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Research paper

Prostaglandin E_2-loaded microspheres as strategy to inhibit phagocytosis and modulate inflammatory mediators release

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ABSTRACT

PGE_2, an arachidonic acid metabolite produced by various type of cells regulates a broad range of physiological activities in the endocrine, cardiovascular, gastrointestinal, and immune systems, and is involved in maintaining the local homeostasis. In the immune system, PGE_2 is mainly produced by APCs and it can suppress the Th1-mediated immune responses. The aim of this study was to develop PGE_2-loaded biodegradable MS that prolong and sustain the *in vivo* release of this mediator. An o/w emulsion solvent extraction–evaporation method was chosen to prepare the MS. We determined their diameters, evaluated the *in vitro* release of PGE_2, using enzyme immunoassay and MS uptake by peritoneal macrophages. To assess the preservation of biological activities of this mediator, we determined the effect of PGE_2 released from MS on LPS-induced TNF-α release by murine peritoneal macrophages. We also analyzed the effect of encapsulated PGE_2 on inflammatory mediators release from HUVECs. Finally, we studied the effect of PGE_2 released from biodegradable MS in sepsis animal model. The use of this formulation can provide an alternative strategy for treating infections, by modulating or inhibiting inflammatory responses, especially when they constitute an exacerbated profile.

1. Introduction

PGE_2, an arachidonic acid metabolite produced by various type of cells, regulates a broad range of physiological activities in the endocrine, cardiovascular, gastrointestinal, neural, reproductive, and immune systems, and is involved in maintaining the local homeostasis [1]. In the immune system, PGE_2 is mainly produced by APCs such as monocytes, macrophages, and dendritic cells. It can suppress most of the Th1-mediated immune responses. In fact, LPS-stimulated macrophages and monocytes can produce PGE_2 [2–4] and it can inhibit the LPS-induced TNF-α release by macrophages through a cAMP-dependent mechanism [5,6]. Also PGE_2 contributes to immune suppression by inhibiting T-cell proliferation as well as various macrophage functions [7–9]. Among the PGs synthesized by COX-2, PGE_2 is perhaps the best studied. It mediates vasodilation, vascular leakiness, pain and may regulate B cell differentiation [10]. However, this mediator may also mediate anti-inflammatory effects [11]. Studies by Zurier and colleagues indicate that PGEs can reduce inflammation in animal models of arthritis and nephritis [12]. The therapeutic value of PGEs in animal and human sepsis models was previously reported [13]. Although PGE_2 is involved in some characteristic features of inflammation, such as edema, it can also reverse acute inflammation through COX inhibition. Indeed, COX-2 inhibitors have been shown to delay the resolution of inflammation [14]. Thus, PGEs is a complex mediator of inflammation, with both inflammatory and anti-inflammatory activities. Regarding PGE_2 effects on human endothelial cells, it causes angiogenesis and phosphorylates eNOS, increasing eNOS activity and NO production. These effects are mediated through activation of cAMP-dependent PKA and PI3K [15–17]. In addition, endothelial COX-2 can be up-regulated *in vitro* by this inflammatory mediator [18,19].

Different studies have shown that systems that control antigen release can increase specific immunity by selectively driving an antigen or gene vector to immune effectors cells [20]. Other applications for this technology include the use of biodegradable

Abbreviations: PGE_2, prostaglandin E_2; APCs, antigen presenting cells; Th1, t-helper 1; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; cAMP, cyclic adenosine monophosphate; COX-2, cyclooxygenase-2; HUVECs, human umbilical vein endothelial cells; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PKA, protein kinase; PI3K, phosphatidylinositol 3-kinase; MS, microspheres.

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polymer systems, which allow the sustained and/or controlled release of the encapsulated substances [21,22].

The aim of this study was to develop PGE_2-loaded biodegradable microspheres that can be administered *in vivo* in order to modulate the immune responses over the course of an inflammatory process. In the first part of our study, we have developed PGE_2-loaded MS, which were prepared using an oil-in-water (o/w) emulsion solvent extraction–evaporation technique. This procedure was previously designed to encapsulate drugs that are either insoluble or poorly soluble in water (lipids), with an efficient yield despite the low concentrations of bioactive agent [23]. Based on the successful encapsulation acquired and the preservation of biological activities of encapsulated PGE_2, the proposed formulation could be employed with other therapies for the treatment of inflammatory and infectious diseases. In this study, we have investigated the effect of encapsulated prostaglandin released from microspheres on the LPS-induced TNF-α release by murine peritoneal macrophages. We analyzed the MS uptake by macrophages under the biological effect of PGE_2 and also investigated the effect of encapsulated or in solution PGE_2 forms on MCP-1 release and NO production by HUVECs. Finally, we studied the effect of PGE_2 released from microspheres in sepsis animal model.

