

Poly- ϵ -caprolactone microspheres containing interferon alpha as alternative formulations for the treatment of chronic hepatitis C

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Interferon-alpha (IFN-alpha) is one of the main drugs used in the treatment of hepatitis C. Use of IFN-alpha has some limitations that result in poor treatment efficacy and low patient compliance. Therefore, the aim of this study was to develop poly- ϵ -caprolactone (PCL) microspheres containing IFN-alpha as an alternative for the treatment of chronic hepatitis C. Microspheres were prepared using the multiple emulsion followed by solvent evaporation technique. Particle size, surface morphology, drug content and encapsulation efficiency of the microspheres produced were evaluated. The stability of the formulation was assessed after 90 days at -20°C. An *in vitro* release study was performed in PBS. *In vitro* cytotoxicity of the formulation was studied using hepatic cell line. The freeze-dried microspheres had mean particle size, IFN-alpha content, and encapsulation efficiency of $38.52 \pm 4.64 \mu\text{m}$, $15.52 \pm 3.28\%$ and $83.93 \pm 5.76\%$, respectively. There were no significant changes during storage and the structural integrity of the protein was not compromised by the preparation technique. A total of 82% of the IFN-alpha was released after 28 days and the developed microspheres did not present cytotoxicity to the hepatic cell line. *In vivo* studies are currently underway to evaluate the biological activity of IFN-alpha encapsulated into microspheres.

Uniterms: Interferon alfa. Hepatitis C/treatment. Poly- ϵ -caprolactone/microspheres/development.

O interferon alfa (IFN-alfa) é um dos principais fármacos utilizados no tratamento de hepatite C, mas o seu uso apresenta limitações que resultam em baixa eficácia do tratamento e não adesão do paciente. Diante disso, este estudo objetiva o desenvolvimento de microesferas de poli- ϵ -caprolactona (PCL) contendo IFN-alfa como alternativa ao tratamento de hepatite C crônica. As microesferas foram preparadas pelo método de emulsão múltipla seguido de evaporação do solvente e caracterizadas quanto ao diâmetro médio das partículas, morfologia da superfície, taxa e eficiência de encapsulamento. A estabilidade da formulação foi acompanhada durante 90 dias a -20 °C. O estudo de liberação *in vitro* foi realizado em PBS. A citotoxicidade da formulação foi avaliada utilizando linhagem de células hepáticas. As microesferas liofilizadas apresentaram diâmetro médio, taxa de encapsulamento e eficiência de encapsulamento de $38,52 \pm 4,64 \mu\text{m}$, $15,52 \pm 3,28\%$ e $83,93 \pm 5,76\%$, respectivamente. Não foram observadas alterações significativas durante o armazenamento e a integridade estrutural da proteína foi mantida após o preparo. Oitenta e dois por cento de IFN-alfa foram liberados em 28 dias e a formulação desenvolvida não apresentou toxicidade para as células testadas. Estudos *in vivo* estão em andamento para avaliar a atividade biológica do IFN-alfa encapsulado nas microesferas.

Unitermos: Interferon alfa. Hepatite C/tratamento. Poli- ϵ -caprolactona/microesferas/desenvolvimento.

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INTRODUCTION

Hepatitis is defined as an inflammatory liver disease which is caused mainly by virus, bacteria, fungus or drugs. Although several virus types may cause hepatitis, only types B, C and D are responsible for chronic hepatitis (Ferreira, Carvalho 1998). The virus responsible for hepatitis C disease belongs to the *Flaviviridae* family and is recognized as the leading cause of chronic hepatitis and liver diseases for which liver graft is indicated. An estimated 170 to 220 million people in the world are infected by this virus worldwide and 3 to 4 million new cases of hepatitis C infection emerge annually. Some 55 to 85 % of patients with the acute infection have an elevated risk of developing hepatocellular injury, cirrhosis, carcinoma or liver failure (Parolin *et al.*, 2006; Siciliano, Boulos, 2004; Weigand *et al.*, 2007).

