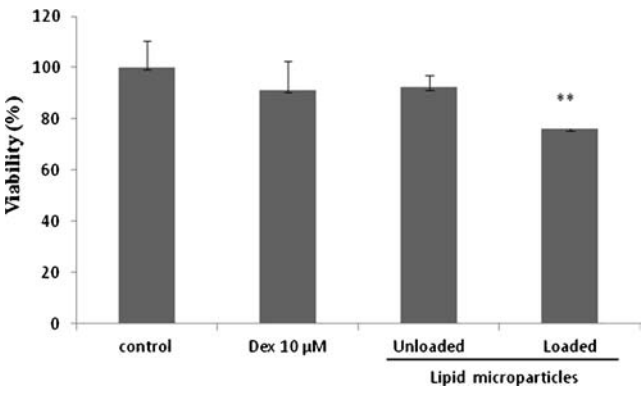

Fig. 13 Cytotoxic effects of lipid microparticles in PC3 cells. The figure shows the cytotoxic effects of dex solution (10 μM), unloaded lipid microparticles and loaded lipid microparticles after 48 h of assay. The measure is as % cell viability.

PVA concentration was reduced up to 0.25%, while the stirring speed during emulsion formation was increased (protocol 6).

Finally, protocol 7 is the same as protocol 6, but the characteristics of the polymer are slightly different. In both cases, poly(D,L-lactide/glycolide) 50:50 copolymers were used but with different molecular weights and, therefore, different inherent viscosities (RG504H > RG502). In addition, the RG504H resomer contains carboxylic acid in chains and hydrates faster, showing a faster degradation *in vitro* than the non-H one. A large increase in microparticle size and a broad size distribution were observed when the RG504H copolymer was used, exceeding 100 μm, probably due to the less diffusion of this higher viscosity polymer through the PVA external solution (32). Furthermore, microparticle drug loading was reduced (Table II), maybe due to the longer contact time between dex and the external phase. Jaraswekin *et al.* (17) found that dex microparticles prepared with higher molecular weight PLGA had a more porous surface and inner structure, and they attributed this observation to the faster polymer precipitation because of its lower solubility: a faster polymer precipitation rate resulted in a more porous structure as a result of the rapid hardening of the polymer surface. In our study, however, modifications in the surface of protocol 7 microparticles, comparing with protocol 6 microparticles, were not observed by SEM (Fig. 1). The obtained results led to the conclusion that the best microencapsulation procedure to elaborate polymeric microparticles was protocol 6, achieving microparticles with an optimal size and morphology for parenteral administration, and with high encapsulation efficiency and drug loading.

The *in vitro* release kinetics of dex from polymeric microparticles showed an efficient dex release control, allowing a prolonged release for more than 1 month. Due to the low molecular weight of dex and the hydrolysis of the aliphatic polyester polymers such as poly(D,L-lactide/glycolide) in aqueous medium, both drug diffusion through the polymeric matrix and microparticle degradation would contribute to drug release from microspheres. A multi-stage release profile was obtained, without showing a significant burst effect. The rapid release stage observed up to day 14, was probably attributable to a great extent to dex diffusion from the outer layers of the microparticles, with a low contribution of microparticle degradation. After that, dex was released slowly, mainly due to microparticle degradation. Banu *et al.* (33) also studied the dex release from PLGA microparticles, and they found severe initial burst effects followed by lag times of 5–10 days depending on the molecular weight of the polymer used. Hickey *et al.* (15) also mentioned a 2-week lag time after an intense burst initial. In microparticles obtained from protocol 6, on the contrary, burst effect was lower than 8%; and the lag time was smoothed, and a significant release of dex was detected which was increasing, fitting to an exponential equation with an estimated dex release rate of 4,3%/day up to day 14. In lipid microparticles low microencapsulation yields were obtained. This is an important disadvantage of the production technique, which is compensated by the high encapsulation efficiency (Table III), achieving a drug loading of about 9 mg of dex/100 mg of microparticles in all batches. The low yield in spray dryer was due to loss of very fine particles as these particles could not settle on the cyclone collector chamber, and the adhesiveness of molten lipids to the glass of the equipment.

When the lipoid percentage increased from 40 to 70%, a significant decrease in microparticle size was detected. The amphiphilic nature of Lipoid® (constituted by phospholipids) could explain this fact. An increase in phospholipid concentration in the solution sprayed into the drying chamber of the spray drier, could generate smaller droplets because of a lower gas-liquid interfacial tension. Once dried, the droplets would become into smaller microparticles. When Lipoid® proportion increased up to 80%, the particles stuck to the glassware, leading to extremely low yields; and an intense agglomeration was detected in the powder collected increasing the microparticle size measured by laser diffraction and the corresponding span value. Blasi *et al.* (15) also observed this fact when preparing by spray drying cetylpalmitate nanoparticles for parenteral administration. Indeed, the higher the lipid _concentration, higher the probability of emulsion growth mechanisms as agglomeration and coalescence during preparation. Additionally, partial melting of the lipid phase during spraying is one of the major reasons for particle growth. In fact, a limitation of spray-drying technique is the use of temperature to remove the solvent, even though the exposition to temperature is very short and that the low latent heat of vaporization, when ethanol/water mixtures are used, drastically reduces the thermal stress of the material. Working with lipid materials, the risk of lipids melting during drying can lead to particle sintering with the increase of the initial particle size.