2. Materials and methods

2.1. Animals

Animals (wild-type (WT) sv129 mice, weighting 18–20 g), subjected to all described procedures in this study were obtained from Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo and were maintained under standard laboratory conditions. All experiments were approved by and conducted in accordance with the guidelines established by the Animal Care Committee of the University.

2.2. Materials

For the purposes of this study, the PGE_2 (dissolved in a stock solution of absolute ethanol) was obtained from Cayman Chemicals (Ann Arbor, MI, USA). LPS of *Escherichia coli* (serotype 0127:B8), tribromoethanol and Griess reagent mixtures were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Poly (vinyl-alcohol) (Mowiols 40–88) was obtained from Aldrich Chemicals (Wankee, WI, USA). Poly (D,L-lactide-co-glycolide) (PLGA) with a co-monomer ratio of 50:50 (lactic/glycolic acid) and molecular weight of 78 kDa was obtained from Boehringer Ingelheim (Ingelheim, Germany). A commercially available PGE_2 enzyme immunoassay (EIA) kit was from Biotrak, Amersham Biosciences UK Limited, Buckinghamshire, UK. Endothelial basal medium (EBM)-2 supplemented with endothelial growth media (EGM)-2 were from Clonetics, Barcelona, Spain. MCP-1 protein was obtained from PeproTech, London, UK. The antibody pair for human human MCP-1 ELISA was from R&D Systems, Madrid. Commercially ELISA antibodies were used to measure TNF-α (Pharmingen, San Diego, California). Neutravidin–horseradish peroxidase was from Perbio Science, Cheshire, UK. K-Blue substrate was from Neogen, Lexington, KY. Methanol, methylene dichloride, acetonitrile and acetic acid (high-performance liquid chromatography grade) were purchased to Merck (Dietikon, Switzerland). Panoptic staining was from Laborclin (Paraná, Brazil).

2.3. Microspheres preparation

Microspheres (MS) containing PGE_2 were prepared using an oil-in-water emulsion solvent extraction-evaporation process [23–25]. In brief, a 0.3 ml internal organic phase (PGE_2 solution 0.25 μg/ml or 7×10^{-3} M) was added to 10 ml of methylene dichloride containing 30 mg of PLGA 50:50. This phase was poured into an external aqueous phase (40 ml of polyvinyl alcohol solution at 3% w/v) and stirred mechanically (RW20; IKA Labortechnik, Staufen, Germany) at 600 rpm for 4 h to extract the organic solvent. Finally, the microparticles formed were washed three times with distilled and sterile water and then freeze-dried until the use.

2.4. Microspheres characterization

The shape and surface of the dried microparticles were observed by scanning electron microscopy (SEM), using a Jeol scanning microscope (JSM 5200, Tokyo, Japan). Particle diameters and distribution of microspheres were characterized using a particle size analyzer (SALD-2101; Shimadzu, Kyoto, Japan). To determine whether the microspheres were contaminated by LPS, a limulus amebocyte lysate test was performed.

2.5. Entrapment efficiency

Quantitation of PGE_2 in solution during its release from microspheres was performed using ELISA. The samples (unloaded and PGE_2-loaded MS) were dissolved in 1 ml of acetonitrile/ethanol (7:3 v/v), and the solvents were evaporated in a vacuum concentrator centrifuge for 4 h. The samples were then resuspended in 0.05 ml of ELISA buffer and diluted (100×). Bound PGE_2 was detected using an antiserum (lyophilized rabbit anti-prostaglandin E_2). Then, a PGE_2 horseradish peroxidase conjugate was added to the plate. After 1 h incubation, the enzyme substrate (tetramethylbenzidine/hydrogen peroxide) was added, and the samples were incubated for 30 min. The enzyme reaction was stopped by adding 0.1 ml of 1 M sulfuric acid to each well. Absorbance was determined in a plate reader set at 450 nm. Sample values were calculated based on a logarithmic equation: $y = -17.696\ Ln(x) + 163.42$; $r^2 = 0.9918$, where y is the percentage of tracer binding, x is the PGE_2 concentration (μg/ml), and r is the coefficient of determination. A standard curve was used to calculate the amount of PGE_2 (concentrations ranged between 50×10^{-6} and 6400×10^{-6} μg/ml).