The treatment of hepatitis C consists, initially, of suppressing viral replication. For this purpose, interferon, ribavirin and PEG-interferon are the main drugs used in clinics (Almeida, Souto, 2007).

Interferon-alpha (IFN-alpha) was approved for clinical use in 1986, being one of the first biotherapeutic products approved by the FDA. It belongs to a class of macromolecules that naturally occurs in the body and is produced by cells of the immune system (Sanchez *et al.*, 2003). During the last 20 years, IFN-alpha has had increasingly broad clinical application, being used in the treatment of conditions such as hepatitis B and C, multiple sclerosis, viral myocarditis, Kaposi's sarcoma, hairy cell leukaemia, condyloma acuminatum and cutaneous T cell lymphomas with considerable success (Beilharz 2000). However, the clinical use of IFN-alpha has some limitations, including a short half-life and rapid loss of biological activity, long-term frequent injections, fluctuation of plasma concentration and serious side effects. These difficulties may result in poor efficacy of the treatment and low compliance among patients (Beilharz, 2000; Sanchez *et al.*, 2003; Hua *et al.*, 2011).

The increasing number of peptides and proteins approved by the regulatory authorities for human use represents an important field of research. However, their use poses major challenges due to a number of obstacles to therapeutic application that hamper effective delivery and release in the body (Almeida, Souto, 2007). To guarantee efficacy of the proteins and peptides, they must target the site of action without being degraded and remain at the site for a long period to promote the desired effect (Segers, Lee, 2007).

Several delivery systems of IFN-alpha have been proposed to improve its efficacy and half-life. Among

these systems, pegylated IFN-alpha has proved highly successful as it can promote the sustained release of this protein over a period of one week. However, according to the literature, although pegylation can improve bio-distribution, evidence suggests that polyethylene glycol (PEG), due to its characteristic of non-biodegradability, may accumulate in the body. Also, it has been suggested that PEG conjugates may suppress protein immunogenicity and antigenicity, which may in turn reduce the conjugate residence time in the blood stream (Hua *et al.*, 2011; Segers, Lee, 2007). Furthermore, it has been described that pegylated IFN-alpha is more prone to induce side effects than IFN-alpha (Li *et al.*, 2011).

Recently, several attempts have been made to develop IFN-alpha delivery systems composed of biodegradable polymers, such as micro and nanoparticles, that can effectively deliver the active protein at a sustained rate, and at a concentration within its therapeutic window (Mohl, Winter, 2004)

These systems are able to maintain adequate serum levels of the drug for long periods of time and protect the macromolecules from degradation before they reach the target site, thus reducing side effects and increasing pharmacological activity (Teixeira *et al.*, 2005). The main objective of the development of delivery systems containing IFN-alpha is to improve patient compliance to the treatment by lowering the number of injections, reducing side effects, and promoting prolonged release of the drug at the site of action (Jorgensen *et al.*, 2006). Among these systems, microparticles may represent a promising alternative as they can facilitate controlled delivery of IFN-alpha by non-invasive routes, such as oral and nasal.

Among the biodegradable polymers, poly- ϵ -caprolactone (PCL) is of particular interest as it allows for a long, sustained and possibly modulated drug release rate (Chawla, Amiji, 2002). PCL is a biodegradable and biocompatible polymer with a very slow degradation rate, making it suitable for long-term delivery. Furthermore, it is biocompatible and extensively used in the pharmaceutical and biomedical fields as a biomaterial (suture, osteosynthesis material, artificial skin, support for cellular regeneration) and for prolonged drug delivery systems targeting specific tissues within the body (Sinha *et al.*, 2004). PCL micro or nanoparticles have indeed been widely explored in recent years for the administration of drugs by different routes and for the treatment of different diseases (Barbault-Foucher *et al.*, 2002; Blanco *et al.*, 2003; Lemmouchi *et al.*, 1998; Sinha *et al.*, 2004; Sun *et al.*, 2006; Verger *et al.*, 1998).