In DSC analysis, thermogram of pure Lipoid® exhibited a single endothermic event at $T_{onset} = 38.5°C$ with an enthalpy (ΔH) of 18.99 J/g, which would correspond to the melting of the aliphatic chains. Gómez-Gaete *et al.* (17) elaborated dipalmitoylphosphatidylcholine microparticles also by spray drying finding that the phospholipid were disposed in a lamellar phase, with a pretransition Tonset of 37,2°C, corresponding to the conversion of the lamellar liquid-crystalline phase. The low melting of Lipoid® also explain the high aggregation state of lipid microparticles observed in SEM images. This may be due to the imaging process, because the sample was subjected to high temperatures.

In order to reduce the sensitivity of the lipid microparticles to temperature, due to the low melting point of Lipoid®, and thus to increase their physical stability, modifications on the nature of the sugar (lactose, mannitol and trehalose in batches 4, 5 and 6, respectively) were performed. Carbohydrates have been used

successfully as cryoprotectants for reducing particle aggregation of lipid coloidal carriers. The best results were obtained with trehalose as protecting agent, in such a way that the batch 6 showed the highest flowability. A thicker sugar layer, which formed a crust after evaporation of the solvent, prevented the aggregation of molten lipid droplets and protected against shearing off from the particle surface (38). This result is probably related to the features of trehalose, such as its low hygroscopicity, the absence of internal hydrogen bonds thus allowing a more flexible formation of hydrogen bonds with phospholipids, and its higher glass transition temperature. The glass transition temperature of trehalose is about 110°C, remaining high even when the sugar is partially rehydrated by exposing it to water, whereas glass transition temperatures of lactose and mannitol are 101°C and 11°C respectively. Trehalose, possessing a higher water solubility than mannitol or lactose, could be used in much higher concentrations. In fact, this sugar has been extensively and successfully used to protect biomolecules during freezing, drying and contact with organic solvents (39). Beside these changes in powder flowability, statistically significant effect was not detected on microparticle size, nor on encapsulation efficacy or drug loading among batches with the three different cryoprotectants.

Dex release from lipid microparticles was faster than polymeric ones, without any differences in kinetic release profiles when different 70% Lipoid® microsphere batches made with different sugars were compared. This suggests that Lipoid® is the unique component affecting dex release. The faster release of dex from lipid microparticles is explained not only by their smaller particle size but also, and more important, by the presence of hydrosoluble excipients. Indeed, according to DSC results, the polymeric microparticles present a matrix structure where the drug is molecularly dispersed in a hydrophobic polymer network. However, lipid microparticles showed an structure mainly amorphous where phospholipids were mixed with trehalose and OVA, which in contact to aqueous medium were dissolved forming canals and favouring drug releaseIn both optimized formulations of polymeric and lipid microparticles, the existence of incompatibilities between drug and excipients was discarded and the absence of the dex characteristic peak in both thermograms indicates that the drug was dissolved or molecularly dispersed within the polymeric or lipid matrix.

The results obtained in the cell proliferation assays demonstrated the cytotoxic effect of dex (10 μm) in PC12 cells.

Furthermore, after a single administration of dex polymeric microparticles, the cellular death rate for 26 days was similar to that obtained with the dex solution administered every 48 h. On the contrary, unloaded polymeric microparticles were not cytotoxic.

Studied references used 1–5 μM concentrations of dex, showing a gene induction that led to an increase in calcium secretion and up-regulation of catecholamine synthesis and storage proteins (24,25), but in any cases described in the literature was apoptosis induced. However, this effect was significantly displayed after raising the dose to 10 μM, since dex solution and loaded polymeric microparticles induced apoptosis around 15.8% and 19.9%, respectively, after 2 days of incubation. After dex solution and dex polymeric microparticle administration, cellular proliferation was inhibited, stopping the cellular cycle during the G2 phase. It was described, in references, the ability of glucocorticoid to stop the cell cycle in osteosarcoma cells, hepatoma, glioma, and breast tumour cells, and to trigger, as a consequence, cellular death. In a recent study carried out on human medullary thyroid cancer cell lines (3), the apoptotic effect of a 1 μM dex solution was demonstrated but however, it was related to a decrease in the transition of G1 to S phase. In this same sense, when T-ALL cells were used, dex induced arrest of the cell cycle at the G0/G1 phase (10). Dex lipid microparticles showed comparable results in terms of proliferation inhibition of PC12 cells to dex solution administered every 48 h in assays up to 3 days; Changes in cell morphology were detected when lipid microparticles were in contact with cells. This cell morphology alteration was believed to result from a physical interaction between lipid microparticles and cells. This interaction could be facilitated by the phospholipidic constituent of the lipid microparticles. To confirm this, the assay was repeated using Transwell: a system that allowed us to separate cells and microparticles, but sharing the same medium and, therefore, allowing the dex released from microparticles to affect cells. This assay was carried within 3 days of treatment, obtaining the same cell viability results but observing that cells did not show any morphological alteration after that time period. This suggests that the cytotoxicity was not caused directly by a physical interaction.