2.6. In vitro PGE_2 release study

PGE_2-loaded microspheres (2 mg) were suspended in 1 ml of PBS/ethanol medium (50:50, v/v), pH 7.4 and incubated at 37 °C on a rotating incubator. Samples were withdrawn at 0, 2, 4, 10, 12 and 24 h and centrifuged (500*g*, 10 min). An equal volume of fresh medium was immediately added to the compartment after each sampling. The concentration of PGE_2 present in the medium was determined by ELISA. Results correspond to the average of three different batches.

2.7. Cell culture

HUVECs were isolated by collagenase treatment [26] and maintained in human endothelial cell – specific EBM-2 supplemented with EGM-2 and 10% fetal calf serum (FCS). Cells up to passage 2 were grown to confluence on 24-well culture plates. Before every experiment, cells were incubated for 16 h in medium containing 1% FCS and then returned to the 10% FCS medium for all experimental incubations. Samples of PGE_2 in solution (5 μg/ml or 1.4×10^{-5} M) and unloaded or PGE_2-loaded MS suspension (1 mg/ml) were added to wells. At the end of the incubation time (4 h), cell-free supernatants were collected and stored at −20 °C for MCP-1 ELISA and nitrite content by Griess reaction.

2.8. Uptake of MS by peritoneal macrophages

Mice peritoneal macrophages were obtained by lavage with 5 ml of PBS/citrate. The isolated cells were centrifuged at 400*g* for 10 min and then resuspended to 3×10^6 cells/ml. Aliquots (1 ml) of cell suspension were added to each well of a 24-well plate and placed in a humidified atmosphere (37 °C, 5% CO_2) for overnight cell adhesion. Non-adherent cells were removed by washing with RPMI-1640 medium, with fetal bovine serum (FBS) and gentamycin (1 μl/ml). Firmly adhered cells in 24-well plates (6×10^5 cells/well) were co-incubated for 2 h with 1 mg/ml of unloaded or PGE_2-loaded MS, using the described culture medium. After incubation, the medium was aspirated and non-ingested MS were washed off with additional medium. Cells were identified by panoptic staining. MS uptake was assessed microscopically by counting the percentage of macrophages that had ingested at least one MS. The number of MS per cell was also evaluated.

2.9. TNF-α release by peritoneal macrophages

Mice peritoneal macrophages (1×10^6 cells/ml) were placed in a 24-well culture plate. After 1 h incubation with 2 mg of unloaded or PGE_2-loaded MS and PGE_2 in solution (3×10^{-7}–10^{-8} M), they were stimulated with LPS (0.5 μg/ml) for 2 h in a humidified atmosphere (37 °C, 5% CO_2). Supernatant dilutions were incubated with specific antiserum in 96-well plates precoated with anti-rabbit IgG antibodies from TNF-α commercial Kit. After overnight incubation at 4 °C, plates were washed and enzyme substrate was added for 60–120 min at 25 °C. The optical density of samples was determined at 450 nm in a microplate reader. Sensitivities were >10 pg/ml.

2.10. NO production by HUVECs

NO production in human endothelial cells was determined by Griess reaction. HUVECs were incubated for 4 h with a suspension (1 mg/ml) of unloaded or PGE_2-loaded MS and PGE_2 in solution (5 μg/ml or 1.4×10^{-5} M), in a humidified atmosphere (37 °C, 5% CO_2). Supernatants (0.1 ml) were incubated with an equal volume of Griess reagent mixtures (1% sulfanilamine, 0.1% *N*-(1-naphtyl)-ethylendiamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. The absorbance was measured in a microplate reader at 540 nm and concentrations calculated from a sodium nitrite standard curve. The data are presented as micromoles of NO_2^- (nitrite) (mean ± the SEM).

2.11. MCP-1 release from HUVECs

Human endothelial cells were cultured in 24-well culture plates and stimulated as described above. After coating the plates overnight with the coating anti-MCP-1 mAb, diluted supernatant samples and standards were added in PBS/0.5% BSA/0.05% sodium azide for 2 h. Biotinylated detector antibodies were added for 2 h, followed by neutravidin–horseradish peroxidase for 1 h. All plate washes were of four cycles in freshly made PBS/0.2% Tween 20. Enhanced K-Blue TMB substrate was added for 30 min and the enzyme reaction stopped by addition of 0.19 M sulphuric acid. Absorbance was read at 450 nm and the data processed by GraphPad Prism software. Results are expressed as pM chemokine in the supernatant. The sensitivity of the assay was >10 pg/ml.