Maintaining activity of the protein during the whole preparative and release period is hard due to its instabil-

ity and physical/chemical interactions. Consequently, as described in previous studies, its co-encapsulation with stabilizing substances can be necessary to assure stability of the peptides and proteins (Li *et al.*, 2011). Substances evaluated to date include human serum albumin (HSA), which has proven effective for protecting interferon against potential chemical and physical degradation mechanisms (Ruiz *et al.*, 2006).

Therefore, the objective of this study was to develop PCL microspheres containing IFN-alpha co-encapsulated with HSA as an alternative to pegylated-interferon for the treatment of chronic hepatitis C. Characterization and *in vitro* cytotoxicity of the formulation and its components in hepatic cell line were studied.

MATERIALS AND METHODS

Materials

Human recombinant IFN-alpha was purchased from Pestka Biomedical Laboratories Interferon Source (USA). Polymer poly-ε-caprolactone [(PCL) molecular weight (Mw) 14kDa] and human serum albumin [(HSA), Fraction V, Mw 66kDa] were obtained from Sigma-Aldrich (Brazil). Poloxamer 188 (Pluronic® F68) was kindly donated by Basf Chemicals (USA). Ultrafiltrated water was obtained from Milli Q plus, Millipore (USA). All other chemicals were of analytical grade.

Preparation of IFN-alpha-loaded microspheres

The multiple emulsion followed by solvent evaporation was the technique used for the preparation of the microspheres. Firstly, a solution of IFN-alpha was diluted in phosphate-buffered saline (PBS, pH 7.4) containing HSA to form the inner aqueous phase. This solution was added to the organic phase, containing PCL dissolved in dichloromethane, with vigorous stirring using an UltraTurrax device (IKA Labortechnik, Germany) to form a single emulsion. This emulsion was added to a water solution containing the surfactant (poloxamer 188) under stirring again using an Ultra Turrax device (IKA Labortechnik, Germany) until multiple emulsion formation. The solvent (dichloromethane) was eliminated by evaporation under magnetic stirring and the microspheres thus obtained were collected by centrifugation (25700xg for 15 minutes, three times). The supernatant obtained was collected for IFN-alpha analysis and the pellets were immediately stored at -20 °C (E-C Apparatus, USA). Prior to lyophilization, the samples stored at -20 °C were placed in liquid nitrogen for 2 minutes. The samples were then placed in the

lyophilization apparatus, which was set at a temperature of -50 °C. After 4 hours, the samples were removed from the equipment and immediately stored at -20 °C. Formulations without IFN-alpha were also prepared by the same technique and served as controls. The composition of the developed formulation is described in Table I.

TABLE I - Composition of the developed microsphere suspensions

Components	Content
IFN-alpha solution (μL)	100
Human serum albumin (mg)	2.5
Poly-ε-caprolactone (mg)	100
Dichloromethane (mL)	2
Poloxamer 188 (mg)	100
Distilled Water (mL)	20

Analysis of IFN-alpha

For determination of drug content and encapsulation efficiency, and also of the amount of IFN-alpha released during the *in vitro* study, the ELISA technique with the Verikine™ Human IFN Alpha Elisa Kit (Pestka Biomedical Laboratories Interferon Source, USA) was used. The method adapted from Yang *et al.* (2010) was followed, as described below.

A known weight of freeze-dried microspheres was added to 10 mL of 6 N HCL solution, which was then placed in a water bath set at 40 °C for 36 hours. A 8 N NaOH solution was added to the resultant solution and the amount of IFN-alpha was then determined in supernatant liquid using the ELISA kit. The amount of IFN alpha was calculated using the equation obtained from a standard curve.

The validation of the method showed the absence of interference of the components used in the microsphere formulations, ruling out the risks of over or under estimation of the concentration of IFN-alpha.

Characterization of IFN-alpha-loaded microspheres developed

Particle size

For this analysis, microspheres under suspension (freeze-dried immediately after preparation) were evaluated. Before analysis, the freeze-dried microspheres were resuspended in water containing 0.03% of polysorbate 80.