This effect could be due to the phospholipids released from the lipid microparticles, which could modify cell membrane permeability, thus, facilitating dex internalization into the cell. In fact, cell morphology alteration observed was believed to be due to the physical interaction between phospholipid constituent of these microparticles and those of cell membranes. However, further research demonstrated that such cytotoxicity was not caused directly by the mentioned physical interaction, given the fact that proliferation assay carried out using a Transwell system, able to separate cells and microparticles while sharing the same medium and thus allowing dex released to act on cell line, did not reveal such cell morphology alteration, while cytotoxicity was kept unchanged.

The most interesting finding was that the apoptosis induced by dex lipid microparticles was around of 17.9% higher (with statistical significance) than that obtained with Dex solution. These results could be due to phospholipid release from lipid microparticles, which could modify cell membrane permeability, thus, facilitating dex internalisation into the cell. Yokoyama *et al.* (40) suggested that corticosteroids incorporated in lipid emulsions were taken up by the macrophages to as

much greater extent that free corticosteroids, resulting in stronger anti-inflammatory activity.

GC apoptosis have been related to mitochondrial alterations, although the mechanism is not yet fully clarified. Dex induces changes in mitochondrial membrane properties that together with the reduced expression of mitochondrial transporters of substrates and proteins may lead to repression of mitochondrial respiratory activity and lower ATP levels that contribute to GC-induced apoptosis (10). This hypothesis is supported by the results obtained on GC resistant PC3 cells, where dex lipid microparticles showed significant antiproliferative effects in contrast to the dex solution. The mechanisms underlying the development of GC resistance are complex and poorly understood. Data currently available suggest that there is not a main mechanism responsible for GC resistance, but several that act either alone or in combination. For example, GC resistance may occur if inactive GCR isoforms are present, if members of the ABC-transporter family are over-expressed or if the apototic pathways are inhibited. An increasing number of reports indicate that activation of the mammalian target of the rapamicin (mTOR) signaling pathway may contribute to GC resistance in hematological malignancies, and so the selective mTOR inhibitor rapamicin capability to re-sensitize the resistant cells to dex treatment has been evaluated (22). They are potential targets of pharmacological actions that could lead to important clinical benefits.

CONCLUSIONS

Two dex controlled-release systems have been developed by using two different biocompatible and biodegradable materials.

On the one hand, polymeric microparticles have been prepared with a synthetic lacti-glycolic copolymer by the oil-in-water-emulsion cosolvent evaporation method. After having evaluated the numerous factors involved, according to our results it is fair to say that PVA concentration in the external aqueous phase represents the best way to increase drug loading while keeping particle size under 100 μm, whereas neither the reduction of the solvent volume or of the aqueous phase volume, nor the use of water-miscible solvents or of higher-viscosity polymers seem to play such an essential role in terms of significantly higher drug loading and encapsulation efficiency. Polymeric microparticles obtained by the optimized procedure exhibited *in vitro* controlled release of dex over more than a month, and PC12 cellular death rate for 26 days similar to that obtained with the dex solution administered every 48 h. Therefore, these polymeric microparticles would be able to avoid, in a subcutaneous or intramuscular administration, the pharmacokinetic peak trough fluctuations characteristic of conventional formulations, improving treatment effectiveness, decreasing GC side effects and improving patient compliance. These results allow us to put forward dexamethasone-loaded polymeric microparticles as an alternative to the current dex administration systems in combined chemotherapy given the importance of an accurate control of dex plasmatic levels to optimize its efficacy.

On the other hand, lipid microparticles have been prepared with egg phospholipids by spray drying. After implementing several changes in formulation process, it can be claimed that phospolipid proportion, seems to be the only variable which affects dex release, faster in all cases than dex release from polymeric microparticles. Loaded lipid microparticles showed higher apoptotic effect in PC12 cells than dex solution; and, unlike dex solution, showed significant antiproliferative effects in PC3, a GC non-sensitive cell line. These results allow us to put forward dexamethasone-loaded lipid microparticles as a potential tool to overcome GC resistance.

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