2.12. Mouse model of cecal ligation and puncture (CLP)

The animals were subjected to CLP surgery, as previously described in detail [27]. Briefly, mice were anesthetized by the i.p. injection of tribromoethanol 2.5%. Under sterile surgical conditions, a 1- to 2-cm midline incision was made on the ventral surface of the abdomen to fully expose the cecum. Then, it was ligated at its base with a 4–0 silk suture (without causing bowel obstruction), and four times punctured with a 21-gauge needle. The abdominal incision was closed using a surgical staple, and 1 ml of sterile normal saline was s.c. administered for fluid resuscitation just after surgery. Sham-operated mice underwent an identical laparotomy, but did not undergo ligation and puncture (control group). Survival was monitored in the different experimental groups for up to 7 days after CLP. The evaluated groups were: sham operated; CLP; CLP + unloaded or PGE_2-loaded MS (0.2 ml, 4 mg/ml, i.p. administered 1 h prior to the surgical procedure) and PGE_2 in solution (0.2 ml, 5 μg/ml, i.p. administered 1 h prior to the surgical procedure).

2.13. Statistical analysis

The ELISA and NO assays were analyzed using one-way analysis of variance (ANOVA) with post-test (Tukey's Multiple Comparison Test). For uptake of MS by macrophages we used Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of PGE_2-loaded MS

Scanning electron micrographies of microspheres are shown in Fig. 1B. Spherical and homogenous microspheres having average diameters of 5.7 μm (Fig. 1A) were achieved. In this study, the formulation developed protected PGE_2 from oxidation elicited by light or water exposure. This method enables to preserve the biological activity of the encapsulated PGE_2 based on the results obtained in this study.

3.2. Entrapment efficiency and in vitro release of PGE_2

The amount of PGE_2 was found to be 12.8×10^{-3} μg/ml (3.8×10^{-8} M) in 2 mg of microspheres. This value represents an encapsulation efficiency of 75.7%.

The 24-h cumulative release and diffusion of PGE_2 from the PLGA microspheres (mean ± SD) is shown in Fig. 1C. In the present study, a burst effect was observed, in which the release of the entrapped mediator reached approximately 25% 4 h later. Thereafter, there was a continuous and slowly release from 4 to 10 h. At the end point of the study (12 h), a 50% release was reached. Under these conditions, a 100% drug release was never attained. Total release is dependent on particle degradation, which may account for the effects observed. Nevertheless, the remaining 50% of the PGE_2 encapsulated could be released over a longer period of time.

3.3. PGE_2 released from MS inhibits the uptake of particles by peritoneal macrophages

PGE_2-loaded MS used in this study had average particle diameters comprised between 5 and 6 μm, as measured by laser light scattering (Fig. 1A). The number of PGE_2-loaded MS engulfed by macrophages was lower than that of unloaded MS (Table 1). No particle uptake was observed at 4 °C (data not shown). The Fig. 2A and B show, respectively, the unloaded and PGE_2-loaded MS uptake by macrophages at 37 °C, after 2 h of incubation. Different cell activation was observed when compared the uptake displayed by macrophages for unloaded and PGE_2-loaded MS.