The particle size of the developed microspheres was evaluated by photon correlation spectroscopy using a Mastersizer apparatus (Mastersizer 2000, Malvern In-

struments, England). For the analysis, water was used as a dispersing agent and the obscuration rate was set at 12%. All analyses were performed at room temperature. The results were expressed as mean particle size \pm standard deviation of values from six different samples.

Zeta potential

The Zeta potential, that measures the surface charge of the particles, was evaluated by dynamic light scattering set at a wavelength of 633 nm at an angle of 90° using a Zetasizer apparatus (Zetasizer HSA 3000, Malvern, Instruments Ltd., UK). All analyses were realized at the temperature of 25 °C and the samples were diluted 40 times to analysis. The results were expressed as zeta potential \pm standard deviation of values from six different samples.

Morphological studies

The surface morphology of the developed microspheres after freeze-drying was observed by scanning electron microscopy (SEM) using a FEI microscope (FEI, USA) operating at 15 kV. Before visualization, the freeze-dried samples were placed into coverslips, which were dried for 72 hours in a vacuum desiccator at room temperature. After drying, the coverslips containing the samples were mounted on aluminum stubs. Prior to microscopic examination, the samples were sputter coated with a gold layer under an argon atmosphere. Microsphere surfaces were viewed at 300 x to 1000 x magnification, and the images transferred to a computer by means of a digital image transfer interface.

Drug content and encapsulation efficiency

The drug content was calculated based on the ratio of amount of IFN- α found in the microspheres to total amount of microspheres used. The encapsulation efficiency was calculated based on the ratio of amount of IFN- α found in the microspheres to total amount of drug added to the formulation.

The amount of IFN- α was determined using the technique described in the section on *Analysis of IFN- α* . All values were expressed as percentage \pm standard deviation from six different samples.

Stability assessment for microspheres developed

Between preparation and beginning of the study, the developed freeze-dried microspheres were stored in impermeable containers that provided a permanent barrier to entry of moisture or solvent.

The particle size, drug content and encapsulation efficiency were monitored over 7, 30, 60 and 90 days after preparation of the freeze-dried microspheres stored

at a temperature of -20° C. Before the analysis of particle size, the freeze-dried microspheres were resuspended in water containing 0.03% polysorbate 80.

The drug content was measured using the method described in the section on *Analysis of IFN- α* .

***In vitro* release study**

A 50 mg amount of IFN- α -loaded freeze-dried microspheres was placed in sterile low-binding microtubes and suspended in 2.0ml of phosphate buffered saline (PBS, pH 7.4) containing 0.02% of polysorbate 80. The microtubes were incubated at 37 °C and centrifuged at 90 rpm. At pre-determined intervals, samples were centrifuged at 2000xg for 15 min and 1.0 mL of the supernatant was collected and replaced with fresh PBS to maintain the sink conditions. The amount of IFN- α released was determined using the method described in the section on *Analysis of IFN- α* .

Evaluation of IFN- α structural integrity

For the evaluation of IFN- α structural integrity after microsphere preparation, during the storage time, and also during the *in vitro* release study, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed using a Bio-Rad apparatus under non-reduced conditions. The samples were prepared under reduced conditions for application onto a gel consisting of 5 and 15% stacking and resolving gel, respectively.

***In vitro* cytotoxicity study**

Microspheres and components

For this study, the following samples were used: microspheres alone, IFN- α -loaded microspheres co-encapsulated with HSA and the components of the formulation (PCL, poloxamer, IFN- α , HSA) at the concentration used in the preparation. The cytotoxicity of these samples was evaluated using a hepatic cell line (HepaRG™, Invitrogen, USA).

Cells were seeded at a density of 1×10^5 /well onto a 96-well tissue culture polystyrene plate at a temperature of 37 °C and under 5% CO₂ atmosphere. After 24 hours of settling, cells were incubated in solutions containing the evaluated samples and also appropriate culture medium containing 1% fetal bovine serum (FBS, Invitrogen, USA). After 24 hours, cell viability was evaluated by using a colorimetric assay based on the mitochondrial conversion of tetrazolium salt, [3-4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide] thiazole blue (MTT, Sigma Chemical,

TABLE II - Characteristics of developed microspheres before and after freeze-drying*

Formulations	Particle size (μm)	IFN-alpha content (%)	Encapsulation efficiency (%)	Zeta Potential (mV)
Before freeze-drying	35.09 ± 3.80 ^a	17.72 ± 2.79 ^b	86.59 ± 6.37 ^c	-37.95 ± 2.19 ^d
After freeze-drying**	38.52 ± 4.64 ^a	15.52 ± 3.28 ^b	83.93 ± 5.76 ^c	-42.55 ± 2.33 ^d

Different letters in the same column means values are significantly different ($p < 0.05$). * All results expressed as mean ± standard deviation, n = 6. ** Results related to freeze-dried microspheres after resuspension in water containing 0.03% polysorbate 80.

USA). Briefly, culture medium was removed, wells were washed once with PBS, and MTT diluted in culture medium containing 1% FBS was added to each well. After 3 h of incubation at 37 °C and 5% CO₂ atmosphere, the plate was centrifuged at 1.000 rpm for 10 minutes in order to precipitate the formazan crystals. The supernatant was removed and 50 μL of dimethylsulphoxide (DMSO) was added to each well to dissolve the formazan crystals. The absorbance values were measured at 595 nm using a microplate reader (Spectramax M5e, Molecular Devices, USA).

As controls for cellular viability, the same cells used in this study received no treatment (positive control) or hydrogen peroxide (30 mM) (negative control). A positive control was considered as 100% of cellular viability. The microspheres or its components were considered cytotoxic if less than 50% of cellular viability was found. The results are expressed as percentage cellular viability ± standard deviation from six different samples.

Microsphere degradation products

Samples of microspheres alone and IFN-alpha-loaded microspheres co-encapsulated with HSA were used after freeze-drying.

For this experiment, hydrolysis and oxidation degradation methods were used to simulate microsphere degradation *in vivo*. A sufficient amount of freeze-dried microspheres was weighed to a final concentration of 0.1 mg/mL in 30 % hydrogen peroxide solution. The samples were incubated for 24 hours at the temperature of 85 °C until complete visual degradation. Subsequently, the solutions containing the biodegradation products obtained were evaluated using the hepatic cell line, following the same procedure as described for *Microspheres and components*.

As controls for cellular viability, the same cells used received no treatment (positive control) or hydrogen peroxide (30 mM) (negative control). A positive control was considered as 100% of cellular viability. The microsphere degradation products were considered cytotoxic if less than 50% of cellular viability was found. The results are expressed as percentage cellular viability ± standard deviation from six different samples.

Statistical analysis

The unpaired t-test was used to analyze differences in particle size, IFN-alpha content and encapsulation efficiency of the developed microspheres. Values of $p < 0.05$ were considered statistically significant.

The One-way ANOVA Kruskal-Wallis test was used to evaluate the differences for each parameter throughout the timeframe of the stability assessment test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Preparation and characterization of IFN-alpha-loaded microspheres

The freeze-dried microspheres co-encapsulated with HSA were successfully prepared by the multiple emulsion followed by solvent evaporation technique and the results of their characterization are shown in Table II. It was observed that the freeze-drying technique did not significantly change microsphere characteristics.

Scanning electron photomicrographs of the microspheres without any protein and those containing IFN-alpha co-encapsulated with HSA are depicted in Figure 1. It was observed that independent of composition, all microspheres were spherical with a smooth surface and homogeneous size. For both samples evaluated, no significant difference in the surface of the microspheres was detected.