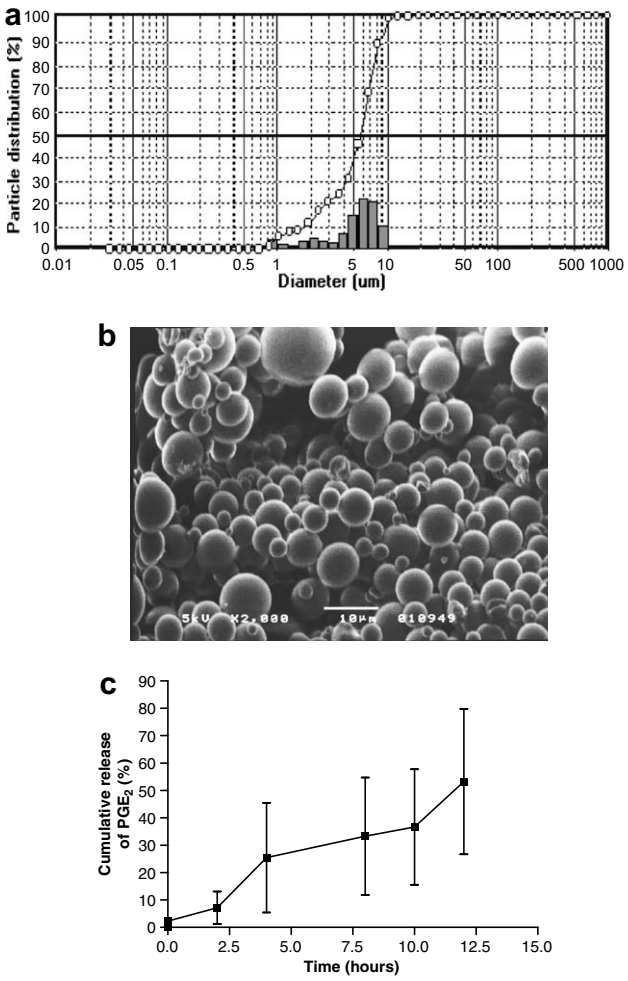

Fig. 1. (A) Size distribution of PGE_2-loaded MS. Mean particle size of 5.7 ± 0.27 μm. (B) PGE_2-loaded MS morphology assessed by SEM. (C) *In vitro* cumulative release of PGE_2 from PLGA microspheres in PBS/ethanol medium, pH 7.4; data are representative of three batches.

Table 1
Uptake of PLGA MS by murine peritoneal macrophages

Tested PLGA MS	% of macrophages containing MS	Number of MS contained per cell
Unloaded MS	96 ± 1.4	3.7 ± 2.3
PGE_2-loaded MS	47 ± 0.5***	2.6 ± 1.5

The cells in complete RPMI medium were incubated for 2 h with 1 mg/ml of unloaded or PGE_2-loaded MS (approximately 2.0×10^{-8} M). Cells were identified with panoptic staining and microscopically counted. The data are presented as means ± the SD ($n = 3$).
*** $P < 0.001$.

In fact, macrophages engulfed less PGE_2-loaded MS than unloaded ones (Fig. 2 B).

3.4. PGE_2 released from MS decreases TNF-α levels produced by LPS-stimulated macrophages

To investigate the effects of PGE_2 on TNF-α release, mice peritoneal macrophages were stimulated with LPS in the presence or absence of two doses of PGE_2 in solution, unloaded or PGE_2-loaded MS. The amount of the cytokine released in the supernatants was assayed by ELISA after the incubation time. When compared to positive control (cells stimulated with LPS) all the groups showed inhibitory effect on cytokine production (Fig. 3). PGE_2 solutions (3×10^{-7} to 10^{-8} M) revealed marked inhibition of TNF-α production. Significant inhibition was also observed with PGE_2 released from microspheres. The amount of cytokine in the cell supernatants of this experimental group was similar to that encountered when the cells were incubated with PGE_2 solution at 3×10^{-7} M (Fig. 3).

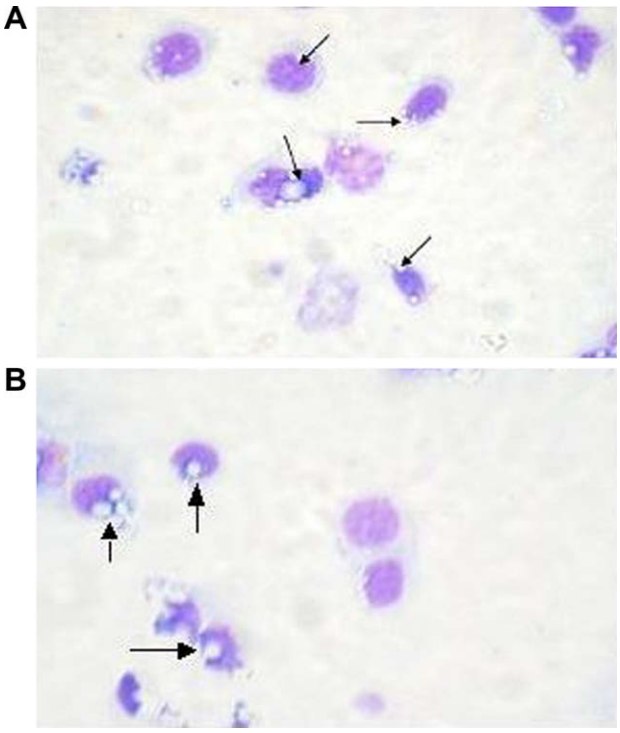

Fig. 2. Light micrographs of peritoneal macrophages, which had ingested (A) unloaded and (B) PGE_2-loaded MS. The black arrows show the engulfed microspheres. Cells were identified by panoptic staining. Magnification: (100×/1.25 oil).