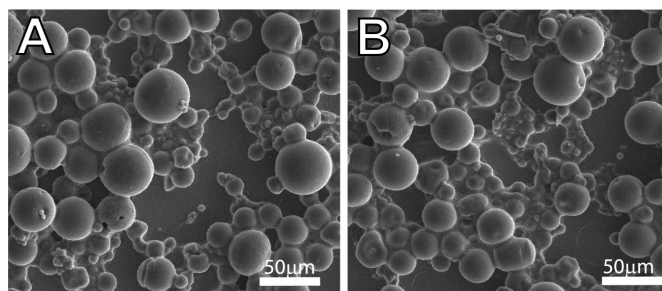


FIGURE 1 - Scanning electron photomicrographs of freeze-dried microspheres without IFN-alpha (A) and freeze-dried microspheres containing IFN-alpha co-encapsulated with HSA (B).

TABLE III - Stability assessment for developed microspheres*#

Formulation	Time (days) [§]	Particle size (µm)	IFN-alpha content (%)	Encapsulation efficiency (%)
Microspheres containing IFN-alpha co-encapsulated with HSA	0	38.41 ± 4.62 ^a	15.94 ± 3.64 ^b	82.48 ± 5.88 ^c
	7	26.97 ± 4.78 ^a	14.24 ± 2.98 ^b	79.91 ± 4.62 ^c
	30	32.29 ± 3.85 ^a	16.14 ± 3.84 ^b	83.01 ± 2.79 ^c
	60	28.93 ± 2.93 ^a	13.79 ± 2.99 ^b	81.56 ± 3.58 ^c
	90	30.66 ± 4.81 ^a	15.03 ± 3.07 ^b	80.03 ± 2.13 ^c

IFN-alpha – interferon alpha. HSA - human serum albumin. Different letters in same column means values are significantly different ($p < 0.05$). * All results expressed as mean ± standard deviation, n = 6. # Results related to freeze-dried microspheres after resuspension in water containing 0.03% polysorbate 80. § Time was determined immediately after preparation, considering freeze-dried microspheres stored at -20 °C.

The stability assessment study showed that the microspheres could be easily resuspended in 0.03% polysorbate 80. There was no significant change in particle size, suggesting they do not agglomerate during storage. (Table III). This was expected because the packaging selected for this product is impermeable and should not allow entry of moisture or any other solvent that could cause microsphere agglomeration or degradation. The structural integrity of the protein does not appear to be compromised by the preparation technique or during storage as evidenced by gel electrophoresis analysis.

***In vitro* release study**

The cumulative release profile of IFN-alpha from the microspheres prepared with PCL co-encapsulated with HSA under *in vitro* sink conditions is plotted in Figure 2.

The results showed a fast initial release and continuous release, during which the drug was released from microspheres in a sustained manner (Figure 2). The structural integrity of the protein does not appear to be compromised by this study as evidenced by gel electrophoresis analysis.

***In vitro* cytotoxicity study**

To evaluate the cytotoxicity of the microspheres and their components, as well as the microspheres' degradation products, the MTT assay on a hepatic cell line was performed. MTT, a tetrazolium salt, is cleaved by mitochondrial dehydrogenase in living cells forming the product formazan, which is dark blue and easily measurable. If the cells sustain damage or are dead, dehydrogenase activity is reduced and lower levels of formazan will be produced compared to live cells (Thomas *et al.*, 2009).

As seen in Figure 3, cell viability after application of the positive control was considered 100% and results

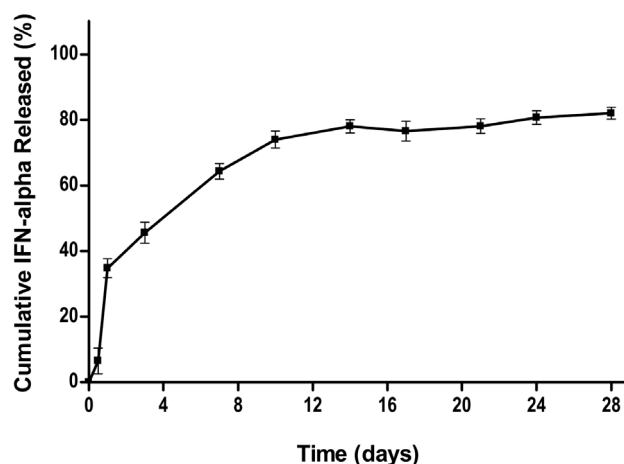


FIGURE 2 - Cumulative interferon-alpha release from poly-ε-caprolactone microspheres co-encapsulated with human serum albumin (Values shown as mean ± standard deviation, n = 6; $p < 0.05$).

obtained for the samples were calculated related to the positive control. The cellular viability after hydrogen peroxide 30 mM (negative control) treatment was near 15%, showing that it is highly cytotoxic to the evaluated cells.

The results obtained in this study showed that the developed microspheres and all their components did not present cytotoxicity to the evaluated cells as all showed a percentage of cellular viability higher than 50% (Figure 3A). Comparing microspheres alone and those containing IFN-alpha co-encapsulated with HSA, it was observed that the cellular viability was slightly higher for the microspheres alone than for those containing the proteins. Nevertheless, cellular viability was still over 50% for both formulations, showing that it is not toxic.

Regarding microsphere degradation products, the cytotoxicity study showed that in the samples submitted to degradation, the products obtained were not toxic to

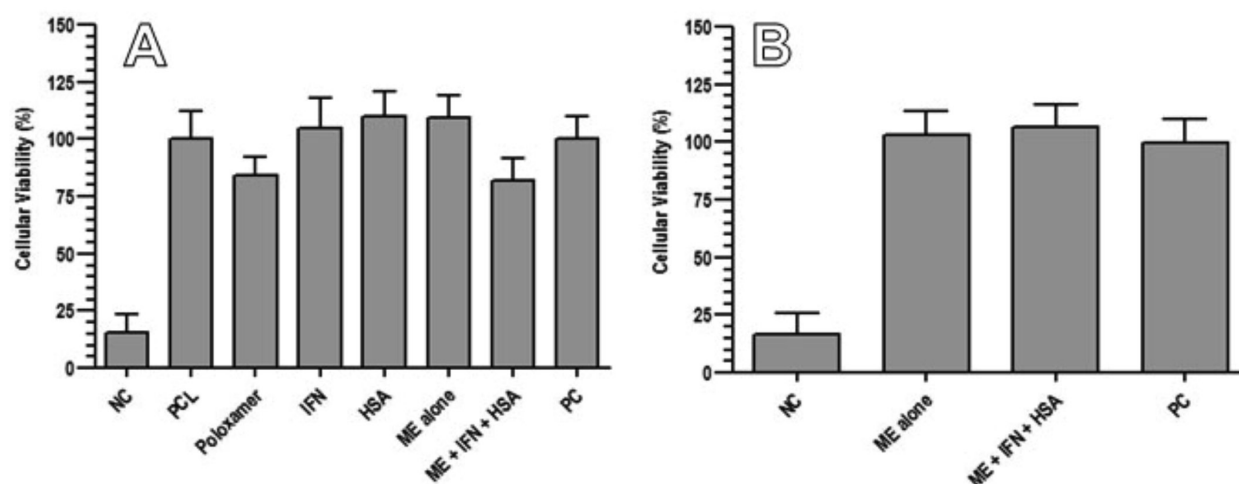


FIGURE 3 – Percentage of hepatic cell line viability after: A – application of developed microspheres and all their components; and B – application of microsphere degradation products. NC: negative control; PCL: poly- ϵ -caprolactone; IFN: interferon-alpha; HSA: human serum albumin; ME alone: microspheres without protein; ME + IFN + HSA: microspheres containing interferon-alpha and human serum albumin; PC: positive control (Values are shown as mean \pm standard deviation, n = 6).

the evaluated cells as the percentage cellular viability was higher than 50% for all the groups (Figure 3B).

DISCUSSION

The multiple emulsion followed by solvent evaporation is the technique most used to encapsulate hydrophilic substances, including proteins, into microspheres, as they can be entrapped in the inner aqueous phase, thus leading to higher drug content results. According to the literature, it can sometimes be necessary to use stabilizing agents in order to maintain the physical stability of proteins (Sanchez *et al.* 2003). Human serum albumin (HSA) at a concentration of 0.1% can protect IFN-alpha against chemical and physical degradation when it is under solution (Ruiz *et al.*, 2006). For this reason, and also because of its availability and low cost, it was decided to co-encapsulate IFN-alpha with HSA.