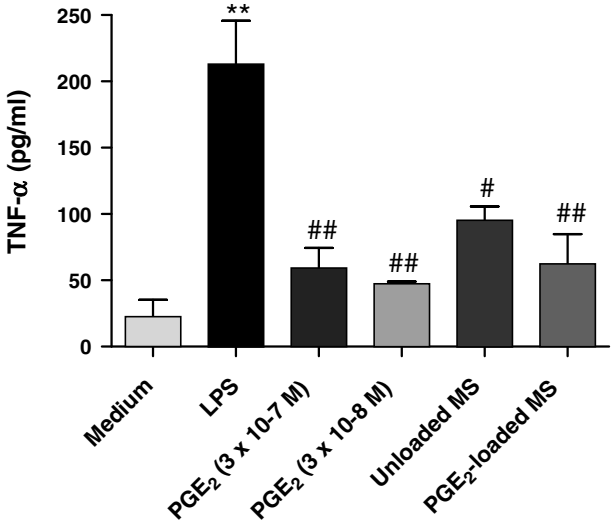

Fig. 3. Effect of PGE_2-loaded MS on LPS-induced TNF-α release from murine peritoneal macrophages. Macrophages were incubated with 2 mg of unloaded or PGE_2-loaded MS and PGE_2 in solution in complete RPMI medium for 1 h. Then, after stimulation with LPS (0.5 μg/ml) for 2 h, cell-free supernatants were collected. TNF-α levels (pg/ml) were determined by ELISA. Results are presented as means ± SEM from three different experiments ($n = 3$); $^{**}P < 0.01$, relative to values in the control (medium) group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, relative to values in LPS-stimulated cells.

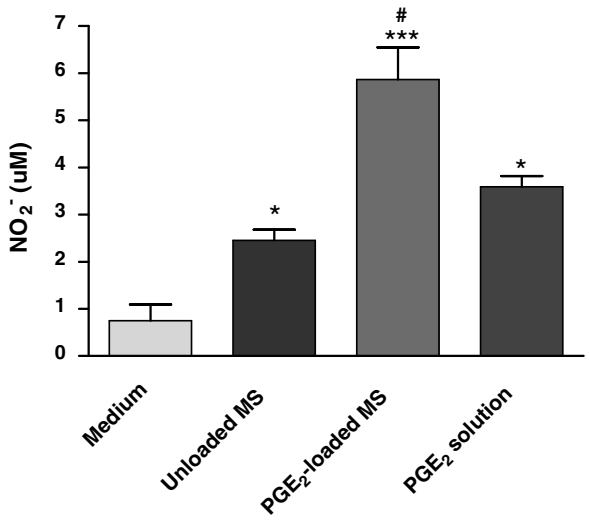

Fig. 4. Effect of PGE_2-loaded MS on NO_2^- production by HUVECs. Cells were incubated for 4 h with 1 mg/ml of unloaded or PGE_2-loaded MS and PGE_2 in solution (5 μg/ml) in EBM-2 medium. Nitrite levels in the supernatants were quantified by Griess reaction. Results are presented as means ± SEM from three different experiments ($n = 3$); $^{*}P < 0.05$ or $^{***}P < 0.001$ relative to values in the control (medium) group. $^{\#}P < 0.05$, relative to values in the unloaded MS group.

3.5. PGE_2-loaded MS increase NO production by HUVECs

Production of NO by human endothelial cells was quantified as described. In HUVECs, all the tested stimuli increased the nitrite levels in the supernatant when compared to the control group (medium) (Fig. 4). Both, PGE_2 in solution and that released from the microspheres were able to significantly increase nitrite levels. In contrast, the unloaded MS only induced a small enhancement of NO_2^- production. Despite these findings, the increase in nitrite levels elicited by PGE_2-loaded MS was statistically different from that provoked by unloaded MS.