In this study, it was observed that microsphere particle size slightly increased after freeze-drying but as this was not significant, it can be assumed that the freeze-drying process does not influence particle size in this formulation, even when it is resuspended (Table II).

When evaluating the content and encapsulation efficiency, the encapsulation efficiency for freeze-dried microspheres was slightly different compared with microspheres that were not freeze-dried (Table II).

In polymeric matrix systems, such as microspheres, the drug is dispersed, homogeneously inside the matrix material. Slow diffusion of the drug through the polymeric system provides its sustained release (Dash and Cudworth

II 1998). Drug release from biodegradable monolithic devices can occur by diffusion, degradation of the polymer, or a combination of these two mechanisms (Jain, 2000).

PCL is a biodegradable polymer that degrades by hydrolysis producing ϵ -hydroxycaproic acid by cleavage of the polymeric chains at the ester linkage (Merkli *et al.*, 1998). Its small polymeric fragments from the matrix undergo phagocytosis and are easily eliminated from the body. The degradation of PCL matrices occurs at very low rates, making it suitable for the sustained and prolonged release of interferons because it can both reduce the number of injections for treatment and increase patient compliance. According to the literature, their degradation products are not toxic, corroborated by the *in vitro* cytotoxicity study performed here showing that the developed system and degradation products were not toxic to the evaluated cells. Further studies involving other cell types and performed *in vivo* will be conducted to confirm the absence of toxicity of the developed formulations.

The *in vitro* release profiles obtained from the IFN-alpha-loaded microspheres co-encapsulated with HSA and prepared with PCL indicated a fast initial release and then continuous release, whereby the drug was released from microspheres in a sustained manner (Figure 2). In the beginning, the profile was characterized by an initial burst in which the drug deposited on the surface was released faster. Subsequently, a period of lower release was observed as a result of heterogeneous bulk erosion combined with the high solubility of IFN-alpha. In this case, water channels can form connecting the surface to the inside of the microspheres, resulting in a slower drug release stage.

Considering that IFN- α is highly soluble, it could freely move from the inside of the microspheres to the aqueous medium where drug release was not predominantly dependent on polymer degradation.

As it has been reported by Nam *et al.* (2000), the mechanism of protein release from biodegradable microspheres is mainly governed by diffusion rather than erosion for up to 6 weeks. In this experiment, the IFN- α was released from the PCL microspheres for 28 days.

The *in vitro* release profiles obtained showed that the microspheres developed are suitable for the prolonged release of IFN- α . The microspheres co-encapsulated with HSA showed a maximum percent release of 82% in 28 days.

The determination of IFN- α by the ELISA technique is possible to indicate its stability because of the specificity and high affinity of antibodies. Clearly, this antigen reactivity may not be related to the biological activity of the protein as, according to the authors, the protein may lose biological activity without losing its epitope reactivity (Sanchez *et al.*, 2003). Hence, *in vivo* studies are being carried out to evaluate IFN- α release from the developed microspheres as well as biological activity.

As a first step, the developed microspheres will be studied for parenteral administration as an alternative to pegylated-interferon. Further experiments will then be conducted to evaluate the potential of these systems for protecting the protein against enzymatic degradation in order to use PCL microspheres for oral administration of IFN- α .

CONCLUSION

This work demonstrated that PCL microspheres are able to release IFN- α for a prolonged period of time. The microspheres, their components, and degradation products did not present cytotoxicity to the hepatic cell line as observed in the test performed. Further studies are being conducted *in vivo* to confirm if the structural integrity of the protein was maintained and also to evaluate the biological activity of IFN- α encapsulated into PCL microspheres.

Based on this preliminary study, it can be concluded that the developed microspheres represent a feasible alternative for the treatment of chronic hepatitis C that will be able to improve the quality of life of this patient group.

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