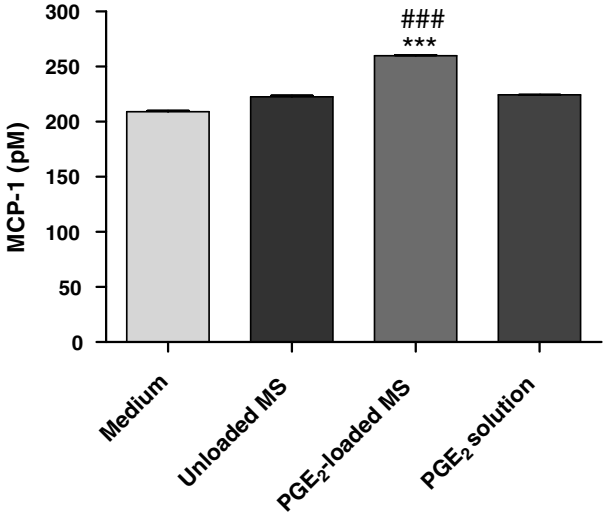

Fig. 5. Effect of PGE_2-loaded MS on MCP-1 release by HUVECs. Cells were incubated for 4 h with 1 mg/ml of unloaded or PGE_2-loaded MS and PGE_2 in solution (5 μg/ml) in EBM-2 medium. MCP-1 levels in the supernatants were determined by ELISA. Results are presented as means ± SEM from three different experiments ($n = 3$); $^{***}P < 0.001$ relative to values in the control (medium) group. $^{\#\#\#}P < 0.001$, relative to values in the unloaded MS group.

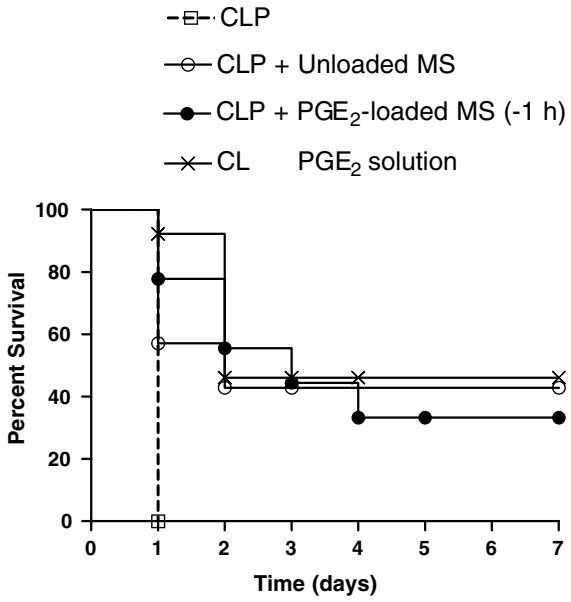

Fig. 6. Effect of PGE_2-loaded MS injection on mice survival after CLP surgery. CLP (control) mice, CLP mice injected with unloaded MS, CLP + PGE_2-loaded MS (−1 h) and CLP + PGE_2 solution. Animals ($n = 7$, per group) subjected to surgery were followed for 7 days and the results were expressed as survival percentage.

3.6. PGE_2-loaded MS increase MCP-1 release by HUVECs

To investigate the effects of PGE_2 on MCP-1 release, human endothelial cells were incubated with unloaded MS, PGE_2-loaded MS and PGE_2 in solution. The amount of the chemokine released in the cells supernatant was determined by ELISA. In HUVECs assay, PGE_2-loaded MS significantly increased MCP-1 levels when compared to medium (Fig. 5). Furthermore, PGE_2-loaded MS induced a significant increase in the chemokine levels when compared to unloaded MS. In contrast, PGE_2 in solution did not cause MCP-1 release from the cells.

3.7. PGE_2 released from MS confers resistance against CLP-induced mortality

Mice survival was monitored in the different experimental groups for up to 7 days after CLP procedure. Interestingly, control group (CLP) had 100% mortality 1 day after surgery, whereas the groups treated with either PGE_2 solution or PGE_2-loaded MS 1 h before surgery, showed, respectively, 7.7% and 22.3% mortality (Fig. 6). In addition, on day 2 after surgery, mice that received PGE_2-loaded MS had 45.5% mortality vs. 58% and 54% observed for CLP + unloaded MS and CLP + PGE_2 solution, respectively. However, after day 3, the CLP + PGE_2 solution group showed 54% mortality vs. 66% achieved for the group treated with PGE_2-loaded MS. As expected, all of the animals subjected to sham operation survived (data not shown).

4. Discussion

The preparation of the PLGA microspheres was based on the emulsion solvent evaporation and extraction method [23–25]. A recent published work from our group [23] showed that another lipid mediator (leukotriene B_4) could be encapsulated with good entrapment efficiency. In this study, the particles average diameters (5.7 μm) and morphology obtained were appropriate for *in vivo* studies, which would allow the delivery of the microspheres by intratracheal or intranasal routes. In this context, particles diameters between 5 and 10 μm (critical sizes) can increase the

phagocytosis process by the cells. Also, especially for intranasal routes a smooth and homogenous surface of the particles achieved in our study can provide less interaction between them and the mucous membrane, increasing the bioavailability to the lungs. In addition, the polymer used to prepare the microspheres, PLGA does not cause tissue injury since it is inert and biodegradable [28]. These characteristic features will allow an appropriate administration of PGE_2-loaded MS to treat inflammation and might modulate cytokine/chemokine release during inflammatory processes. Furthermore, the decreased uptake of PGE_2-loaded MS by macrophages compared to that observed with unloaded MS (Table 1) could contribute to the development of futures studies where the immunosuppressive and/or immunomodulatory action of this mediator during the course of inflammatory diseases will be investigated.

Many studies have used chromatographic methods such as HPLC to detect arachidonic acid derived eicosanoids in solution, including prostaglandins [29]. However, the disadvantages of these methods are mainly the poor sensitivity or detection limits for the prostanoids molecules such as prostaglandins. In our study, we have employed a competitive ELISA assay, which has better sensitivity for quantifying the mediator released from MS than conventional HPLC [30,31].

In vitro release profile and bioactivity studies achieved confirmed the sustained release of PGE_2 over a period of hours. Taking into account the size of the microspheres used for the formulations to be administered intratracheal or intranasally and the rate at PGE_2 is released, we can estimate other doses that can be administered in future *in vivo* studies. In this context, our findings could lead to the development of an appropriate formulation that increases the bioavailability of this mediator during the course of the inflammatory and infectious process. Moreover, this technique provides a strategy for delivering PGE_2 to modulate cytokines and/or chemokines release during infections. The continuous PGE_2 release from the biodegradable microspheres was able to inhibit TNF-α production by LPS-stimulated macrophages (Fig. 3), demonstrating that the biological activity of the mediator can be preserved while it is encapsulated. Moreover, the initial burst release (25%) achieved in our study could contribute to the earlier effects observed for phagocytosis assay and NO production when the cells were incubated with the microspheres. In fact, during 2 h incubation, the phagocytosis assay has shown that the release of PGE_2 to the medium and/or into the cell cytoplasm can decrease the number of engulfed microspheres by peritoneal macrophages (Fig. 2B).

Furthermore, the encapsulated and released PGE_2 from microspheres is able to stimulate human endothelial cells since it can provoke the generation and release of NO and MCP-1 (Figs. 4 and 5, respectively). Therefore, PGE_2 in solution did not provoke similar responses, suggesting its molecule degradation during the assay. On LPS-stimulated macrophages, PGE_2 decreased TNF-α release exerting its expected immunosuppressor effect. By contrast, on HUVECs, the released mediator increased NO and MCP-1 levels and thus revealing an unexpected but interesting inflammatory effect.

Our data from the experimental model of sepsis employed suggest that PGE_2 has an important role during the first hours and days since the mortality in these groups is clearly diminished. Both PGE_2 solution and released from microspheres were able to prolong animal survival as described. Taking together, these results and that obtained from LPS-stimulated peritoneal macrophages (Fig. 3) suggest that PGE_2 released from microspheres prolonged animal survival during the sepsis by a TNF-α inhibition mechanism. As previously reported [13], PGEs have therapeutic value in animal and human sepsis models. In this context, our findings indicate that the controlled release of PGE_2 from microspheres can constitute a useful strategy for the treatment of infections, especially when administered during the first hours of the inflammatory process (Fig. 6). Moreover, PGE_2 administration in its solution form can provoke loss of renal function and microvascular blood flow [32] and also arterial thrombosis [33]. This fact corroborates the importance to develop a formulation that can release PGE_2 in a different manner and intensity compared to conventional administrations.

In conclusion, in this study, we have developed PGE_2-loaded biodegradable MS using an oil-in-water emulsion solvent extraction–evaporation process. This system allowed us to obtain an appropriated encapsulation and good entrapment efficiency. In addition, the method employed preserved PGE_2 bioactivity as demonstrated by different *in vitro* and *in vivo* studies. In this regard, PGE_2 released from microspheres inhibited the particles' phagocytosis and LPS-induced TNF-α release by murine peritoneal macrophages. Moreover, the formulation was efficient in stimulating human endothelial cells to produce high levels of nitrites and MCP-1. Further studies are required to develop strategies to control PGE_2 release from microspheres and to adjust the dose for *in vivo* administrations. The proposed formulation could be used alone or in combination with other therapies to modulate cytokines and/or chemokines release and could constitute an alternative therapy for the control of different inflammatory and infectious disorders.

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